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Escuela de Ciencias Biológicas e Ingeniería

Enzyme-based biosensors for pesticide detection

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniero Biomédico.

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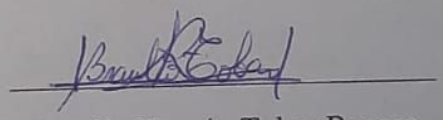

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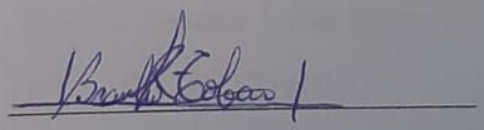

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Resumen

La industria agrícola en Ecuador es la industria no petrolera de mayor recaudación bruta. Con el fin de mantener altos niveles de calidad y producción, el control de plagas y enfermedades es necesario. En Ecuador, las plagas son atacadas principalmente con pesticidas, en lugar de control biológico. Esto crea una dependencia de pesticidas en la agricultura que podría potencialmente convertirse en un problema de salud pública para las comunidades de agricultores e incluso para los consumidores. La detección de plaguicidas es posible con el desarrollo de biosensores enzimáticos. Un biosensor basado en enzimas permite la detección de cualquier analito que sea catalizado por la enzima o inhiba la actividad catalítica de la enzima. El biosensado es un método de bajo costo y alta sensibilidad con un diseño portátil simple. Esta tesis es una revisión de las enzimas que se han utilizado como elemento biosensible en la construcción de biosensores basados en enzimas. Los biosensores se han clasificado según el mecanismo de trabajo aplicado para la detección: inhibición o catálisis. Existen biosensores ópticos, potenciométricos o voltamétricos que podrían aplicarse para la detección de plaguicidas comunes utilizados en Ecuador. Por lo tanto, es tanto un desafío como una oportunidad para desarrollar y comercializar biosensores enzimáticos en Ecuador.

Palabras claves: Pesticidas, Biosensores enzimáticos

Abstract

The agricultural industry in Ecuador is the highest non-petroleum grossing industry. In order to maintain high quality and production levels, pest and disease control are needed. In Ecuador, pests are mainly attacked using pesticides, rather than biological control. This creates a pesticide dependence in agriculture that could potentially become a public health issue for the farmer communities and even the consumers. Pesticide screening is possible with the development of enzyme based-biosensors. An enzyme-based biosensor permits the detection of any analyte that is catalyzed by the enzyme or inhibits the catalytic activity of the enzyme. It is a low cost, high sensitivity method with simple portable design. This thesis is a review of the enzymes that have been used as biosensing element in the construction of enzyme-based biosensors. Biosensors have been classified according to the work mechanism applied for the detection: inhibition or catalytic-based. There are optical, potentiometric or voltammetric biosensors that could be applied for the detection of common pesticides used in Ecuador. It is therefore both a challenge and an opportunity to develop and commercialize enzyme-based biosensors in Ecuador.

Keywords: Pesticides, Enzyme-Based Biosensors

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Introduction

Pesticides

Pesticides are chemical compounds used widely in agriculture to control, destroy or kill weeds, bacteria, insects, fungi, rodents, and other pests (1–6). However, most pesticide usage entails hazardous consequences for living organisms. In fact, it is a major concern in environmental chemistry the presence of pesticides in the food, water and soil (4,7). Pesticide residues affect ecosystems due to the bioaccumulation and long-term effects in both animals and humans (2–4). The exposure to pesticides leads to several health disorders such as skin rashes, infertility, cancer, respiratory and neurological diseases (1,2,8,9).

Pesticides are the leading method of poisoning in the developing world (6,10,11). In 2018, it was estimated that annually there were around 1-5 million cases of pesticide poisoning among agricultural workers (3,6,12,13). From these cases, 20,000 people die from poisoned food consumption only (6,14). Besides, there are numerous cases of developing both chronic and acute illnesses due to prolonged exposure to pesticides (5,15). The International Agency for Cancer Research (IARC) has studied a wide range of fungicides, herbicides, insecticides and other similar chemicals to conclude the presence of carcinogenic compounds in various pesticides (10). Some of the chemicals associated with cancer have been lindane, insecticide used for plague control; methoxychlor, an organochloride insecticide used to protect crops, and phenoxy acid herbicides (10,16).

Another concern of the negative role of pesticide is the induction of congenital malformations and the genotoxic potential. Experimental data revealed the presence of different agrochemical ingredients with mutagenic properties in the pesticides. This could lead to gene mutation, DNA damage, reduced fertility and chromosomal alterations (10,17). Nevertheless, the genotoxic potential, showed in the experiment for agrochemical ingredients, was generally low (10). Furthermore, it must be considered that the exposures to pesticides are to mixtures of these ingredients. So, the genotoxic potential of a single compound will not have the same effect in humans.

Problem Statement

The agricultural industry in Ecuador is the highest non-petroleum grossing industry. Agriculture is a fundamental pillar of the Ecuadorian economy. Ecuador is the world leader in banana production. In 2019, it exported almost 25% of the bananas in the world, just followed by Europe with a 20%. Similarly, it is one of the largest cocoa producers in the world. In 2014, Ecuador registered a total production of 240 thousand tons of cocoa. In addition, Ecuador is the third-largest exporter in cut flower industry in the world.

Nowadays, pest and disease control in crops plays a big role in the maintenance of both quality and quantity of the agricultural industry. This along with a demanding industry, produce a pesticide dependence with the potential of becoming a main concern in public health, for the farmers and the consumers. Therefore, there is an urgent necessity to measure the levels of pesticides. Biosensors permit the detection of any analyte using a biological element in a cost-effective manner. Enzyme-based biosensors have been used for pesticide detection because it is a low cost, high sensitivity method with simple portable design. This thesis will be a review of enzyme-based biosensors and their possible application to the Ecuadorian agricultural industry.

Objectives

- To search in the literature for enzymes that have been used in biosensor development
- To determine the main characteristics that affect the performance of a biosensor
- To find a suitable biosensor for the Ecuadorian agricultural industry

Types of pesticides

Pesticides can be classified according to the targeted pest or based on their chemical compounds. Pesticides based on the target organism are acaricides, algaecides, avicides, bactericides, fungicides, herbicides, insecticides, molluscicides, nematicides, rodenticides, slimicides, virucides and weedicides (3,8,18,19). However, as a result of the interest to the biosensor performance, this thesis will mainly focus on the classification based on the chemical constituents of the pesticide. Pesticides classified by their chemical structures are organochlorine, organophosphate, carbamate, inorganic pesticides and synthetic pyrethroids (2,3,18).

Organochlorine pesticides are highly polluting to the environment and human health. Some of the traits of organochlorine pesticides are their toxicity on nontarget organisms and their tendency of bioaccumulation (20,21). Their method of operation is to interfere in the sodium/potassium balance of the target's nerve fiber; therefore, the nerve will constantly be excited. Among the most used pesticides are the Lindane, Aldrin or dichlorodiphenyltrichloroethane (DDT) (3,21). Nowadays, the use of these pesticides has been significantly reduced in most of the developed countries, and have been largely replaced by more effective organophosphates and carbamates (22).

Organophosphorus pesticides impose fewer toxicological effects than organochlorine pesticides, so they were considered harmless in comparison. Nonetheless, after years of overuse, the bioaccumulation exerted alarming toxicological effects on nontarget organisms, reaching an annual estimation of 3 million poisoning and 200,000 deaths (3,23). Organophosphate operate by blocking the activity of enzyme acetylcholinesterase, this will permit acetylcholine to transfer nonstop impulses, affecting the nerves and producing paralysis on the targeted pest (21,24). Similar to organophosphorus pesticides, carbamate inhibits the acetylcholinesterase. Yet, the toxicological potential of carbamates is less significant than the organophosphates (3). Temik, Furadan and Sevin are examples of commercial carbamates, while some commonly used organophosphates includes parathion, malathion or fenitrothion (25–28).

Pesticide detection

There is an imperative need for establishing low levels of pesticides in the food. It is necessary to have means of pesticide detection in food and environmental samples. Early methods for pesticide analysis relied on conventional techniques such as liquid chromatography, gas chromatography, ELISA, high-performance liquid chromatography and mass spectrometry detection (3,29,30). These methods offer reliability and sensibility, but they are not appropriate for on-field detection. Not to mention that are time consuming, need expensive equipment and trained technicians (2,31,32). For instance, when using spectrophotometric methods with complex color reactions, the tests tend to last longer and the matrix interfere with the results. Besides, there are some pesticides that get decomposed when are subjected at injection head temperatures of chromatographic techniques (31). Therefore, current investigations focus their research

towards biosensor development. Nowadays, the application of biosensors has been directed towards the food industry, health care and environmental practices (33,34).

Biosensors

A biosensor is a device that uses an immobilized biological element that will sense the presence of an analyte, connected to a transducer that converts the chemical signal to a measurable electrical signal (35). Aside from the lower cost in comparison to conventional techniques, biosensors exhibit high specificity, high sensitivity, portability, user-friendly operation, compact size and the ability to perform real time analysis (31,36,37). Their biological base allows a qualitative toxicological measurement rather than just a quantitative obtained by the conventional methods. In the last decade, there has been serious improvement in biosensor fabrication by utilizing diverse biosensing elements or developing different methods of immobilization for the biological element (1,38,39). Among the innovations applied in biosensors are the use of nanotechnology and immune sensors, which use antibodies to detect the signal, similar as immunoassays (40).

Biosensors can be classified by their signal transduction technique or by the bio recognition element used for the pesticide detection. According to the technique used for the signal transduction, biosensors can be electrochemical, mechanical, optical or piezoelectrical (41). Most biosensors use electrochemical transducer, which permit the development of simple design, small size, low cost, portable biosensors that have high sensitivity (1,42–45). Alternatively, the biorecognition elements used for the biosensor could be antibodies, aptamers, DNA sequences, enzymes, and fragments of microorganisms or whole cells (1). This thesis will focus on the biosensors with enzymes used as biorecognition element.

The electrochemical detection system, which is the most used, could be potentiometric, amperometric or conductometric. Potentiometric systems rely on in the variation of the potential with its corresponding current change due to the reduction and oxidation of the electrochemical reaction (46). Amperometric detection is based on the current change produce by the variations in the chemical concentration (47,48). Conductometric measurements depend on the conductivity and resistivity of the analyte. So, it is affected by the number of ions, pH, and temperature. The optical techniques rather than directly measuring electrons, uses the photons produced for electrical

transduction. Some of the most important parameters in photometric measurements are intensity, absorption, reflectance, quenching, decaying time and radiant energy transfer (49).

Advancements in this technology are made mainly by improving the biological components and implementing novel microsystems technologies. Due to its high chemical specificity and the biocatalytic signal amplification property, enzymes are among the most selected option as biosensing element of the pesticide biosensor (4). In enzyme-based biosensors, the transducer will receive a signal from the enzyme, proportional to the concentration of the target analyte. This signal may result from any reaction catalyzed by the enzyme such as light emission, heat emission, change in the pH, absorption or reflectance. Then, the transducer will convert the signal to a quantifiable reaction, such as current, potential or even an optical signal (50). The converted signal can be amplified, processed or stored afterwards.

In biosensors technology development, the biosensing element requires to be immobilized with the transducer interface. A proper immobilization will assure the interaction between the biorecognition material and the transducer; hence, the quality of the biosensor (51). *Figure 1* shows the most used methods for immobilization are physical adsorption at a solid plane, cross-linkage between molecules, covalent attachment to a surface, affinity-based linkage and the entrapment in a membrane system (3,52).

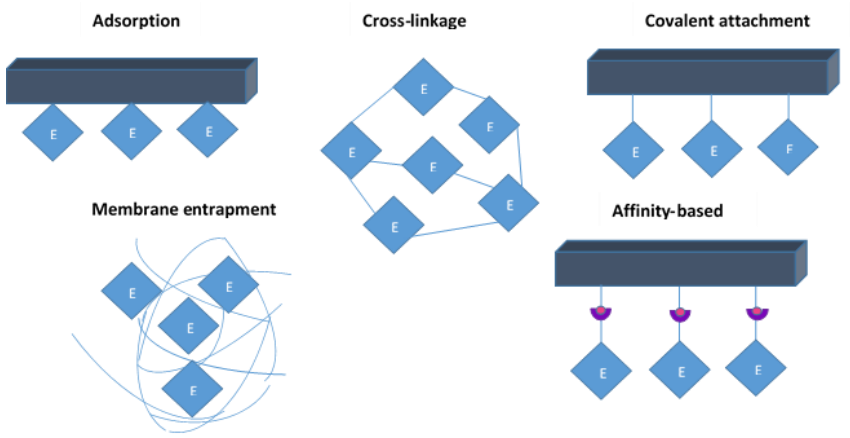

Figure 1. Schematic representation of the main methods of immobilization

Enzyme based biosensors

Enzymes are natural catalyzers of reactions occurring within an organism that are inhibited or catalyzed depending the metabolic situation. Enzymatic biosensors are

fabricated based on these two principles. The biosensor could measure the inhibition of an enzyme, in which the pesticide will work as an inhibitor, or measure the resulting compound of the catalytic reaction, in which the pesticide will be the substrate.

Inhibition-based Biosensors

Cholinesterase-Based Biosensors

Inhibition-based biosensors are the most common method used for enzyme biosensor development. They are mainly based on the use of the enzyme cholinesterase (ChE) which are acetylcholinesterase (AChE), primarily found in red blood cell membranes, and butyrylcholinesterase (BChE), primarily found in blood plasma (4,53,54). ChE enzyme have several inhibitors such as heavy metals, nicotine or fluoride although the main inhibitors relevant to biosensors are organophosphate pesticides and carbamate insecticides (37,55,56). In fact, the most developed organophosphorus detectors have been AChE biosensors. AChE is a natural ChE enzyme that have three amino acids at its active site: aspartic acid, histidine and serine (57). The quaternary ammonium group of AChE is attracted to the binding site, so the hydroxyl group of the serine deprotonate and hydrolyzes the compound (57,58). In the case of organophosphorus pesticides, the nucleophilic serine covalently binds to the phosphorus atom of the organophosphate. This binding is responsible of blocking the serine and inactivating the enzyme (59).

Equation 1 shows the AChE enzyme hydrolyzing acetyl esters and forms choline and acetic acid, while butyrylcholinesterase forms butyric acid, as shown in Equation 2.

$$Acetylcholine + H_2O \xrightarrow{AChE} Choline + Acetic\ acid \quad (1)$$

$$Butyrylcholine + H_2O \xrightarrow{BChE} Choline + Butyric\ acid \quad (2)$$

The acid formation produces a variation in the pH that can be measured using electrochemical methods, pH sensitive fluorescence indicators or pH sensitive spectrophotometric indicators (60–62). Other approach is to use artificial substitutes for AChE and BChE are acetylthiocholine and butyrylthiocholine, respectively, which produces electroactive thiocholine rather than natural choline that is not electrochemically active (1). In general, thiocholine oxidation occur when a voltage is applied and could be measured. If there is an inhibitor, the conversion of the acetylthiocholine is reduced, thus

the electrochemical reaction also decreases (63). Moreover, the anodic oxidation current will decrease in presence of pesticides in samples.

There have been reports of cholinesterase enzyme isolation from several organisms since 1950s, and usage in biosensors since 1980s (3,64). Since then, there have been constant improvements, applying diverse detection methods, immobilization techniques or biosensor configuration. An important drawback of ChE biosensor is that the enzyme is affected by other compounds that are not pesticides, such as heavy metals, nicotine or fluoride. Therefore, biosensors will not have high selectivity. Among the alternatives, to compensate the lack of selectivity, are the development of genetically modified AChE enzyme that outperform the natural enzyme in terms of enzymatic activity, stability and sensitivity of organophosphorus compounds (3). Different kind of transducers have been also tested, mostly electrode-based transducer, as amperometric or potentiometric transducers (41). Amperometric transducers measures and analyzes the byproducts of the oxidation of choline produced when the enzyme hydrolyses the acetylcholine (47,48). Whereas, potentiometric transducer measures the variation of the pH to determine the concentration of pesticide in the sample (46,65). Optical transducers, even if less used, remain as an alternative for construction of AChE based biosensors. Another approach for biosensor development is combining cholinesterase with choline oxidase (ChOx) in a bi-enzymatic biosensor (66). Acetylcholinesterase will convert acetylcholine into choline. The role of the ChOx is to produce H_2O_2 in the presence of choline, which can be oxidized and measured. When AChE is inhibited, ChOx will be inhibited as a consequence of the absence of choline production. Meng et al. developed a bi-enzymatic optical biosensor using both AChE and ChOx as biological receptor with CdTe quantum dots as fluorescent probes for optical transduction. The authors take advantage of the fluorescence quenching of the quantum dots in the presence of H_2O_2 to develop a biosensor that will diminish the quenching rate when there are increasing amounts of organophosphates. In the experiment, they used paraoxon, dichlorvos and parathion, reporting the limit of detection of dichlorvos at 4.49 nM.

Peroxidase-Based Biosensors

Peroxidase is another enzyme opted for the construction of enzyme-based biosensors. Peroxidases are present in various biological processes and their main function is the catalysis of the oxidation of both organic and inorganic substrates, using the oxidative property of the peroxides (67). Due to the fact that peroxidases are a large

group of enzymes, there have been various approaches for the biosensor development. For the application in the biosensor, the substrate used to obtain the base signal are quinones. Once the base signal is determined, the inhibitor effect of the pesticide will decrease the current response indirectly proportional to the pesticide concentration.

Moccelini et al. described a peroxidase-based biosensor that detects thiodicarb, which is a carbamate pesticide (68). The researchers extracted the peroxidase enzyme from alfalfa sprout and used hydroquinone to obtain the base signal. The enzyme was immobilized on a gold electrode in self assembled monolayers. It was reported that the biosensor limit of detection was 57.5 µM and the results obtained were similar at a 95% confident level to the results of the high-performance liquid chromatography procedure. Another method implemented for a peroxidase-based biosensor was presented in the work of Oliveira, where they extracted the peroxidase from the atemoya and immobilized it on a modified nanoclay and mineral oil (69). As in the previous example, hydroquinone was used as the substrate to obtain the base signal. Yet, this biosensor was developed for the determination of glyphosate, rather than the carbamate. The sensor was tested with spiked water samples and reported a detection limit of 0.17 nM in glyphosate. As a final note, in order to develop a peroxidase-based biosensor, it has to be considered that peroxidase is not only inhibited by carbamate and glyphosate, but also by sulfides or heavy metals.

Tyrosinase-Based Biosensors

Tyrosinase enzyme is an oxidase responsible for the control of melanin production. It is a copper-containing enzyme found in many bacteria species and in plant and animal tissues as well. As shown in equation 3 and 4, tyrosinase is involved in two consecutive melanin synthesis reactions. First, it catalyzes the hydroxylation of a monophenol to a o-diphenol.

$$Monophenol + O_2 \xrightarrow{Cresolate\ activity} Catechol \quad (3)$$

Then, the o-diphenol is oxidized to its corresponding o-quinone. The resulting o-quinone will eventually form melanin, in animal tissue, or enzymatic browning in fruits.

$$Catechol + O_2 \xleftrightarrow{Catecholase\ activity} O\ quinone \quad (4)$$

Tyrosinases have two different binding sites, a substrate site with affinity for aromatic compounds, and an oxygen site with affinity for metal binding agents. The enzyme main substrates are phenols such as dopamine and tyrosine and it is inhibited by

different environmental pollutants, for instance, atrazine, hydrazine, carbamate pesticides and cyanide (70–73).

Kim et al. proposed an electrochemical biosensor to detect 2,4-dichlorophenoxyaceticacid (2,4-D), which is a systemic herbicide (73). The investigation focused on developing a biosensor that do not require a substrate to activate nor maintain the activity of the enzyme after immobilization. Rather than using a substrate continuously, they used reduced pyrroloquinoline quinone (PQQ) covalently bound to modified gold nanoparticles, as shown in *figure 2*. The results showed an enhancement in the sensitivity, a maintenance of the tyrosinase activity and a limit of detection of 2.98 $x10^{-6}$ nM of 2,4 D.

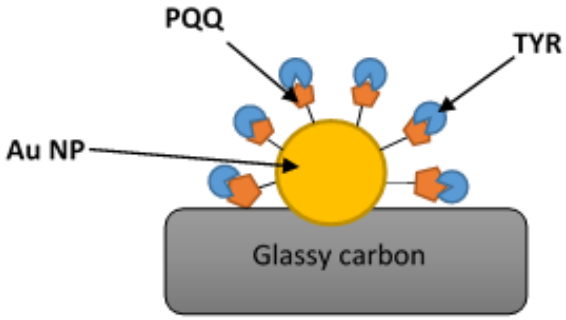

Figure 2. Schematic diagram of the tyrosinase-based biosensor.

Another tyrosinase-based biosensor was constructed by Tanimoto & Ferreira to determine the concentration of carbamates and organophosphorus pesticides in river water samples (70). The tyrosinase was obtained from mushrooms and it was immobilized to a composite electrode by cross-linking with glutaraldehyde and bovine serum albumin. Catechol was used as the substrate and the electrochemical reduction of the formed o-quinone was the measurement to determine the pesticides inhibitory effects. This research measured the inhibition effects of methyl parathion, diazinon, carbaryl and carbofuran and the results determined that methyl parathion and carbofuran act as competitive inhibitor, whilst diazinon and carbaryl act as mixed inhibitors. The main issue with tyrosinase biosensors is their poor specificity due to the interference of the various substrates and inhibitors. Furthermore, the enzyme tends to be unstable, so it reduces tyrosinase-based biosensors lifetime. Nevertheless, tyrosinase is able to endure high temperatures and the solvents used to dissolve the pesticides.

Alkaline Phosphatase-Based Biosensor

Alkaline phosphatase (ALP), also known as basic phosphatase, is responsible of the dephosphorylation of organic and inorganic compounds and performs optimally at alkaline pH levels. In equation 5, there is the phosphate and alcohol product of the dephosphorylation of a monoester.

$$Phosphate\ monoester + H_2O \rightarrow alcohol + phosphate \quad (5)$$

ALP has different inhibitors such as organophosphorus pesticides, inorganic salts, or heavy metals. Consequently, the enzyme has been used in the development of biosensors for toxicity screening. ALP is present in both prokaryote and eukaryote organisms and has numerous substrates, which gives the enzyme versatility for its use in biosensor construction.

In 2018, it was published the development of an ALP-based biosensor for the detection of chlorpyrifos (74). The enzyme was extracted from algae and the biosensor functioned using the inhibition of Ap-algae in presence of the insecticide. Normally, AP-algae dephosphorylate the phosphate of 2-phospho-L-ascorbic acid, which is the substrate. The dephosphorylation will release L-ascorbic acid that will be examined with voltammetric methods. In the presence of chlorpyrifos, the enzyme activity will be inhibited, thus the base current will decrease. The biosensor was constructed with ZnO nanoparticles on a glassy carbon electrode. The role of the nanoparticles was to increase the conductivity between the immobilized enzyme and the electrode. Pabbi et al. reported selective chlorpyrifos detection at concentrations up to 10 nM in samples with acephate, malathion, triazophos and some alkali metals, with null interference. This represent a great biosensor in terms of selectivity and reliability.

Another approach for the biosensor construction was made by García et al. (75). They developed a fluorometric alkaline phosphatase-based biosensor for the detection of heavy metals (Ag^+ and CN^-) and organochlorine (tetradifon), carbamate (metham-sodium) and organophosphorus (fenitrothion) pesticides. The enzyme was immobilized through microencapsulation in sol-gel matrices of tetramethyl orthosilicate. The reaction catalyzed by the ALP enzyme was the hydrolysis of 1-naphthyl phosphate into fluorescent 1-napthol, which was inhibited by the pollutants. It was reported that the detection limits of the biosensor were 4.1 µM for tetradifon and 91.2 µM for metham-sodium.

Acid Phosphatase-Based Biosensor

Acid phosphatase (AP) have been also used in biosensors for the detection of pesticides and heavy metals. For instance, the work of Mazzei et al. described a bi-enzymatic biosensor that coupled AP with glucose oxidase (GOx) for the toxic screening of malathion, methyl parathion and paraoxon (76). The biosensor was developed by physicochemical immobilization of AP with GOx over an amperometric H_2O_2 electrode and the substrate used was glucose-6-phosphate (G6P). AP phosphorylates G6P to produce inorganic phosphate and glucose, as shown in equation 6.

$$Glucose - 6 - phosphate + H_2O \xrightarrow{AP} inorganic\ phosphate + glucose \quad (6)$$

Then, GOx oxidizes glucose into gluconolactone, which will be measured to determine the base signal by amperometric methods (7). The detection limit varied depending the type of pesticide, malathion and paraoxon reported detection limits of 1.5 μgL^{-1}; while the methyl parathion reported a limit of detection of 0.5 μgL^{-1}.

$$Glucose + O_2 \xrightarrow{GOx} Gluconolactone + H_2O \quad (7)$$

Other biosensors based on the inhibition of AP enzyme were developed in different studies for the determination of heavy metals levels (77). In their research, acid phosphatase was immobilized with bovine serum albumin and glutaraldehyde to a screen-printed carbon electrode by cross-linking. They used phospho-L-ascorbic acid as the substrate and measured the response using amperometric methods. This response decreased in the presence of As(V), which was the inhibitor. The experiment was performed using ground water samples and it reported detection limits of the As(V) up to 0.11 µM.

Tagad et al. also developed a biosensor for heavy metal detection (78). In their study, the describe a portable optical acid phosphatase-based biosensor for Hg^{2+} detection. AP enzyme was extracted from *Macrotyloma uniflorum* and was immobilized by covalent linkage in glutaraldehyde gelatin. The substrate used was p-nitrophenyl phosphate, which produce p-nitrophenol as a result of the enzymatic reaction. The light intensity transmitted from the reaction was measured in volts and changed in presence of Hg^{2+}. The response of the sensor was determined between 0.01-10 mM, with a detection limit of 0.01 mM.

Laccase-Based Biosensor

Laccases belong to the group of blue copper-containing oxidases and their main function is to catalyze the substrate oxidation and reduce molecular oxygen to water. Laccases are found in plants, fungi, insects and bacteria (79). Oliveira et al. worked in two laccase-based biosensors and in both papers, they extracted laccase from the fungi *Trametes versicolor* (80,81). Laccase was directly immobilized on graphene doped carbon electrode with Prussian blue film electrodeposited on it. The role of the Prussian blue was to reduce the resistance and the capacitance of the enzyme biosensor. The substrate used was 4-aminophenol and it was monitored by cyclic voltammetry and square-wave voltammetry. The laccase-based biosensor detected carbamate pesticides with a detection limit range from $5.2x10^{-3}$ µM to 0.1 µM. Another laccase-based biosensor, developed by the same research group, had the enzyme immobilized in a multi-walled carbon nanotubes (MWCNT) electrode. They used the same substrate as before, 4-aminophenol, and also detected the inhibition produced by the primicarb with square-voltammetry. This biosensor detected the primicarb in a range from 0.99 to 11.5 µM.

Ribeiro et al. developed a laccase-based biosensor for the detection of the insecticide formetanate hydrochloride (FMT) (82). Laccase immobilization was done by cross-linking with glutaraldehyde on a modified gold electrode. The FMT inhibition of the laccase activity happened in the presence of phenolic composites and was determined by square-wave voltammetry. The biosensor was tested in mango and grapes samples with a limit of detection of 95 nM. Another pesticide that have been detected by laccase-based biosensor is catechol. In 2020, Zhang et al. reported a laccase biosensor fabricated using MoS_2 nanosheets and gold nanoparticles (83). MoS_2 have been proved to be biocompatible, while also have abundant position for the laccase immobilization due to its large specific surface area. Gold nanoparticles role was to enhance both the conductivity of MoS_2 and the detection sensitivity of the biosensor. The detection limit of the biosensor was reported to be 2 µM of catechol.

Urease-Based Biosensor

Urease function is to catalyze the hydrolysis reaction of urea to carbon dioxide and ammonia, as equation 8 displays.

$$Urea + H_2O \xrightarrow{Urease} CO_2 + ammonia \quad (8)$$

Subsequently, the carbamate produced is degraded by hydrolysis to carbonic acid and another molecule of ammonia. Urease is found in bacteria, fungi and plants. It is inhibited by pesticides and heavy metals that have been used to develop urease-based biosensors. In 2013, Braham et al. described the construction of a potentiometric biosensor using urease inhibition as the working principle (84). The enzyme was immobilized by cross-linking with glutaraldehyde, bovine serum albumin and Fe_3O_4 coated nanoparticles to an insulator-semiconductor electrode. The substrate used was urea and the pesticide detected was atrazine molecules. The limit of detection of atrazine was about 0.13 µM. In other study, another research group. proposed a potentiometric biosensor for the detection of glyphosate. Unlike the previous model, the enzyme was immobilized on gold nanoparticles and an agarose-guar gum membrane (85). The gold nanoparticles enhance the enzyme activity and the conductivity of the biosensor. The amount of ammonium produced by the catalyzed reaction of the urease enzyme was measured by direct potentiometry, and was reduced in the presence of glyphosate. The detection limit of the glyphosate was 5 µM.

Aldehyde Dehydrogenase-Based Biosensor

Aldehyde dehydrogenase (AlDH) is responsible to catalyze the oxidation of aldehydes to carboxylic acids with two cofactors: nicotinamide adenine dinucleotide (NAD^+) or nicotinamide adenine dinucleotide phosphate ($NADP^+$), as seen in equation 9. The active site of the enzyme binds to an aldehyde substrate and to one of the cofactors, either NAD^+ or $NADP^+$ to form NADH along with a proton.

$$Aldehyde + NAD^+ \xrightarrow{AlDH} Acid + NADH + H^+ \quad (9)$$

Noguer & Marty developed various biosensors for the detection of dithiocarbamate fungicides using the working principle of the AlDH inhibition. AlDH substrate was propionaldehyde, which produced NADH due to the oxidation cause by the enzyme. The pesticide inhibits the current formed by the product of the oxidation reaction and was measured by potentiometry (86–88). They developed several bi-enzymatic electrochemical biosensors that had aldehyde dehydrogenase and diaphorase immobilized to a screen printed electrode using photocrosslinkable poly(vinyl alcohol) bearing styryl pyridinium. Since the beginning, the biosensors reported better sensitivity results compared with conventional methods such as spectrophotometry or chromatography with a detection of 16 nM of dithiocarbamate over the 4.33 µM detection of conventional

methods. The limits of the biosensor were further studied with more dithiocarbamate pesticides such as zineb, maneb, metam sodium and nabam, with results that concluded a higher sensitivity in aldehyde dehydrogenase biosensors.

Glutathione S-Transferase-Based Biosensor

Glutathione S-Transferase (GST), also known as ligandin, is a cytosolic enzyme responsible for the catalysis of glutathione to xenobiotic substrates. It has been reported that among various other inhibitors, GST is specifically inhibited by captan, which is a fungicide (89). Consequently, Choi et al. developed a GST-based optical biosensor to detect captan in water. The enzyme was immobilized in gel film using gel entrapment technique. The biosensor had two substrates, 1-chloro-2,4-dinitrobenzen (CDNB) and glutathione (GSH), which produced s-(2,4-dinitrobenzen) glutathione that emitted a yellow light. In the presence of the captan, there was a decrease of the yellow product that was detected through the absorbance of the product. The study reported that the biosensor was able to detect captan at a concentration of 6.65 µM. Another reported GST inhibitor is molinate, which is a thiocarbamate herbicide (90). Oliveira et al. constructed an electrochemical biosensor to measure molinate concentration in environmental water. GST was immobilized by aminosilane-glutaraldehyde covalent attachment to carbon electrode. The substrate used to determine the standard enzymatic activity were GSH and CDNB in an ethanolic solution. The inhibition produced by molinate in the water was measured by differential pulse voltammetry and the biosensor exhibited a detection limit of 0.35 µM.

Glutathione S-transferase I is an isoenzyme of GST with the characteristic of catalyzing triazine (91). Andreou and Clonis developed a fiber-optic GST-I based biosensor for the detection of atrazine, a triazine herbicide. GST-I was cloned and expressed in *E. choli* model and immobilized on a hydrophilic outer membrane of polyvinylidene fluoride. The middle membrane had a sol-gel with bromocresol green (BCG), which is a dye used as a pH indicator. Since the biosensors used atrazine as a substrate and not to inhibit the enzymatic activity, this a catalytic-based biosensor. The biosensor limit of detection was 0.84 µM for atrazine without any interference reported from other pesticides presented in the water samples.

Catalytic-Based Biosensor

Organophosphorus Hydrolase-Based Biosensor

A more direct method for enzyme-based biosensor detection is the development of biosensor based on the activity of the enzyme catalyzing the pesticide. Organophosphorus hydrolase (OPH), also known as phosphotriesterase, is a bacterial enzyme that degrades organophosphorus pesticides. Rather than being inhibited by the pesticide, the pesticide acts as a substrate for OPH. OPH-based biosensors have several advantages over inhibition-based biosensors. It is a rapid, direct method of biosensing, highly selective in comparison, since it mainly catalyzes OP pesticides, and it is suitable for continuous monitoring (3,92). Organophosphorus hydrolase is able to catalyze the hydrolysis of various substrate containing P—O, P—CN, P—F, and P—S bonds, producing an alcohol with two protons in the process (93). Since the alcohol produced is frequently chromophoric and electroactive, the enzyme tends to be connected with an optical transducer to measure the amount of chromophore produced, or with a pH indicator to determine the protons generated in the catalysis (92). Due to the OPH enzymatic activity hydrolyzing organophosphorus pesticides, the enzyme has been studied for its use in bioremediation.

OPH enzyme has nonspecific substrate binding site. Hence, organophosphorus hydrolase enzyme has a vast substrate specificity. OPH catalytic properties will be affected by the environmental factors such as temperature, concentration of the substrate and pH. For instance, a rise in temperature will continuously increase the kinetic energy of the molecules involved in the reaction (49). Therefore, the enzymatic activity will keep increasing until it gets to a threshold. Once the threshold is surpassed, the temperature breaks the hydrophobic bonds that keep the secondary structure of the active site. This will denature the enzyme and result in a loss of the catalytic activity. The optimal conditions of OPH enzyme is 35 ºC (94). At this point the enzyme displays it maximum activity.

Lee et al. developed an OPH-based amperometric biosensor to determine the levels of organophosphate nerve agents (95). The biosensor was developed on a mesoporous carbon (MC) and carbon black (CB) platform, which reported better sensitivity to phenolic compounds released from the OPH reaction. Additionally, the MC/CB layer exhibited higher amperometric response in comparison to carbon nanotube electrodes. At the most optimal conditions, the biosensor had a limit of detection of 0.12

µM for paraoxon. Another approach for the detection of paraoxon using OPH enzyme was taken by Khaksarinejad et al. In their work, they constructed an optical biosensor that was developed over a nanomagnet-silica core shell where the enzyme was immobilized. They used coumarin 1, which not only is a competitive inhibitor of the OPH, but also is fluorescence emitter (96). As shown in *figure 3*, Coumarin 1 was collocated at the enzyme's active site and emitted intense radiation when excited. As coumarin 1 is a competitive inhibitor, in the presence of paraoxon the emitted radiation significantly reduced. The concentration of the organophosphate in the sample was proportional to the reduction of the fluorescence. The detection limit of the biosensor was $5x10^{-6}$ µM for paraoxon.

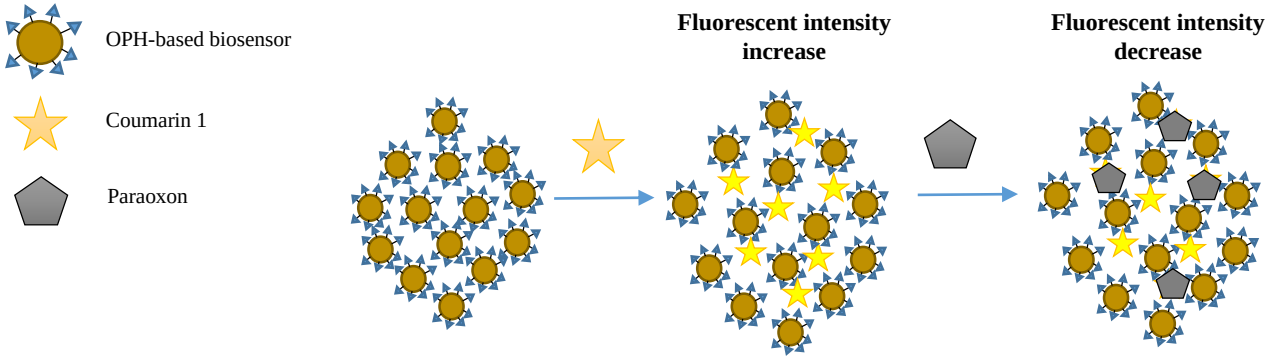

Figure 3. Schematic presentation of the nanobiosensor mechanism for the detection of paraoxon.

Methyl parathion hydrolase-based biosensors

Methyl parathion hydrolase (MPH) is an aryldialkyl phosphatase part of the organophosphorus hydrolase family (49). It catalyzes the specific oxidation of methyl parathion, which is an organophosphate insecticide used in agriculture. Due to MPH high selectivity toward methyl parathion, Chen et al. fabricated an electrochemical MPH based nanocomposite biosensor (97). The enzyme was immobilized over a nanocomposite film of gold nanoparticles on silica with MWCNT. The nanocomposites allowed a high specific surface area in the sensing film and a higher conductivity as well. The response was measured using square wave voltammetry and the detection limit reported was $1.13x10^{-3}$ nM for methyl parathion. Another MPH-based biosensor was designed using AuNP electrodes (98). The electrode was developed by mixing AuNP with aryldiazonium salt to enhance the electron transfer efficiency between the enzyme and the electrode. The electrode was also modified with PEG molecules to avoid non-specific adsorption and with 4-carboxyphenyl to maximize the AuNP role as electronic bridge. The enzyme was

covalently immobilized to the electrode and the methyl parathion detection was measured when 4-nitrophenol was produced as the product of the hydrolysis of the methyl parathion using amperometry. The detection limit for the pesticide reported was 0.27 nM, which is a very low value. Nevertheless, the main advantage of using MPH in a biosensor is the high selectivity and specificity in the detection of methyl parathion in a sample.

Discussion

There are several enzymes used for biosensor development. Since the pesticide components are toxic and biohazardous, their interaction with an enzyme is frequently disruptive and inhibitory, with some few exceptions. Most biosensors use this inhibition mechanism as the working principle to the development of the device. Basically, the process to determine the pesticide level is to get a main signal with an enzyme-substrate complex, then measure the reduction of that signal due to the presence of the inhibitor, and match it with the concentration of the pesticide. The main signal is obtained from the enzyme catalyzing the substrate reaction at the most optimal conditions. Temperature, pH, and level of substrate are factors that affect the enzyme performance. Nevertheless, in terms of biosensor development, the immobilization technique used, the type of transducer and the electrode configuration are decisive to determine the efficiency of a biosensor.

Paraoxon, for instance, have three reported optical biosensors using the same OPH enzyme as the biosensing agent with three very different detection limits, as shown in Table 1. Simonian et al. described a biosensor developed using OPH isolated from an *E. coli* strain and immobilize it by covalently linkage to nanogold in configuration of OPH/mono-sulfo-NHS-nanogold conjugate. Then, the conjugate was incubated with a fluorescent inhibitor used as a decoy and measured with UV-vis spectrum. The limit of detection was $20x10^{-6}$ M, which is not the best result for an OPH-based biosensor. Yet, this type of detection has some interesting characteristics. Since the optical signal results from the competitiveness between the OP compound and the decoy, the detection will not be affected by the OPH ability to hydrolyze the pesticide. Instead, it will depend on the affinity between the decoy and the enzyme active site. In order to have a better performance of the biosensor, the decoy should have high affinity to the OPH. A high affinity will increase the linear range because more substrate will be needed to displace it from the active site. On the other hand, if the decoy has low affinity, it could be easily

displaced by a non-desired molecule diminishing the biosensor selectivity. Overall, it was a solid biosensor that could be improved with decoy modifications.

Enzyme	Target	Transducer	Biosensor configuration	Linear range [M]	LOD [M]	Reference
OPH	Paraoxon	Optical	OPH/mono-sulfo-NHS–nanogold	$2.0x10^{-5}$ - $2.4x10^{-4}$	$2x10^{-5}$	(99)
ALP	Paraoxon	Optical	B-PUHT	N/G	$4.4x10^{-6}$	(100)
AChE	Parathion	Optical	PCCA	N/G	$4.3x10^{-15}$	(9)
AChE/ChOx	Parathion Dichlorvos Parathion	Optical	PAH/CdTe PAH/PSS PAH/ChOx/AChE	10^{-12}-10^{-6}	$2.8x10^{-12}$ $2.1x10^{-12}$ $4.8x10^{-12}$	(101)
GST-I	Atrazine	Optical	Glass/sol-gel indicator/Durapore membrane	$2.5x10^{-6}$-$1.3x10^{-4}$	$0.8x10^{-6}$	(91)
GST	Captan	Optical	GST/gel film	0-$6.7x10^{-6}$	N/G	(89)
OPH	Paraoxon	Optical	Fe_3O_4-SiO_2/APTES/GTA/OPH	$5.0x10^{-12}$-$2.5x10^{-7}$	$5.0x10^{-12}$	(96)
ChE	Carbaryl Dichlorvos	Optical	glass/sol-gel-indicator/Durapore	$5.5x10^{-7}$-$4.0x10^{-5}$ $2.3x10^{-8}$-$1.4x10^{-7}$	$5.4x10^{-7}$ $2.4x10^{-8}$	(61)
AChE	Dichlorvos	Optical	TEOS/sol-gel/AChE	$2.3x10^{-6}$-$3.2x10^{-5}$	$2.3x10^{-6}$	(62)
AChE/CHOx	Dichlorvos	Optical	CdTe QDs	$4.5x\ 10^{-9}$-$6.8x10^{-6}$	$4.5x10^{-9}$	(66)
ALP	Tetradifon Metham-sodium	Optical	1-naphthyl-phosphate/ALP	$3.5\ x10^{-6}$-$2.8x10^{-5}$ $1.9\ x10^{-4}$-$7.7x10^{-4}$	$4.9x10^{-6}$ $2.9x10^{-4}$	(75)
AP	Hg^{2+}	Optical	AP/PDMS	$0.01x10^{-3}$-$10x10^{-3}$	$0.01x10^{-3}$	(78)
AlDH	Zineb Paraoxon	Optical	AlDH/PVA-SbQ	N/G	$3.3x10^{-8}$ $1.8x10^{-9}$	(87)
OPH	Paraoxon	Optical	OPH-(7-isothiocyanato-4-methylcoumarin)	10^{-9}-10^{-5}	10^{-9}	(93)

Table 1. Optical enzyme-based biosensors. N/G: Not given.

Orbulescu et al. biosensor had the enzyme immobilized with a different configuration. The configuration was a covalent immobilization of OPH-coumarin to silanized quartz slides. Coumarin is an UV-excitable fluorophore that emits in a similar range of the p-nitrophenol absorbance, ~430 nm and ~400nm respectively, which is able to quench the coumarin emission. In a sample with paraoxon, the OPH will hydrolyze and form p-nitrophenol. Since OPH was labeled with coumarin, paraoxon hydrolysis results in less emission monitored by UV-vis and fluorescence spectroscopy. The results showed a detection limit of $5x10^{-9}$ M using fluorescence spectroscopy, which is much lower than the Simonian biosensor. This biosensor had a more direct approach to detect paraoxon concentration. Rather than relying in the affinity of the fluorophore with the enzyme, Orbulescu et al. depended on the amount of p-nitrophenol produced after the hydrolysis and measured the quenching of the fluorophore. The advantage of this method is that the fluorophore can be selected only by its spectroscopic properties, while Simonian method needs a fluorophore that not only have great spectroscopic properties, but also high affinity to OPH.

The third biosensor was constructed by Khaksarinejad team. The biosensor had a similar working principle as Orbulescu., but a different configuration. Khaksarinejad et al. immobilized the enzyme on a nanomagnet-silica core shell that intensified the emission of the Coumarin. The F_3O_4 NPs also improved the OPH enzymatic activity and, along the intensification of the emission, enhanced significantly the functionality of the biosensor. The limit of detection was in order of 10^{-12} M paraoxon, which is incredibly low. This biosensor has great sensitivity, but if the intensity of the fluorochrome increase, a lower detection limit could be achieved. In comparison, Khaksarinejad biosensor showed the highest sensitivity and the lowest detection limit of the OPH-based optical biosensors.

In Ecuador, the main exported non-petroleum product is banana, which is from the musaceae family and affected by various diseases. Arguably, the most lethal disease in banana plantations is black sigatoka. Black sigatoka is a fungus that attack the foliar tissue disrupting photosynthesis and the plant growth. Chemical treatment for this disease is the use of chlorothalonil with glyphosate, which is a herbicide, over the death leaves of the affected plant to stop sporulation to other plants. As shown in Table 2, Glyphosate is an inhibitor of some enzymes that have been used in biosensor construction. G.C Oliveira et al. developed a simple peroxidase-based biosensor suitable for the detection of glyphosate in Ecuador. The enzyme was immobilized on a carbon paste electrode and hand mixed with MWCNT and graphite powder for 15 minutes. The mixture was then packed in a plastic syringe with a copper wire connected to establish contact. The biosensor was inhibition based, so it determined a base signal with a substrate and then measured the decrease of the current response in the presence of the glyphosate. It was reported a limit of detection of $1.8x10^{-7}$ M, in a range of $5.9x10^{-7}$ to $2.7x10^{-5}$ M using square wave voltammetry. The study also report that the biosensor maintains its electrochemical properties for a period of eight weeks. This biosensor has a simple construction, low-cost, shows stability for a period of 8 weeks, while also have a low detection limit and monitor glyphosate in a water sample without significant interference. It is a portable biosensor with high sensitivity that could be easily used in the biggest industry of Ecuador, suitable for medium and big producers to assure non-hazardous condition for the inhabitants and themselves.

Enzyme	Target	Transducer	Biosensor configuration	Linear range [M]	LOD [M]	Reference
MPH	Methyl parathion	Amperometric	AuNP/GC	$7.6x10^{-10}$-$3.8x10^{-7}$	$2.7x10^{-10}$	(98)
MPH	Methyl parathion	Voltammetric	MPH/SP@AuNPs MWCNTs/GCE	$3.8x10^{-9}$-$1.9x10^{-5}$	$1.1x10^{-9}$	(97)
GST	Molinate	Voltammetric	GCE/APTES/GA/GST	$1.0x10^{-6}$-$4.2x10^{-5}$	$3.42x10^{-7}$	(90)
GST	Captan	Voltammetric	SAM modified gold/GST	$8.3x10^{-7}$-$5.3x10^{-5}$	$8.3x10^{-7}$	(102)
OPH	Paraoxon	Amperometric	MC/CB/GC/CNT/OPH	$2.0x10^{-6}$-$8.0x10^{-6}$	$0.1x10^{-6}$	(95)
AChE B	Pirimiphos methyl Omethoate Paraoxon	Voltammetric	AChE B screen-printed electrode	N/G	$3.5x10^{-12}$ $1.2x10^{-7}$ $1.8\ x10^{-9}$	(37)
AChE	Chlorpyrifos-ethyl-oxon	Amperometric	Ag/AgCL/PVA-SbQ/AChE	N/G	$3.0x10^{-9}$	(56)
AChE	Diazinon Carbofuran	Voltammetric	Au/MBT/POMA-PSSA/AChE	N/G	$2.3x10^{-10}$ $2.7x10^{-10}$	(58)
AChE	Organophosphates	Potentiometric	PVC/plasma-polymerized ethylenediamine	10^{-6}-10^{-1}	$2.0x10^{-6}$	(60)
Peroxidase	Glyphosate	Voltammetric	Graphite MWCNT/peroxidase	$5.9x10^{-7}$-$2.7x10^{-5}$	$1.8x10^{-7}$	(69)
Peroxidase	Thiodicarb	Voltammetric	Au/peroxidase/SAMs	$2.3x10^{-6}$-$4.4x10^{-5}$	$5.8x10^{-7}$	(68)
Tyrosinase	Methyl parathion Diazinon Carbofuran Carbaryl	Amperometric	SP CoPc-CGCE	$2.3x10^{-8}$-$3.8x10^{-7}$ $6.2x10^{-8}$-$1.6x10^{-7}$ $2.3x10^{-8}$-$4.1x10^{-7}$ $5.0x10^{-8}$-$2.5x10^{-7}$	$2.3x10^{-8}$ $6.2x10^{-8}$ $2.3x10^{-8}$ $5.0x10^{-8}$	(70)
Tyrosinase	Dichlorvos	Voltammetric	GC-NQS-Tyr-PPy	$6.0x10^{-8}$-$8.0x10^{-6}$	$6.0x10^{-8}$	(71)
Tyrosinase	2,4-D	Amperometric	PQQ-Tyr/AuNP-GC	$3x10^{-15}$-$4.5x10^{-14}$	$3.0x10^{-15}$	(73)
PPO	Thiodicarb	Voltammetric	Graphite powder: Nujol: CHcych-PPO	$3.8x10^{-7}$-$2.2x10^{-6}$	$1.6x10^{-7}$	(72)
ALP	Chlorpyrifos	Voltammetric	ALP-algae/ZnO/GC	10^{-9}-10^{-1}	10^{-9}	(74)
AP/GOx	Aldicarb	Amperometric	GOx: potato tissue/AP	$2.4x10^{-7}$-$6.6x10^{-7}$	$2.1x10^{-7}$	(76)
Laccase	Ziram Carbofuran	Voltammetric	LACC/PB/GPE	$2.5x10^{-8}$-$5.7x10^{-7}$ $5.0x10^{-7}$-$5.9x10^{-6}$	$5.2x10^{-9}$ $1x10^{-7}$	(80)
Laccase	Primicarb	Voltammetric	MWCPE electrode	$9.9x10^{-7}$-$1.2x10^{-5}$	$1.8x10^{-7}$	(81)
Laccase	Catechol	Voltammetric	MoS_2-AuNPs-Lac/GCE	$2x10^{-6}$-$2.0x10^{-3}$	$2x10^{-6}$	(83)
Urease	Atrazin	Potentiometric	IS/NPs-$(PAH/PSS)_3$/PAH/urease	10^{-2}-10^{-7}	$0.1x10^{-6}$	(84)
Urease	Glyphosate	Potentiometric	Au NP/agarose-guar gum entrapped urease	$3.0x10^{-6}$-$3.0x10^{-4}$	$3.0x10^{-6}$	(85)

Table 2. Electrochemical enzyme-based biosensors. N/G: Not given.

Conclusions

Biosensors pave the way to a controlled environmental monitoring. There are reports of different immobilization techniques, enzymes used as biological elements, configuration of electrodes or transducers. The variety of enzymes allows to design biosensors for broad families of pollutants. Organophosphorus, organochlorides, carbamates, insecticides, fungicides or herbicides, any kind of pesticide can be screen using the right enzyme. Enzyme-based biosensors working principle is to measure the

signal through an immobilized enzyme connected to a transducer. Most enzymes are inhibited in the presence of pesticides. Hence, most biosensors depend on inhibition-based configurations, where the signal is measured at optimal conditions for the enzyme by electrochemical or optical methods and then the signal reduces in the presence of the inhibitor. The development of nanoparticles in enzymatic configuration enhance the biosensor characteristic, since they enable the transfer of electrons from the enzyme to transducer. This property increases sensitivity and response time.

In terms of specificity, there is still room for improvement. AChE-based biosensors or tyrosinase-based biosensors are mainly used for rapid screening because the huge number of inhibitors for each enzyme. This could be solved with new immobilization techniques or configurations. Regarding its use in environmental field, biosensors are mostly experimental and quite few, compared to fields like medicine. In Ecuador, due to its high agricultural development, pesticide screening is a needed tool to ensure a safe environment for the inhabitants and the farmers. It is both a challenge and an opportunity to develop and commercialize enzyme-based biosensors in Ecuador.

Abbreviations

IARC: International Agency for Cancer Research

DDT: Dichlorodiphenyltrichloroethane

ChE: Cholinesterase

AChE: Acetylcholinesterase

BChE: Butyrylcholinesterase

ChOx: Choline Oxidase

Tyr: Tyrosinase

2,4-D: 2,4-dichlorophenoxyaceticacid

PQQ: Pyrroloquinoline quinone

ALP: Alkaline phosphatase

AP: Acid phosphatase

GOx: Glucose oxidase

G6P: Glucose-6-phosphate

MWCNT: Multi-walled carbon nanotubes

FMT: Formetanate hydrochloride

AlDH: Aldehyde dehydrogenase

NAD^+: Nicotinamide adenine dinucleotide

$NADP^+$: Nicotinamide adenine dinucleotide phosphate

GST: Glutathione S-Transferase

CDNB: 1-chloro-2,4-dinitrobenzen

GSH: Glutathione

BCG: Bromocresol green

OPH: Organophosphorus hydrolase

MC: mesoporous carbon

CB: carbon black

MPH: Methyl parathion hydrolase

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