

Bacterial Spore Based Biosensor for Detection of Contaminants in Milk

Kumar N*, Thakur G, Raghu HV, Singh N, Sharma PK, Singh VK, Khan A, Balhara M, Avinash, Lawaniya R, Kouser S, Tehri N, Gopaul Rajesh and Shivani Arora

Food Safety and Microbial Biosensor Laboratory, Dairy Microbiology Division, National Dairy Research Institute, Karnal, Haryana, India

Abstract

Milk and milk products comprise essential nutrients for all age groups. Hence ensuring the quality and safety of dairy products offered to the consumers is mandatory objective of the dairy industry. Bacterial spores one of the hardest forms of life can be exploited as biosensing element over the cell based detection system because of longer shelf life of the spores. Bacterial spores in the presence of favorable conditions germinate into metabolically active vegetative cells. The ability of spores to carry out cycle between the spore state and germinated cell contributes to their attractiveness as biosensing systems. The presence of contaminants such as antibiotics, aflatoxin and bacteria affects the life cycle events of bacterial spores and hence can be exploited as platform for detection of aforementioned in milk. This review focuses on the application of bacterial spores as biosensing system for contaminants in milk system. Furthermore, we have focused our attention on the discussion of principal concepts of spore structure and spore germination and examples of spore based detection systems that have been achieved up until now to detect potential contaminants in milk in our laboratory.

Keywords: Spore; Contaminants; Milk; Biosensing element

Introduction

Milk, being a major constituent of the human diet, its quality assurance is considered essential to the health and welfare of community. Now a day's consumer demand for a product that has consistent quality, good taste and a longer shelf life and most importantly safe for consumption will ultimately benefit dairy producers by increasing consumption of this type of high quality products. Therefore the standards for a number of microbial and non-microbial contaminants have been specified for the first time as a legal requirement by Food Safety & Standards Authority of India (FSSAI) for milk and milk products in India. The testing of milk and milk products for presence of contaminating agents has become a mandatory practice for dairy industry before dispatching the products into the global market. Till date the conventional testing methods are considered as gold standards and are of first choice of the quality control laboratory of every dairy industry. But these conventional procedures are laborious to perform and more importantly requires 2-7 days on an average for complete testing of a milk product. But the industry cannot hold the product for so long time and hence by the time product is pushed into the market. The only thing that industry can do is banning the product and recalling the product. To overcome this unmanageable situation dairy industry is looking for alternatives to conventional methods which are rapid, cost effective, and easy to perform and significantly validated with approved standard methods. Our laboratory is playing a prominent role in this field by developing spore based detection system for monitoring microbial and non-microbial contaminants in milk and milk products and thus paving the way for ensuring fresh and healthy milk for consumption.

Biosensor

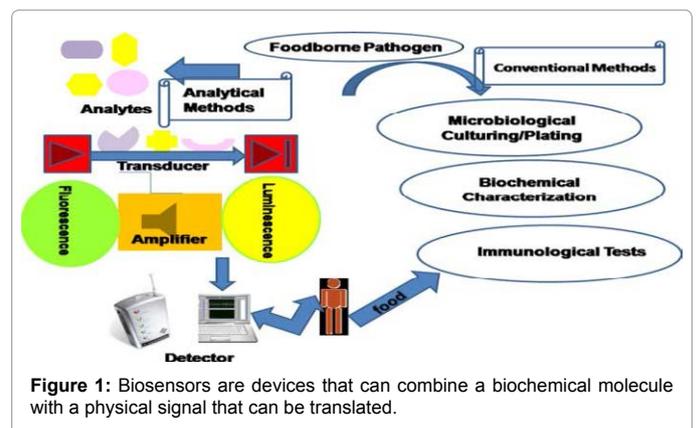
Biosensors are devices that can combine a biochemical molecule with a physical signal that can be translated into an indication of the safety or quality of the food which is sensitive, selective, rapid, cost effective, and portable can be an alternative for existing conventional techniques. But still operation of biosensors is a challenging task for their utility owing to the cost and shelf life of bio-recognition molecule (Figure 1).

Classification of biosensor

It may be classified according to the biological specificity conferring mechanism or to the mode of signal transduction, or alternatively a combination of both. Further the classification biosensors are depicted in Table 1.

Spore

The basic structure: The members of genera *Clostridium* and *Bacillus* have the aptitude to form endospores during stress and starvation conditions. The dormant spore state of spores is very much



*Corresponding author: N Kumar, Food Safety and Microbial Biosensor Laboratory, Dairy Microbiology Division, National Dairy Research Institute, Karnal, Haryana, India, Tel: +91-184-2259187; Fax: +91-184-2250042; E-mail: nrshgoyal@yahoo.com

Received September 06, 2013; Accepted October 28, 2013; Published November 05, 2013

Citation: Kumar N, Thakur G, Raghu HV, Singh N, Sharma PK, et al. (2013) Bacterial Spore Based Biosensor for Detection of Contaminants in Milk. J Food Process Technol 4: 277. doi:10.4172/2157-7110.1000277

Copyright: © 2013 Kumar N, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Signal Transduction	
Electrochemical	Amperometric; Conductometric; Impidometric; 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	
Antibodies	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.

stable allowing them to survive even for millions of years. The typical structure of spore is responsible for its survival ability even millions of years in dormant state [1] as shown in Figure 2. The spore structure comprises of number of layers namely outermost cortex made up modified peptidoglycan. The next layer to cortex is multilayered protein shell known as coat divided into inner coat and an outer coat. The inner coat is casing the nucleic material in condensed state complexed with small acid soluble proteins SASPs, which make up more than 20% of the spore protein composition [2].

The role of SASPs is to protect the nucleic material against the stress conditions of surrounding adverse environment such as extreme temperatures, pHs, humidity levels and radiations such as UV and gamma radiation [3]. Even though spores appear dormant and rugged, the spores persistently scrutinize the environment for the presence of nutrients (sugars & amino acids) that triggers germination and finish with vegetative cells [4].

Spore germination: The irreversible process of spore germination is usually triggered by presence of signaling molecules in the environment marking the conversion of dormant spores into metabolically active vegetative cell. The signaling molecules which naturally induce germination are nutrients termed as germinants. The chemical nature of these germinants include are single amino acids, sugars or purine nucleosides, combinations of nutrients can also lead to spore germination as mixture asparagine, glucose, fructose and K^+ (AGFK) triggers spore germination in *B. subtilis* [5]. The germinants are species and strain specific for spores. Nutrient induced germination involve specific receptors (GRs; so named for the best studied GR in *B. subtilis*, GerA) localized in the inner membrane which are activated by germinants by allosteric interaction which are located in the inner-membrane of the spore [6-8]. The binding of germinants to specific receptors initiates a series of events such as loss of refractivity well synchronized with the release of Ca^{2+} DPA, a rapid efflux of monovalent cations (H^+ , Na^+ and K^+) and water to the core resulting in a partly dehydration [9,10]. Afterward, there occurs removal of physical constraints i.e. spore layers by release of cortex lytic enzymes and allow core to expand [11]. The cortex degradation is followed reactivation of enzymes and the synthesis of ATP from the 3-PGA (3-phosphoglyceric acid) precursor by the rehydrated core. The SASP (Small Acid Soluble Proteins) are degraded to release the DNA and for initiation RNA, protein and DNA synthesis in the further outgrowth phase [4,12]. The various events that occur during spore germination such as release of Ca^{2+} DPA, loss of refractivity or release

of germination mediated enzymes can be used as parameters to detect the onset of spore germination.

Exploiting spore as biosensing element: The presence or absence of microbial and non microbial contaminants affects the spore germination phenomenon in two different ways. The presence of non microbial contaminants i.e. antimicrobials such as antibiotics, aflatoxin and pesticides in milk impede the spore germination and restrict spore from releasing germination mediated enzymes and Ca-DPA even if germinant is present, this phenomenon is known as spore germination inhibition principle. While the microbial contaminants such as Enterococci and *Listeria monocytogenes* in milk act on specific complex sugars and convert them to simple sugars by their specific marker enzymes action. These simple sugars act as germinants and lead to spore germination as the presence of microbial contaminants in milk argument the spore germination and can be detected. This principle is known as germinant-germinogenic substrate principle.

Advantages of using spore as biosensing elements: The spore based biosensing systems are much superior in terms their activity and viability. Firstly spore based biosensing system has a long shelf life, as according to the studies of [13] analytical performance of the spore-based sensing systems, was retained up to a period of 8 months when kept as dried spores at room temperature. Secondly, the spore germination process completes within minutes of sensing germinants in the environment so it can produce a real time response for detection of analyte. Thirdly, the spore production is a low priced process and its immobilization is an effortless process which curtails the cost of biorecognition molecule employed in a biosensor. Based on above characteristics spores were employed as vehicles to preserve, store and transport the whole-cell bacterial biosensing systems [14].

Detection of antimicrobial agents in milk using spores as biosensing element: The prevalence of antimicrobial agents in milk is rising day by day. Antibiotics are a group of antimicrobials that are routinely administered to dairy animals as therapeutics and to prevent outbreak of diseases. This antimicrobial agent pave its way into the milk due to usage of unapproved antibiotics, extra label dosages, failure to observe withdrawal periods, and lack of proper treatment records. The residuals of used antibiotics comes into milk causing consumers various problems such as allergic reactions, decreased antimicrobial susceptibility in bacteria of medical importance and the potential spread of antibiotic resistance. In addition, antibiotic residues inhibit the growth of lactic acid bacteria, which is a problem for fermented milks manufacturer. Another antimicrobial agent which is of concern is Aflatoxins which are well-known hepatocarcinogens,

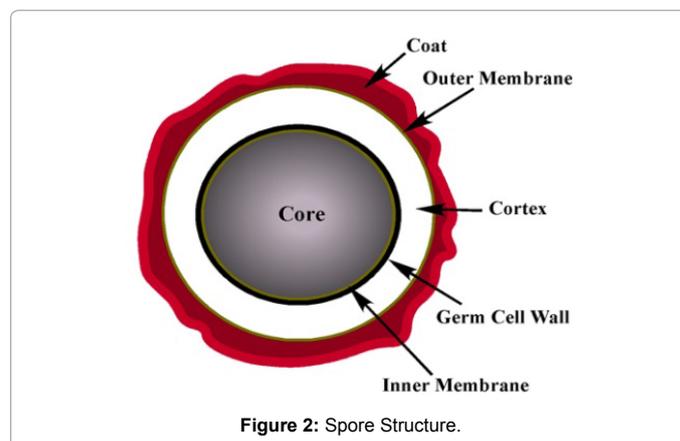


Figure 2: Spore Structure.

mutagens, and immunosuppressive agents produced as secondary metabolites by *Aspergillus flavus* or *A. parasiticus* [15]. Aflatoxin M1 (AFM1) is hydroxylated metabolite of AFB1 and exhibits a high level of genotoxic activity with serious health risk in dairy food chain because of this mycotoxin accumulates and damages DNA [16]. The regulatory authorities such as European Union and Codex Alimentarius, 2001 has given legal standards for presence of this antimicrobial in milk and milk products i.e., 0.5 ppb and 0.05 ppb respectively.

Detection of aflatoxin M₁ in milk: For detection of aflatoxin M₁ in milk a spore inhibition based-enzyme substrate assay (SIB-ESA) has been developed and patented (Patent Reg # 3064/ DEL/ 2010) [17]. The system comprises of spores of *Bacillus* spp. lyophilized/ immobilized in micro centrifuge tube/ sensor disk to which milk and substrate is added. In case where analyte is absent in milk system, specific germination mediated enzyme(s) are released by spores as milk act as germinant. The released enzymes act specifically on chromogenic or fluorogenic substrate resulting in colored reaction or fluorescence as end product which is measured semi-quantitatively by either visually or using optical system at specific excitation/emission spectra (Figure 3). The milk containing analyte halts the spore germination phenomenon and no color development or fluorogenic reaction is detected. The developed system is capable of detecting the analyte at Codex recommended concentrations of aflatoxin M₁ (0.5 ppb) and works well with raw, pasteurized and dried milk products [18,19].

Detection of β-lactam antibiotics in milk: A similar system for monitoring of β-lactam antibiotics in milk has been invented (Patent Reg No. 115/DEL/2009) [20]. It is based on the principle of resistance mechanism of some β-lactamase generating *Bacillus* spp. Some spore forming bacteria such as *B. cereus* and *B. licheniformis* produce β-lactamase enzyme due to induction by β-lactam antibiotics and the enzyme production is proportional to the concentration of inducer present in milk. A real time microbial assay based on β-lactamase enzyme using starch iodine as colour indicator has been developed. The microbial assay is working on principle of non competitive enzyme action on inducer (β-Lactam) resulting in indirect reduction of starch iodine mixture through penicilloic acid. A comparison of

the intensity of the test reaction with that of a control was taken as criteria to determine whether the sample is positive or negative. The assay can detect specifically β-lactam groups in spiked milk within 15-20 min at regulatory codex limits with negligible sensitivity towards non β-lactam groups. The presence of Inhibitors other than antibiotic residues in milk did not interfere with the working principle [21] (Figure 4).

Spore germination based assay for monitoring antibiotic residues:

Assay involves the transformation of dormant spores of *Bacillus stearothermophilus* 953 into active vegetative cells. The inhibition of germination process specifically in presence of antibiotic residues was used as a novel approach for monitoring target contaminants in milk. The indicator organism i.e., *B. stearothermophilus* 953 was initially allowed to sporulate by seeding in sporulation medium and incubating at 55°C for 18 ± 2 h. The spores exhibited a typical chain behavior as revealed through phase contrast microscopy. The minimal medium inoculated with activated spores was incubated at 64°C for 2-3 h for germination and outgrowth in presence of specific germinant mixture containing dextrose, whey powder and skimmed milk powder added in specific ratio along with reconstituted milk as negative control and test milk samples. The change in color of the medium from purple to yellow was used as criteria for detection of antibiotic residues in milk (Figure 5). The efficiency of the developed assay was evaluated through a surveillance study on 228 samples of raw, pasteurized and dried milks and results were compared with AOAC approved microbial receptor assay. The presence of antibiotic level was 10.08% at Codex maximum residual limit having false positive result only in 0.43% of the samples. The results of the present investigation suggest that developed spore based assay can be a practical solution to dairy industry for its application at farm level, milk processing units, independent testing and R&D Centres in order to comply with the legal requirements set by Codex.

Detection of microbial contaminants in milk: Each year various food borne illness outbreaks and food recalls are reported because of actual or potential bacterial contamination. In view of this Food Safety Standards Authority of India (FSSAI) has made it mandatory for the

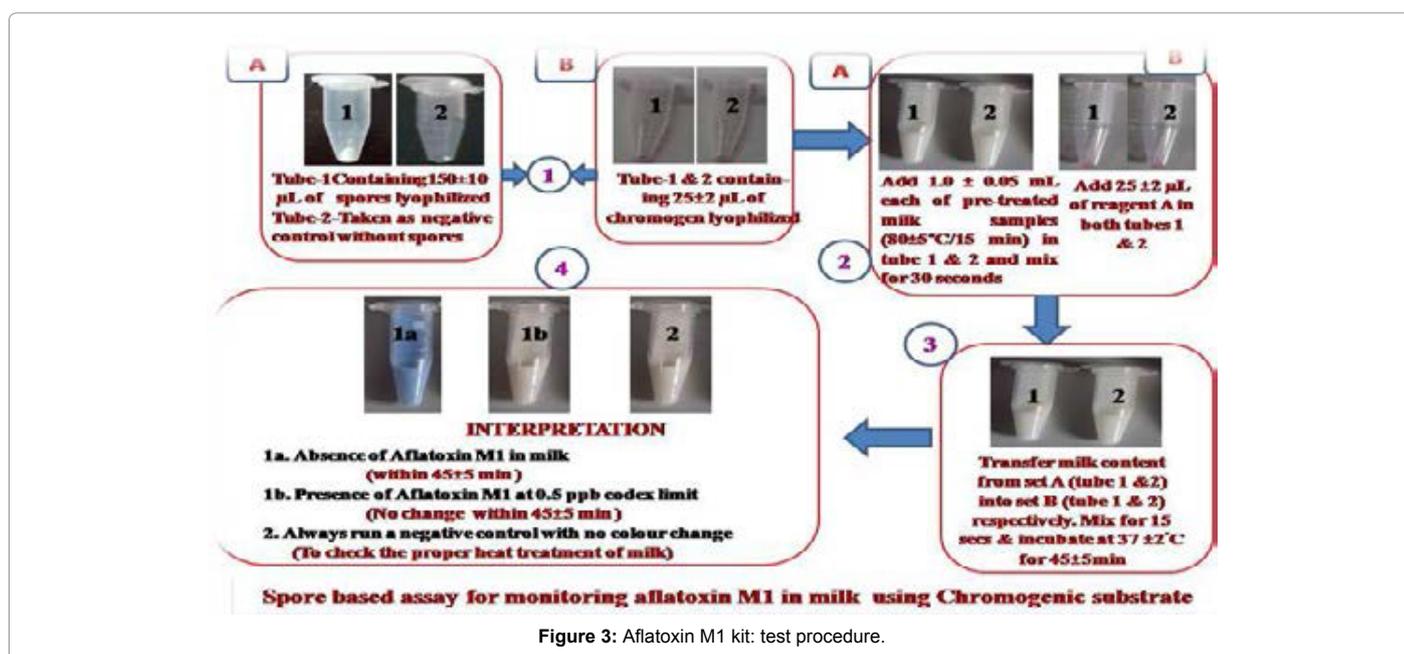


Figure 3: Aflatoxin M1 kit: test procedure.

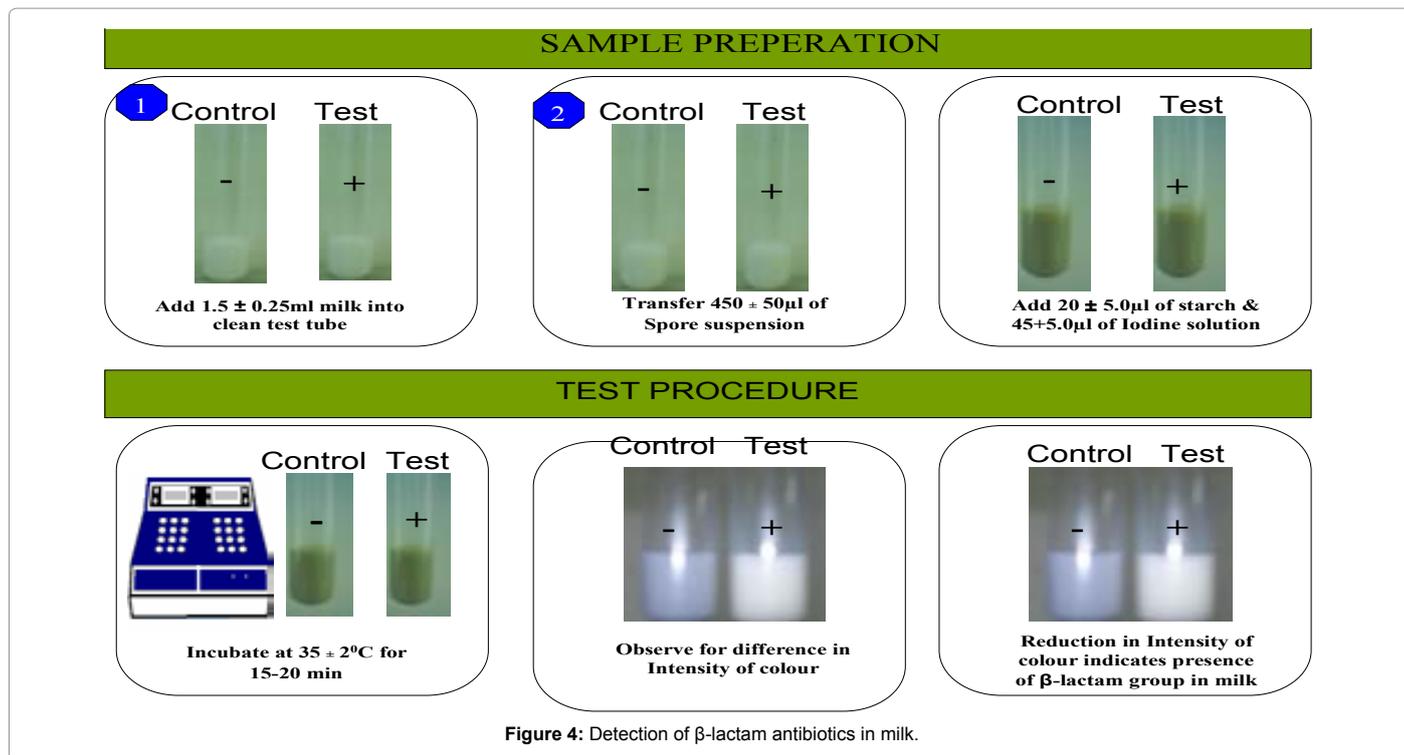


Figure 4: Detection of β -lactam antibiotics in milk.

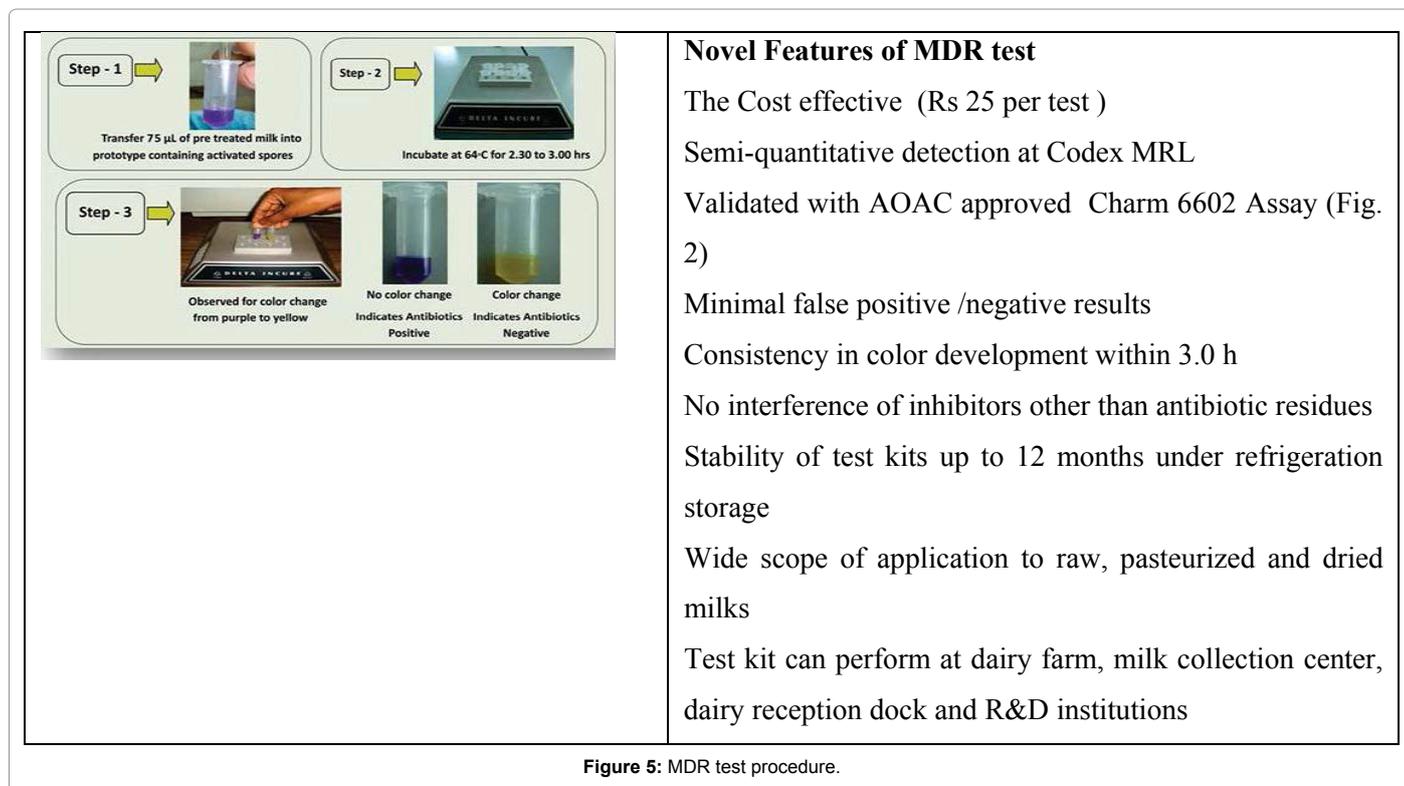


Figure 5: MDR test procedure.

food and drugs department of every state to test for harmful bacteria like *E. coli*, *S. aureus* and *Listeria monocytogenes* before pushing it into the market. The producers will have to declare on their packets that the milk is certified free of the any microbial contamination. The conventional methods used for isolation and detection of microbial

contamination although known as gold standards are laborious and time consuming. So dairy industry can't rely on these methods and is looking for alternative methods which are rapid, specific and well validated with approved standard ISO methods. In our laboratory spore based biosensing assays have been develop for detection of

Enterococci and *L. monocytogenes* based on germinant-germinogenic substrate principle.

Spore based biosensor for detection of *L. monocytogenes*: The spore based detection system consist of two stage assay firstly the primary enrichment of milk sample in a developed selective medium, *Listeria* selective enrichment medium (LSEM) which allow selectively the growth of *L. monocytogenes* while inhibit all other potential contaminants [22]. The enrichment period depends on initial no. of cells present in the milk and milk products. So, during this step there will be a change in color of the medium i.e. from yellow to black/blue, indicating the presence of *Listeria* spp. based in marker enzyme activity and is used as an indication for detection of *Listeria* spp. (Figure 6). This enrichment phase is termed as Stage-1. After enrichment of cells, cells are pellet out and washed with buffer thrice to remove medium components. The pure cells obtained are used to perform spore based

assay which consist of incubating cells with *Bacillus* spores and specific combination of complex sugars acting as germinogenic substrates. These sugars combination is specific for specific marker enzymes *L. monocytogenes*. The marker enzymatic activity of *L. monocytogenes* acts on complex sugars to convert it into simple sugars which can act as germinants. The germinants prop up germination activity in *Bacillus* spores which is detected by cleavage of fluorogenic substrate specific for germination mediated enzymes i.e. diacetate fluorescein (DAF). The whole assay can be carried out in compatible 96 well plate fluorometric microtitre assay. The detection time for assay is 20 hrs for detecting up to 1 log cells milk sample and assay can be applied to different milk and milk products.

Spore based biosensor for detection of Enterococci: Spore based detection system for rapid detection of Enterococci is based on targeting β -D-glucosidase as marker enzyme and its specific action on marker-

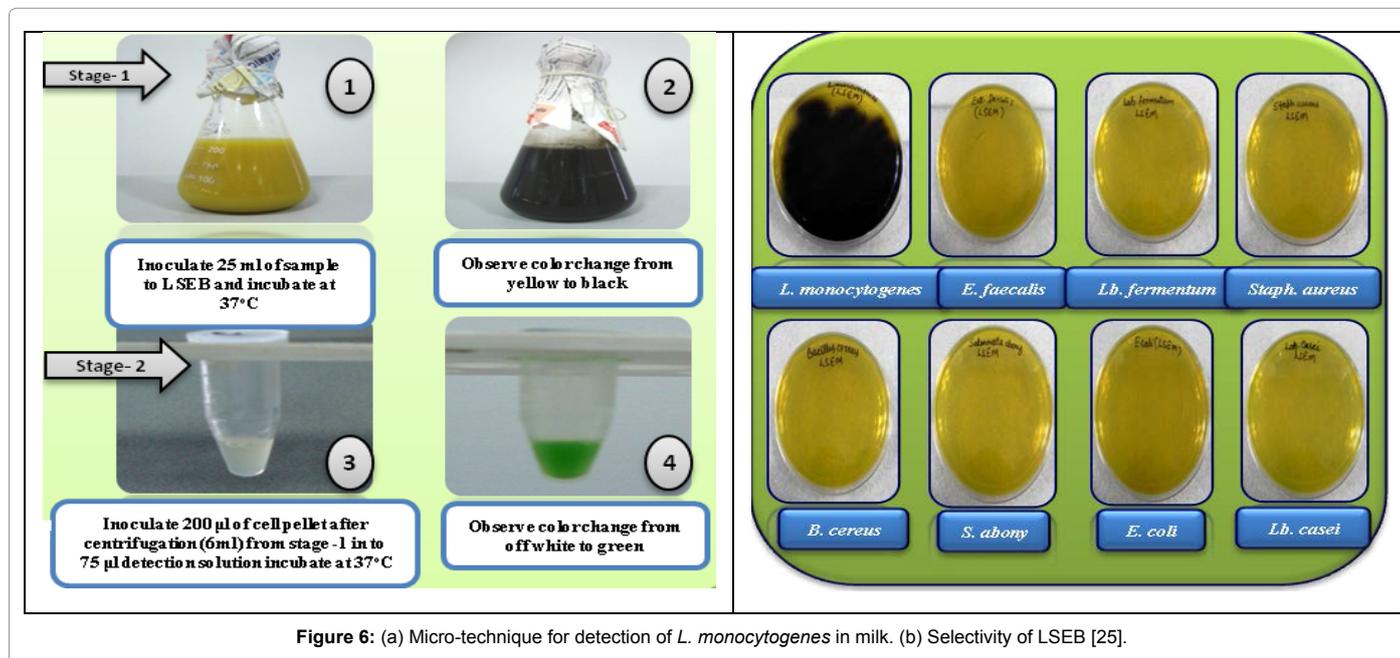


Figure 6: (a) Micro-technique for detection of *L. monocytogenes* in milk. (b) Selectivity of LSEB [25].

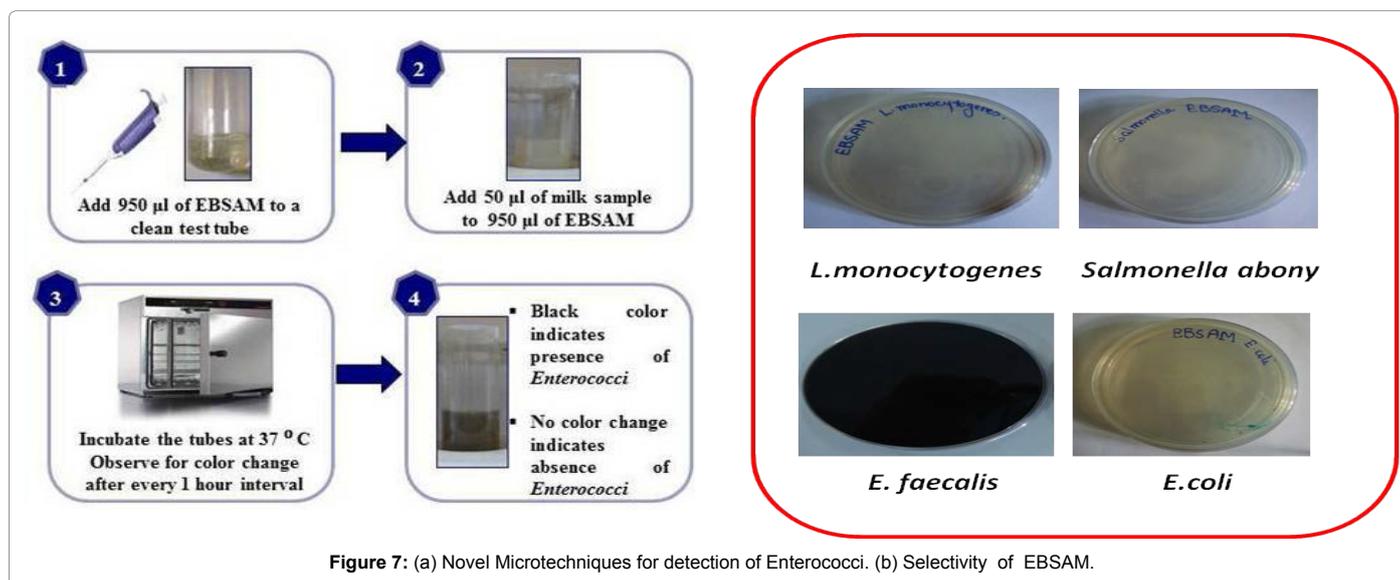


Figure 7: (a) Novel Microtechniques for detection of Enterococci. (b) Selectivity of EBSAM.

enzyme substrate i.e. esculin resulting in germinant stimulus for spores produced by specific strain of *B. megaterium*. The developed spore based bioassay consists of target bacteria, microbial spores suspended in buffer, marker-enzyme substrate and a fluorogenic substrate. The detection principle is based on quantification of fluorescent signal produced as result of DAF hydrolysis by germination mediated marker enzyme released from bacterial spores germination triggered by β -D-glucosidase activity on non-specific germinant substrate. The spore based bio-assay developed has sensitivity of 5.5 ± 0.4 log cells after pre-enrichment of milk sample in specific enrichment broth i.e. sodium Azide and esculin based medium (SAEBM) within real time of 8 ± 2 hrs [23,24] (Figure 7).

Conclusion

The living phase of bacterial spores revolves around two phases i.e. dormant state and metabolically active vegetative state. This conversion from one phase to another phase is completed only if spore senses favorable conditions in the environment and presence or absence of microbial or non microbial contaminants directly or indirectly affect this conversion. So this phenomenon can be targeted to sense the presence of contaminants in milk and hence develop spore based biosensor systems. A number of spore based sensing system have been developed to detect aflatoxin, antibiotics and microbial pathogens in milk. These biosensing systems are superior over existing methods in terms of better sensitivity, low cost and help in rapid analysis of milk and milk products. The spore based biosensor is a novel strategy being exploited to ensure safe and healthy milk to each consumer.

References

1. Desnous C, Guillaume D, Clivio P (2010) Spore photoproduct: a key to bacterial eternal life. Chem Rev 110: 1213-1232.
2. Setlow P (2007) I will survive: DNA protection in bacterial spores. Trends Microbiol 15: 172-180.
3. Zhang P, Garner W, Yi X, Yu J, Li YQ, et al. (2010) Factors affecting variability in time between addition of nutrient germinants and rapid dipicolinic acid release during germination of spores of *Bacillus* species. J Bacteriol 192: 3608-3619.
4. Setlow P (2003) Spore germination. Curr Opin Microbiol 6: 550-556.
5. Paidhungat M, Setlow P (2002) Spore germination and outgrowth. *Bacillus subtilis* and its relatives: from genes to cells. American Society for Microbiology, Washington, DC, USA, 537-548.
6. Hudson KD, Corfe BM, Kemp EH, Feavers IM, Coote PJ, et al. (2001) Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. J Bacteriol 183: 4317-4322.
7. Paidhungat M, Ragkousi K, Setlow P (2001) Genetic requirements for induction of germination of spores of *Bacillus subtilis* by Ca^{2+} -dipicolinate. J Bacteriol 183: 4886-4893.
8. Wolgamott GD, Durham NN (1971) Initiation of spore germination in *Bacillus cereus*: a proposed allosteric receptor. Can J Microbiol 17: 1043-1048.
9. Chen D, Huang SS, Li YQ (2006) Real-time detection of kinetic germination and heterogeneity of single *Bacillus* spores by laser tweezers Raman spectroscopy. Anal Chem 78: 6936-6941.
10. Hashimoto T, Frieben WR, Conti SF (1969) Germination of single bacterial spores. J Bacteriol 98: 1011-1020.
11. Popham DL, Helin J, Costello CE, Setlow P (1996) Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. Proc Natl Acad Sci U S A 93: 15405-15410.
12. Cowan AE, Koppel DE, Setlow B, Setlow P (2003) A soluble protein is immobile in dormant spores of *Bacillus subtilis* but is mobile in germinated spores: implications for spore dormancy. Proc Natl Acad Sci U S A 100: 4209-4214.
13. Sangal A (2010) Stability of Spore-Based Sensing Systems. Unpublished M.Sc., University of Kentucky, Kentucky.
14. Date A, Pasini P, Daunert S (2007) Construction of spores for portable bacterial whole-cell biosensing systems. Anal Chem 79: 9391-9397.
15. Prandini A, Tansini G, Sigolo S, Filippi L, Laporta M, et al. (2009) On the occurrence of aflatoxin M1 in milk and dairy products. Food Chem Toxicol 47: 984-991.
16. Shundo L, Navas SA, Lamardo LCA, Ruvieri V, Sabino M (2009) Estimate of aflatoxin M₁ exposure in milk and occurrence in Brazil. Food Control 20: 655-657.
17. Development of spore inhibition based "enzyme substrate assay" (SIB-ESA) for monitoring aflatoxin M1 in milk. Indian Patent Reg No. 3064/DEL/2010. Mumbai, Office of the Controller General of Patents, Designs & Trade Marks.
18. Kumar N, Kaur G, Thakur G, Raghu HV, Singh N, et al. (2012) Real time detection of enterococci in dairy foods using spore germination based bioassay. Office of the Controller General of Patents, Designs & Trade Marks, Mumbai.
19. Singh NA, Kumar N, Raghu HV, Sharma PK, Singh VK, et al. (2013) Spore inhibition-based enzyme substrate assay for monitoring of aflatoxin M₁ in milk. Toxicol Environ Chem 95: 765-777.
20. Kumar N, Das S, Manju G (2009) A kit for detection of b-lactam antibiotic group in milk using bacterial spore as biosensor. Indian Patent Reg No. 115/DEL/2009. Office of the Controller General of Patents, Designs & Trade Marks, Mumbai Kumar N, Singh NA, Singh VK, Bhand S, Malik RK (2010).
21. Gaare M, Kumar N, Raghu HV, Khan A, Singh VK (2012) Specific detection of β -lactam antibiotics in milk by spore based assay. International Research Journal of Microbiology 3: 2141-5463.
22. Mandeep (2013) Development of enzyme substrate assay for detection of *Listeria monocytogenes* in milk. National Dairy Research Institute, Deemed University (ICAR), Karnal.
23. Kaur G (2011) Spore Germination: an Innovative Approach for Detecting Enterococci in Milk. National Dairy Research Institute, Deemed University (ICAR), Karnal.
24. Deshmukh SS (2013) Screening of Enterococci in milk using developed media and functionalized biochip. National Dairy Research Institute, Deemed University (ICAR), Karnal.
25. Kouser S (2013) Evaluation of "two stage enzyme assay" for detection of *L. monocytogenes* in milk and milk products. National Dairy Research Institute, Deemed University (ICAR), Karnal.