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Review article

DETECTION OF FOOD BORNE PATHOGENS

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ABSTRACT
Food safety is a global health issue and the foodborne diseases create a major threat to human health. Therefore, detection of microbial pathogens in food is the key to the problems related to health risks. Conventional molecular methods of detection such as colony counting methods, immunology-based methods and polymerase chain reaction may take up several hours to days to confirm the presence of the pathogen. New modified molecular methods like DNA microarray, antibody array and rapid analytical technique like biosensors show potential approaches for the detection and quantification of pathogens. Biosensor technology offers several benefits over conventional methods such as simplicity of use, specificity for the target analyte, less time consumption, capability for continuous monitoring, potentiality of coupling to low-cost, portable instrumentation. This review offers an overview in the dynamic progress in foodborne pathogen detection, identification and quantification.

KEYWORDS: Polymerase chain reaction, ELISA, Biosensor, electrode, antibody immobilization

INTRODUCTION

Nowadays concern for food safety and quality has gained an immense importance in food industry. The probability for contamination of food has increased by the spread of food borne pathogens. Any pathogenic microorganism in food can lead to severe health related problems in animals and humans and can cause widespread damage (Arora *et al*., 2011). The ability of some microorganisms to evolve rapidly allows them to survive under stressful conditions also (Nayak *et al*., 2009). Most prominent foodborne pathogens are *Escherichia coli*, some strains of *Staphylococcus aureus*, *Shigella* spp., *Bacillus anthracis* (produces anthrax toxin), *Campylobacter jejuni*, *Clostridium perfringens*, *Clostridium botulinum* (produces a powerful paralytic toxin botulin), *Salmonella* spp. *Listeria monocytogenes*, *Vibrio cholera*, *Yersinia enterocolitica* and *Coxiella burnetii*. These bacteria mostly produce toxins and other cell metabolites which cause deadly diseases (Feng, 2001; Moss and Adams, 2008a, b). Common foods which get easily contaminated are: milk, cheese, meat, chicken, fish, raw vegetables, fruits etc (Loir *et al*., 2003). In such condition the control measures that we currently undertake, seem time-consuming and inappropriate. The traditional methods of detection need around 1- 2 days for the target pathogen to be identified. Ever since these traditional methods evolved into molecular diagnostics rapid and accurate identification has been possible (Weinstein *et al*., 1995; Carroll *et al*., 1996; Mata *et al*., 2004; Kamesh and Roman, 2005).

The best upcoming technology to combat this problem is the use of biological sensors that provide us with a tool to rapidly detect the presence and quantify the amount of microorganisms in the food samples (Nayak *et al*., 2009). For the availability of fresh products in the food industry, biosensor research has been focused on the contaminant detection, content verification, monitoring of raw materials conversion and product freshness (Collings and Caruso, 1997). To meet the expectations, analytical methods for pathogen detection in food must have the specificity to distinguish between different bacteria, the adaptability to detect different analytes and the sensitivity to detect bacteria directly in food samples without any pre enrichments (Invitski *et al*., 1999). Dr. Leland C Clark, the father of biosensors, established an 'enzyme electrode' in 1960 for the measurement of glucose levels using immobilized glucose oxidase enzyme. Other enzyme electrodes developed later were urease for urea detection, glutamate dehydrogenase, lactate dehydrogenase *etc*. (Turner, 2007). Further emphasis was given to more sensitive recognition elements including antigens, antibodies, nucleic acids, whole cells and proteins and the signal was measured by electrochemical, piezoelectric and magnetic transducers (Nayak *et al*., 2009).Viable microbes produce metabolites, such as carbon dioxide, ammonia, acids, or they are bioluminescent as exemplified by Vibrio fischeri, which can be used to monitor viability. Many microbial biosensors are based on light emission from luminescent or fluorescent bacteria that are genetically engineered to express fluorescent or luminescent proteins, such as Green Fluorescent Protein (GFP) or Luciferase protein ((D'Souza, 2001; Baeumner *et al*., 2003). So far, microbial biosensors and bioassays have been applied prevalently in the detection of food additives and food contaminants than in direct monitoring of food pathogens themselves. The previous research studies associated with conventional methods of detection such as polymerase chain reaction (PCR) (Burtscher and Wuertz, 2003), colony counting methods (Allen *et al*., 2004), immunological assays, Enzyme Linked Immunosorbant Assay method (ELISA) (Van Dyck *et al*., 2001) (Fig. 1) have several shortcomings compared to the analytical methods of detection.

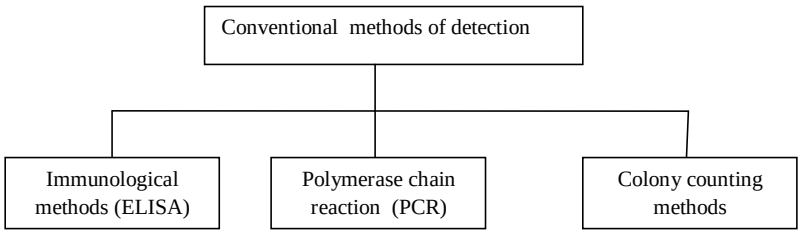


FIGURE 1. Conventional methods of pathogen detection

Conventional methods of detection

Conventional methods include Colony counting methods, Polymerase chain reaction and Immunological methods.

Culture and colony counting methods

The culturing and plating method is the oldest bacterial detection technique and remains the standard detection and quantification method. However, other techniques are necessary because culturing methods are excessively time-consuming. For bacterial detection, 4–9 days are needed to obtain a negative result and between 14 and 16 days for confirmation of a positive result (Brooks *et al*., 2004). This is an obvious inconvenience in many industrial applications, particularly in the food sector. Different selective media are used to detect particular bacterial species. They contain inhibitors (in order to stop or delay the growth of non-targeted strains) or particular substrates that only the targeted bacteria can degrade or that confers a particular color to the growing colonies (Fratamico and Strobaugh, 1998).

Polymerase chain reaction

The research worker nowadays are undertaking studies of the characteristics of the microorganisms that can survive even in pesticide contaminated site (Rohilla and Salar, 2012) at the molecular level towards generating informations and data desired for successful fabrication of rapid molecular methods of detection (Majumdar *et al*., 2012). Polymerase chain reaction (PCR) is a nucleic acid amplification method. It was developed in the mid 80s (Mullis *et al*., 1986) and it is widely used in bacterial detection. It is based on the isolation, amplification and quantification of a short DNA sequence including the targeted bacteria's genetic material. Examples of different PCR methods developed for bacterial detection are: (i) real-time PCR (Lazaro *et al*., 2005), (ii) multiplex PCR (Jofre *et al*., 2005) and (iii) reverse transcriptase PCR (RT-PCR). There are also methods coupling PCR to other techniques such as, for example surface acoustic wave sensor (SAW) (Deisingh and Thompson, 2004) or evanescent wave biosensors (Simpson and Lim, 2005). The PCR is a lot less time-consuming than other techniques, like culturing and plating. It takes from 5 to 24 h to produce a detection result but this depends on the specific PCR variation used and this does not include any previous enrichment steps. PCR method consists of different cycles of denaturation by heat of the extracted and purified DNA, followed by an extension phase using specific primers and a thermostable polymerization enzyme. Then each new double stranded DNA acts as target for a new cycle and exponential amplification is thus obtained. The presence of the amplified sequence is subsequently detected by gel electrophoresis. Amongst the different PCR variants, multiplex PCR is very useful as it allows the simultaneous detection of several organisms by introducing different primers to amplify DNA regions coding for specific genes of each bacterial strain targeted (Touron *et al*., 2005). Real-time PCR permits to obtain quicker results without too much manipulation. This technique bases its detection in the fluorescent emission by a specific dye. It attaches itself to the targeted amplicon given that fluorescence intensity is proportional to the amount of amplified product (Cady *et al*., 2005) it is possible to follow the amplification in real time, thus eliminating laborious post-amplification processing steps such as gel electrophoresis. One of the limitations of PCR techniques lies in that the user cannot discriminate between viable and non-viable cells because DNA is always present whether the cell is dead or alive. Reverse transcriptase PCR (RT-PCR) was developed in order to detect viable cells only (Yaron and Matthews, 2002). RT is an enzyme able to synthesize single-stranded DNA from RNA in the 5´–3´ direction. Several genes specifically present during the bacteria's growth phase can then be detected. This technique gives sensitive results without any time-consuming pre-enrichment step (Deisingh and Thompson, 2004). PCR may also be found coupled to other techniques. Examples are "the most probable number counting method" (MPNPCR) (Blais *et al*., 2004) surface plasmon resonance and PCR acoustic wave sensors (Deisingh and Thompson, 2004), Light Cycler real –time PCR (LC-PCR) and PCR-enzyme-linked immunosorbent assay (PCR-ELISA) (Perelle *et al*., 2004), the sandwich hybridization assays (SHAs) (Leskela *et al*., 2005) or the FISH (fluorescence in situ hybridization) detection test (Lehtola *et al*., 2005).

An oligonucleotide probe that becomes fluorescent upon hybridization to the target DNA (molecular beacon; MB) was evaluated in a real-time polymerase chain reaction (PCR) assay to detect the presence of *Salmonella* species. As low as 1–4 colony-forming units (CFU) per PCR reaction could be detected (Liming and Bhagwat, 2004). The MB beacon probe used in this study was able to detect *Salmonella* spp. from variety of fresh and fresh-cut produce at a very low level of contamination (i.e., at 1–3 CFU/25 g of produce). Attachment of *Salmonella* sp. to food matrices plays a crucial role in their virulence and enables to cause disease at a lower infection dose (Waterman and Small 1998). Moreover, attachment also plays a crucial role in recovery of pathogens from food sources and hence may significantly influence the sensitivity of PCR-based detection protocols.

Recently, Heller (Heller *et al*., 2003) compared different DNA isolation methods to detect Shiga toxin producing *Escherichia coli* O157:H7 isolates from various foods by fluorogenic linear probe (TaqMan) real-time PCR and reported a detection limit of $5.3X10^3$ CFU/g of salad green or ground beef. Fortin (Fortin *et al*., 2001) reported the

InstaGene matrix to be superior to conventional (phenol–chloroform) DNA isolation procedures, and reported a sensitivity of 1 CFU of *E. coli* O157:H7 per milliliter of raw milk or apple juice by MB probe real-time PCR, which is similar to the detection limits observed in this study. Both studies utilized fluorogenic probes, and the combination of superior DNA isolation and sensitive MB probes apparently achieved a better pathogen surveillance capability.

For further use in surveillance and outbreak epidemiology another efficient molecular-based technology such as DNA microarray is used that offers an alternative to screen simultaneously for multiple virulence markers and to assist in the identification of the virulence types of foodborne pathogens (Call, 2005; Uttamchandani *et al*., 2009).

A novel colorimetric method for detection of pathogenic *E.coli* on DNA mircoarrays (photopolymerization)

Photopolymerization is a novel colorimetric detection method for genotyping *E.coli* O157 strains (Kuck and Taylor, 2008; Sikes *et al*., 2008; Dawson *et al*., 2009). For photopolymerization to occur, a streptavidin conjugated photoinitiator is used to specifically label the microarrays that have been hybridized with the biotin-labeled, single-stranded DNA targets. After irradiating at a wavelength absorbed by the photoinitiator (λ 404 nm), polymer forms exclusively in only a few minutes where the probe sequences were spotted on the microarray (Kuck and Taylor, 2008; Dawson *et al*., 2009) polymer formation can be observed after staining.

Photopolymerization is a simple, rapid, and quantitative DNA microarray-based detection method for assessing the genetic composition of *E. coli* O157 strains by examining genes encoding Shiga toxin and selected virulence determinants that have been associated with pathogenic *E. coli*. This approach facilitates the evaluation of the potential virulence of *E. coli* O157 strains and provides relevant information to the food industry for determining the prevalence and risks of pathogenic *E. coli* in food production environments (Quinones *et al*., 2011).

Immunology-based methods

The field of immunology-based methods for bacteria detection provides very powerful analytical tools for a wide range of targets. For example, immunomagnetic separation (IMS) (Mine, 1997; Perez *et al*., 1998) a pre-treatment and/or preconcentration step can be used to capture and extract the targeted pathogen from the bacterial suspension by introducing antibody coated magnetic beads in it (Gu *et al*., 2006). IMS can then be combined with almost any detection method, *e.g*., optical, magnetic force microscopy, magds\flneto resistance (Bead Array Counter) (Baselt *et al*., 1998) and hall effect (Besse *et al*., 2002*)* amongst others. Other detection methods are only based on immunological techniques; in this case the enzyme-linked immunosorbent assay (ELISA) (Crowther, 1995) test is the most established technique nowadays as well as the source of inspiration for many biosensor applications. ELISAs combine the specificity of antibodies and the sensitivity of simple enzyme assays by using antibodies or antigens coupled to an easily assayed enzyme. Antibody arrays have been developed to detect two foodborne pathogenic bacteria (*Escherichia coli* O157:H7 and *Salmonella* spp.) simultaneously using chemiluminescent detecting system. Solid supports using nitrocellulose membrane and poly-l-lysine (PLL) glass slide were compared and optimized for antibody array construction. This study revealed that the PLL slide was a more suitable support due to highly accurate results and the absence of non-specific background. Phosphate-buffered saline (PBS, pH 7.2) and 3% skim milk in PBS buffer were optimal spotting and blocking reagents, respectively. Same sensitivity was achieved for bacterial detection as in a conventional ELISA, 10^5-10^6 CFU/ml for the *E. coli* O157:H7 and 10^6-10^7 CFU/ml for *Salmonella* detections. This antibody array has advantages of a much shorter assay time of 1h and needs small amounts of antibodies. The assay procedure was successfully applied to bacteria added (Karoonuthaisiri *et al*., 2009). Electrochemical measurements combined with molecular detection methods have been proved to be a rapid and reproducible tool for detection of pathogens. Sensitivity and efficacy have been improved for the safety and quality assurance of food. Electrochemical detection system could detect the bacterial contamination at a low level with minimum assay time by the help of a mediator in the bacterial suspension.

An amperometric electrochemical immunoassay for detection and quantification of *Staphylococcus aureus* (*S. aureus*) present in food samples has been established. The method was based on sandwich enzyme immunoassay (EIA) technique where the enzyme label was used to catalyze the dephosphorylation of substrate $NADP^+$ to NAD^+. NAD^+ so formed then catalytically activated an NAD^+- specific redox cycle by incorporating an enzyme amplification step with ethanol, diaphorase and alcohol dehydrogenase. The NADH so formed further reduced the mediator, ferricyanide which induced a redox cycle at Platinum (Pt) electrode. The response obtained had a linear relationship to the increasing concentration *S.aureus* in pure culture as well as in artificially contaminated food samples. The study was also extended for naturally contaminated milk samples and *S.aureus* contamination for this case gave similar result as of the known strain. The detection limit was found to be 10 CFU/ml of *S. aureus* (Majumdar *et al*., 2012).

Another electrochemical approach was made for quantification of catalase positive pathogenic bacteria, *Staphylococcus aureus* by monitoring hydrogen peroxide consumption at Pt microelectrode using Amperometric Biosensor Detector. Hydrogen peroxide consumption was measured amperometrically at +650mV. Different parameters such as hydrogen peroxide concentration, reaction time with hydrogen peroxide, pH and temperature were optimized. The developed methodology allowed the detection of *Staphylococcus aureus* at concentration levels of approximately 10 CFU/mL in assay time of 10 min without any pre-enrichment procedure. This electrochemical approach was also successfully applied to detect *S. aureus* in various food samples (Majumdar *et al*., 2013)

Biosensor

A biosensor is an analytical device which incorporates a biological sensing element integrated within a physicochemical transducer. The aim of a biosensor is to produce an electronic signal proportional to the specific interaction of analytes with the sensing element (Turner,

2000). This means that biosensors essentially transform biomolecular interactions into a digital signal and thereby can be used for detection of analytes ranging from small molecules to whole of pathogenic microorganisms (Skottrup *et al*., 2008). The amplifier in the biosensor responds to the small input signal from the transducer and delivers a large output signal that contains the essential waveform features of an input signal. The amplified signal is then processed by the signal processor where it can later be stored, displayed and analysed (Velusamya *et al*., 2010). Biosensor has been proved to be rapid, economic, consistent assessment technique, which has expanded in many food industries.

Elements of Biosensor

Biosensor has two components, one is bioreceptor and another is transducer (Fig 2).

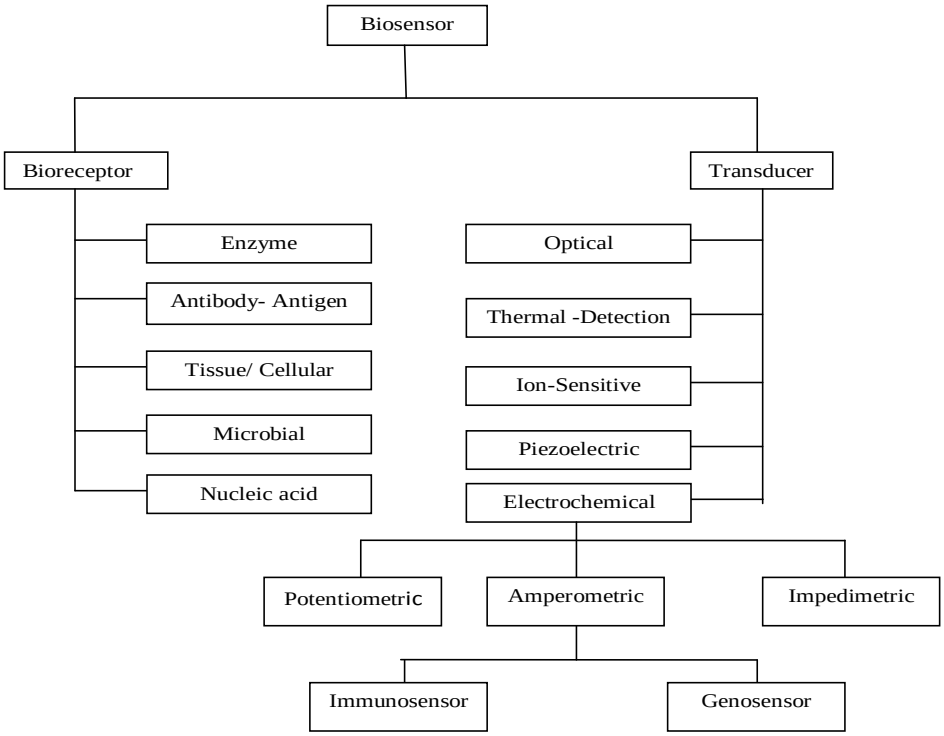


FIGURE 2. Schematic representation of classification of biosensor

Bioreceptors

These are the key to the specificity for biosensor technologies. They are responsible for binding the analyte of interest to the sensor for the measurement. These bioreceptors can take many forms and the different bioreceptors that have been used are as numerous as the different analytes that have been monitored using biosensors. Bioreceptors are further classified into five major different categories such as enzyme, antibody-antigen, tissue/cellular, microbial, nucleic acid (Vo-Dinh and Cullum, 2000).

Enzyme

Enzymes act as bioreceptors based on their specific binding capabilities and catalytic activity. Enzymes are mostly proteins except a small group of catalytic ribonucleic acid molecules. Enzymes require an additional chemical component called as cofactor, which may be either one or more inorganic ions such as Fe^{2+}, Mg^{2+}, Mn^{2+} or Zn^{2+} or a more complex organic or metaloorganic molecule called coenzyme. If an enzyme is denatured its catalytic activity gets destroyed. The activity of an enzyme gets modulated when ligand binds to the receptor. This enzyme activity gets enhanced by an enzyme cascade, which leads to complex reactions in the cell. Thus enzyme-coupled receptors are used to modify the recognition mechanisms (Diamond, 1998).

Antibody-Antigen

Antibodies are biological molecules that exihibit specific binding capabilities for specific structures. An antibody (Ab) is a biological molecule made up of amino acid sequences. For an immune response to be generated against a particular molecule, a molecular size greater than 5000 Da are generally necessary. The way in which an antigen and its specific antibody bind to each other is analogous to a lock and key fit, as an antibody "fits" its unique antigen in a highly specific manner. This unique property of antibodies is the key to their usefulness in immunosensors where only the specific analyte of interest *i.e.* the antigen, fits into the antibody binding site (Vo-Dinh, and Cullum, 2000).

Tissue/Cellular

Cellular structures and cells comprise of bioreceptors used in the development of biosensors. These bioreceptors are either based on biorecognition by an entire cell/ microorganism or a cellular component that is capable of specific binding to certain species. Many cell organelles can be isolated and used as bioreceptors. Whole mammalian tissue slices or in vitro cultured mammalian cells are used as biosensing elements in bioreceptors. Plant tissues are used in plant based biosensors because they are effective catalysts as a result of the enzymatic pathways they possess (Diamond, 1998).

Microbial

Microbial biosensors incorporate a microorganism sensing element which specifically recognizes species of interest. These biosensors are less sensitive to inhibition by solutes and are more tolerant of suboptimal pH and temperature values than enzyme electrodes. They have longer life time. These microorganisms offer a form of bioreceptor that allows a whole class of compounds to be monitored. Microorganisms such as bacteria and fungi have been used as indicators of toxicity or for the measurement of specific substances (Vo-Dinh and Cullum, 2000).

Nucleic acid

Nucleic acids have received increasing interest as bioreceptors for biosensor technologies. The complementarity of Adinine: Thymine (A:T) and Cytosine: Guanine (C:G) pairing in DNA forms the basis for the specificity of biorecognition in DNA biosensors, often referred to as genosensors. If the sequence of bases composing a certain part of the DNA molecule is known, then the complementary sequence can be called as probe (Vo-Dinh and Cullum, 2000). Gene probes are used in detection of disease causing microorganisms in water supplies, food or in plant, animal or human tissues. The specificity of nucleic acid probes relies on the ability of different nucleotides to form bonds only with an appropriate counterpart. The detection of specific DNA sequences provides the basis for detecting a wide variety of bacterial pathogens (Invitski *et al*., 1999). Previously, a radioactively labelled probe was used in DNA hybridization test for bacteria in foods (Feng, 1992). The main disadvantages of radiolabelled probes are the short shelf life of P^{32}-labelled probes, expensive, hazardous and disposal problems associated with radioactive wastes. This method of detecting bacteria by hybridization requires the presence of at least 10^5–10^6 bacteria in the sample to obtain a positive signal which needs pre–enrichment of the target organism without which DNA hybridization approach does not provide the required sensitivity to detect bacteria at required level (Tietjen and Fung, 1995). However, the process of gene amplification i.e polymerase chain reaction (PCR) increases the sensitivity of DNA probes many times (Sailki *et al*., 1985). PCR uses the heat stable DNA polymerase of *Thermus aquaticus* and allows short lengths of double stranded DNA (template) to be copied millions of times in vitro. This PCR–gene probe based assay has high potential for improving detection of food borne pathogen. This is extremely sensitive and specific method. Since sensitivity is not a limiting factor, a promising alternative way to conduct nucleic acid based assays is by using non-radioactive labeled probes, which is associated with the development of biosensor technologies (Wang *et al*., 1997a; Wang *et al*., 1997b; Zhai *et al*., 1997).

Transducer

A transducer should be capable of converting the biorecognition event into a measurable signal. Typically, this is done by measuring the change that occurs in the bioreceptor reaction.

TYPES OF BIOSENSOR

Optical biosensor

Fibre optic was the first commercially available optical biosensors in which pathogens or toxins are fluorescently labelled, which when bound to the surface of the biosensor gets an excitation by the laser wave (635 nm) generating fluorescent signals (10, Taitt *et al*., 2005). NAD(P)+ /NAD(P)H-dependent dehydrogenases are obvious candidates for these biosensors, because NAD(P)H is known to absorb light strongly at 340 nm and fluoresce at 460 nm. Several NAD (P) +/ NAD (P)H-dependent dehydrogenase enzymes exist in variety of food and beverage-related compounds (Luong *et al*., 1997). Optical biosensors have been designed for rapid detection of pathogens (Baeumner, 2003); toxins (Bae *et al*., 2004) and various contaminants (Willardson *et al*., 1998; Tschmelak *et al*., 2004) in food. In recent times antibody coupled fibre optic biosensors have been developed for detection of pathogens like *Salmonella, Listeria, Staphylococcus* sp., *E.coli* (Arora *et al*., 2011).

Thermal - Detection biosensor

This constitutes of enzymes with temperature sensors, when the analyte comes in contact with the enzyme, the heat reaction of the enzyme is measured which is calibrated further against the analyte concentration (Kovacs, 1998). The sensitivity of the technique is as low as 10^{-5} M of substrate concentration which is quite satisfactory for analysis of several food components (Luong *et al*., 1997).

Ion-Sensitive biosensor

Ion sensitive field effect transistor (ISFET) is built on a standard technology which produces source drain and gate regions. The gate uses an ion sensitive membrane that renders ISFET capable of biochemical recognition in the presence of the analyte with the increase in local ion concentration. The hardware component consists of an electrode system that could be conventional platinum or Ag/AgCl microelectrode and a field effect transistor with an ion sensitive gate or gas-sensing electrode (Mohanty, 2001).

Electrochemical biosensor

Electrochemical signal detectors measure an electrochemical response. They have some advantages over other analytical transducing system, such as comparable instrumental sensitivity, possibility to operate in turbid media and possibility of miniaturization which allows even small sample volume (1-20 µl) to be analysed (Jenkins *et al*., 1988). Based on their operating principle, the electrochemical biosensor can employ Potentiometric, Amperometric, Conductimetric and Impedimetric transducers converting the chemical information into a measurable amperometric signal (Pohanka and Skladal, 2008).

Potentiometric biosensor

Potentiometric biosensor is based on ion-selective electrodes (ISE) and ion-sensitive field effect transistors (ISFET). The primary output signal is possibly due to ions accumulated at the ion selective membrane interface (Pohanka and Skladal, 2008). Current flowing through the electrode is equal or near to zero. The electrode follows the presence of the monitored ion resulting from the enzyme reaction (Kauffmann and Guilbault, 1991). A Potentiometric biosensor with a molecularly imprinted polymer constructed for the herbicide atrazine assay allows detection from $3X10^{-5}$ to $1X10^{-5}$ (D'Agostino *et al*., 2006), molecularly imprinted polymer was also used for tracking the level of neurotransmitter serotonin (Kitade *et*

al., 2004). Another Potentiometric biosensor with co-immobilized urease and creatinase on the polyammonium membrane was used for creatine analysis (Karakus *et al*., 2006). The LAPS (Light Addressable Potentiomentric Sensor) biosensor was used for the *Escherichia coli* assay allowing detection limit as low as 10 cells/ml when the specific primary capture antibody was immobilized on the LAPS flow through cell & the secondary antibody labeled by urease was used for sandwich complex formation (Pohanka and Skladal, 2008). These are portable and inexpensive. One major disadvantage associated with these biosensors is the poor selectivity in some food samples (Arora *et al*., 2011).

Amperometric biosensor

Amperometric biosensors are quite sensitive and give more stable output than the potentiometric ones (Ghindilis *et al*., 1998). The working electrode of the amperometric biosensor is usually either a noble metal or a screen printed layer covered by the biorecognition component (Wang, 1999). At the applied potential, conversion of electroactive species generated in the enzyme layer occurs at the electrode and the resulting current (typically nA to μA range) is measured (Mehrvar and Abdi, 2004). Amperometic biosensors can work in two or three electrode configurations. The two electrode system consists of reference and working (containing immobilized biorecognition component) electrodes. The main disadvantage of the two electrode configuration is limited control of the potential on the working surface with higher currents, and because of this, the linear range could be shortened. But with third auxillary electrode being employed this problem was solved. Voltage was applied between the reference and the working electrode and current flows between working and auxiliary electrode (Fig. 3). The amperometric biosensors are used on a large scale for analytes such as glucose, lactate (Ohnuki *et al*., 2007) and sialic acid (Marzouk *et al*., 2007). These biosensors generally rely on the enzyme system involving horseradish peroxidase (HRP) and alkaline phosphatase (AP) that catalytically converts electrochemically inactive analytes into active products. These biosensors are used as immunosensors (antibody based) or genosensors (DNA - based) in food borne pathogen detection (Arora *et al*., 2011).

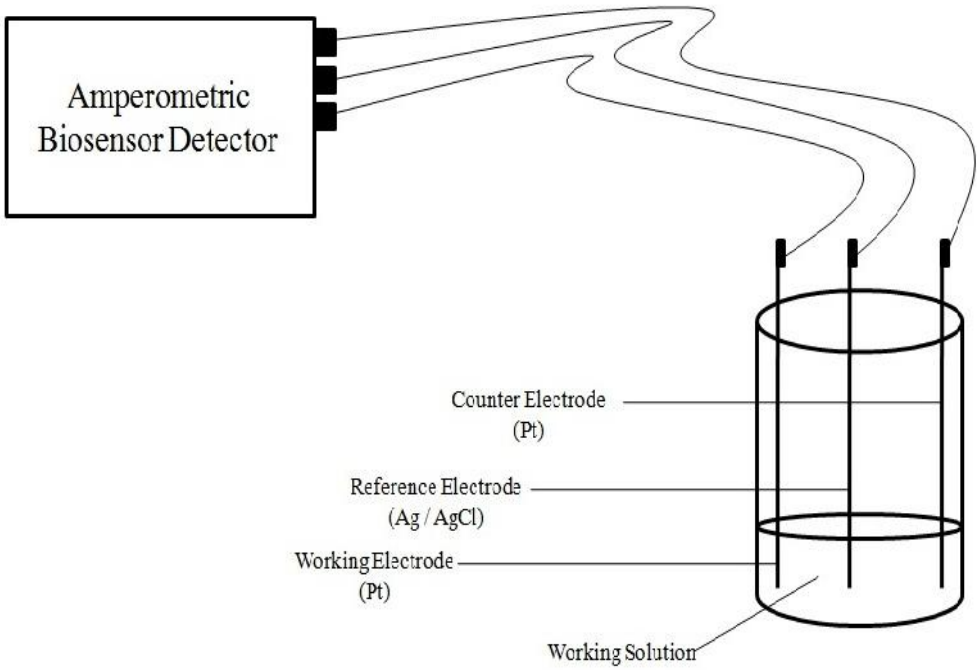
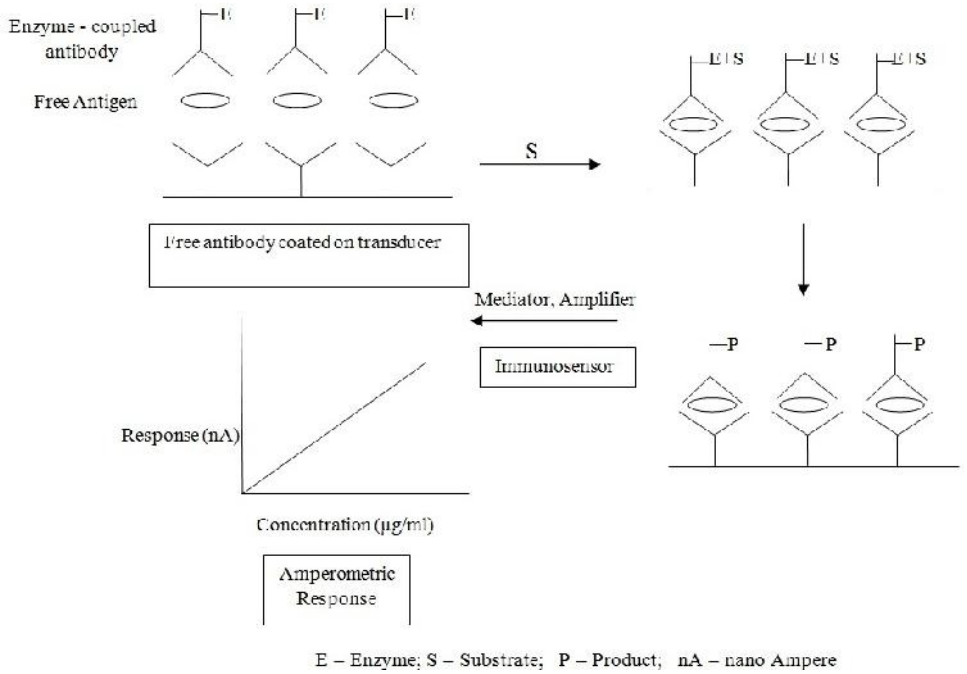


FIGURE 3. Schematic representation for construction of Amperometric biosensor detector

Detection with antibodies specific for particular pathogen is the basic approach maintained in immunosensors (Fig. 4). Here, an enzyme - substrate catalysis in conjugation with an antibody produces products such as pH change, ions, oxygen consumption which are capable of generating an electrical signal on a transducer.

In DNA–based biosensors, short nucleic acid sequences called as probe specific for a particular bacterium were immobilized on the surface of a transducer. Binding between the two complementary sequences does the confirmation for the presence of the target bacteria (Fig. 5). This procedure is termed as hybridization (Arora *et al*., 2011).



E – Enzyme; S – Substrate; P – Product, nA – nano Ampere

FIGURE 4. Schematic representation of an electrochemical immunoassay technique for pathogen detection

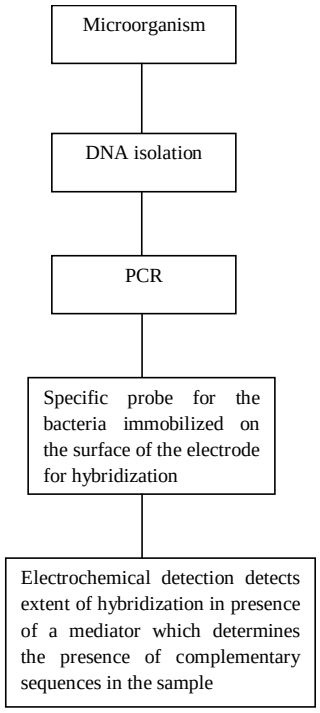


FIGURE 5. Schematic representation of DNA- based biosensor

Impedimetric biosensor

It is a powerful technique used for the detection of electrochemical systems. Such devices follow either impedance (Z) or its component resistance (R) and capacitance (C); inductance typically has only a minimal influence in a typical electrochemical set up. The expression of impedance is as follows:

$Z^2 = R^2 + (1/2\ \pi\ FC)^2$, F stands for frequency

The inverse value of resistance is called conductance and for this reason such systems are also called as conductometric. Impedance biosensor includes two electrodes with applied alternating voltage; amplitudes from a few to 100 mV are used (Pohanka and Skladal, 2008). Analysis is done on the basis of changes in conductance, capacitance and impedance. With microbial metabolic process, conductance and capacitance increase whereas impedance decreases (Invitski *et al*., 2000). Impedance biosensors have been successfully used for microorganism growth monitoring due to the production of conductive metabolites (Silley and Forsythe, 1996). Impedimetric biosensors are less frequent compared to potentiometric and amperometric biosensors. A number of

samples can be analyzed at a single time using impedance microbial technology but the sensitivity of the sensor towards the analyte is less compared to other sensors (Arora *et al*., 2011). Still, there have been some promising approaches, such as hybridization of DNA fragments previously amplified by a polymerase chain reaction (PCR) has been monitored by an impedance assay (Davis *et al*., 2007). A model impedance immunosensor containing electrodeposited polypyrrole film with captured avidin connected through biotin to anti-human IgG was able to detect antibodies as low as 10 pg/ml present in a sample (Ouerghi *et al*., 2002). The applications of new materials (bacteriophage and lectin, the use of nanomaterials) and microfluidics techniques have provided unprecedented opportunities for the development of high-performance impedance bacteria biosensors (Wang *et al*., 2012).

Piezoelectric biosensor

This is also used for pathogen detection. The surface of piezoelectric sensor is coated with bacterial specific antibodies. The binding of bacteria with antibodies results in increased mass of quartz crystal and proportionate decrease in oscillation frequency, which is detected by the quartz crystal microbalance (QCM) on transducer surface (O'sullivan and Guilbault, 1999).

Peptides and nucleic acids are most commonly used as probes in biosensors because of their versatility in forming various tertiary structures. The interaction between the probe and the analyte can be detected by various sensor platforms, including quartz crystal microbalances, surface acoustical waves, surface plasmon resonance, amperometrics, and magnetoelastics. The field of biosensors is constantly evolving to develop devices that have higher sensitivity and specificity and are smaller, portable, and cost-effective.

Applications

It has become possible to expand the range of analytes with more improved sensitivity, specificity and less time consumption for detection of the target analyte in food. Biosensors have immense scope in maintaining food quality and safety.

Biosensors for food borne pathogens

Electrochemical biosensors such as antibody based immunosensors are commonly used for detecting microorganisms in food (Ricci *et al*., 2007). *Escherichia coli, Salmonella typhimurium, Listeria monocytogenes etc*. are common food borne pathogens. Food constituents like milk, meat, cheese, vegetables get contaminated by such microorganisms easily. An analytical approach to amplify the signal output which is more specific and sensitive has been studied. Immobilizing the electrode surface with specific antibody reduces the possibility for nonspecific binding between antibody and bacteria contaminated food samples leading to the amplified response output. In this study, an amperometric immunosensor based on antibody immobilization onto the platinum (Pt) electrode surface by cross-linkage via glutaraldehyde (GA) pre-coated with polyethyleneimine (PEI) layer for the detection of *Staphylococcus aureus* in food samples has been developed. Immobilization of antibodies on to the sensor surfaces lead to a change in response for control (absence of test bacteria) and samples (presence of test bacteria). The changes were quantified by the increase in amperometric response. Response of the sensors to increasing concentrations (10^1-10^8CFU/ml) of pure culture of *S. aureus* NCIM 2602 as well as *S. aureus* inoculated food samples (milk, cheese and meat) was studied and for all the samples similar response pattern was observed. The amperometric response obtained between the increasing concentrations of test bacteria and current output showed good linearity, achieving detection limit down to 10 CFU/ml. Further Scanning Electron Microscopy studies justified the response obtained for amperometric measurements (Majumdar *et al*., 2013).

Gfeller (Gfeller *et al*., 2005) made use of an oscillating cantilever for the detection of *E. coli* in less than 1 hour. In this type of biosensor, measurement was done by the change of resonance frequency of the cantilever array. This change was because of an increase in mass caused due to adsorption of the pathogen on the cantilever. To avoid the interference by any undesired environmental changes a reference cantilever was used. Further binding of bacteria to the surface patterned with specific antibodies has also been described by St. John (St. John *et al*., 1998).

Minunni (Minunni *et al*., 1996) defined a method for detection of *Listeria* in milk in the range of 2.5 $X10^5$ to $2.5X10^7$ cells/crystal with an assay time of 15 min using quartz crystal microbalance displacement assay method. The antibody specific for binding of bacteria was immobilized on the gold coating of the quartz crystal plate and the antigen - antibody binding was monitored real time using a liquid flow cell. Banada (Banada *et al*., 2009) used light scattering sensors for the detection of microorganisms in vegetable and meat samples in the detection limit of single cell/ 25 g of test sample. The forward scattering was able to detect the presence of bacteria based on the distinct colony/ scatter signature (Nayak *et al*., 2009).

Biosensors in agriculture

With increased threat of bioterrorism, the need for biosecurity has become an essential issue to save the nation. The need for biosecurity is necessary when agricultural produce or any living object is to be transported across the international borders. Biosensors may play a major role in this field as they provide rapid and specific detection compared to the older techniques. A biosensor has been developed for the detection of the fungus *Phakopsora pachyrhizi* that causes Asian rust or Soybean rust, using the SPR technique. In this case, antibody against *Phakopsora pachyrhizi* was used as the analyte. The biosensor had a response range of 3.5–28 mg/ml of antigen solution (leaf extract) and a detection limit of 800 ng/ml (Mendes *et al*., 2009). BSA was used as the blocking agent to avoid any non-specific binding between antigen and antibody to occur. Such rapid and simple methods can be developed for world's most acute crop diseases thus preventing major damage (Nayak *et al*., 2009).

CONCLUSION

As the world becomes more concerned about the impact of food on human health, the safety against biowar and the demand for rapid detection techniques has increased commercially. Though conventional pathogen detection methods are sensitive, they lag behind the analytical methods by detection time as they are time-consuming and

laborious for the detection and identification of microbial contaminants in food. Analytical methods are able to detect pathogens in very low concentrations of the samples and must be suitable for in situ real-time monitoring as well. Analytical method involves the development of biological sensors for the detection of pathogens. Biosensors offer rapid, real time and multiple analyses from food samples. Biosensors that rapidly detect total microbial contamination are essential tools for the food quality assurance. There are multiple biosensors that simultaneously detect multiple analyses with minimum interferences and have great applications in the medical diagnostics, food quality control, environmental monitoring and other industries. These greatly reduce time consumption, manpower and cost of identifying and monitoring different substances for example in food, potable water, waste water, rivers, reservoirs etc. Simple, affordable, reliable and portable biosensors have been crucial requirements of consumer's demands. These have been designed to provide detection limits as low as possible with minimum interferences from species in bulk samples.

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