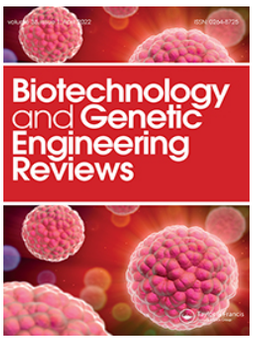

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REVIEW ARTICLE

Challenges and opportunities of using immobilized lipase as biosensor

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ABSTRACT

Over the years, the science of biosensors has evolved significantly. The first or earliest generation of biosensors only detected either the decrease or increase of product or reactant-based natural mediators as the pathway for electron transfer. The subsequent second-generation biosensors were biomolecule based and used artificial redox mediators, such as organic dyes to detect and to increase the reproducibility and sensitivity of the result. However, the recent generation of biosensors work mostly on the principle of electron mobility, with different criteria, such as selectivity, precision, sensitivity, etc., can be used to quantify, efficiently. This review deals with exploring the scope and applications of Immobilized lipase biosensors. Generally, Triglycerides or TG molecules are either detected using Gas Chromatography or, using a chemical or an enzymatic assay. Immobilization of lipase on solid supports has led to increased stability and reusability of the enzyme in non-aqueous solvents. With better enzyme performance, efficient product recovery, and separation from the reaction, immobilized lipase biosensors are garnering increasing interest worldwide. Along with so many advantages including but not limiting to ones mentioned earlier, immobilized lipase-based biosensors come with their own set of challenges, such as the partitioning of the analyte with aqueous medium, slower reaction rate, etc., they have been discussed in the following review. Alongside, we also review the development of a new generation of biosensors and bioelectronic devices based on nanotechnology.

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Biosensors; lipase; immobilization; selectivity; sensitivity; reproducibility

1. Introduction

There is a tremendous demand in analysis and detection of biological samples in the present pandemic situation in more accurate, cheaper, and faster ways. Biosensors are one of the critical components in this field. All over the world, scientists are trying to develop and improve the efficiency of the various components of the biosensor for its better performance. The

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biosensor is a device that senses a chemical element and consists of a biological component and a detector. The sensing part, or biological component, primarily consists of immobilized enzymes. Increasing triglyceride (TG) level leads to hyperlipidemia, a significant concern for many cardiac patients. Apart from coronary diseases, several liver disorders are also associated with TG. Hence, accurate TG detection in body and food sources is essential to avoid any health issues. The best detection mechanism employed immobilized lipase as the sensing element in a biosensor. Apart from TG, immobilized lipase biosensors are also used to detect components of pesticides, such as diazinon, malathion, etc.

Lipases are enzymes that belong to the lipase-esterase super family, a type of catalytic enzyme that mainly deals with the hydrolysis of lipids. The frequently used lipases are triacylglycerol acyl hydrolases (EC 3.1.1.3). Lipase is successfully immobilized in various matrices from natural sources to chemically modified nanoparticles using different immobilization techniques. Immobilized lipases catalyzed reactions such as acetylation (Jiang & Fang, 2020), hydrolysis of carboxylic esters (Jiang & Fang, 2020; Miao et al., 2019), and transesterification (De Oliveira et al., 2019). Lipases are structurally close to cholinesterase (acetylcholinesterase and butyrylcholinesterase) and are widely applicable for bio-assays, biosensor development or construction, and sensitivity towards neurotoxic compounds (Pohanka, 2015). Monteiro et al. (2021), in their review have highlighted that *Candida antarctica* lipase B (CALB) has the capability to specifically affect the sn-2 position of triglycerides, making it a candidate of special focus. G.V. Lima et al. (2017), have shown a straight-forward two-step process for synthesis of (S)-Pindolol using lipases from *Pseudomonas fluorescens* and *Candida rugosa,* respectively.

Lipase as an enzyme has been elaborately been used in various industries, such as textile, personal care, food processing and dairy, etc., and even by converting glycerol into value added industrial products (P.J.M. Lima et al., 2021). The attention towards Lipase driven biosensors has been gradually growing over the last few decades. Valério et al. (2021), in their review have noted that *Rhizopus sp*. strains, under different growing conditions, produce different isolipases and hence can play a crucial role in lipase mediated industries. From 1970 to almost 2020, the whole idea around biosensors has evolved from just being an input detector contained in an enclosure with capabilities for bio-based detection to generate an electric signal and a modulator or modifier that converts this electric signal into a tangible, recordable, presentable output. These outputs can range from being in the form of light, marking, color, or sound and alpha-numeric bits of information or peaks, etc., to a much more sophisticated and simplified version of a transducer-enabled device that physically or chemically operates to detect, observe, study, or record information based on biological samples (Aykaç

et al., 2021; Baronas et al., 2021; Naresh & Lee, 2021). Their utilization in bioassay and biosensors is highly advanced because of their application in military nerve agents.

Although various reports are available on immobilizing lipase on nanoparticles and other immobilized enzyme-nano-composites as biosensors, a comprehensive review of the present scenario using lipase as biosensor is very scarce in the literature. In this article, we started with a brief description of the evolution of current-generation biosensors. Then, we have summarized the critical industrial applications and immobilization methods associated with lipase. Finally, in detail, we review the recent developments in the field of enzyme-based biosensors, primarily focusing on immobilized lipase as biosensing elements. While reviewing the challenges and prospects of immobilized lipase as a biosensor, we observed tremendous opportunity to develop a highly sensitive, reproducible, cost-effective biosensor using immobilized lipase for commercial purposes.

2. Generations of biosensor

Over the years, biosensors have been anything but detection devices limited to scoped biological analysis. Proteins or proteic compounds have opened up newer avenues for sensors with potentials of better performance in terms of sensing utility, performance, and output metrics and improved accuracy and precision for detection while bringing down the costs significantly, especially in the case of amperometric biosensors. The first generation of biomolecules-based biosensors could only detect either the increase or the decrease of a product-based or a reactant-based natural mediator as a pathway for electron transfer. The first of these to be developed was in 1962 to measure blood oxygen content using the Clark oxygen electrode by Champ Lyons and Professor Leland Clark. The blood is mixed with a haemolysing solution, releasing the oxygen and carbon dioxide content locked in the sample. The acidified solution diffuses through a permeating layer composed of Silastic membrane along with 0.5 mg of Teflon into a solution, alkaline in nature, to measure the sample's carbon dioxide content. This allows the monitoring of carbon dioxide concentration based on the drop in values of pH. Alongside, using hydrophobic membranes such as polyethylene, the quantities of glucose in biological samples were estimated to deduce the dissolved oxygen concentrations (Clark & Lyons, 1962). Figure 1(a) illustrates the essential components of the first-generation biosensor.

The subsequent second-generation, as illustrated through Figure 1(b), biomolecule-based biosensors used artificial redox mediators (Jędrzak et al., 2018; Kuznowicz et al., 2021; Pohanka, 2021) such as organic dyes like Methyl Violet, phenazines, and methylene blue (Hirakawa & Mori, 2021),

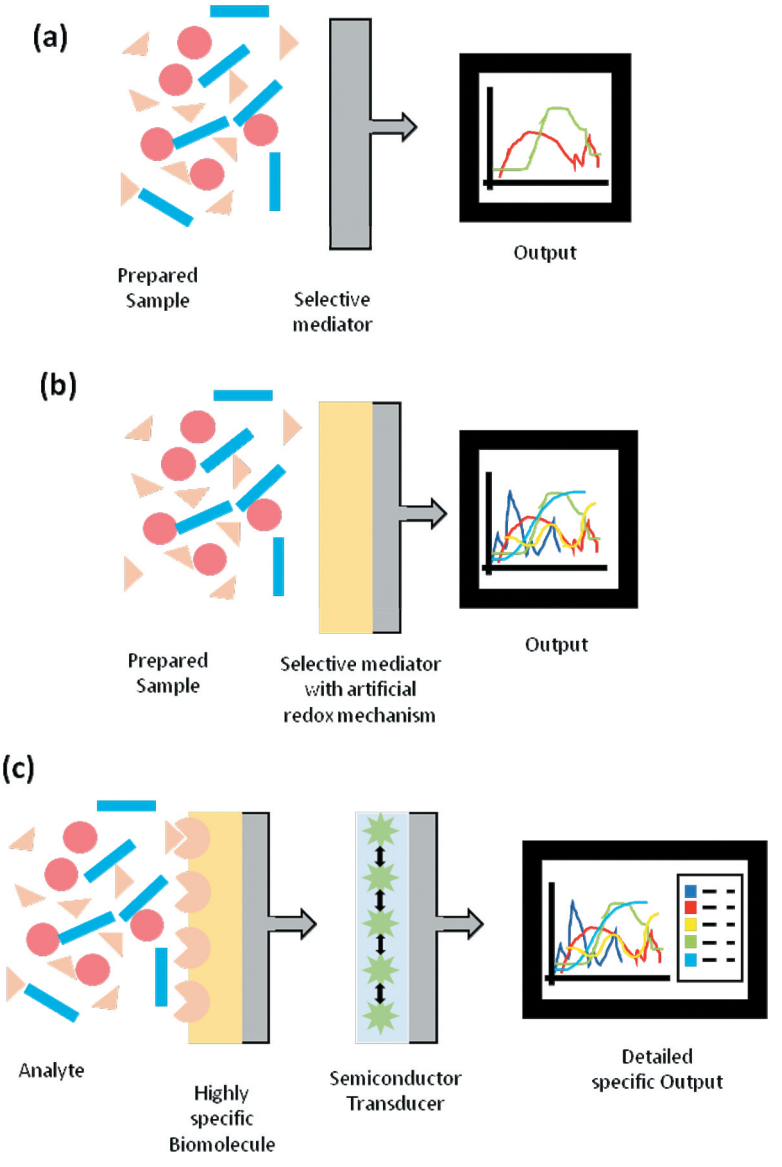

Figure 1. Generations of biosensors through the years. (a) First generation biosensor, (b) second generation biosensor, (c) third generation biosensor.

and ferrocene and its derivatives, tetrathiafulvalene (TTT), tetracyanoquinodimethane (TCNQ), quinones and ferricyanide, ferrocyanide that increase the reproducibility and sensitivity of the result (Aggas et al., 2020). All these organic charge relay/transfer complexes have been used individually or in combination for various biosensing applications (Akyilmaz et al., 2017; Şahin, 2020). Then came the quinones and their derivatives that are known for their features, such as biocompatibility, processes for functionalization and ease of synthesis (Abdulbari &

Table 1. General classification of biosensors.

Biomolecule	Transducer
Enzyme	Electrochemical
Nucleic Acid	Amperometric
Whole-Cell	Conductometric
Tissue	Potentiometric
Organelle Based	Optical
Immuno-sensor	• Surface Plasmon Resonance • Absorbance • Fluorescence • Chemiluminescence • Colorimetry • Piezoelectric • Acoustic • Ultrasonic • Thermal • Ion sensitivity

(Asal et al., 2019)

Basheer, 2017), while ferricyanide and ferrocyanide served as an excellent choice for electron acceptors of cathodic nature and are widely preferred as biosensor mediators due to reversibility and rapid oxidation of ferrocyanide, forming ferricyanide (Penteado et al., 2017).

In the recent two decades, the current generation of biosensors (Figure 1c), also known as the modern third generation of biosensors, have seen tremendous growth, primarily due to research and development, fabrication, commercialization, and demand-driven opportunities. Compared to the earlier generations of sensors and biosensors, the current generation is attracting much scientific attention and initiatives to overcome the challenges posed. However, despite all the significant growth and advantages, the previous generation of sensors and biosensors has shown many drawbacks. Therefore, a lot of work is now focussed on the development of more straightforward, easier to use, and better devices using a technical emphasis on conductive polymers, nanoparticles, nanowires, nanofibers, and nano-materials, polymeric suspensions, ionic solutions/fluids, enzymes, and enzyme-based functionalized nanostructures, PCR-based assays, and carbon nanostructures (Botewad et al., 2021; Halilović et al., 2019). Table 1 summarizes the general classification of biosensors based on detection methods that combine one or more of these nano-materials.

3. Importance of nano-material in the lipase biosensor industry

The use of nano-materials as enzyme immobilization carriers has recently attracted attention. Immobilized lipases are much sought after by industries as well as researchers. The various applications of immobilized lipases on functionalized nano-materials appear promising (Kim et al., 2015). Lipase is often used as a bio-element for providing specific analysis. Immobilized

lipases are used as biosensors to accurately and efficiently determine the quantitative presence of triacylglycerol. It is also used to analyze Dichlorvos insecticide residues in vegetables (Karousos & Reddy, 2002). The development of newer generations of bioelectronics is based on nanotechnology biosensors. These devices have higher sensitivities, show enhanced stability, and they pose 'an excellent scenario' for business in the market, according to Abu-Salah et al. (2010) as cited in Chandra et al. (2020). These properties promote the electrochemical reaction, commanding nano-barcode for biomaterials, and signal amplification of bio-recognition events to directly wiring enzymes to electrode surfaces using nanoscale materials. Carbon nanotubes (CNTs) and gold nanoparticles have been reported to be of regular use in enzyme biosensors (Chandra et al., 2020; Zhang et al., 2019). $NiZnFe_2O_4$ nanoparticles of superparamagnetic nature, coated with TEOS and BQ activated with immobilized PFL at pH 7 for 24 h have resulted in synthesis of optimal biocatalysts (Rios et al., 2019) (a). Breger et al. (2020), have demonstrated success in Förster Resonance Energy Transfer (FRET)-based Quantum Dot biosensor originating from a custom-synthesized ester substrate displaying a peptide at one end and a dye acceptor at the other. Fernandez-Lopez et al., (2015), have reported that Mn2+ and Ca2+ cations at concentrations of 5 mM, are able to greatly stabilize the lipases from *Rhizomucor miehei* (RML) and *Candida rugosa* (CRL) when they are present during the inactivation, but only if the enzymes are immobilized on octyl-agarose.

4. Lipase immobilization

4.1. Methods

There are various strategies to perform chemically covalent-oriented immobilization. For example, residues of cysteine consisting of a thiol group is suited well for oriented form of immobilization; site-specific form of immobilization is achieved with the help of sulfhydryl chemistry by disulphide bonds formation using cysteine. Despite the increased stability of the immobilized biomolecule, thiol groups accessibility, limit the process's efficiency (Liu & Yu, 2016). Molecularly Imprinted Polymers (MIP) are materials of synthetic origin that possess highly specific recognition sites of artificial origin, similar to antibodies, that bind precisely with the target analytes (Xu et al., 2020). The MIP strategy-based oriented form of immobilization depends on selection of physical shape or covalent interactions of chemical nature, or even both (Vasapollo et al., 2011). Various other strategies are at the hands of researchers, for the immobilization of biomolecules. However, the technique is chosen because the process significantly affects the biosensor's bioactivity, storage potential and shelf life, selectivity,

Table 2. Immobilization methods used for Biomolecules.

Method	Interaction	Advantages	Disadvantages
Covalent Binding	Irreversible	• Stability: High • Binding: Strong	• Cost: High • Loss of enzyme activity
Crosslinking	Irreversible	• Stability: High • Binding: Strong	• Loss of enzyme activity • Toxicity of crosslinker
Entrapment	Irreversible	• Stability: High • Enzyme activity is well preserved. • Pore size: Changeable	• Destruction of biomolecule shape • Leakage of biomolecule • Limitations in Mass transfer
Adsorption	Reversible	• Chemical compounds not needed	• Reproducibility: Low • Usage time: Short • Orientation: Random • Sensitive to ionic strength and pH
Affinity	Reversible	• Configuration: Oriented • Selectivity: High • Sensitivity: High	• Cost: High • Necessary to label

(Asal et al., 2019; Nguyen et al., 2019)

stability, accuracy and time of response (Gonçalves et al., 2019; Gupta & Rathod, 2020; Khosla et al., 2017; Liu et al., 2018; Shuai et al., 2017). Most widely used techniques for immobilization include covalent bonding, crosslinking, adsorption, entrapment, and affinity binding. In Table 2 all these immobilization methods have been summarized. Immobilization methods may or may not be combined, based on the nature of the biomolecule, to develop biosensors with high efficiency, presenting the benefits offered by different techniques (Bhardwaj, 2014). Enzyme and antibody immobilization is mostly performed by adsorbing them followed by crosslinking them (Chang et al., 2017; Plekhanova & Reshetilov, 2019).

Ma et al. (2018) performed immobilization by adsorption of lipases on the surface of metal-organic frameworks, majorly crystals of a network formed with metal ions assembly and linking it with organic molecules. The matrix formed because of the combination provides numerous advantages, including high porosity, flexible morphology, various functionality, thermal and chemical stability, and biocompatibility. In this study, Zeolitic imidazolate frameworks (ZIF) and ZIF-8 modified with ammonium hydroxide An-ZIF-8 are used to detect methyl parathion present in pesticides. De Moura Barboza et al. (2019) used a similar process of immobilization by adsorption with a different matrix that was lamellar zinc hydroxy-nitrate 'decorated with gold nanoparticles (HZnL/AuNPs)', as they quote, for detection of carbendazim (CBZ) in pesticides. A unique method was designed by Zehani et al. (2014) for the detection of Diazinon in pesticides. Lipases were immobilized using enzyme crosslinking with glutaraldehyde vapor and bovine serum albumin. De Souza et al. (2020), have highlighted maximum conversion of rac-indanyl acetate to (R)-indanol with upto 97% excess of enantiomers using covalently immobilized *Candida antarctica* lipase B (CALB) on cashew apple bagasse activated with 10% glycidol–ethylenediamine–glutaraldehyde (GEG).

Escamilla-Mejía et al. (2015) immobilized lipases by physical entrapment process using Nafion. This anionic polymer allows the passive transfer of hydrogen ions and protects the surface of electrodes from interfering particles in samples, such as proteins, carbohydrates, and anions. Hasanah et al. (2019) used pectin-hydrogel-based membrane as the immobilization matrix and for pH indicator, they chose chromoionophore ETH 5294 (CI). The first layer in the hydrogel membrane consisted of pectin, crosslinked with $CaCl_2$ and the CI. Followed by the second layer where a TG specific lipase was entrapped and immobilized. A different method was used by Bodade et al. (2017) in which lipase was immobilized by adsorption on the surface of the Chitosan-nano CuO/Au electrode and then used for detection of triglycerides. F.T. Cavalcante et al. (2021) and (2021b), have highlighted the increasing growth in terms of new materials for enzyme carriers, along with improved and newer methods for immobilization principles resulting in more robust, eco-friendlier, and cheaper biocatalysts. Covalent co-immobilization of *Candida antarctica* lipase A (CALA) and *Candida antarctica* lipase B (CALB) on chitosan activated by Glutaraldehyde stabilized by Taguchi Method, exhibited upto 6 times longer half-life than the free enzymes at pH 7 in the temperature range 50°C to 80°C (Moreira et al., 2022)

4.2. Support materials

Support materials refer to solids, insoluble matrixes where biomolecules may be attached to or immobilized upon or onto (Elnashar, 2010). Widely implemented for the conservation of the enzyme's tertiary structure during immobilization, the support material helps in making the enzymes more stable and reactive. An essential characteristic of ideal support materials is to be able to demonstrate good levels of chemical and mechanical strength to influence and alter the immobilized biomolecules' properties (Elnashar, 2010; Sirisha et al., 2016). The support to be considered optimum, is expected to exhibit inertness towards enzymes and the reaction mixture, should cost low, renitent, easily accessible, non-toxic, and most importantly, biocompatible. Generally, the support is preferred to be of a mesoporous material by origin, which can provide a wider surface area and relatively higher porosity to facilitate increased reaction spaces and hence the amount of the immobilized biomolecule while ensuring its safety and protection from external obstructions (Mohamad et al., 2015). Since functional groups are the sites where a biomolecule binds, the support materials is expected to contain easily modifiable groups (Elnashar, 2010). The integration of nanotechnology and biosensor technology has allowed LODs to be lower than ever reported earlier (Bhalla et al., 2016).

Various other hybrid support materials are produced by combination of different organic, and inorganic materials in various ratios, to integrate the properties individually unique to them. Such a synthetic polymer with greater pH dynamics, thermal, and mechanical stability, further enhanced by joining with an enhanced biocompatible biopolymer shows higher affinities towards enzymes. Additionally, controlling their hydrophilicity and the flexibility of forming into various shapes and sizes makes it easy to achieve immobilization through adsorption, or covalent binding, or entrapment methods. Moreover, their synthesis is easier, and a broad range of functional groups can be found on their surfaces, including hydroxyl (-OH) groups, carbonyl (C = O) groups, carboxyl (COOH) groups, amine ($-NH_2$) groups, and epoxy groups, which can increase the support's affinity (Zdarta et al., 2018).

Manoel et al. (2016) produced different polymeric supports as core and shell that exhibit different composition and morphologies, with divinylbenzene (DVB) and styrene (S) as co-monomers to immobilize lipase enzyme from *Themomyces lanuginosus* (TLL), *Rhizomucor miehie* (RML), the form B of lipase from *Candida antarctica* (CALB). They have shown that the so produced biocatalyst exhibits higher activity than commercially available biocatalysts with various substrates.

5. Present trends in enzyme immobilized biosensors

5.1. Modern biosensors

The recent generation of biosensors comes with redox enzyme-based biosensing mechanisms based on direct electron transfer. Redox enzymes are immobilized and then mounted on the surface of the electrode in this biosensor generation, making the direct transfer of electrons possible between the transducer and the enzyme (Arora, 2013). Different parameters such as accuracy, precision, sensitivity, selectivity, reusability, limit of detection (LOD), working range, and time factors are used to quantify their efficiency (Ghanam et al., 2021).

5.2. Critical characteristics to biomolecule-based biosensors

Ideally, a few key features and characteristics are ensured to be fulfilled for a biomolecular biosensor to be successful (Figure 2). The ability of biomolecules to precisely identify and detect the presence of a specific analyte without reacting to several others present in a given sample mix, also known as its selectivity, is an essential feature of an enzyme immobilized biosensor. Along with selectivity, an ideal enzyme immobilized biosensor is precise, referring to its ability to produce the same results for each

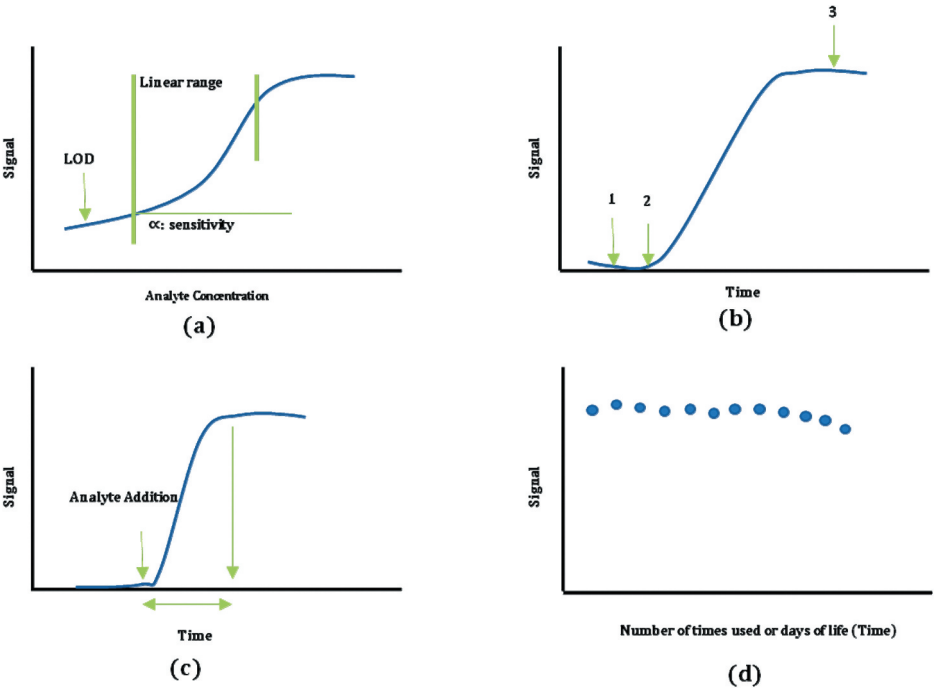

Figure 2. General characteristics of bio-molecule-based biosensors (a) LOD (limit of detection), linear range and sensitivity; (b) selectivity (selective to analyte); (c) response time; (d) reusability (number of use) and shelf life (days).

measurement on repeated usage. It should be highly sensitive (given by the calibration curve slope). That is, the analyte quantity required for every successful measurement should be as minimal as possible. The range of operation is preferred to be wide (linear range), usually defined as the range of analyte concentration required by the sensor to detect or the minimum quantity of the analyte that can present an identifiable output signal, and a lower LOD or Limit of Detection. LOD is dependent on the affinity of recognition between the analyte and the element, expressed in terms of 'K_d' or the dissociation constant. Lower K_d values signify high affinity of recognition between the analyte and the element. Another critical factor in the recent decades for the success of an enzyme immobilized biosensor is its reusability which is the direct result of a high precision usage on every use (Shruthi et al., 2014).

Response time, generation and regeneration time, and storage potential or shelf life are also crucial factors of time for biosensors. The response time can be defined as the duration (as short as possible) required for the biosensor system to reach equilibrium to obtain a reliable measurement. Post sample interaction; the sensor would again need some time to go back to its working state; this duration is termed as regeneration time, which also

is preferred to be a short interval like that of response time (Ghanam et al., 2021; Shruthi et al., 2014). The shelf life can be expressed as the time range during which the biosensor can consistently analyze, measure, and consistently achieve reliable results. To make the product a commercially and industrially viable one, it should be as long as possible.

A successful biosensor also exhibits stability in pH and temperature for all its scheduled reactions. This feature allows detection without any specified pretreatment or, at the most, a minimal treatment. The thus formed biosensor should be inexpensive, tiny, or portable, user-friendly, and reusable. If the biosensor is invasive, the probe should be as small as possible to reduce bulk tissue damage. It should also present more characteristics in biocompatibility, non – antigenic, and non – toxic in action.

And most importantly, the sensor or the invasive component should also be sterilizable. The response generated by a biosensor should be accurate, precise, and linear over its operating range. The reaction caused should preferably be noise-free, which is often a problem for tools measuring voltage. The source of noise could be the sensor, or the measuring instrument, or some external sources. The noise must be minimal to conduct correct measurements. When it is challenging to avoid electrical noise, some compensatory mechanisms must be adjusted (Ghanam et al., 2021). There are also recorded instances where one specific parameter in the biosensor is enhanced or improved, there stands a possibility of variation or decrease for another parameter, primarily due to the newly enhanced parameter (Wongkaew et al., 2018).

5.3. Enzyme interactions

Lipases are enzymes that belong to the lipase-esterase super family, a type of catalytic enzyme that mainly deals with the hydrolysis of lipids. The frequently used lipases are triacylglycerol acyl hydrolases (EC 3.1.1.3). Lipases are structurally close to cholinesterase (acetylcholinesterase and butyrylcholinesterase) and are widely applicable for bio-assays, biosensor development or construction, and sensitivity towards neurotoxic compounds (Pohanka, 2019). Lipase is classified as a serine hydrolase characterized as having a catalytic triad consisting of nucleophilic serine and an aspartic or glutamic acid bonded via a hydrogen bond histidine. In closed conformation, the access for the solvent and the substrate to the active site is blocked, and the enzyme is inactivated. When exposed to an appropriate hydrophobic solvent or solvent interface, the active site cap moves into an open conformation, and access to the active site is allowed. This eyelid movement exposes the hydrophobic fragments of some specific amino acids to the solvent (Rios et al., 2019; Rios et al., 2018).

The choice of biomolecules is made based on the analyte to be detected. Other determinants of the selection could also depend on (but not limited to) factors such as the storage stability in high temperatures and various pH states, hydrophobic and oxidizing environments (Chang et al., 2017; Plekhanova & Reshetilov, 2019). Pure enzymes are extensively chosen in modern third-generation biosensor development owing to their high specificity. These enzymes' high specificity property enables packing incredible amounts of enzymatic activity onto a small surface of the transducer surface area. Apart from high cost of purified enzyme (Bisen, 2014), due to challenges relating to solvent tolerance, pH, and temperature stability, these sensors' enzymatic action is sometimes rendered hampered (Warner & Andreescu, 2016).

5.4. Factors on which enzymes depend

In some instances, enzymes might require coenzymes or cofactors, and in their absence, the activity of the enzyme may drop or even be lost while immobilizing onto a transducer (Chhillar et al., 2019; Shruthi et al., 2014). The immobilization of whole cells for inherent enzymes is another avenue that has presented a viable alternative for pure enzymes. The lengthy, expensive, and complex procedures for enzyme purification can be avoided, and the enzyme can be retained at the best potential in its natural and original environment, safer from inactivation (Chang et al., 2017; Plekhanova & Reshetilov, 2019). Since the development of the whole cell-based biosensors by Diviès in 1975, immobilized cell biosensors have shown various possible applications (Monošík et al., 2012). For detection of arsenic ranging between 0.74 and 69 µg/l, *Escherichia coli* or *E. coli* was used (Sharma et al., 2013). Again, for detection of arsenate (<10 ug/L), *E. coli* has been employed (Hooda et al., 2021), and also for detection of tetracyclines (45 nM; Kaur et al., 2018; De Lima et al., 2018). The only significant limitations presented by these sensors are the undesired side reactions arising due to other cellular enzymes and the longer duration of response and recovery times (Shruthi et al., 2014). Other than enzymes and whole cells, the biomolecule biosensor genre also includes tissue-based biosensors, antibodies-based biosensors, and nucleic acid-based biosensors.

6. Lipase biosensors

The broad commercial availability of lipase makes it a frequently used component in biosensors. Biosensors typically contain three essential components: a bio-receptor, transducer, and an electronic system comprising a signal amplifier, processor, and display (Kaur & Kaur, 2019). The bio-

receptor is the component where the interaction with the chosen analyte takes place to produce an effect. The bioreceptor needs to have a high selectivity for the analyte among a matrix of other biomolecules. The transducer component is either a semiconducting material or a nano-material. Based on the different bio-transducers, biosensors can be optical, electrochemical, thermal, or piezoelectric. The transducer is always in proximity with the bioreceptor layer. A schematic example for a lipase biosensor is shown in Figure 3.

Several lipase-based biosensors have been studied and observed. For example, an immobilized lipase-based biosensor was developed by Ma et al. (2018), which used 30 mg/L of *Burkholderia cepacia* lipase (BCL) on 750 μM substrate of p-nitrophenyl pesticides (methyl parathion), and the nano-materials used for immobilization included Zeolitic imidazolate frameworks (ZIF) and amine-functionalized ZIF-8. It was used in direct as well as sensitive detection for methyl parathion. Another biosensor developed to quantify triglycerides in food samples is reported to be potentiometric. It is based on the use of lipase derived from *Candida rugosa* immobilized in a Nafion membrane on a graphite-epoxy transducer (Escamilla-Mejía et al., 2015).

A novel electrochemical biosensor using lipase-based assay is used to quantify target DNA. 100 μL of pancreatic lipase is used for hydrolysis of ester bonds present on one side of the substrate (probe DNA). The hydrolysis of the esters leads to the release of ferrocene from the magnetic nanoparticles attached to the other end of the probe. The amount of ferrocene released in the solution is in direct proportion to the concentration of the target DNA and can be measured by the electrochemical current generated (Chen et al., 2014).

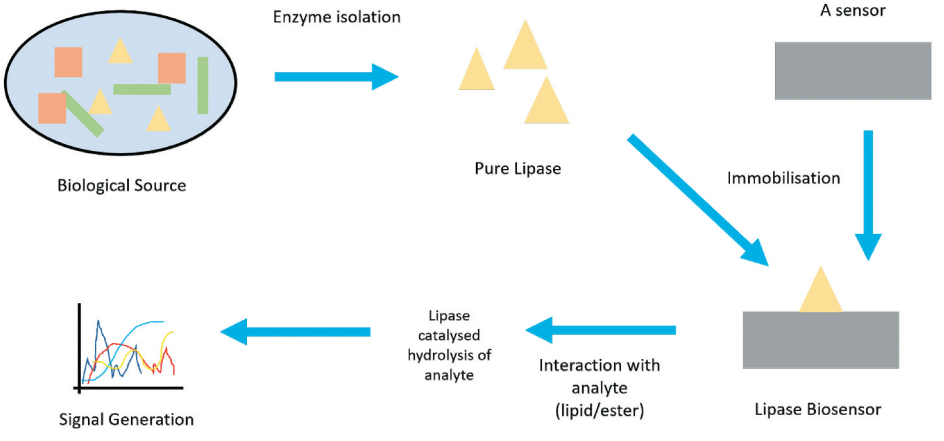

Figure 3. A schematic representation of the working principle of lipase-biosensor.

Multiple triglycerides-sensing lipase biosensors have been studied. One such biosensor uses electrodes made from Chitosan and CuO nanoparticles by a spin coating method. These electrodes help provide a favourable immobilization environment for lipase as it a specific enzyme for the detection of triglyceride. Immobilization of lipase results in enhanced electron transfer at the interface (Bodade et al., 2017). We also found reports on novel optical biosensors to detect triglycerides. It uses 60 μg of *Candida antarctica* lipase entrapped on the pectin hydrogel membrane. It also consisted of chromoionophore ETH 5294 (CI). At an optimum condition of 50 mM of phosphate buffer, 60 μg of enzyme loading, and pH of 7, the biosensor shows enhanced sensitivity of 0.001(ΔA/(mg/dL); (Hasanah et al., 2019). One of the biosensors used consisted of extracellular microbial lipase produced by *Ceratobasidium sp.2*, an endophytic fungus. Lamellar zinc hydroxide, decorated with gold nanoparticles was used for immobilization, followed by incorporating into carbon paste consisting of multi-walled carbon nanotubes (MWCNTs) for detection of carbendazim (De Moura Barboza et al., 2019).

Zehani et al. (2014) developed biosensors using lipases from a microbial source (*Candida Rugosa)* and from an animal source (porcine pancreas) to detect Diazinon in an aqueous medium. These enzymes were immobilized on the functionalized gold electrode surface. Though both biosensors showed lower detection limits, the CRL (*Candida Rugosa* Lipase) biosensor showed better performance than PPL (Porcine Pancreatic Lipase) biosensor in terms of sensitivity. Owing to its specificity towards organophosphate pesticides detection, the CRL biosensor is ideal for monitoring and treating industrial wastewaters containing organophosphate pesticides. Aside from the fact that immobilisation of an enzyme may marginally affect their activity in comparison to that of the free enzyme, the above cases are evidence to the fact that immobilization leads to manifold advantages.

Almost all the biosensors studied in the literature showed reasonable Limit of Detection (LOD), selectivity, and good Relative Standard Deviation (RSD) values. However, it must be noticed that immobilized lipases showed enhanced activity than non – mobilized lipases. The biosensors with immobilized lipases showed lower LOD values ranging from 0.01 to 20 μM, whereas in some cases, the calculated LOD values were between 84.45 and 253.03 μM (Table 3). The biosensor made by immobilizing lipase on lamellar zinc particles to detect carbendazim pesticides showed the best results out of all the pesticides detecting biosensors studied. It has a meagre LOD value of 0.016 μM and good reusability and reproducibility. Reproducibility tests based on five biosensors independently prepared and used, have shown promising results with RSD values of 3.6% below than that for conditions optimized. For successive fifteen measurements through the same biosensor, the RSD obtained was

Table 3. Comparative study of biosensor parameters.

Lipase Used	Substrate	Working Electrode	Limit of detection (LOD) (μM[#])	Reusability (Days)	Reproducibility (RSD%)a	References
Burkholderia cepacia lipase (BCL)	p-nitrophenyl pesticides (methyl parathion)	Glassy Carbon	0.28 to 0.51	20	Not Mentioned	(Ma et al., 2018)
Not Mentioned	Chlorfenvinphos and Malathion	Carbon paste electrode (cavity of 3.0 mm diameter)	84.45 to 253.03	Not Mentioned	Not Mentioned	(Reddy et al., 2014)
Candida rugosa	Triglycerides (TGs)	Graphite-epoxy transducer or biosensor	20	Not Mentioned	5.27 to 9.02	(Escamilla-Mejía et al., 2015)
Candida rugosa (CRL), and Porcine pancreas (PPL)	Diazinon	Gold	0.01 to 0.1	25	2 to 5	(Zehani et al., 2014)
Ceratobasidium sp.2	Carbendazim (CBZ)	Biosensor	0.016	140	3.6	(De Moura Barboza et al., 2019)
Candida antartica	Triglycerides (TGs)	Biosensor (lipase coating on pH optical sensor)	0.16	25	2.5	(Hasanah et al., 2019)
Aspergillus Niger	Triglycerides (TGs)	LIP/Chit-nano CuO/Au bioelectrode	0.14	Not Mentioned	Not Mentioned	(Bodade et al., 2017)

aRSD stands for Relative Standard Deviation; [#]μM represents micro molar concentration

below 4.4%. Studies for carbendazim recovery from water samples with the help of the biosensor showed that RSD is ranged from 98.5% to 105.4% (De Moura Barboza et al., 2019).

Among the immobilized lipase biosensors that detect triglycerides, the hydrogel pectin-based triglyceride optical biosensor shows a low LOD of 0.16 μM as well as good reproducibility. The RSD value for this biosensor reached up to about 2.5%, and its absorbance decreased by 10.2% after 15 days of storage, which shows its stability up to 15–20 days (Hasanah et al., 2019).

7. Challenges of lipase biosensors

The methods mentioned in earlier sections show that the most common method is the adsorption of enzymes on a matrix. Still, there are some disadvantages of using it as there is a chance of desorption from the surface leading to a loss in activity that could be a reason for the varied results of biosensors adopting the same detection process. Another method that showed promising results along with adsorption on the surface of lamellar zinc hydroxy-nitrate molecules was crosslinking enzymes with glutaraldehyde vapor and bovine serum albumin. It also has a disadvantage as crosslinking might cause an alteration in the enzyme's active site, leading to a loss in enzyme activity. Thus, we must choose an appropriate immobilization method and a suitable matrix or support material for immobilization as the combination of two may change the results. It has also been reported by way of studying the commercially available Novozym 435, which entails immobilization of *Candida antarctica* lipase B (CALB) on microporous acrylic polymer resin, which the success in industrial application of Novozym 435 is limited, owing to challenges, such as support disintegration, protein desorption, diffusion limitations and dependence on co-solvent for better reaction rates, etc. (Verdasco-Martin et al., 2016).

The biosensors that were studied in the review had LOD values in the range of micromolar. This value is challenging because the components measured using the lipase biosensors, such as toxic pesticide content and triglyceride content, are very critical to handle. The LOD value of the biosensors should be lowered down in the nanomolar range. Another challenge of lipase biosensors includes their usability. After 20 to 25 days of use, the response of the biosensors decreased to 80% of the initial response, thus restricting its use up to 15 days after storage only (Table 3). Therefore, it is a significant challenge to develop such lipase biosensors, which are accurate, long-lasting, and efficient for a longer time.

8. Future scope of immobilized lipase as biosensors

Genetic engineering techniques can drastically boost the exploration of newer lipases and develop new enzyme properties. Such engineered products would be easier to screen, characterize, isolate, purify, and immobilize onto supports regarding the chosen application for the immobilized enzyme. Thus, several previously deemed not viable reactions or not industrially suited for product synthesis have become an attraction and a possibility for the near future. Newer avenues combined with cost reduction in catalysed reaction systems through immobilization present opportunities to develop various further studies.

With the surging soil pollution by pesticides, insecticides, and chemical fertilizers, the ecosystem is constantly threatened. These chemicals can directly affect the neural system of humans, even if present in trace amounts. Lipase biosensors can be used for the rapid detection of pesticides in a food sample or water sample. These biosensors also offer to detect triglycerides (TG) present in a sample. The detection of TG can help to detect any underlying cardiac diseases in the patient. Researching newer applications for support material obtained from inexpensive sources that are more efficient and advantageous could assume a critical position in the industry for biocatalysts. There will be a major need for rapid detection of specific compounds in samples in the coming future. Considering the performance efficacy, reusability, and cost-effectiveness, immobilized lipase as biosensors is a good choice.

9. Conclusion

Globalization and the consequential growth in research and development focusing the enzymes segment keep the industrial enzyme market continuously rising. Newer advancements, product launches, and increasingly pacing lifestyle continue to push the market growth in the foreseeable future. The demand for immobilized enzyme-based biosensors is driven by an ever-expanding population and the widespread need for sustainable solutions. Biosensors have recently been accepted as one of the most accurate means of detection devices. Various industries employ them to be precise, specific, sensitive, and sustainably efficient instruments. What is even more attractive to the researchers is the use of immobilized enzymes in biosensors. Immobilized enzymes, in comparison to free enzymes, offer more catalytic activity. In this regard, it is of utmost importance that factors like support material employed and the technique or strategy for immobilization applied must be thoughtfully selected and experimentally studied, and deemed fit for us to ensure a suitable final biocatalyst or product.

This review has observed that biosensors with immobilized lipase systems offer promising results for detecting pesticides and triglycerides. However, even though most immobilized lipase-based biosensors showed very low LOD values, more research is needed to enhance reusability and reproducibility. It has also been observed that the involvement of nanotechnology has opened newer dimensions in the development of potential supports and reaction mechanisms for enzyme immobilization. Although, it was observed that several researchers and authors believe that choosing a nanoparticle or a nanoparticle-based composite as a support material for enzyme immobilization depends directly on the reaction being considered for use in the immobilization procedure.

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