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Review Article

Application of Biosensors for Detection of Contaminants in Milk and Milk Products

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Abstract

There are certain thermoduric organisms in milk and milk products which survive pasteurization temperature. Also, besides pasteurization, there are certain pathogenic organisms which gets activated during storage and packaging of milk products. Therefore, it is very necessary to keep the quality and nutritional aspects of food safe to consume it for the human being before it gets packaged. The advancement in technology is being carried out to in dairy industry, which has emerged out with the use of biosensors for determining the quality of milk and milk products. Biosensors are devices that can combine a biochemical molecule with a physical signal that can be translated into an indication of the safety or quality of food. These devices are evolving as substitutes for the existing conventional techniques. There are different types of biosensors depending upon the substrate to be used. Example- Electrical biosensors, immobilized based biosensors, Optical biosensors, Nano-material based biosensors and microbial biosensors. Various techniques like enzyme substrate assay and spore based biosensors have been experimented at various research institutes in India for detection of thermoduric organisms like *Micrococci spp, Listeria spp* and broad spectrum antibiotics in milk and milk products. These technologies have come out to be cost- effective, highly- sensitive, wider applicability and a good replacement for the existing conventional techniques. Conclusion: India is the major producer and exporter of milk and milk products. The use of this technology can be beneficial for keeping the nutritional and quality aspects of milk and other food products. This will also reduce packaging costs and there is a potential in carrying out online monitoring of raw materials, trace compounds, vitamins, flavors, additives and contaminants in future.

Keywords: Biosensors; Milk and Milk Products; Thermoduric Organisms; Enzyme Substrate Assay; Spore Based Biosensors

Introduction

Biosensors are devices that can combine a biochemical molecule with a physical signal that can be translated into an indication of the safety or quality of the food. Biosensors help carrying out the procedures that are sensitive, selective, rapid, cost effective and portable. These devises are evolving as excellent substitutes for the existing conventional techniques (Figure 1) [1].

Types of biosensors

The classification of biosensors is given in table 1 below:

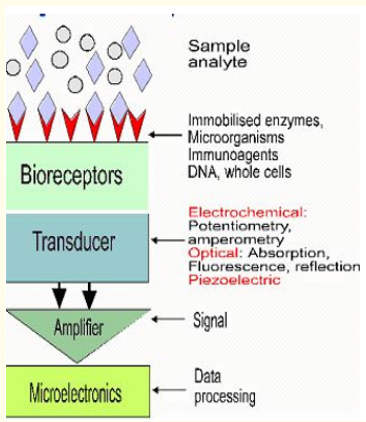


Figure 1: Biosensor.

Signal Transduction	
Electrochemical	Amperometric, Conductometric, Impedimetric, Potentiometric
Optical	Absorption, Fluorescence/ Phosphorescence, Bio/Chemiluminescence, Reflectance, Raman scattering, Refractive index
Mass sensitive	Surface acoustic wave biosensors, Cantilever biosensors, Piezoelectric biosensors
Thermometric	Calorimetric biosensors
Recognition molecule	
Antibiotics	Monoclonal, Polyclonal
Protein receptors	Metatropic receptors, Ionotropic receptors
Whole cells	Microbial sensors, Mammalian cells, Tissues, Bacterial endospores
Nucleic acids	Hybridization, Low weight compound interaction
Enzymes	

Table 1: Classification of biosensors.

Objectives

a) To develop quick, sensitive and reliable techniques for monitoring of dairy products.

b) Ensuring quality and safety of dairy products.

Review of Literature

Types of biosensors

The two main elements in a biosensor are a biological recognition element or bioreceptors and a signal transducer. On the basis of these two elements, biosensors are classified.

Bioreceptor

The bio-receptor is a bio-molecule that recognizes the target analyte and can be divided into three distinct groups: bio-catalytic, bio-affinity and microbe-based systems.

- Bio-catalysis- based biosensors depend on the use of pure or crude enzymes to moderate a chemical reaction. Enzyme inhibition is required for a large number of environmental pollutants such as antibiotic/drug residues, aflatoxin M1, pesticides and heavy metals in food system. Such methods require the use of chromogen/fluorogens for measuring the presence of target contaminants in food. This works on the principle of non-competitive enzyme action on inducer resulting in indirect reduction of starch iodine mixture through penicilloic acid.

- Bio-affinity based biosensors- These biosensors rely on the use of proteins, DNA or microbial receptor to recognize and bind a particular target.

- Microbial biosensors-These biosensors involve application of microorganisms or their spores as biological recognition element. They generally involve the measurement of microbial respiration, or its inhibition by the analyte of interest [2].

The basic requirement of a biosensor is that the biological material should bring the physico-chemical changes in close proximity of a transducer. Thus, immobilization technology has played a major role in this. The biological material is immobilized directly on the transducer or on membranes which can be mounted on the transducer. Immobilization not only helps in forming the required closed proximity between the biomaterial and the transducer but also helps in stabilizing it for reuse.

- Immobilization-based biosensors-Some of the widely used immobilization techniques include absorption, entrapment, covalent binding and cross-linking. Novel techniques have been developed for immobilizing viable or non-viable cells through adhesion on a variety of polymeric surfaces including glass, cotton fabric and synthetic polymeric membranes using poly ethyl- enimine (PEI) [2].

Signal transducer

A signal transducer is the second essential component of a biosensor. It converts the recognition event into a measurable signal. The transducer can take many forms depending upon the parameters being measured. The most well developed classes of transducers are potentiometric, amperometric, conductometric, optical, acoustic or piezoelectric etc. These utilize various electrochemical responses to measure changes in the electrical properties of the biological recognition element. Based on this, the biosensors are:

- Optical biosensors-These employ linear optical phenomena including fluorescence, phosphorescence, polarization, rotation, interference, surface Plasmon resonance (SPR), total internal reflection fluorescence (TIRF). For non-linear phenomena includes second harmonic generation [2].

Application of biosensors for quality assurance of dairy products

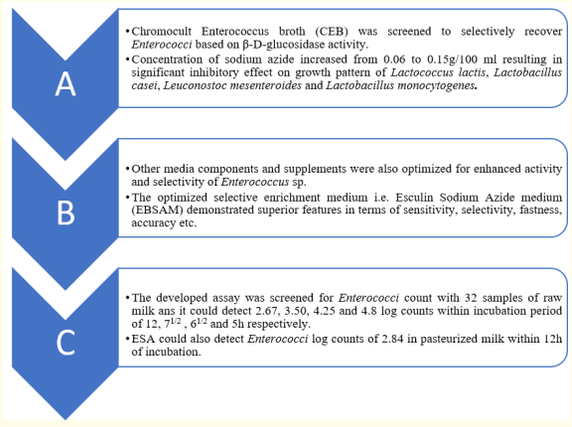
Detection of *Enterococci* in milk

Enterococci are ubiquitous bacteria present in the environment and in the gastrointestinal tract of healthy animals and humans. In milk products, they are used as probiotics resulting in positive effects on human digestibility. As adjunct starter cultures, enterococci release natural antimicrobial substances inhibiting adulteration due to food-borne pathogens. In dairy products, both *Enterococcus faecalis* and *Enterococcus faecium* species are relatively heat resistant. Also, most of the enterococci are resistant to freezing. Therefore, some investigators have associated food poisoning outbreaks with enterococcal bacteria [3].

Development of enzyme substrate assay (ESA) for monitoring Enterococci in milk [4]

Process

An Enzyme Substrate Assay (ESA) based on β-D-glucosidase activity was developed at NDRI, Karnal to meet the emerging demand of dairy industry (Figure 2).



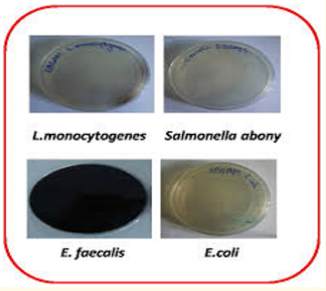


Figure 2: Selectivity of EBSAM.

Novel features of the process [2]

- Cost effective (.97/- per test)
- Better sensitivity
- Consistency in colour development within 5 - 12h.
- Lab Validation with conventional method (ISO 7899-2: 1984)
- Wide spectrum of application for raw, pasteurized and dry milk.
- The test can be applied for assessing the hygiene status of equipment's/utensils, air, water, personnel and plant environment.
- Patents have been filed and a non-exclusive license cost of the technology is~ 3.5 lakhs.

Spore based biosensor for detection of *Enterococci* in milk [5]:

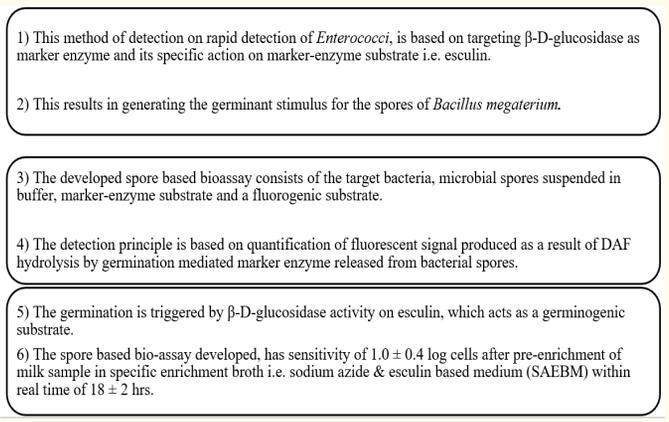


Figure 3

Advantages of using spore based biosensors

- Spore based biosensing system has a long shelf life. The analytical performance of the spore-based sensing sys tems has been retained for as long as for 8 months, when kept as dried spores at room temperature [6].
- The spore germination process completes within min utes of sensing the germinants in the environment. This can produce a real time response for detection of analyte [7].
- Using the spore as biosensing element is a low priced process as the spores are required in micro liters only and they can be immobilized effortlessly. This helps in curtailing the cost of biosensor [8].

- Based on above characteristics spores have been em ployed as vehicles to preserve, store and trans port the whole-cell bacterial biosensing systems [9].

Detection of *Listeria monocytogenes* in milk

Currently, *Listeria monocytogenes* is considered one of the most important pathogens responsible for food-borne infection. It is often incriminated in outbreaks of human listeriosis [10]. Pregnant women, infants, immune-compromised and the elderly people are at greatest risk for listeriosis [11]. In dairy industry, Listeria can contaminate directly or indirectly the products and the environment through contaminated raw milk, resulting in huge losses both in terms of public health and economy. This sensitizes the scientific and medical communities to focus on the safety of these products (Marnissi., *et al*, 2013).

A Novel enzyme-substrate based bio-assay for real time detection of *Listeria monocytogenes* in milk [2]:

This test is based on the principle of targeting enzyme- substrate reaction using chromogenic substrate for specific unique marker enzymes of target bacteria to release free chromogen that can be visually detected by colour change or by calorimetrically activity after initial enrichment of the bacterium in novel selective medium.

Novel features

- Cost effective (91.01/- per test)
- Better sensitivity
- Consistency in colour development within 5 - 24 h.
- Lab Validation with conventional method (ISO: 14988 [Part- 2]: 2002/ ISO: 11290-2:1998).
- Wide spectrum of application for raw, pasteurized and dry milk.
- The test can be applied for assessing the hygiene status of equipment's/utensils, air, water, personnel and plant environment.
- Patents have been filed and a non-exclusive license cost of the technology is- 3.5 lakhs.

Spore based biosensor for detection of *Listeria monocytogenes* [12]:

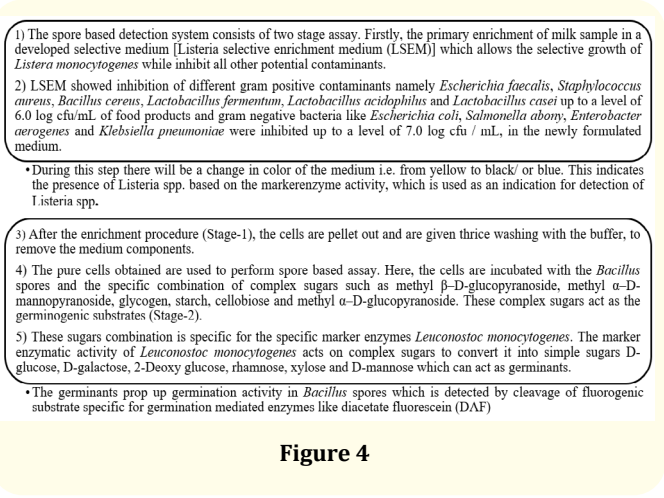


Figure 4

Advantages

1. The detection time for assay is 20 hrs. for detecting up to 1 log cells in the milk sample.
2. The assay can be applied to different milk and milk prod ucts.
3. The developed assay is much superior over culture based methods or PCR as it does not require further biochemical tests, moreover, the assay is based on the specific marker enzyme activity, which detects only viable cells and not dead cells.

Detection of aflatoxin M1 in milk

Aflatoxin M1 (AFM1) is a potent carcinogen, teratogen and mutagen found in the milk when lactating animals consume feed contaminated with aflatoxin B1(AFB1) [13]. When animals consume feed contaminated with AFB1, it is bio-transformed to AFM1 by the hepatic microsomal mixed-functionoxidase system and gets absorbed in the milk of mammals [14,15]. The residues of AFM1 are stable enough to survive in raw and processed milk, hence they are known as milk toxins [16]. The carcinogenicity of AFM1 is nearly 2 - 10% higher than the original form AFB1. Moreover, AFM1 together with aflatoxins B2 and G1 can cause DNA damage, gene mutations, chromosomal anomalies, immuno-suppression and cell transformation in humans [17,18].

Thus, for detection of aflatoxin M1 in milk, a spore inhibition based –enzyme substrate assay (SIB-ESA) has been developed at NDRI, Karnal and patented (Patent Reg # 3064/ DEL/ 2010) [5].

Process [19,20]:

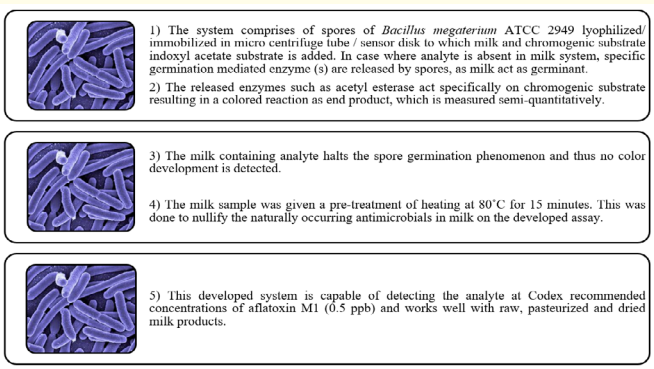


Figure 5

Detection of antibiotics in milk

Antibiotics are widely used in dairy cattle management for the treatment and prevention of diseases and as dietary supplements. They may be administered orally as feed additives or directly by injection [21]. The presence of antibiotic residues at levels higher than the MRL in foods may cause public health hazards including toxicological, microbiological, immunological and pharmacological hazards [21].

Detection of broad spectrum antibiotic residues in milk

Principle

Assay involves the transformation of dormant spores of *Bacillus stearothermophilus* 953, into active vegetative cells. The germination process of the spores, specifically in presence of antibiotic residues is inhibited. This principle was used as a novel approach for monitoring target contaminants in milk [2].

Process

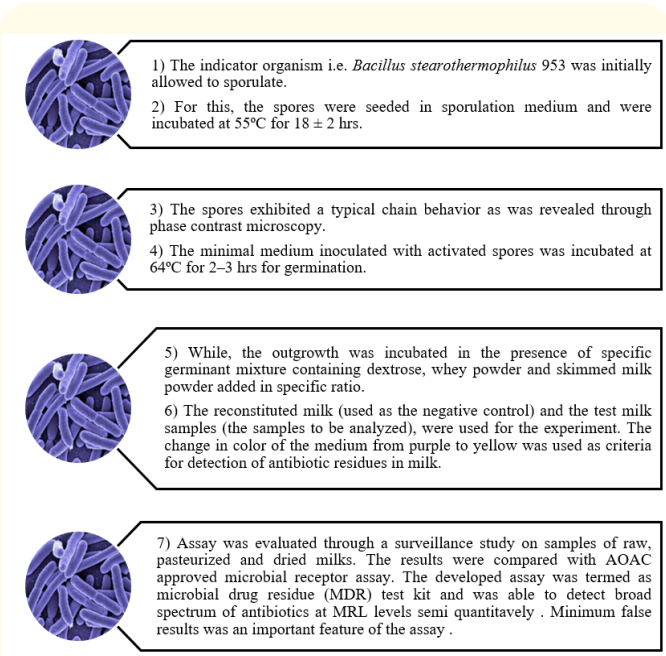


Figure 6

Detection of tetracycline in milk using spore [22]

In cattle, tetracycline antibiotic can be used when treating general, respiratory, urinary, and local infections [23]. Goldfrank., *et al.* [24] stated that tetracyclines have been reported to cause hypouricemia, hypokalemia, proximal and distal renal tubular acidosis in humans.

1. A new strategy was developed for establishing an indirect tetracycline assay using SPR based on the resistance mechanism of tetracycline in Gram-negative bacteria. The estimated limit of detection of tetracycline in raw milk was 15 µgl-1.
2. To determine sulfamethazine residues in milk, an SPR sensor was built by covalently immobilizing sulfamethazine onto a carboxymethyl dextran- modified gold film on the surface.

Process [19,20]:

1. Polyclonal sulfamethazine antibodies were added to sam ples, and free antibodies were detected.
2. No cross-reactivity of antibodies with other antibiotics was found.
3. An immunosensor was developed for the detection of sul fonamide antibiotics in milk.
4. The immunosensor combines generic immunoreagents with a wave-guide-interrogated immune sensor label-free optical biosensor.
5. It was reported that the developed system allowed dis crimination between milk contaminated with sulphon amide antibiotics (sulfapyridine) at or above the maximum residue level (MRL) (100 μgl^{-1}).

Detection of bacterial contamination in sterile UHT milk with a D- and L-Lactate Biosensor [25]

Monitoring bacterial contamination in milk is especially important in the control of long life milk treated in ultra-high temperature (UHT).

Principle

D-lactate oxidase is unavailable commercially and therefore, D-lactate can only be measured through the use of D-lactate dehydrogenase. Thus, this biosensor is developed based on the principle- "Reactions catalyzed by the dehydrogenase enzymes depend on the presence of the NAD+ coenzyme to produce NADH which is the electrochemically active substance". L-lactate is measured by acceleration of milk fermentation of any selected bacteria at elevated temperature. The progress of fermentation is generally followed by measuring pH. If pH decreases, this indicates bacterial contamination [26-31].

Process

1. Biosensor based on L- and D-lactate directly in milk was incubated with Staphylococcus aureus. The biosensor measures the reduced form of nicotinamide ad enine dinucleotide (NADH) which is formed at a graphite electrode held at +500mV with a silver/silver chloride reference electrode. Also, it produced a slight pH varition which indicated bacterial contamination in milk.
2. This biosensor is well- developed using micro dialysis fiber and a pre-electrolysis cell. Micro dialysis is a sampling procedure to filter out all molecules with high molecular weight (proteins, etc.) and pre-electrolysis was optimized to eliminate all residual elec troactive compounds.

Table 2 below showing the pH variation of D- and L-lactate concentrations, measured after fermentation with different bacterial species.

Innoculum	Measurement		
	pH	L-lactate (mM)	D-lactate (mM)
Control	6.6	0.32	0
Staphylococcus faecalis	5.56	21.6	-
Bacillus coagulans	6.21	4.76	-
Enterobacter sakazakii	5.62	0.4	10.7
Staphylococcus aureus	6.79	0.12	0.72
Bacillus sphericus	6.56	0.31	0

Table 2: pH D- and L- Lactate concentrations measured after fermentation.

Hurdles to application of biosensors include

- Diversity and complexity of samples.
- Relatively high development costs for single analyte systems.
- Limited shelf and operational life.

Conclusion

India is the major producer and exporter of milk and milk products. The use of this technology can be beneficial for keeping the nutritional and quality aspects of milk and other food products. This will also reduce packaging costs and there is a potential in carrying out online monitoring of raw materials, trace compounds, vitamins, flavours, additives and contaminants in future.

Future Trends

There are number of areas where the unique capabilities of biosensors might be exploited to meet the requirements of environmental monitoring. Therefore, advances in areas such as multi-pollutant screening could allow these techniques to be more competitive. The present scenario demands for increased range of detectable analytes with portable device structure.

Therefore, solution to these resulting issues will require further convergence with associated technologies such as biochemistry, polymer chemistry, electronics, micro-fluidics and separation technology. Micro-electro-mechanical systems or MEMS technology is one of the promising areas that may be going to fulfill these demands in future. The technology is an integration of mechanical elements, sensors, acutators and electronics on a common silicon substrate through micro fabrication technology. Biochips and sen-

sor arrays for detection of wide range of hazardous chemical and biological agents can be made out of these MEMS based devices, making it feasible for simultaneous detection of multiple analytes.

This also brings the lab-on-chip concept. However, Immobilization and stabilization of bio-molecules on these Nano-devices may be a greater challenge. Some of the works in these areas have already been initiated. Utilization of molecular recognition ability of biomolecules like avidin-biotin or streptavidin-biotin in conjunction with a lithographic technique is being investigated for the micro immobilization of enzymes on silicon wafers for biosensor applications. Immobilization of enzymes on silicon supports has attracted attention in biosensor chip technology and a variety of classical techniques have been proposed.

There are interesting possibilities within the field of biosensors. Given the existing advances in biological sciences, coupled with advances in various other scientific and engineering disciplines, it is imminent that many analytical applications will be replaced by biosensors. A fruitful fusion between biological sciences and other disciplines will help to realize the full potential of this technology in the future [2].

There is a big potential for on-line monitoring of raw materials, trace compounds, vitamins, flavours, additives and contaminants. In future, on-line use of biosensors provides feedback control of both the component and microbial levels of these and similar processes by continual on-line monitoring. Quality control in food and dairy industry still relies on human senses such as smell, sight and taste. Quality control of microbial spoilage, oxidative rancidity and fruit ripening by tasting may be replaced by biosensors. Though biosensors are not cheap today but in due course of time due to their wide applications may become cheap. The cost of biosensor depends upon its design for the specific parameter to be analyze and the biological component to be used. Looking to the scope, need, applications and advantages of biosensors in food and dairy industry these are economically viable.

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Volume 1 Issue 12 December 2018