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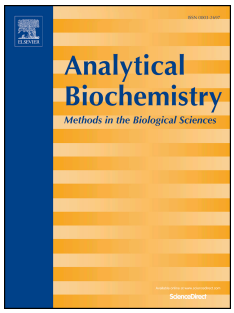

Biosensors based on β-galactosidase enzyme: Recent advances and perspectives

Shiv K. Sharma, Roger M. Leblanc

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Biosensors based on β-galactosidase enzyme: recent advances and perspectives

Shiv K. Sharma[a] and **Roger M. Leblanc**[a,*]

[a]*Department of Chemistry, 1301 Memorial Drive, University of Miami, Coral Gables, Florida 33146, USA*
**Corresponding author: rml@miami.edu Phone: (+1)305-284-2194 (R. M. Leblanc)*

ABSTRACT

Many industries are striving for the development of more reliable and robust β-galactosidase biosensors that exhibit high response rate, increased detection limit and enriched useful lifetime. In a newfangled technological atmosphere, a trivial advantage or disadvantage of the developed biosensor may escort to the survival and extinction of the industry. Several alternative strategies to immobilize β-galactosidase enzyme for their utilization in biosensors have been developed in recent years in the quest of maximum utility by controlling the defects seen in the previous biosensors. The overwhelming call for on-line measurement of different sample constituents has directed science and industry to search for best practical solutions and biosensors are witnessed as the best prospect. The main objective of this paper is to serve as a narrow footbridge by comparing the literary works on the β-galactosidase biosensors, critically analyze their use in the construction of best biosensor by showing the pros and cons of the predicted methods for the practical use of biosensors.

Key Words: Biosensor, Immobilization, Lactose, β-Galactosidase

Abbreviations: *GaO=Galactose Oxidase, GO=Glucose Oxidase, P3HT=Poly(3-hexyl thiophene), FIA= Flow Injection Analysis, GCE=Glassy Carbon Electrode*

Contents

1. Introduction

Biosensors are the analytical hybrid devices which correspond to the substance being measured by transforming information to an analytically useful signal through a biochemical mechanism in which the sensing element is biological in nature [1]. In other words, biosensors are devices that include a biological sensing element connected to a transducer [2]. The history of biosensors starts from simple laboratory level and has emerged to field testing and commercialization in almost every part of the world. There has been significant development in this area in which even gene expression patterns, genome mapping and detection of genetic mutations is feasible due to the DNA microchip technology. The first used biosensors were called the enzyme electrodes [3]. Since then biosensors have been studied and developed continuously till this day. A patient can extract one drop of blood and readout the glucose concentration in less than one minute. The biosensors are not only used in identifying and quantifying the analytes, but also used in screening of specific molecules present in complex compounds even in a very low concentration. This property has gained their utility in analytical research, food and chemical industries [4], pollution checking [5], process monitoring and in clinical diagnosis [6]. Biosensors can be considered as a complementary tool due to their simplicity, rapid response, relative low cost, continuous monitoring and ability to be miniaturized [7].

The evolution in β-galactosidase biosensors can be categorized in three divergent generations that describes the stages of integration, and differentiate between modes of signal convey in a redox enzyme and electrode. Fig. 1 shows schematic representation and features of three different generations of biosensors. The signal transfer between natural secondary substrates, products, and an electrode is regarded as the first generation. Whereas, second generation biosensors consist of artificial electron mediators instead of natural co-substrates and third generation biosensors involve direct electron transfer among redox-active biomolecule and the electrode surface [8]. The main drawback of first generation biosensors was associated with high applied potentials. This problem was controlled by the use of mediators (second generation). However, use of mediators facilitated various interfering reactions, so direct electron transfer (DET) in third generation biosensor opens the avenue to highly selective biosensors virtually free of interference.

β-Galactosidase (E.C. 3.2.1.23) is a crucial enzyme that hydrolyzes D-galactosyl residues like lactose or β-galactose comprising chromogenic or fluorogenic substrates from oligosaccharides and polymers [9]. β-galactosidase has been employed in many food industries to hydrolyze lactose, enhance digestibility, sweetness, solubility and flavor of dairy products [10, 11]. Fig. 2 summarizes the importance of β-galactosidase enzyme. The lack of this enzyme or reduction in enzyme activity in the body causes problem in lactose digestion. There are vast numbers of people worldwide suffering from lactose intolerance. A study has shown that 65 % of the world population experiences from various degrees of lactose intolerance [12]. Symptoms of lactose intolerance include diarrhea, nausea, muscular spasm, swelling, borborygmi and chronic flatulence [13]. The results of lactose malabsorption are dermal disease, rheumatological complaints, persistent exhaustion, and lack of growth and development in children. It should be noted that lactose intolerance are not synonymous to lactose malabsorption [14].

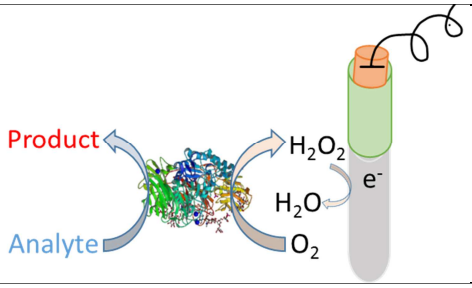
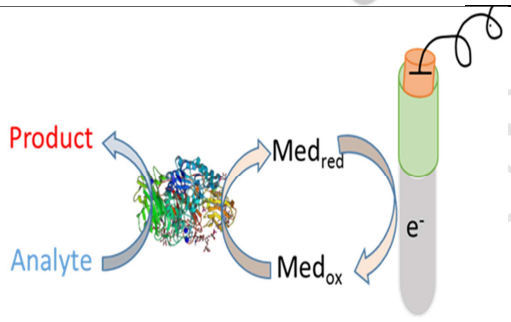
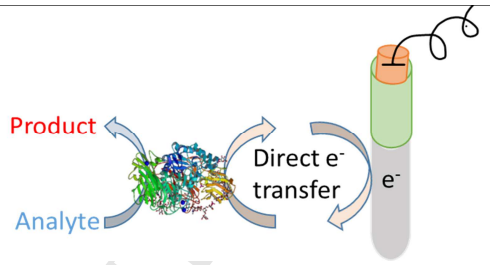
Generation	Schematic	Feature
First		The electrons are transferred to molecular oxygen and the resulting decrease in the oxygen concentration and/or the produced H_2O_2 is measured.
Second		Use artificial mediators or nanomaterials to transport electrons to the electrode.
Third		The electrons are transferred directly from enzyme to the electrode without any intermediate stages or use of mediators.

Fig. 1. Generations of β-galactosidase biosensors

Manipulation of precise biosensors to quantify lactose furnishes a pristine tool in the sector of dairy products. Previously, methods to evaluate lactose required oxidation of aldehyde groups, reaction with phenolic compounds, a few of these methods are polarimetry, infrared spectroscopy, liquid and gas chromatography, and enzymatic assays. But, now different types of amperometric, potentiometric, and conductometric biosensors have been used to measure the lactose.

Several reviews have been published in different enzyme biosensors, but broad studies of the β-galactosidase based biosensors have not been made so far. The substantial use of these biosensors in medicine, dairy industries, laboratory and many other fields demand the latest research and development. This review paper discusses the latest trends in the β-galactosidase based biosensors, future perspectives and different strategies of immobilization which ultimately lead to an enhancement in the performance of the biosensor.

1.1. Significance of use of enzymes as biological element

High performance liquid chromatography (HPLC) or gas chromatography (GC) techniques, as non-enzymatic techniques, have been used for a long time for the determination of nutrients, metabolic products, and secreted recombinant proteins [15]. These methods were somehow sensitive but not specific to the substrates. Besides that, they were expensive and time consuming. Similarly, other classical analytical methods like, titrimetric, and spectrometric methods were also used. The above methods were able to detect picomole levels but relied on less precise and incommodious derivatization methods. These instruments are now-a-days restricted to off-line analysis due to the complexity of the instruments. In contrast to this, biosensors, show promise for gaining the analytic data in a quicker, easier and economic fashion as compared to prevailing assays [16]. The biosensor principles are frequently used as the on-line analysis of specific components. These biosensors are comprised of the suitable biochemical receptor either an enzyme or antibody, which is in immobilized form [2, 17]. The biochemical reaction between analyte and enzyme is monitored by a transducer which are available as an amperometric or potentiometric electrode, a thermistor, fluorimeter and photometer in the market. The main problem with these biosensors is non-sterilizability due to heat sensitivity of the biochemical receptor. This problem was solved by FIA devices which consist of a system where receptors are coupled to the process via suitable interfaces that remove the samples automatically maintaining the sterile integrity of the process. Also, FIA provides lowest standard deviation and coefficient of variations for the various range of samples. Besides this, many researchers are frequently using multi enzyme biosensor for enhanced specificity, sensitivity, multifunctionality and miniaturization. For example, microdialysis-coupled flow injection method can be employed in multienzyme amperometric biosensor such that quantity of different components of milk products like, glucose, galactose, and lactose can be determined at once easily. Allosteric regulation of enzymes is also prevailing to curb them which ultimately changes their functions. Apart from these methods, Langmuir-Blodgett film of immobilization offers monomolecular adsorption of enzymes to the biosensor electrode which can’t be achieved by other methods.

The biological component used in the sensor is important because its interaction with the substrate is highly specific and avoids interferences from other substances which interfere in many analytical methods. Besides enzyme, biological components like antibodies, nucleic acids, micro-organisms and tissues are used in biosensors. The performance factor of the biosensor is accounted in terms of selectivity, sensitivity range and accuracy. The ability to discriminate between different substrates is regarded as the most important characteristics of the biosensors. This ability is directly related to the function of the biological component. The sensitivity and accuracy has been known to the femtomolar range and ± 5 %, respectively. Beside this, the essence of the solution such as pH, temperature, and ionic strength; the response, recovery and working time also affect the performance of the biosensors.

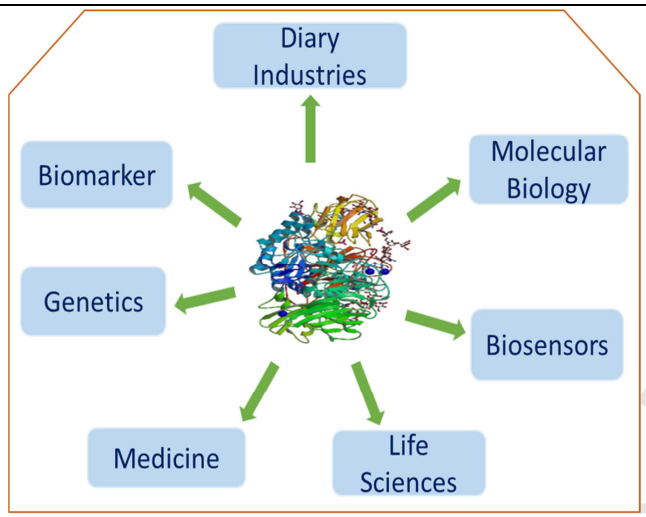


Fig. 2. Summary of importance of β-galactosidase

Enzymes are the most regularly used biological elements in biosensors because of their specificity to bind with the substrate. They are either used in purified form, or may be present in microorganisms or in slices of intact tissue. The enzyme needs to be closely connected to the transducer and the process is known as immobilization. It has been validated that immobilized enzymes are more beneficial than free enzymes [18]. Typically, the enzyme binds to the substrate very easily. They are highly selective and have catalytic activity, thus improving their sensitivity. In addition, enzyme stability has improved due to the immobilization of enzymes on the transducer surface [19]. However, they are difficult to extract, isolate and purify which increases their cost. There is also chance of losing of enzyme activity during immobilization. They are subject to deactivation after short time period [20]. Moreover, there is a high chance of burying the active site of the enzyme intensely within the neighboring protein shell which often makes direct ET not feasible and artificial redox mediators may be indispensable.

2. Immobilization of β-galactosidase enzyme in biosensors

While designing biosensors, the immobilization of enzymes on the transducer surface is an important and decisive step. Many studies have been performed on the immobilization of enzymes due to the known benefits of the enhancement in the property of the enzyme which is convenient in the handling, ease of separation, reduced product cost and conceivable increase in thermal and pH stability [21-23]. β-galactosidase has been extensively utilized as a prototype enzyme due to its adaptability as a biosensor for lactose. Fig. 3 shows simple schematics of enzyme immobilization techniques.

The selection of immobilization technique affects biosensor performances like sensitivity, selectivity and stability as it affects the enzyme orientation, loading, mobility, stability and structure. The immobilization of enzyme on a solid support has been substantially employed in industrial bioprocesses since it avoids or minimizes the undesired existence of the enzyme in the product formed. Furthermore, the variety of support used and the method of immobilization is very crucial because immobilized enzyme in this technique reduces the operational cost by facilitating the reuse of the enzyme [24, 25]. It has also been found that immobilization

facilitates enzyme recovery which also helps to reduce the cost because the enzyme can be used repeatedly [26]. Also, it can preclude intramolecular process like autolysis, proteolysis and aggregation. Different immobilization techniques have been developed that are associated with adsorption, covalent bonding, entrapment, cross-linking or affinity [7, 27-34]. Nanoparticles based enzyme immobilization and their potential applications in biosensors have been described in a review paper [35]. Furthermore, different kinds of techniques applied for the immobilization of β-galactosidase and its significances in food industry has been explained in detail in a review article [36]. The 3D direct immobilization of β-galactosidase from *E. coli* on the silicon surface through glutaraldehyde has been described [27]. It was found that the immobilized enzyme was stable and preserved about 80 % of its inceptive activity after 10 days at 24 °C. The authors have predicted that the capability to induce 3D structures with amplified packing capacity for biosensing molecules renders the potential to enhance biosensor activity.

To obtain the best immobilized enzyme, it is highly suggested that the characteristic of support and enzyme should be known. 3D conformation of an enzyme is known to be liable to alterations because it is balanced by the optimization of counter forces like, hydrophilic interactions, Coulombic force, hydrogen bonds, and van der Waals interactions. Also, it is very important to note that enzymes can pivot inside out while using a hydrophobic support, losing its activity because proteins in general are hydrophobic in their interior and hydrophilic in the exterior [37]. These immobilization techniques have been used for β-galactosidase to advance electrochemical, optical, or gravimetric enzymatic biosensors.

2.1. Immobilization of β-galactosidase by simple adsorption

The adsorption technique of immobilization is relatively easier and less disruptive to the enzyme but life span is short. Immobilization of thermostable β-galactosidase has been performed on epoxy reinforce showing its efficiency for lactose hydrolysis and galactooligosaccharides (GOS) biosynthesis [38]. It has been shown that the increase in stability of enzymes due to multipoint immobilization is directly associated to a reduced pliability of protein upon denaturation conditions [39]. The immobilization of β-galactosidase on aborigine ZnO and ZnO- nanoparticles (ZnO-NP) by simple adsorption mechanism has been demonstrated [40]. This study showed that native ZnO and ZnO-NP revealed 60 and 85 % immobilization yield, respectively. Hybrid biosensors have been advanced for the quantification of lactose [41] by adsorbing Lactozym and β-galactosidase onto diethylaminoethyl cellulose (DE-52). These biosensors showed Nernstian characteristics. These two biosensors showed the lifetime of two weeks and two months, individually. An amperometric biosensor has been developed for the lactose determination by the immobilization of β-galactosidase and GOD onto a GCE coated with mercury thin film [42]. In this study, gelatin and glutaraldehyde were used onto mercury thin film electrode to immobilize enzyme. The response to lactose was determined in the range of 1×10^{-4} and 3.5×10^{-3} M, and the sensitivity was 52.1 nAM^{-1} retaining stability till 40 effective measurements, but the authors have not compared the correlation of their data with HPLC data.

Another method for lactose determination in milk has been developed based on oxidation of lactose by cellobiose dehydrogenase immobilized in an enzyme reactor [43]. The biosensor worked in FIA system and the assay time was calculated as 2 min $sample^{-1}$, and the linear response for lactose was between 0.05 and 30.00 mM. A descriptive review [44] on recent advances of biosensor technology used in threat agent identification and medicine has proved that the biosensors are not only of great significance because of their tendency to solve analytical

problems but are also used to cope with the challenges in defense, homeland security, agriculture, food safety, environmental surveilling, medicinal field, pharmacology, industry, and many more.

β-Galactosidase can be exploited as a marker to fabricate a quick detection assay for diagnosis of *E. coli*. In this perspective, a porous silicon-based chemiluminescence biosensor has been developed [45] which identified the emission of light once β-galactosidase enzyme catalyzes its substrate dioxetane having a sensitivity of 10–100 CFU of *E. coli*. A lactose biosensor has been developed [46] on the essence of a thin-film conductometric sensor in association with two prominent enzymatic activities of β-galactosidase and GOD. The results showed a linear calibration curve for lactose concentration between 60 and 800 μM (Fig. 4). Detection limit of this conductometric biosensor was found far lower in comparison to the biosensors developed by Sharma et al. [47] and Amárita et al. [41]. Similar to, but not limited to the, above work another project has been performed using β-galactosidase and GOD [48]. In this study, the biosensor was incorporated into FIA system that permitted determination of lactose on-line within three minutes. This biosensor was utilized to record lactose concentration while producing β-galactosidase from cheese whey by the use of yeast *Kluyveromyces marxianus*. The sensor exhibited reliable stability for four months and after 7,000 approximate measurements showing linearity in analytical curve for range of lactose concentration from 1 to 30 g/L. However, the precision of measurements in a standard lactose solution has not been performed in this study.

2.2. Immobilization of β-galactosidase by physical entrapment

There are many immobilization techniques entailing physical entrapment or chemical interactions between the enzyme and the support. It has been shown that the entrapment of crosslinked concanavalin A–β galactosidase (con A-B gal) in calcium alginate beads can be a beneficial method for continuous transformation of lactose into glucose and galactose [49]. It has been observed that con A–β gal complex conserved 92 % of the initial enzyme activity whereas crosslinked complex revealed 88 % activity. It was observed that the temperature and pH-optima of the different immobilized β-galactosidase preparations were similar to its soluble analog and the entrapped crosslinked complex maintained about 50 % activity in about one hour subjection with 4.0 M urea at room temperature. Likewise, entrapped crosslinked con A–β gal complex preserved 81 and 62 % of the original enzymatic activity with 5 % $CaCl_2$ and 5 % galactose, respectively. This study showed that entrapped complex maintained 95 % activity after seventh run and 93 % of its original activity for 2 months storage at 4 °C which is apparently good for the development of biosensor for the determination of lactose. In this method, the standard deviation values for lactose, glucose and galactose has not been discussed so far.

2.3. Immobilization of β-galactosidase by covalent attachment

Among different methods, covalent attachment is most durable and has been established to last for about 4-14 months. An amperometric biosensor has been developed by Pilloton et al. to quantify lactose concentration using the principle of FIA [50]. The sensor was selective and rapid, but it had some problems, such as samples must be diluted and the system itself was costly. Another work to analyze lactose using FIA principle has been carried out by Ngo and co-workers [51]. This approach was more sensitive than previous method probably due to the involvement of the immobilization of three enzymes (β-galactosidase, GO and peroxidase) on to a 2-fluoro-1-methylpyridinium salt activated Fractogel, a non-compressive support material that

forms stable enzyme support linkage and excellent flow properties which is required in flow injection methods. Magnetic Fe_3O_4–chitosan nanoparticles have been proved as the effective support for the immobilization of β-galactosidase [52]. In this work, the optimization of the immobilization process was executed by inspecting immobilization & cross-linking time, enzyme-glutaraldehyde concentration, and initial pH values of glutaraldehyde and the enzyme solution. This resulted in the immobilized enzyme presenting a fine storage length, pH and thermal stability which are considered necessary for the construction of biosensor.

A magnetoelastic biosensor has been constructed by covalently immobilizing β-galactosidase onto a gold-coated magnetoelastic film via a self-assembly monolayer (SAM) of ω-carboxylic acid alkyl-thiol containing terminal carboxylic group [53]. In this study, wireless biosensors were made by utilizing an idea in which the magnetoelastic alloy films render the ability to fabricate wireless sensing mechanisms usually used in anti-theft devices. This work demonstrated that it was possible to flourish enzymatic sensors that can be planted and handled in fastened, opaque vesselss for *in situ* identification of substrates or inhibitors.

2.4. Immobilization of β-galactosidase by encapsulation

In this technique, an inert membrane is used to trap the enzyme on the transducer. In doing so, there is close attachment between the transducer and the enzyme making it adaptable and reliable. In the meantime, the specificity of the enzyme is maintained and there is good stability over temperature, pH and substrate concentration ranges. An excellent review article has been presented in the encapsulation of different enzymes [54]. Boissiere et al. have successfully demonstrated the encapsulation of catalytically active β-galactosidase [55]. This approach has enabled the ability to curb the feature of the hybrid composites by changing the polysaccharide from alginate to gelatin.

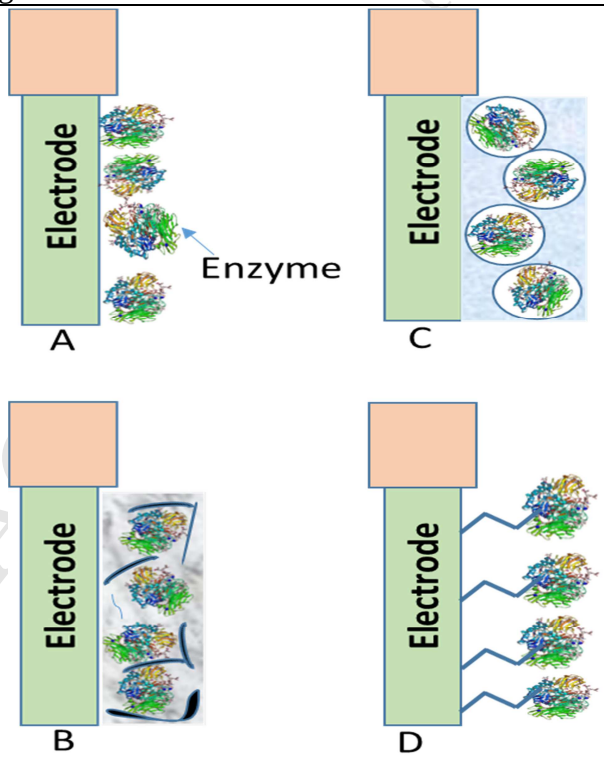


Fig. 3: Schematics of enzyme immobilization techniques: (A) Physical adsorption, (B) Entrapment, (C) Encapsulation, (D) Covalent attachment

2.5. Immobilization by the solid-phase method

β-Galactosidase from *Kluyveromyces lactis*, immobilized by the solid-phase method by direct or tethered connection to the Sepharose 4B matrix has been described [56]. Here, enzymes which were immobilized on beaded agarose that acted on low molecular weight substrate showed resembling activity whether they were PEG tethered or not. It was also shown that the activity for an immobilized enzyme with a high molecular weight substrate was significantly increased using a heterobifunctional PEG tether.

An approach for the evaluation of glucose and lactose in food products with the utilization of biosensors based on Berlin Blue has been developed [57] in which the biosensor revealed increased sensitivity, low detection limits, and rapid response. It was found that the lactose biosensor response was linear over the concentration range 1×10^{-5} - 1×10^{-2} M. A comparison of two biosensors demonstrated that the lactose biosensor ranked below the glucose biosensor in all parameters. The reason for this finding was explained based on occurrence of consecutive enzymatic reactions in the determination of lactose, because glucose, an intermediate product formed in the decomposition of lactose, diffuses to the active center of GOD within the membranes. Previously, a similar experiment was performed in which an amperometric biosensor was developed by jointly immobilizing the GOD and β-galactosidase [58]. The operation of this sensor was solely based on the detection of hydrogen peroxide by the use of platinum electrode. In this case, the analytical range was observed from 2×10^{-3} to 1×10^{-2} M and the detection limit was 1×10^{-3} M.

2.6. Nanostructure immobilization of β-galactosidase

Nanoparticles based enzyme immobilization has drawn increased attention in recent years as it furnishes substantial surface area for binding larger number of enzyme to the matrix, impedes unfolding of the protein and allows increased flexibility for conformational transformation essential for enzyme activity [59, 60]. Also, nanostructures are said to possess special features to equilibrate predominant components that determine biocatalyst efficiency, encompassing specific surface area, mass transfer resistance and efficacious enzyme packing. Besides that, immobilized enzymes on nanoparticles reveal increased stability in a broad range of temperature and pH, contrast to free enzymes. Several nanostructures have been used in the immobilization of β-galactosidase to develop a biosensor [61]. The sensing strategies based on magnetic nanoparticles (MNPs) have different analytical merits such as amplified sensitivity, reduced limit of detection, increased signal-to-noise ratio, and shorter time of analysis [62, 63]. It has been investigated that the application of macro and nanoparticle of chitosan for the immobilization of β-galactosidase revealed exceptional working stability at 37 °C, being capable to hydrolyze 83.2 initially and 75.9 % lactose after 50 cycles of continuous use [64]. An intense study in the immobilization of β-galactosidase on a biaffinity support, concanavalin A layered Al_2O_3 nanoparticles was conducted [65]. It was noticed that the immobilized enzyme had high activity (91 % after 4th repeated use and 85 % after 6th repeated use) as no modification and distortion took place at the active site of the enzyme. This observation gave a better idea that active sites were less blocked by nanomatrix and permitting more free access to the incoming substrates when used in biosensor. Another work was done by the same authors in which the enzyme from

Aspergillus oryzae has been immobilized on concanavalin A-layered calcium alginate-cellulose beads where they have shown the relevance in lactose hydrolysis in continuous spiral bed reactors [66]. It would be better for reader if the correlation of obtained data was compared with HPLC data. El-Assar immobilized β-galactosidase on amino functionalized poly (acyonitrileco-methyl methacrylate) poly (AN-co-MMA) nanofibers in which 35 % of its initial activity was retained when stored at 4 °C but 64 % of its initial activity was retained after ten successive reuse [67]. In this experiment, an increase in thermal and pH stabilities upon immobilization was observed.

β-galactosidase from *Kluyveromyces lactis* has been covalently immobilized to functionalized silicon dioxide nanoparticles (10–20 nm) showing 87 % immobilization yield [68]. In this case, the immobilized nanoparticle–enzyme conjugate maintained more than 50 % enzyme activity up to the eleventh cycle. The authors have not explained the reason for significant decrease in enzyme activity. Immobilization of β-galactosidase on ZnO nanoparticles for the primary purpose of lactose hydrolysis has been done [69]. Here, the cross-linked adsorbed enzyme preserved 90 % activity after one-month storage, while the original enzyme showed only 74 % activity under equivalent incubation conditions. The cross-linked β-galactosidase exhibited activity till seventh cycle and sustained 88.02 % activity up to third cycle.

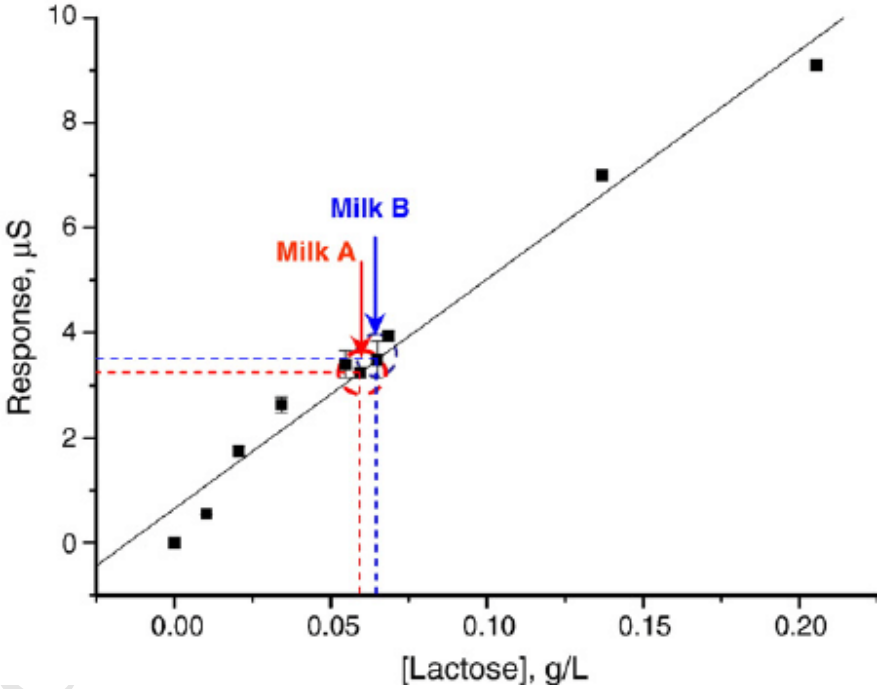


Fig. 4. Use of linear calibration curve to estimate the lactose concentration corresponding to conductometric biosensor response. Two milk samples (A and B) having equivalent lactose concentration of 0.0594 g/L × 1000 and 0.0649 g/L × 1000 respectively were employed in the experiment [46]. (Reprinted with permission, copyright 2008, Elsevier)

2.7. β-Galactosidase immobilized by layer-by-layer (LBL) and Langmuir-Blodgett films

LBL assembly has been determined as a valid technique to immobilize enzymes with conserved activity due to its simplicity and flexibility [70]. A biosensor has been reported

regarding the benefits in maintaining enzyme activity of poly (ethylene imine) (PEI) [71]. In this work, β-galactosidase was immobilized in LBL films with PEI and poly-vinyl sulfonate was fabricated on an indium tin oxide (ITO) electrode modified with a layer of Prussian blue showing lactose determination with a sensitivity of 0.31 μA mM^{-1} cm^{-2} and a detection limit of 1.13 mM L^{-1}. Another amperometric lactose biosensor has been produced by immobilizing β-galactosidase and GaO in Langmuir–Blodgett films of poly (3-hexyl thiophene)/stearic acid [47]. The enzyme immobilized LB film was employed as the working electrode and platinum as the reference electrode. It was shown that β-galactosidase/GaO immobilized P3HT/SA films can be utilized for the assessment of lactose from 1 to 6 g/dl and have a shelf-life more than 120 days by retaining 95 % enzyme activity (Fig. 5). An incorporated amperometric biosensor for the quantification of lactose has been reported [72]. The developed bioelectrode was based on the use of 3-mercaptopropionic acid (MPA) self-assembled monolayer (SAM)-modified gold electrode on which β-galactosidase, GOD, peroxidase (HRP) and mediator tetrathiafulvalene (TTF) were co-immobilized by a dialysis membrane. Similar to other processes, β-galactosidase catalyzed the hydrolysis of lactose, and yielded glucose which was catalytically oxidized to gluconic acid and H_2O_2, which was decreased in the existence of HRP but the life time of the biosensor was only 28 days. A linear calibration plot was acquired for lactose over the 1.5×10^{-6} to 1.2×10^{-4} M concentration range, with a limit of detection of 4.6×10^{-7} M. It was reported that, the bioelectrode exhibited an appropriate accomplishment in FIA systems in association with amperometric detection.

The immobilization of β-galactosidase has been demonstrated on an ionic liquid-cellulose film that showed enhanced pliability and formability [73]. Polyamine was used in making the film and the film was treated with glutaraldehyde to produce aldehyde groups for further enzyme attachment. In doing so, 60 % of the initial enzyme activity was preserved and was employed in 16 replicated batches of lactose hydrolysis at 7°C. Another method of immobilization of β-galactosidase on the surface of concanavalin has been developed [74] in which a layered calcium alginate–starch beads were suggested as therapeutic agent for lactose intolerant patients. In this study, authors have observed significantly increased stability in opposition to the circumstances of digestive system like, salivary amylase, pH, pepsin and trypsin; though soluble and immobilized β-galactosidase revealed identical pH-optima.

3. Use of β-galactosidase in allosteric biosensors

It is a well-known fact that enzymes are very important in bodily activities like digestion, respiration and other cellular functions. Therefore, proper regulation of these enzymes is essential. It is a true that an unregulated enzyme may devise a good deal of something or not a thing at all, both of these instances can have intense consequences on how the body executes. A crucial mechanism employed to curb enzymes is allosteric regulation. Allostery is defined as the condition of a protein (enzyme) in which the structure and activity of the enzyme is remodeled by the binding of a metabolic molecule at a site other than the chemically active one. Beside the active site, numerous enzymes have different areas known as allosteric sites which are localized in a varying spot from the active site. The alteration of enzyme's shape and conformation occurs when a molecule binds to an allosteric site which ultimately changes enzymatic functions [75]. Hence, allosteric enzyme regulation is when a molecule holds to a spot other than the active spot and alters the properties of the enzyme by altering its conformation. Different allosteric enzymes have been designed, developed and used in biosensors [76-78]. It is to be noted that the natural

allosteric enzymes can't be directly employed as biosensors since most of their modulators are deprived of analytical appeal [76]. The regulatory feature of allosteric enzymes offers huge perspectives to using as biosensor recognition elements [79]. It has been found that the regulatory subunit works as the recognition element influencing, either in favorable or unfavorable fashion via conformational alteration, the catalytic site helping as the transducing element [80]. Allosteric responses in enzyme engineering can efficiently be triggered to act as molecular biosensors by binding the antibody efficiently [76]. For the detection of HIV antibody, an engineered enzyme biorecognition element has been employed [81] in serum involving two overlapping epitopes (P1 and P2 from the gp41 envelope glycoprotein) incorporated adjacent to β-galactosidase active site, resulting in a hybrid enzyme.

As the *E. Coli* β-galactosidase provides quick response enzymatically to antiviral antibodies, the allosteric feedback of a β-galactosidase molecular sensor, consisting a B-cell epitope from HIV, has been excellently scrutinized upon binding of an effector monoclonal antibody within a broad range of standard concentrations of both enzyme and substrate [82]. The enhancement of the enzymatic response of the sensor and the allosteric nature of the biosensor has been confirmed by an experiment in which the binding of the effector, anti-P1 monoclonal antibody, to a protein NF795gpc is observed by ELISA and plotted against enzyme activation [81]. In this experiment, the dose dependence of the signal and linear loss of saturation between 5 and 15 ng/μl of antibody was taken as a key feature in justifying the result.

4. Multi enzyme biosensor

Different researchers have been using multi enzyme biosensors in the past. This is very important for the practical biosensors for high specificity, high sensitivity, miniaturization and multi functionality. In 1990, a bi-enzyme amperometric biosensor based on the GOD-β-galactosidase sequence for the detection of lactose was developed [83]. The researchers observed that the response of the bi-enzyme probe depended linearly on lactose concentration between 0.02 and 3.00 mM dm^{-3}. The use of biosensor to various milk and foodstuff samples produced good correlations toward enzymatic photometric (y = (0.956x-1.67) mM dm^{-3}) and infrared detection (y = (1.0772x-0.3909) %). The authors have employed their work in the determination of urine lactose and have predicted its helpfulness in the diagnosis of *mastitis* in cows. A year later, biosensors for the hand by hand quantification of lactose and glucose, starch and glucose, and sucrose and glucose were developed [84]. The developed biosensor composed of β-galactosidase, mutarotase, invertase and GOD using oxygen as the detector, and linear range was determined to be 58.5 to 181.3 mM.

Later, multi enzyme biosensor was developed using field effect transistor as a detector [85] using β-galactosidase, GaO and NAD as a cofactor. The linear range of the detector was 0.0 to 1.5 mM. In the same year, a screen-printed enzyme electrode for the evaluation of lactose was developed [86]. The authors designed an amperometric lactose sensing electrode based on β-galactosidase and GOD using thick-film technology. It was observed that the sensor prevailed stability for 3 months having a linear range from 0.0-4.7 mM. Similarly, a bio-field effect transistor (FET) sensor was produced for quantification of lactose based on co-immobilized β-galactosidase/glucose dehydrogenase [87]. The bi-enzyme system was co-immobilized on the surface of the FET gate. It was claimed that the sensor furnished a quick and reproducible response to lactose and was stable for 20 days of continuous measurement. The short response time, according to authors, was due to the vicinity of two enzymes as a result of crosslinking during immobilization and the convection current created by the stirrer which promoted the

diffusion of acidic products far from the gate. In 1998, a multi enzyme biosensor was developed based on Clark-type oxygen electrode in the determination of lactose, glucose and sucrose [88]. The sensitivity of the sensor was 50 nA/mM, range of the calibration was 0.0-0.8 mM, the response time was 2 min and response after 1 week of storage was 62 % of the initial response. In the same year, Liu et al. developed an amperometric biosensor sensitive to glucose and lactose based on co-immobilization of ferrocene, GOD, β-galactosidase and mutarotase in β-cyclodextrin polymer [89]. In this work, ferrocene was incorporated in the pockets of the β-cyclodextrin polymer through a host-guest chemical reaction. The measurements were conducted by the use of cyclic voltammetry and amperometry to show the efficacy of e^- transfer between immobilized GOD and a GCE via ferrocene. The linear range was found to be 50×10^{-6} to 13.5×10^{-3} M and the biosensor maintained 85.7 % of initial sensitivity after 2 months of storage.

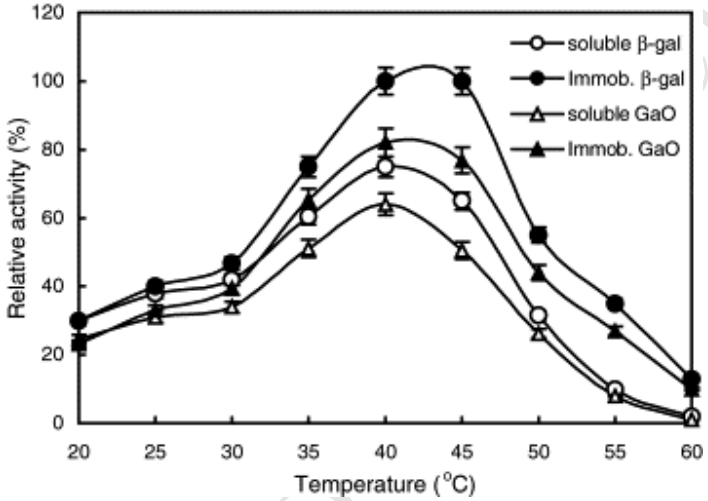


Fig. 5. Determination of enzymatic activity of β-galactosidase and GaO in immobilized state (LB films) and soluble state (native) at different temperatures [47]. (Reprinted with permission, copyright 2004, Elsevier).

An amperometric biosensor was developed for lactose determination in raw milk through the simultaneous immobilization of β-galactosidase and GaO on a derivatized polyethersulphone membrane [90]. It was stable for 20 days and the sensitivity and reproducibility were 6.81 and 0.72 nAM^{-1}, respectively. Another amperometric lactose biosensor was produced based either on co-immobilization of two enzymes (GaO with peroxidase) or co-immobilization of three enzymes (β-galactosidase, GaO and peroxidase) [91]. It was observed that the presence of β-galactosidase highly enhanced the sensor's sensitivity, but its linear range was slimmer as compared to the sensor without β-galactosidase. The authors determined the response time between 60 and 75 s and a detection limit for lactose determination from 44 to 339 mM. Their data was in good correlation with the HPLC data and the precision of measurements of standard lactose solution for the trienzymatic and bienzymatic sensors was 2.19 and 2.02 %, respectively. A lactose biosensor was emerged based on β-galactosidase and GOD [92] in which alternating current electrophoretic deposition (AC-EPD) method was used to fix enzymes on the surface of the biosensor. It was found that the sensor had a sensitivity up to 111 nA/mM mm^2 with larger linear range up to 14 mM lactose, fast response time (~8 s) and good stability without engaging any stabilizers or outer polymer membrane.

A three-cascaded-enzymes biosensor based on a modified GCE has been developed using β-galactosidase, GOD, and horseradish peroxidase [93]. Enzymes were immobilized on the GC

electrodes by using the polyethyleneimine (PEI). This biosensor showed tendency to quantify lactose concentrations up to micromolar range having a rapid response (2 to 4 s) but its stability has not been explained so far. Rajendran and Irudayaraj developed a microdialysis-coupled flow injection amperometric biosensor for the diagnosis of the level of glucose, galactose, and lactose in milk [94]. The sensor was dependent on enzyme catalyzed reaction in association with the three different analytical methods, viz microdialysis sampling, amperometric detection, and FIA. The sensor revealed a linear response between 0.05 and 10.00 mM for glucose, 0.1 and 20.0 mM for galactose, and 0.2 and 20.0 mM for lactose. The relative standard deviation values of the sensor measurements for glucose, galactose, and lactose were found to be 3– 4 % (n = 3).

Table 1. Selected list of β-galactosidase biosensor showing its source and specific properties

Biosensor/ Transducer	**Method of immobilization**	**Immobilizing agents**	**Source of β-galactosidase**	**Linear response rate and other properties**	**Reference**
Ingold potentiometric	Physical entrapment	Diethylaminoethyl cellulose (DE-52) and agar	*Enterobacter agglomerans*	0.0146-0.2920 mM, Stability: 2 months	[41]
Chronoamperometric	Coated with mercury thin film	Gelatin and glutaraldehyde onto mercury thin film GCE	*Aspergillus oryzae*	Linear range: 1×10^{-4} -3.5 $\times 10^{-3}$ M.	[42]
Chemiluminescence	Physical Adsorption	Dioxetane substrate/ porous silicon	*E. Coli*	Lower Detection limit: 10–100 CFU of *E. coli*	[45]
Amperometric	Immobilization in LB films of P3HT/SA	P3HT/stearic acid (SA)	Sigma Chemicals, USA (*E. coli)*	0.029-0.175mM, Half -life: above 120 days	[47]
Conductometric	Deposition on electrodes	Phosphate buffer, glycerol, gluteraldehyde	Sigma Chemicals, USA (*E. coli)*	30 and 600 μM	[46]
Amperometric	Adsorption	Perfluorosulfonated polymer	Unknown	Detection limit: 1×10^{-5}-1×10^{-2} M, Analysis time: 2-3 min	[57]
Amperometric	Adsorption	Thin-layer plexi-cells on natural protein membranes	Unknown	Analytical range: 1×10^{-5}-1×10^{-2} M, Detection limit: 1×10^{-3} M	[58]
Amperometric	Immobilized in layer-by-layer (LbL) films	PEI and PVS on an ITO electrode modified with a layer of Prussian Blue (PB)	*Aspergillus oryzae*	Sensitivity: 0.31μ A $mmol^{-1}$ cm^{-2} Detection limit: 1.13 mM L^{-1}	[71]
Amperometric	Adsorption	MPA, Mediator: (TTF) coimmobilized by a dialysis membrane	*E. coli*	1.5×10^{-6} to 1.2×10^{-4} M, Detection limit: 4.6×10^{-7} M	[72]
Amperometric	Adsorption (bi-enzyme)	Gelatin	*E. Coli*	0.02 and 3.00 mmol dm^{-3} of lactose	[83]
Unknown	Adsorption	Cellulose triacetate, gluteraldehyde	Unknown	58.5 to 181.3 mM	[84]
Unknown	Not specified	pH sensitive gates of an eight-channel field transistor(FET)	*E. Coli*	0.0-1.5 mM	[85]
Amperometric	Thin-film technology	Gluteraldehyde	*E. Coli*	0.0-4.7 mM	[86]

Bio-FET sensor	Crosslinking	Gluteraldehyde	*E. Coli*	Response rate: 120 s Stability: 20 days	[87]
Unknown	Crosslinking and co-immobilization	Gelatin crosslinked with glutardialdehyde	*Kluyveromyces marxianus*,	0-0.41 mM	[88]
Amperometric	Cross-linking with Gluteraldehyde and β-cyclodextrin polymer	Ferrocene and β-cyclodextrin	Sigma Chemicals, USA (*E. coli*)	50×10^{-6} to 13.5×10^{-3} M	[89]
Amperometric	Deposition/adsorption	Derivatised polyethersulphone membrane	*Aspergillus oryzae*	Sensitivity: 6.81 $nA.M^{-1}$,	[90]
Amperometric	Deposition/adsorption	Ferrocene and DEAE-dextran as stabilizer	*E. Coli*	Detection Limit: 44 to 339 mM	[91]
Amperometric	AC - Electrodeposition	Not needed	*Aspergillus oryzae*	Sensitivity: 111 nA/mM mm^2, Linear range: 14 mM lactose	[92]
Amperometric	Electrochemical deposition/ cross linking with gluteraldehyde	PEI + gluteraldehyde	*E. Coli*	Linear range: 2.9×10^{-5} to 9.9×10^{-4} M, Response Rate: 2 to 4 s, Stability: 140 days	[93]
Amperometric: Microdialysis-coupled flow injection	Enzyme injection and electrochemical	0.1 M phosphate buffer at pH 7.0, containing 10 mM NaCl	*E. Coli*	Response rate: 0.05 - 20 mM	[94]

5. Other β-galactosidase Biosensors

An accessible, sensitive, and uncomplicated biosensor based on β-galactosidase activity has been developed for the determination of toxoflavin, a toxic component to various plants, fungi, animals, and bacteria [95]. This biosensor was composed of a sensor strain (COK71), substrates (X-gal or ONPG), and culture medium, devoid of complicated preparation process. It has been claimed that the method was simple, dependable, and samples containing 50-500 nM of toxoflavin could be inspected. The limit of detection for toxoflavin in the thin layer chromatography (TLC) assay was determined to be 5–10 μM. The identification of wastewater toxicity was carried out by using bioluminescent bacterial biosensors integrated with estrogenic assays [96]. In this work, bacteria based toxicity screening and endocrine disruption via β-galactosidase activity in recombinant yeast with yeast estrogen screening assays were combined for monitoring water samples. Although, this method can't identify every toxin present in water and is limited to a set of water samples but this strategy of integrating two methods seems to outweigh more elaborated bioassay system by increasing the promptness and easy conduct.

Alcalá et al. have cloned and fabricated a recombinant SD6 scFv fragment in *E. coli* targeted against a sensing peptide, exhibited on a prototype β-galactosidase based biosensor that enhanced the enzyme activity up to about 200 % [97]. The use of immunoassay to biosensors has been developed to exploit in the point-of-care setting that preferably demands immunoassay free of separation steps and with little volumes of both sample and reagents [98]. The expedience of cloned enzyme donor immunoassay was probed for utilization to biosensors. This technique was based on β-galactosidase, which was genetically engineered by others into two indolent fragments, enzyme donor (ED) and enzyme acceptor (EA). Combination of the ED and EA fragments in the assay resulted in development of active enzyme, which acted on the substrate to produce a perceivable signal. The reporter gene lacZ, coding for the enzyme β-galactosidase,

which produced a color reaction or an electrochemical reaction with suitable substrates, was also employed for the construction of sensors for mercury, arsenite and antimonite. The bacteria utilized in these investigations was genetically engineered to generate the enzyme β-galactosidase in response to these ions [99, 100].

To analyze the lactose in the milk samples, a wireless biosensor was produced by co-immobilizing β-galactosidase, GOD, and catalase onto a magnetoelastic ribbon-like sensor which was pre-plastered with a layer of pH-sensitive polymer [101]. It has been found that the GLA-catalyzed hydrolyzation of lactose generated β-d-glucose, which was further oxidized to gluconic acid under the catalysis of GOD, promoted in the pH-responsive polymer contracting, and thus enhanced the resonance frequency of the sensor. It was observed that the variations in frequency were linearly proportional to the concentration of lactose from 2 to 16 mM with a detection limit of 0.72 mM. It has been claimed that the suggested wireless sensor can be developed for assays of lactose in fastened non-transparent vessels. Whether a biosensor is developed using the single enzyme or multi enzyme, they usually have the same components. Fig. 6 shows the components of the β-galactosidase biosensor.

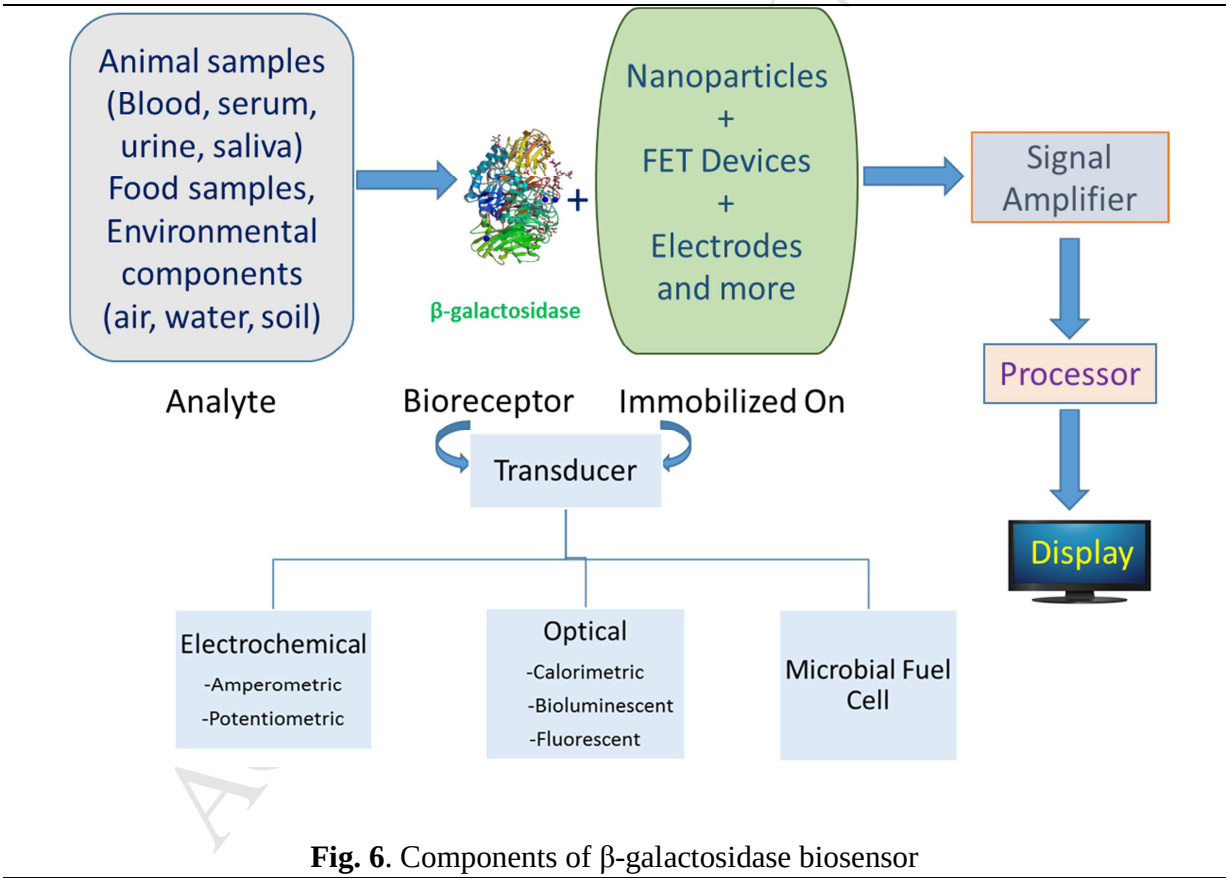


Fig. 6. Components of β-galactosidase biosensor

Whole cell biosensors are also familiar in the biosensing field though they are complicated and the life time of the cells is limited as compared to the enzymes. Detection of pesticide chlorpyrifos (CPF), a neurotoxin, by genetic-based whole cell biosensor has been studied by Loprasert and co-workers [102]. In this study, two reporter systems namely β-galactosidase and sulfatase has been compared and data showed that sulfatase system was more responsive than β-galactosidase system.

In another context, a method has been developed, claimed as a first discovery, in which the β-galactosidase biosensor can be converted into another kind of enzyme biosensor that can catalyze sucrose rather than lactose [103]. In this research work, the impact of glutaraldehyde on the conformational properties of *Kluyveromyces lactis* β-galactosidase has been studied. It has been shown that the optimum substrate of the enzyme interchanged from lactose to sucrose with a certain concentration of glutaraldehyde. An electrochemical biosensor to detect the activity of protein kinase A (PKA) was developed by using β-galactosidase, gold nanoparticles-carbon nanospheres and phos-tag-biotin [104]. Here, β-galactosidase was used to catalyze the hydrolysis of p-aminophenyl galactopyranoside (PAPG) to p-aminophenol (PAP) under neutral conditions and which ultimately helped in inhibition of PKA activities. Recently, another method of inhibiting antibiotics contaminating environmental water by the use of paper based biosensor has been proposed [105]. This biosensor was based on the identification of variation of color instigated by β-galactosidase. Although, this method has relatively higher detection limit as compared to the previous method but it seems promising in preventing the occurrence of antibiotic-resistant bacteria in water samples.

6. Conclusion and future perspectives

In the study, it has been found that enzyme based amperometric biosensors are reliable analytical devices with enhanced selectivity that can be handled by amateur manpower. Several potentiometric biosensors have been reported in the literature, but they have been substituted by amperometric biosensors due to their superior resolution. However, potentiometric biosensors have specific merits because they allow the settlement of problems not resolved by amperometric biosensors. Also, hybrid biosensors are less discerning than enzymatic ones, but the selection of the type of biosensor should be devised per several supplementary factors such as accessibility of biocatalysts and the stability and sensitivity demand. The use of β-galactosidase biosensor in measurement of lactose has been helpful in the identification of cows/buffalos that are suffering from *mastitis* because the milk from these animals has low lactose level. Apart from this, it has been helpful in treatment of waste water as lactose is a major parameter in waste water. Clinically, the β-galactosidase biosensor has been helpful in determination of lactose level in blood. An excess amount of lactose in blood reveals gastrointestinal malignancy. It has been reported that till now the biosensor with the highest sensitivity consists of a gold disk electrode modified with mercaptopropionic acid-self-assembly monolayer (MPA-SAM) in which β-galactosidase, GOD and peroxidase are immobilized with a dialysis membrane. However, the ITO-coated glass plate electrode in which β-galactosidase and GOD are immobilized in LB films shows shelf-life above 120 days.

The increased demand of biosensors as important tools in immunoassays, toxicology analysis, forensics, gene identification, high quality food processing and drug screening have generated an increased need for sophisticated techniques to be used in biosensors. The present trend of taking the blood, urine and other biological samples away from the patient for classical analysis, which may not be accomplished for hours or even days, needs to be replaced as soon as possible. When a patient is in intensive care the application of on-the-spot biosensors could enable analytical results to be obtained within minutes at most. We believe that there is a considerable scope to design smart and efficient sensing systems. Due to increased customer

demand for high food quality, real time testing of fabrication processes which use shrewd and facile methods to operate sensors is an alternative avenue to solve this demand. For this, the investigation of automated on-line lactose biosensor in the milking parlor is very important. Apart from this, there is a need to improve the uniformity and performance in the biosensor electrodes. This is probably due to the lack of efficient automated immobilization techniques. We can also think of implanted biosensors for continuous monitoring of a metabolite, which would be important in controlled drug delivery system. By doing this the blood glucose level of a diabetic patient would be monitored continuously and insulin would be released automatically once glucose level reached a certain level. Beside this, the pollution level in air, water, and soils could be monitored in real-time without exaggerating the situation. In addition, several works have been done to attain the aspired performance of biosensors but these possibilities are restricted to the realm of academic papers due to unsolved problems like degradation of the sensing layer in *in vivo* application, long term stability of biomaterial, non-uniform adsorption, creation of anti-interference layers, etc. The authors hope that this review will help to boost worthwhile discourse to induce novel propositions and approaches towards the evolution, establishment and optimization of the β-galactosidase based biosensors.

Conflict of Interest

Authors declare no conflict of interest.

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