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STABILITY OF SPORE-BASED SENSING SYSTEMS

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ABSTRACT OF THESIS

STABILITY OF SPORE-BASED SENSING SYSTEMS

The full exploitation of bacterial whole-cell biosensing systems in field applications requires the survival of bacterial cells and long term-preservation of their sensing ability during transportation and on-site storage of such analytical systems. Specifically, there is a need for rapid, simple and inexpensive biosensing systems for monitoring human health and the environment in remote areas which often suffer from harsh atmospheric conditions and inadequate commercial distribution and storage facilities. Our laboratory has previously reported the successful use of bacterial spores as vehicles for the long-term preservation and storage of whole-cell biosensing systems at room temperature.

In the present research, we have accomplished a year-long study to investigate the effect of extreme climatic conditions on the stability of spores-based whole-cell biosensing systems. The spores were stored in laboratory conditions that simulated those found in real harsh environments and germination ability and analytical performance of the spore-based sensing systems upon storage in such conditions was monitored. Our results proved that the intrinsic resistance of spores to harsh environmental conditions helped maintain the integrity of the sensor bacteria. The revived active cells actually retained their analytical performance during the course of the twelve-month storage study.

KEYWORDS: Whole-Cell Biosensing Systems, Bacterial Spores, Extreme Environmental Conditions, Biosensor Storage and Preservation, On-Site Applications.

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THESIS

ABHISHEK SANGAL

The Graduate School

University of Kentucky

2010

STABILITY OF SPORE-BASED SENSING SYSTEMS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master's of Science in the
College of Arts and Sciences
at the University of Kentucky

By

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Lexington, Kentucky

Director: Dr. Sylvia Daunert, Professor of Chemistry

Lexington, Kentucky

2010

Dedicated to

My beloved Mother

Shashi Prabha Sangal

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

With the increase in public awareness about environmental hazards, including soil, water and air contamination, much focus has been put on developing techniques to detect potentially harmful pollutants and other chemicals in the environment. There are two general methods for detecting pollutants in the environment – physicochemical and biological methods. Whereas physicochemical methods involve the use of mainly chromatographic analytical equipment such as gas chromatography (GC) and high performance liquid chromatography (HPLC), biological methods involve the use of one or more biological components. Owing to the high cost and technical complications associated with conventional chromatographic methods [1], much of the attention is now being given to development of biological sensors. Moreover, physicochemical methods often overestimate the bioavailability of the pollutants and provide inaccurate toxicological information, since metals and xenobiotics often exist in an insoluble form in the environment [2]. For these reasons, biosensors, especially those based on whole cells, have found a place for monitoring and evaluating the toxicological activities of environmental samples. The term 'biosensor' is given to a measuring device which utilizes a biological component as the recognition element coupled to a transducing element. The biological component senses a chemical or physical change in the immediate environment and the transducing element converts that change in the environment into the measurable signals [3]. The biological sensing element confers selectivity, while the transducer brings about the sensitivity in the biosensing system. Over the years, many different kinds of biological components, such as antibodies,

binding proteins, enzymes, receptors and whole cells, have been utilized to develop biosensors [4]. Whereas each of the different biomolecules confer high specificity, selectivity and rapid detection times, the lack of functional information such as analyte bioavailability and the high cost of extraction and purification procedures associated with these biomolecules pose a challenge to their practical utility in biosensing applications. On the other hand, genetically engineered whole-cell biosensing systems are robust, easy to prepare, provide information about bioavailability of the analyte and are easy to store. These characteristic features of whole-cell biosensing systems have made them a popular tool in environmental applications.

1.1 WHOLE-CELL BACTERIAL BIOSENSING SYSTEMS

The physical habitat of bacteria in soil, air, and water has led bacteria to develop a natural resistance against the pollutants present in the environment. This resistance of bacteria is seen most often in the form of genes on bacterial plasmids or less frequently in the form of genes on the bacterial chromosomes which code for mechanisms that allow the bacteria to sense and either efflux the toxic compounds out of the cell or detoxify them [5]. This constant sensing of their immediate environment makes bacteria ideal for determination of the bioavailability and toxicity of pollutants. Because of this natural toxic metal and organic compounds resistance machinery present in bacterial cells, nowadays, whole-cell bacterial biosensing systems are the focus of attention for the analysis of environmental samples. Development of a whole-cell biosensing system typically makes use of recombinant DNA technology to construct a plasmid in which the

sensing element is coupled to a reporter gene. The sensing element in bacterial cells consists of a regulatory protein and promoter sequence of DNA and brings about the selectivity in the system. The expression of fused reporter gene is either enhanced or decreased in presence of the toxin in the form of a detectable signal which brings about the system's sensitivity. The promoter is commonly located upstream of the reporter gene. The expression of the reporter gene is controlled by the regulatory protein and is expressed in a dose-dependent fashion in the presence of the specific analyte. Numerous biosensing systems have been developed to detect environmental pollutants like toxic metals such as arsenic, antimony, mercury, chromium and lead [3, 6-11] and organic aromatic compounds such as polychlorinated biphenyls [12], chlorocatechols [13], dihydroxylated biphenyls [14], as well as biologically relevant molecules such as tetracyclines [15] and N-acylhomoserine lactones [16].

Whole-cell bacterial biosensing systems commonly employ either the constitutive or inducible expression of a reporter gene within the bacterial cells [17]. In the constitutive expression systems, the promoter is constantly active and the reporter gene is always expressed under normal conditions, which results in the production of the reporter protein and its corresponding signal. However, in the presence of the analyte, which in this case is usually toxic to the cells, the metabolic activity of the cells decreases and the expression of the reporter gene is either inhibited or reduced. The reduction in the signal intensity of the reporter protein is measured and correlated as the toxicity of the sample (Figure 1.1). Inducible expression systems, on the other hand, work opposite to those with constitutive expression. In inducible expression systems, the promoter region of the regulatory operon system is usually bound to the regulatory protein and hence prevents

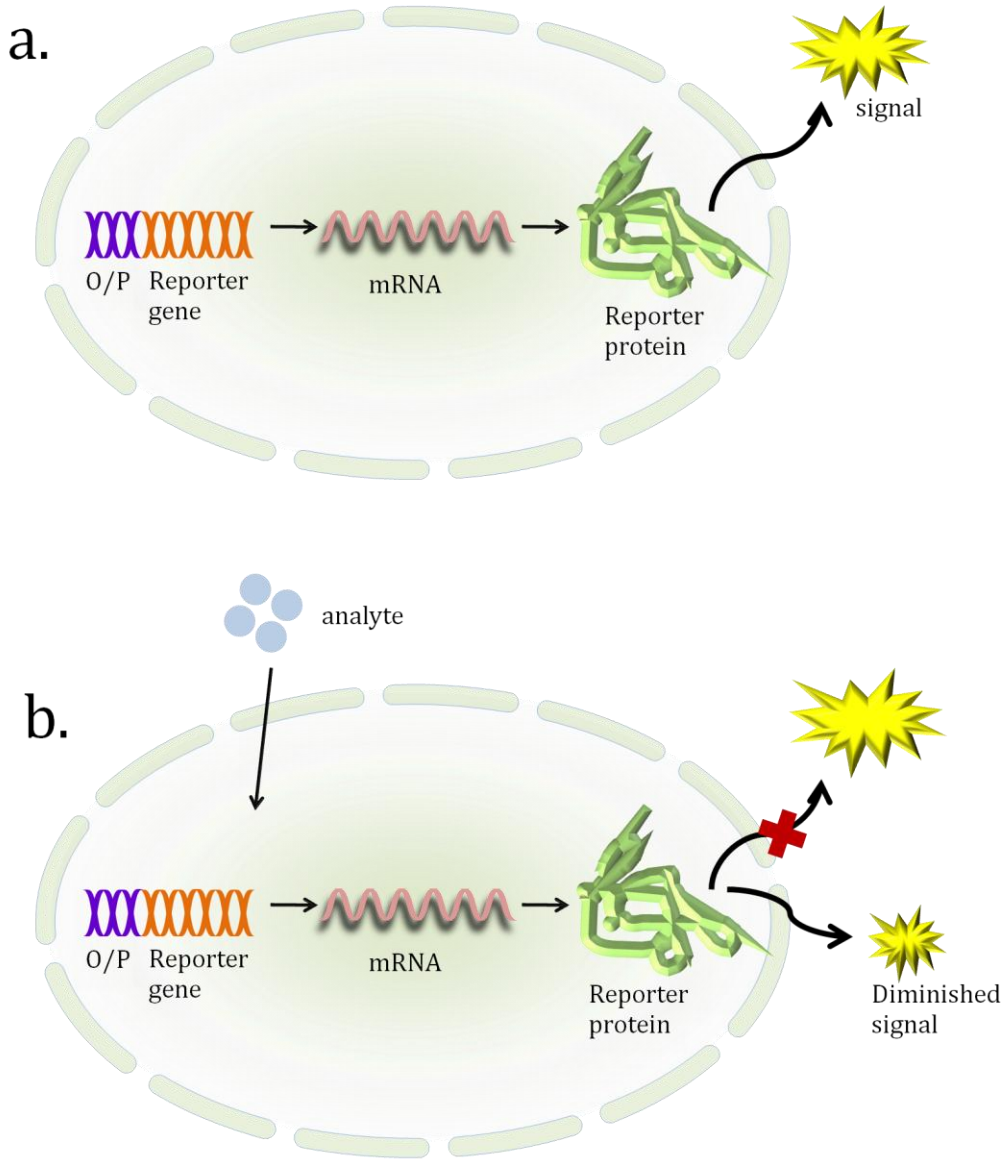

Figure 1.1. Constitutive expression in bacterial whole-cell sensing systems. (a) The reporter protein is placed under the transcriptional control of a constitutively active promoter. This leads to continuous expression of the reporter gene and a steady signal is measured. (b) In the presence of analyte/toxic chemicals, the expression of reporter gene decreases or is inhibited, thus giving a reduced or no signal intensity.

the expression of reporter gene which is placed under the transcriptional control of promoter. In the presence of specific chemicals, the cells experience stress or respond to the analyte and the promoter region become free. This leads to the expression of the reporter gene. The expression of the reporter gene depends completely on the amount of the analyte present in the cell and hence the signal intensity can be correlated to analyte concentrations of the sample (Figure 1.2).

Whole-cell based biosensing systems possess certain advantages over other types of biosensors including: (a) living cells determine only the bioavailable fraction of compounds and may give a more accurate response in terms of the toxicity of the sample; (b) these systems provide an inexpensive and simple way of determining contaminants; (c) there is no need for extensive extraction and purification procedures while using intact cells as the biosensing system; (d) whole-cell sensing does not require expensive instrumentation and highly trained technical personnel; and (e) these systems are amenable to miniaturization and automation, thus enabling multiplex analysis and high throughput screening.

Despite the advantages of whole cell biosensors and their precedence over other types of sensors, the use of these living biosensors is still a challenging task for their utility in on-site applications. The main hurdle in the applicability of these systems for testing real samples in the field is the shelf life associated with the living cells. For the on-site application of the whole-cell biosensing systems, the sensing cells need to be active and viable during the course of transportation and the storage period. A lot of research is being carried out on ways to package the biosensing cells so as to increase

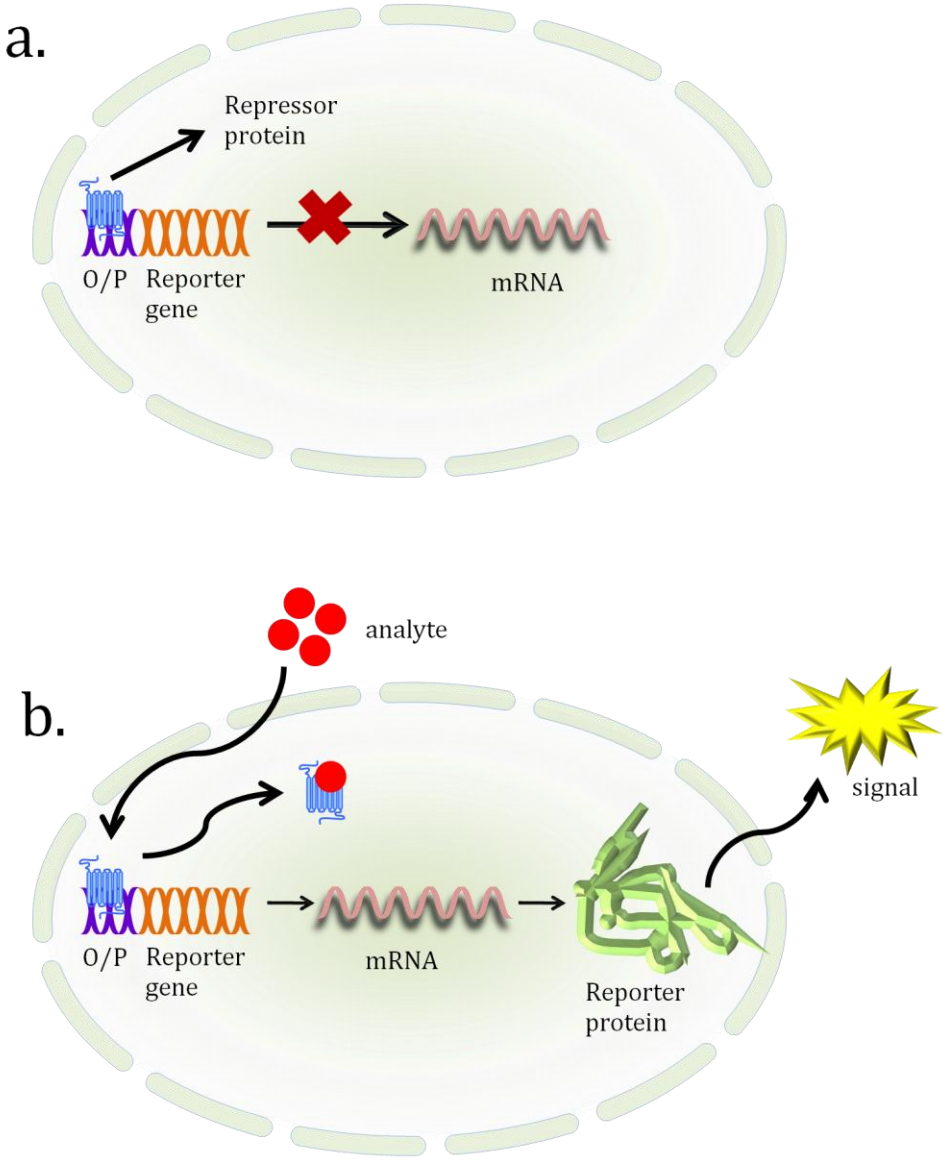

Figure 1.2. Inducible expression in bacterial sensing systems. (a) In the absence of analyte, the promoter region is bound by the regulatory protein. Due to this binding, the transcription of the reporter gene is inhibited. (b) When analyte is present it binds to the regulatory protein causing it to be released from the promoter site. The DNA polymerase activity starts and leads to the expression of the reporter gene.

the longevity of their shelf life. Various preservation techniques have been employed to maintain the bacterial cells in a viable and active state. Some of the common techniques include freeze drying, immobilization of living cells in inorganic and organic polymers, and bacterial sporulation.

1.2 FREEZE DRYING

Water is an essential solvent for life. However, a variety of organisms, including bacteria, fungi, yeast, plants and certain nematodes, can survive a severe limitation of water [18-20]. This phenomenon in which these dry organisms survive through the unfavorable condition of complete dehydration in a state of arrested metabolism is termed as anhydrobiosis. In the presence of water, the organisms swell and resume active life. It has been reported that small carbohydrate molecules such as trehalose, sucrose, and maltose act as chemical protectants of dry cells [21] and that these desiccation tolerant organisms carry high concentration of these sugar molecules [18, 22]. The insights on survival strategies of anhydrobiotic organisms have made it possible to preserve and stabilize biomolecules and microbes that do not normally survive dehydration. The method most commonly used nowadays to preserve biologically important molecules and organisms by drying them is freeze drying.

Freeze drying or lyophilization is a dehydration process used to remove the water content from biological materials so that these materials remain stabilized during the storage period or during transportation. The whole process is divided into three stages; freezing, primary drying and secondary drying [23]. The process starts with the freezing

of the material at temperatures below the triple point of the water, usually between -50^{o}C to -80^{o}C. This helps in separating the solutes from solvent. Once the water is separated from the solutes, it is removed by the process of sublimation in the primary drying stage. In the frozen state, heat is applied and the pressure of the system is reduced so that water gets converted from the solid phase to the gas phase avoiding solid-liquid and liquid-gas transition. The remaining unfrozen water content which is present on the surface of the material is dried in the secondary drying stage by increasing the temperature. After the process of lyophilization, the resulting anhydrous biological product is sealed in containers to protect it from the environment for long periods of time. Upon rehydration of this product, the biological materials show good activity.

Freeze drying is one of the earliest methods used to store and preserve biosensors for a long period of time and also during the transportation. The main advantages of the freeze drying storage method include the increased shelf life of the organisms and easy restoration of active cells by rehydrating the freeze dried product. Numerous studies have been reported that make use of freeze drying as a strategy for the long term preservation of biosensors and use these freeze dried sensing cells for constructing portable biosensors for on-site analysis [24-28]. These studies suggest that the storage time using the freeze drying method ranges from ten days up to several months and the stability of the sensing cells also depends upon the storage temperature of the dried product. Although freeze drying is a widely used preservation method, it may lead to protein denaturation and DNA/ RNA damage that result in decrease in bacterial cell viability [29-31]. Billi et al. [32] developed a recombinant *E. coli* strain for the synthesis of sucrose and a survival study of this strain revealed that after freeze drying and immediate rehydration, only

2.5% of cells were viable. Louis et al. [33] also have reported similar results where the freeze drying of *E. coli.* cells resulted in the loss of 1 to 4 logs of viability within 6 weeks of drying. Another major disadvantage of freeze drying is that it is a very complex and costly technique. The previous studies suggest that dried product remains stable for a longer period of time at a storage temperature of -20^{o}C or lower. This condition hampers the use of freeze dried sensing cells in the field applications where it is not possible to maintain such low temperature. In a recent study, Buchinger et al. customized the freeze drying protocol for the long term stabilization of enzymes of liver S-9 fraction at ambient temperatures [34]. They have reported that the metabolization performance of S-9 fraction (when 250 mM of trehalose was added as a cryoprotectant) after storage at room temperature for 42 days was the same as compared to the S-9 fraction (in the absence of cryoprotectant) that was stored at -80^{o}C. To the best of our knowledge, this is the first study of its kind to store the freeze dried products at ambient temperatures. Although this result is encouraging, improvements in optimizing the protocols are needed for the long term preservation of biosensing cells at ambient temperatures with preserved cell viability. Sensitivity of the dried product to moisture also adds to its disadvantage and may spoil the sensing cells during the storage period. Although freeze drying enables easy and inexpensive shipping of biosensing cells at room temperature, cost and complexity of the process represent some of the disadvantages of this method.

1.3 IMMOBILIZATION OF BIOSENSING CELLS

Another method that is extensively used to store and preserve whole cell biosensing systems is the immobilization of such cells into different supporting materials that can preserve the viability of the cells. Such immobilization techniques include attachment of cells on LB agar matrix and sol gel immobilization. The criteria of choosing the right matrix for the immobilization of sensing cells is that the matrix should keep the cells isolated from each other. This prevents self aggregation of bacterial cells and protects these cells from the microbial attack.

Agar is a gelatinous substance found in the cell wall of certain marine algae of class Rhodophyceae. It is a polymer made of subunits of sugar galactose and finds its use in culture medium for microorganisms. Due to its gelatinous nature, its resistance to degradation by bacteria, and strong acid and mechanical stability, agar has been an attractive matrix for the immobilization of whole-cell biosensing systems. Kim et al. [35] developed a whole cell biosensor to detect the presence of hydrogen peroxide, phenol and mitomycin C in water samples and used LB-agar matrix for the immobilization of genetically engineered cells in 96 well microtiter plates. The engineered cells were suspended in LB-agar and were vigorously shaken to prevent cell aggregation and were transferred to the wells of the microtiter plate where the matrix containing the cells solidified (Figure 1.3). In their long term stability studies, Kim and Gu found that these immobilized cells were able to show dose dependent curves for the toxic materials for two weeks when kept at 4^{o}C. However, the signal intensities decreased in each case after two weeks as compared to the light intensities of their response initially. The loss in the response was attributed to the loss of metabolic activity and viability of the sensing cells.

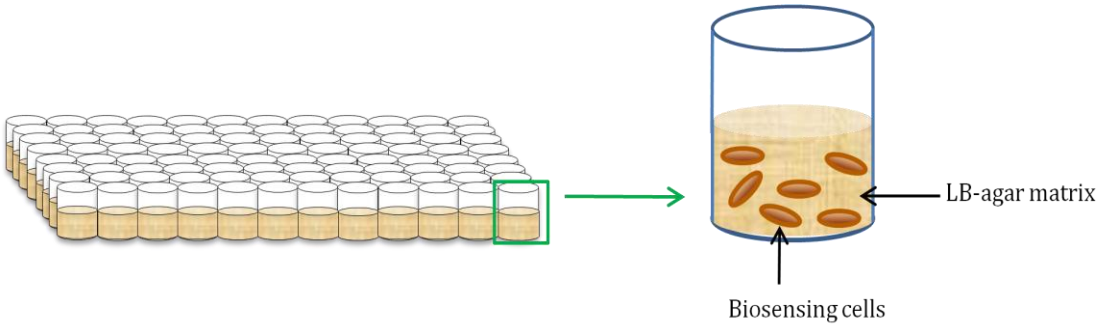

Figure 1.3. Bioluminescent bacterial cells immobilized in LB-agar matrix in a microtiter plate.

In another study, Park et al. [36] immobilized a recombinant strain of *Salmonella typhimurium* on an agar matrix to study the activity of the sensing cells to detect mitomycin C after long storage periods. The recombinant cells contained the plasmid that had inducible SOS promoter fused to promoterless *luxCBADFE* operon. Mitomycin C stimulated SOS response in the sensing cells with the production of bioluminescence. The authors stored the biosensor immobilized on agar in a microtiter plate for 6 weeks at 4^{o}C. They reported that the biosensor immobilized on agar and stored at 4^{o} C did not show any significant difference in activity after 4 weeks as compared to the freshly prepared cells. However, after 6 weeks of storage period, there was a decrease in bioluminescence values of the system.

The sol-gel process involves the addition of a colloidal suspension of a polymer-forming precursor to a solution of an active material. This active material gets entrapped in the sol-gel matrix. The mild conditions associated with sol-gel chemistry, such as low temperatures, high surface area and porosity, aqueous solvents, neutral pH, and adequate ionic strength; make these materials a cytocompatible scaffold to immobilize living cells [37-38]. The entrapped biomaterials within the matrix still retain their activities and react with substrate species that diffuse through the pores of sol-gel matrix.

Immobilization of living cells within sol-gel derived silica matrices has been extensively used for a long time. In the 1980's, Carturan et al. [39] immobilized *Saccharomyces cerevisiae* cells in a multilayered sol-gel derived thin film. Branyik et al. [40] demonstrated the immobilization of both yeast (*Candida tropicalis*) and bacterial (*Psuedomonas species 2*) cells in the tetraethoxysilane (TEOS) silica gels and the use of

these gels in bioremediation. A general method of preparing living cells entrapped in sol-gel matrices is shown in figure 1.4.

Recently, silicate gels have been applied as the immobilization tool to entrap whole-cell biosensing systems in the gel matrix for the preservation and storage of the genetically engineered bacteria. High chemical and biological stability of these gels and excellent transparency of silicate gels make them favorable encapsulating matrices for biological sensors. Yu et al. [41] reported that encapsulation of whole-cell biosensor within the silicate gels maintained an activity of 95% for two months. They developed a whole-cell biosensor to detect organophosphates using genetically engineered *Moraxella* spp. bacterial cells. When the biosensing cells were encapsulated in sodium silicate sol-gel matrix and stored at 4^{o}C, the system showed a reduction of only 5% activity after 2 months as compared to reduction of 30% activity by the freely suspended sensing cells. Premkumar et al. [42] demonstrated the immobilization of genetically engineered bioluminescent bacteria *Escherichia coli* by encapsulating the cells in sol-gel derived silicates. The sol-gel mixture was prepared by mixing tetramethylorthosilicate (TMOS), hydrochloric acid, and distilled water at an optimal pH of 7. A suspension of bacterial cells in LB media was thoroughly mixed with the sol-gel matrix and films of *E. coli*–silicate hybrid were prepared by coating the mixture on the glass plates. These films were then used to detect the toxic compounds such as phenol and hydrogen peroxide. The silicate cages prevented both escape and proliferation of the entrapped bacteria and allowed free sample diffusion and the expression and detection of reporter proteins. The advantage of the entrapment of bioluminescent cells in sol-gel is the multiple use of same

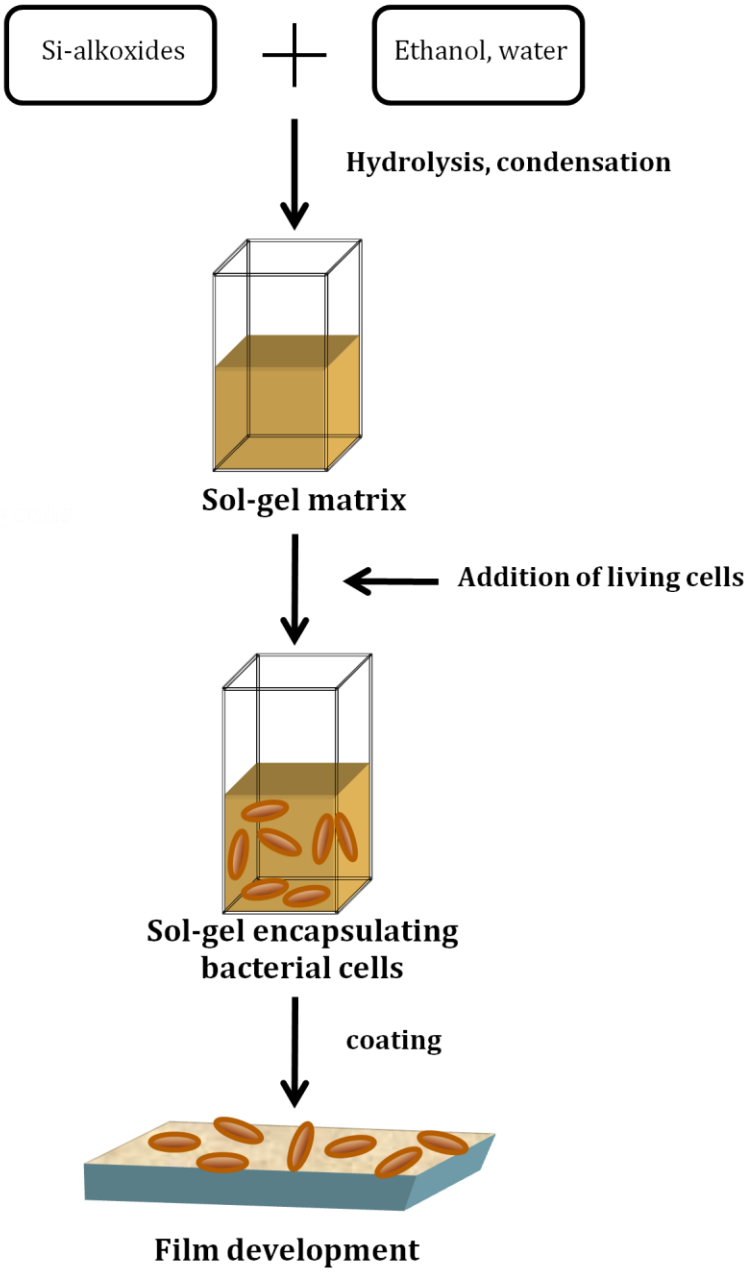

Figure 1.4. Preparation of bacterial cells encapsulating sol-gel thin films.

sensing element a number of times. The shelf life of the bacterial cells in the sol-gel matrix was shown to be 3 months when kept at 4^{o}C. The sol gel encapsulated biosensors are normally kept and stored in wet conditions. Drying the cells result in permanent loss of activity of the biosensing cells. However, in an attempt to freeze dry their system, they reported that the freeze drying of their sensing system led to disintegration of silicate films and loss of viability and activity of biosensing cells. Continuous efforts are being made to make sol-gel encapsulation an efficient technique for the preservation and long term stabilization of biosensors. For the successful freeze drying of gel encapsulated biosensing cells with maintained activity, Tessema et al. [43] used optimal concentrations of trehalose and glycerin in the sol-gel silicate precursors and demonstrated that presence of these chemicals prevented the inactivation of bacterial cells during the freeze drying process. Their results show that a 10% or more concentration of trehalose stabilized the freeze dried biosensing cells in the polymer gels for up to 6 weeks at -20^{o}C and up to 3 weeks at 0^{o}C. The sensing cells, however, were poorly stable at room temperature.

Long term storage of sol-gel encapsulated biosensing systems is limited by the increase in cross linking of the gel network over time. The increase in cross linking results in a decrease of the average pore size and expulsion of the solvent from the matrix. This has a considerable effect on the immediate environment of the cells and all these effects contribute to the stability and activity of the biosensing cells. Although research is still being carried out to make the sol gel encapsulation process an efficient method to preserve and store whole-cell biosensing systems, this method also lacks practical utility in terms of low stability period, high cost of production and maintenance.

1.4 BACTERIAL SPORULATION

The methods described above provide a promising way to store, transport and preserve living microorganisms and whole-cell based biosensors. Research is still being carried out to make these methods more efficient and convenient for practical utility. However, these methods have, till now, not exceeded the storage time of more than few months. Long term preservation and storage of the sensing cells is very important for on-site applications. Additionally, these methods are not useful when the sensing cells are to be transported to locations with unfavorable conditions such as extreme temperatures, very high or low humidity, extreme pHs, and presence of chemicals in the environment. Therefore, with a need to develop an inexpensive and easy to use method to preserve, store, and transport the whole-cell biosensing systems for extended periods, even in unfavorable conditions, our laboratory has made use of certain species of spore forming bacteria and established the concept of spore based whole-cell sensing systems.

Certain species of gram positive bacteria, such as *Bacillus* and *Clostridium*, and few gram negative bacteria, such as *Sporomusa malonica, Sporomusa ovata* and *Coxiella burnetti* are able to survive in harsh and unfavorable climatic conditions and under the conditions of stress and starvation [44-45]. These bacteria undergo the process of sporulation in response to the adverse changes in their immediate environment and generate endospores. Endospores are dormant and metabolically inactive bodies that preserve the genetic material of bacteria within certain protective layers of proteins and sugars (Figure 1.5). Endospores are one of the hardiest forms of life on earth and are resistant to very high and very low temperatures and pressures, UV radiation, various noxious chemical substances, and many other extreme environmental conditions. Upon

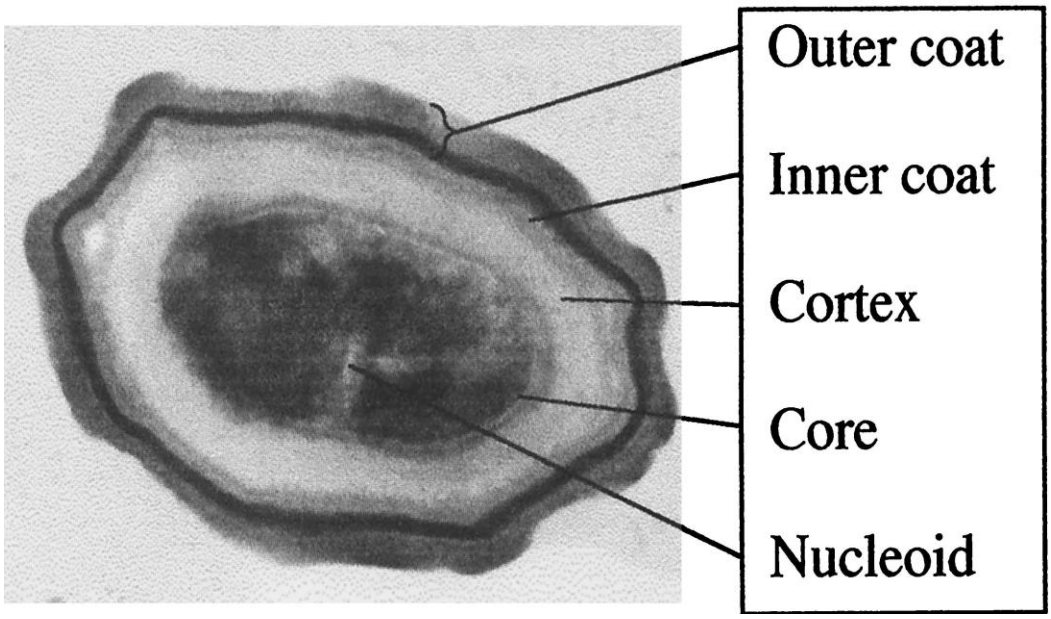

Figure 1.5. Electron micrograph of the cross section of a spore of *Bacillus subtilis*. The DNA is contained in the nucleoid within the spore core. The spore core is surrounded by different protein and peptidoglycan layers. (Adapted from Nicholson et al. [44])

the arrival of favorable conditions, the endospores germinate to form metabolically active vegetative cells. It has been reported that bacterial spores are probably the longest lived cellular structures known with their age ranging from several of years to hundreds of thousands of years [46-47].

The process of sporulation involves various cellular activities such as DNA replication, sporulation-specific asymmetric cell division, changes in gene expression, and specific signal transduction mechanisms [48] (Figure 1.6). An endospore is formed within the mother cell in a compartmentalized structure known as forespore. It starts with the replication of DNA into two daughter nuclei. The daughter nuclei move towards the opposite poles and then the cell divides forming two asymmetrical compartments - a large cell, called the sporangium or mother cell and a much small cell called forespore. The smaller forespore contains one of the daughter nuclei along with some small acid soluble proteins that tightly bind and condense the DNA and are responsible for resistance to ultraviolet light and DNA damaging materials [49]. The forespore then develops a very thick layer of loosely linked peptidoglycans, known as the cortex. The cortex mainly serves to keep the forespore dehydrated and aid in temperature resistance. Then layers of different coat proteins develop over the cortex to form the inner coat and the outer coat. These protein layers provide much of the chemical and enzymatic resistance to the genetic material. Although endospores are a dormant form of bacteria, these structures sense certain germinants in their environment and respond by germinating to vegetative active cells.

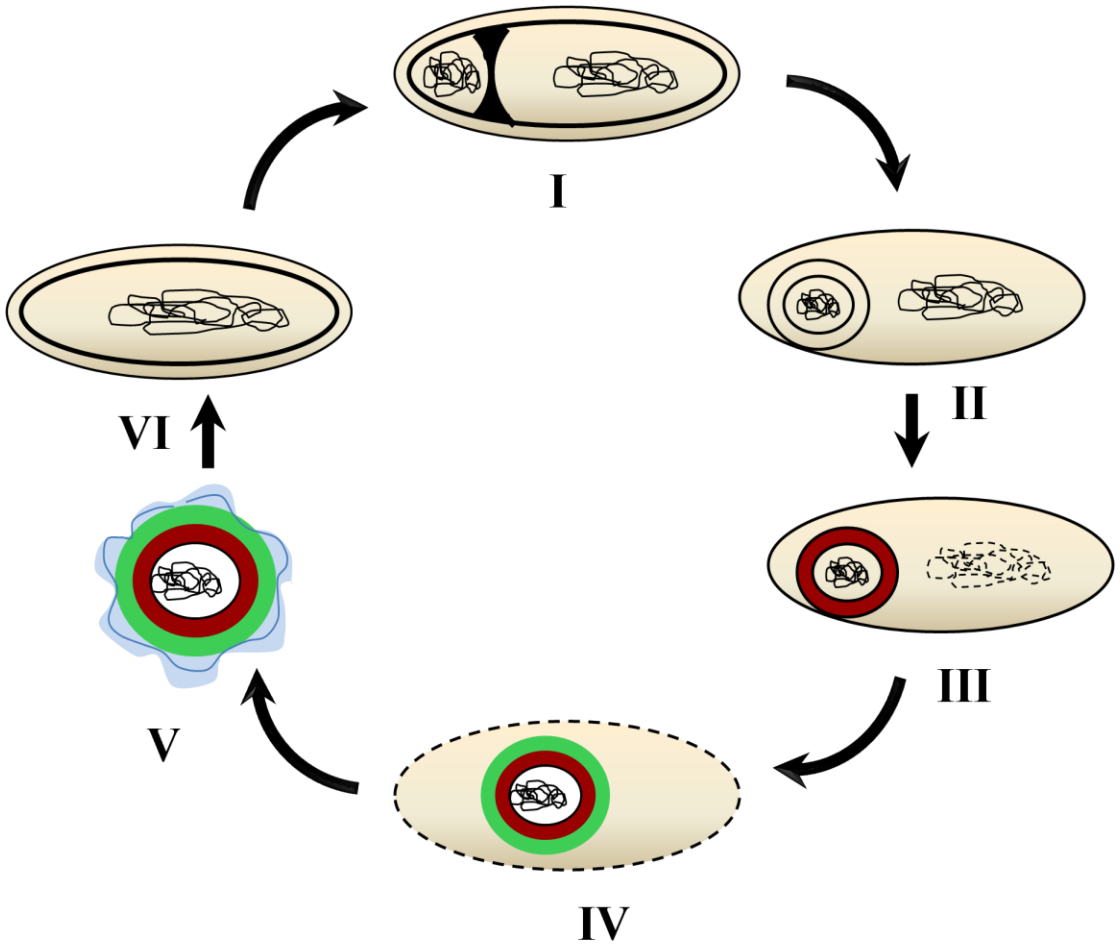

Figure 1.6. Schematic presentation of endospore development in spore forming *Bacillus* bacteria. (I) Under the conditions of starvation and stress, the DNA undergoes replication to form two daughter nuclei separated by cytoplasmic memberane. (II) One of the daughter nuclei is then protected by 2 layers of cytoplasmic memberane – an inner and an outer memberane. (III) A thick layer of peptidoglycans, known as cortex, fills the space between the two memberanes. (IV) A layer of proteins surround the cortex and is known as spore coat. The cell disrupts and the spore becomes free. (V) In some bacteria, an outer protective layer, known as exosporium, also exists. (VI) Under the favorable conditions, the coatings break open to form vegetative bacterial cells.

The good sensing ability of bacterial endospores has led them to be used as biosensors. Rotman et al. [50] used microbial spores as nanodetectors for bacterial detection with the help of certain enzyme activities. Their spore based biosensing system used spores suspended in buffer containing diacetyl fluorescein and an enzymatic substrate that could produce a spore germinant as the result of a specific enzymatic activity. They used L-alanoyl L-alanine as enzymatic substrate for the detection of bacteria exhibiting alanine aminopeptidase activity. In the presence of this enzyme, L-alanoyl L-alanine will hydrolyze into L-alanine, a germinant of bacterial spores. The germinanting spores, in turn, will convert diacetyl fluorescein into easily detectable fluorescent products. They further used this approach to identify bacterially contaminated platelet concentrates before transfusion [51]. However, the high resistance capability and extreme long life of endospores was not utilized until recently when the bacterial spores were used as vehicles to preserve, store and transport the whole-cell bacterial biosensing systems. Date et al. [6] employed spore forming *Bacillus* species to package the whole-cell biosensing systems to increase the shelf life of these systems and also to be able to use these systems in harsh environments for in the field applications. As a proof of concept, two different *Bacillus* species, namely *Bacillus subtilis* and *Bacillus megaterium*, and two different analytes, namely arsenic and zinc, were used. The authors demonstrated that the activity and viability, in terms of analytical performance of their spore-based sensing systems, was retained up to a period of 8 months when kept as dried spores at room temperature. The authors also demonstrated that the activity of the sensing cells is retained even after three sporulation-germination cycles (Figure 1.7). A similar kind of study was subsequently done by Fantino et al. [52] where a whole-cell bacterial

sensor based on immobilized spores, also called sposensor, was developed for the colorimetric detection of toxic compounds.

Thus, spore based whole-cell bacterial sensing systems have evolved as very robust, easy to use, simple, and inexpensive method for long term preservation, storage and transport of biosensing systems.

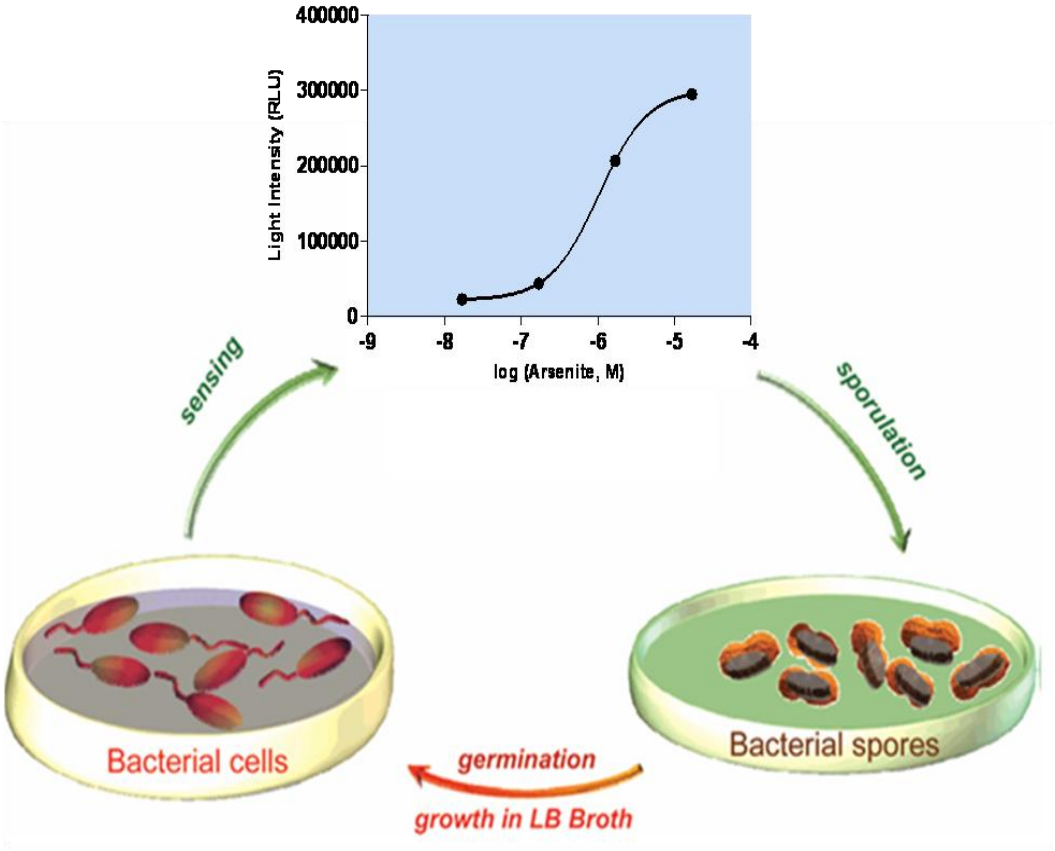

Figure 1.7. Schematic representation of sporulation-germination cycles for the spore-based whole-cell sensing systems. The metabolically active vegetative cells are used to sense the toxic analytes. These sensing cells can then be stored and preserved in the form of dormant bacterial spores. Upon providing the nutrients, these spores germinate to form vegetative cells again. Adapted from Date et al. [6])

CHAPTER TWO

STABILITY OF SPORE-BASED SENSING SYSTEMS

The practical utility of whole-cell biosensors in field applications requires the bacteria to be cost-effectively formulated in a way that maintains their viability and sensing capability, while enabling their long-term storage and transportation [53]. The different approaches, such as freeze frying, vacuum drying, and encapsulation of bacteria in organic and inorganic polymers, to keep the biosensing microorganisms alive and active have been critically reviewed by several authors [54-55]. All these methods suffer in their practical applications in the way that they either are costly and complex or the performance of the biosensing system is proven to decline after relatively short periods of time, in the order of a few weeks or months [55]. Also, none of these methods have been evaluated for their effectiveness in preserving biosensors in real life harsh environmental conditions found in different regions. The main disadvantages of whole-cell biosensing systems that prevent their extensive application to field analysis are: (a) their limited stability over time towards physical and chemical factors such as temperature, light, humidity/drought and pH; (b) their consequent reduced reproducibility; (c) increased cost of technology arising from expensive storing and shipping methods. From our point of view, maximum utility of the biosensing systems can be achieved only when the bacterial cells maintain their stability and analytical performance for a long period of time, in the field in various environmental conditions, while preserving the overall low cost of the technology.

Previous work in our laboratory led to a new preservation strategy consisting in

the development of spore-based whole-cell biosensing systems, in which the sensors were implemented into bacterial strains, such as certain *Bacillus* species, that are able to form spores [6]. Spores are dry, tough and non-reproductive dormant structures that preserve the DNA of the bacteria during times of environmental stress. Upon the return of favorable conditions, spores transform into vegetative cells regaining their full metabolic activities [45]. It was initially demonstrated that such spore-based biosensing systems maintained their analytical performance for a period of six to eight months when stored at room temperature [6]. A follow-up study proved that the storage period could successfully be extended to two years. Our method provided a simple, inexpensive, stable and resistant way of storage and transport of the developed bacterial sensor strains.

In continuation to our efforts to provide portable, rugged and robust biosensing systems that are cost-effective and can be used for on-site applications in a variety of environments, we have now studied the effect of harsh climatic conditions on the stability of spore-based biosensing systems. Different biomes on the earth have completely diverse climatic conditions, which are characterized by extremely low or high temperatures and varying degrees of humidity; examples of such environments include hot deserts, tundra regions and tropical rainforests. Importantly, these extreme conditions are often found in remote areas far from analytical laboratories and in developing countries that lack adequate storage and distribution facilities. Specifically, we have employed a spore-based arsenic/antimony biosensing system as a model and have exposed it to extreme conditions that simulate those found in real harsh environments. Such conditions included wet and dry heat, cold and desiccation. We have compared the

analytical performance of the biosensing system before and after storage in the above conditions at one-month intervals for a period of twelve months.

Our results show that bacterial spores can be used as a vehicle to store bacterial biosensing systems for a long period of time in extremely harsh environmental conditions. Also, the small size of spores and ease of their germination to vegetative active cells can facilitate implementation of portable analytical devices for on field sensing applications.

2.1 EXPERIMENTAL SECTION

2.1.1 Reagents

Potassium antimonyl tartrate hydrate, sodium m-arsenite, sodium arsenate dibasic heptahydrate and erythromycin were purchased from Sigma Aldrich (Milwaukee, WI). Luria Bertani (LB) broth, Lennox was obtained from Fisher Scientific (Bridgewater, NJ). All chemicals were reagent grade and were used as received. The luminescent substrate for β-galactosidase, Beta-Glo reagent, was purchased from Promega (Madison, WI). All solutions were prepared using reverse osmosis water (Milli-Q water purification system, Millipore, Bedford, MA).

2.1.2 Bacterial strain and plasmid

The bacterial strain used in this study was *B. subtilis* strain ars-23, which contains plasmid pMUTin-23 [56]. The plasmid carries the *ars* operon that confers resistance to arsenic [As (III) and As (V) states] and antimony [Sb (III) state] as well as the *lacZ*

reporter gene encoding *β*-galactosidase under the control of the regulatory sequences of the *ars* operon.

2.1.3 Apparatus

All luminescent measurements were performed on a Polarstar Optima microplate luminometer from BMG Labtech (Durham, NC) using Costar 3912 96-well flat bottomed white polystyrene microplates (Corning Inc., Corning, NY). All experiments were conducted at room temperature unless specified otherwise. All luminescence intensities reported are the average of five replicates and are expressed in relative light units (RLU). The Hitachi S-3200 scanning electron microscope (SEM) at the University of Kentucky Electron Microscopy Center was used to take SEM images of spores.

2.1.4 Bioreporter assay

A single colony of *B. subtilis* ars-23 strain was selected and added to 300 mL of LB broth containing 34 μg/mL erythromycin. The cells were grown overnight in an incubator shaker at 37 °C, 250 rpm until the optimum optical density at 600 nm (OD_{600}) of 0.6-0.7 was achieved. Aliquots of 50 μL of the culture medium containing the cells were then transferred to the wells of 96-well microtiter plates. Standard solutions of potassium antimonyl tartrate, sodium arsenite, and sodium arsenate of concentrations ranging from 1×10^{-3} M to 1×10^{-9} M were prepared by serial dilutions of 1×10^{-2} M stock solutions of these analytes with reverse osmosis water. All stock solutions of the analytes employed in this study were freshly prepared in reverse osmosis water for each set of monthly assays. 10 μL of analyte standard solutions and 10 μL of reverse osmosis water as blank was added in five replicates to the cell suspension in the microplate wells

and incubated at 37 °C, 250 rpm for 1 hour. Next, these cells were equilibrated to room temperature, and then 60 μL of Beta-Glo reagent (prepared following the manufacturer's instructions) was added to each well. The reagent and the bacteria were mixed using a plate shaker and incubated for 30 minutes at room temperature. The luminescence signal was then measured using the microplate luminometer with an integration time of 3 seconds per well.

2.1.5 Formation of bacterial spores

The standard protocol described by Date *et al.* [6] was employed to produce bacterial spores. Briefly, immediately after the initial assay was performed to evaluate the analytical performance of the biosensing system, the remaining bacterial cells were placed in sporulation medium and subjected to vigorous shaking at 250 rpm in an incubator shaker at 37 °C for 4 days. The optical density at 600 nm of the obtained spore suspension was recorded to be 0.34. The suspension was then centrifuged at 10,000 x g for 10 minutes at 4 °C, washed twice and resuspended in reverse osmosis water. To release the spores from the outer cell wall, the spores were subjected to sonication for 5 minutes. The spores were then separated from the cell wall debris by centrifuging the suspension at 10,000 x g for 10 minutes at 4 °C. The supernatant containing the cell wall debris was discarded and the spore pellet was resuspended in 120 mL of reverse osmosis water. The OD_{600} of the spore suspension in reverse osmosis water was recorded to be 0.82, which can be employed as an indication of the number of spores present in the suspension. A total of 72 aliquots of 1.5 mL each of the spore suspension in water were transferred into microcentrifuge tubes. The tubes were centrifuged at 10,000 x g for 10 minutes at room temperature. The supernatants were discarded and the pelleted spores

were kept in four different storage conditions, which were set up in the laboratory to simulate real harsh environmental conditions, as described in the following section.

2.1.6 Storage conditions

To check the stability of the spore-based sensing system over time, four different storage conditions were set up in the laboratory that simulated the harsh conditions found in certain real environments. Specifically, the following conditions were examined in the study. (a) Dry heat: located between 15° to 35° latitude north and south of the equator, most hot deserts experience an average day temperature of 38 °C with very low humidity (around 250 mm of rain per year) [57]. These conditions were simulated in the lab by using an oven set at 37 °C. (b) Wet heat: wet heat is found in tropical areas with average temperature of 27 °C and relative humidity between 77 and 88 % (> 2,000 mm of rain per year) [58]. The climatic conditions in tropical environments were simulated in the lab by using a water bath set at 28 °C. A humidity meter (Hygro-thermometer, Extech Instruments, Waltham, MA) was attached to the water bath to monitor the relative humidity level, which proved to be constantly greater than 90%. (c) Cold: to resemble the climatic conditions in arctic and polar areas, where the temperature ranges from -22 °C to -25 °C for most part of the year and year long, respectively, a -20 °C laboratory freezer was used [58]. (d) Desiccation: very dry climatic conditions with an average temperature of 25 °C were simulated in the lab by using a typical laboratory desiccator kept at room temperature. All the instruments used to simulate real environmental conditions were exclusively dedicated to these experiments. The four climatic conditions and their laboratory simulations are summarized in Table 2.1.

2.1.7 Monitoring of the biosensing system's analytical performance

Eighteen microcentrifuge tubes containing bacterial spores were stored in each of the above conditions for up to 12 months. One tube was taken each month from each of the experimental settings and spores were germinated to vegetative cells. The spores were resuspended in 750 µL of LB broth, then transferred into a conical flask containing 300 mL of LB broth with 34 µg/mL erythromycin and finally incubated in an incubator shaker at 37 °C at 250 rpm. The sensing cells were grown till the OD_{600} became 0.6-0.7 (approximately 15 hours) and then evaluated for their ability to respond to the three analytes: arsenite, arsenate and antimonite. The bioreporter assay was performed using the above protocol.

2.2 RESULTS AND DISCUSSION

In previous work performed in our laboratory [6], we demonstrated that bacterial spores are viable vehicles for the long-term preservation of whole-cell sensing systems at room temperature. In order to prove long-term stability and on-site applicability of spore-based biosensing systems in real harsh environmental conditions, we have studied the response and analytical performance of the sensing bacteria upon storage of the spores in simulated climatic conditions resembling those found in rough environments.

We chose an arsenic/antimony whole-cell biosensing system as a model of the spore-based storage approach. Arsenic occurs mainly as inorganic species in the environment and is present predominantly in pentavalent arsenate (As^{V}) and trivalent arsenite (As^{III}) states [59-62]. Arsenic causes a variety of adverse toxic effects on humans such as respiratory, cardiovascular and carcinogenic effects [63]. Specifically,

	Real environmental conditions	Laboratory simulations
Dry heat	Avg. T = 38 °C Precipitation = ~ 250 mm/year	Oven at 37 °C
Wet heat	Avg. T = 27 °C Precipitation = > 2,000 mm/year (RH = 77 – 88 %)	Water bath (28 °C; RH > 90 %)
Cold	T = -22 °C to -25 °C	-20 °C Freezer
Desiccation	Avg. T = 25 °C Extremely low humidity	Desiccator at room temperature

Table 2.1. Real harsh environmental conditions and their simulations in the laboratory.

RH: relative humidity.

we used *B. subtilis* strain ars-23 bearing plasmid pMUTin-23, which carries sensing elements from the *ars* operon coupled with the reporter gene *lacZ* [56]. The *ars* operon in *B. subtilis* consists of an operator/promoter (*O/P*) sequence and a regulatory gene *arsR*, open reading frame *ORF2* and two structural genes *arsB* and *arsC* downstream of the *O/P* region. While the *arsR* gene encodes the ArsR protein that regulates the expression of the *ars* operon, the *arsB* and *arsC* genes encode the proteins that form the efflux pump to extrude arsenite and antimonite out of the cell. ArsR is a DNA-binding inducer-dependent repressor protein that binds to the *O/P* region of the *ars* operon in the absence of inducer. Upon binding to the inducer (here, arsenite, arsenate and antimonite), ArsR changes its conformation leading to its release from the DNA binding site, thus enabling expression of the structural genes needed for the resistance mechanism. ArsB is an inner membrane protein forming the part of the extrusion pump that transports arsenite and antimonite out of the cell. However, this protein is incapable of effluxing arsenate. ArsC is a cytoplasmic reductase enzyme that reduces arsenate (As^{V}) to arsenite (As^{III}), which is then pumped out by ArsB. The *lacZ* reporter gene present in pMUTin-23 is fused in–frame to the *ORF2* gene and thus placed downstream of *arsR* and upstream of *arsB* genes. This keeps the reporter gene under the control of ArsR protein. *lacZ* encodes *β*-galactosidase, which is used as the reporter enzyme. When the ArsR protein releases the DNA binding site, the *β*-galactosidase enzyme is expressed and its activity can be recorded as a signal by using luminescent substrates. Figure 2.1 shows the response mechanism of the biosensing system in the presence of analyte.

Bacillus species are representative examples of bacterial adaptation during times of stress and starvation. When starved of nutrients or subjected to stress, such as,

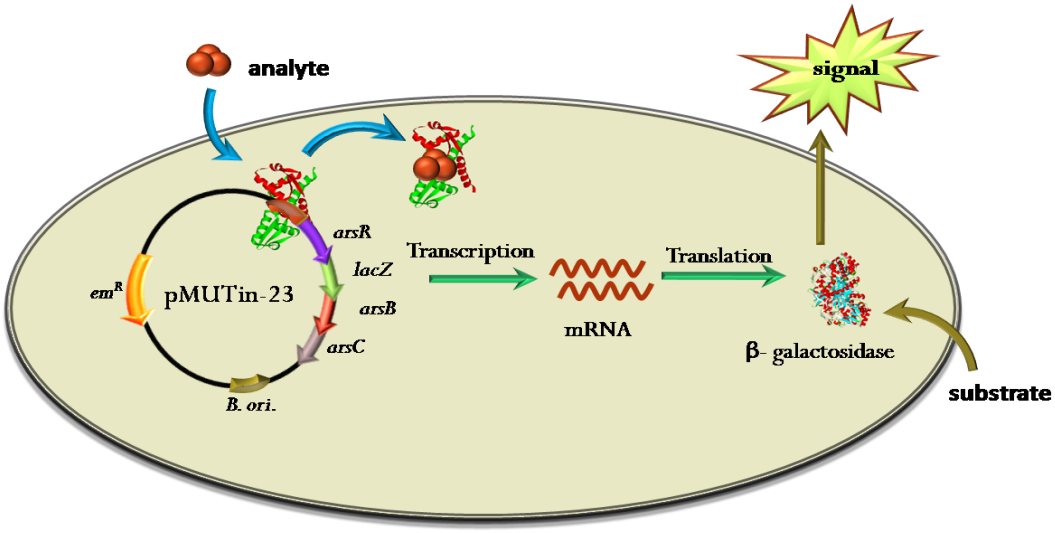

Figure 2.1. Schematic representation of the arsenite/antimonite biosensing system. Induction of the sensing system by the analyte activates the expression of β-galactosidase reporter enzyme. A bright luminescent signal results from the addition of an external substrate.

extreme temperatures and harsh environment, *B. subtilis* replicates the DNA and packages one of the two daughter DNA molecules into a tough and resistant multilayered coat called endospore. Thus, the spore locks the DNA and protects the integrity of the bacterial genetic material till conditions are suitable for germination. Upon induction by nutrients and a variety of non-nutrient agents, these dormant spores germinate to active vegetative cells. For more details on the genes controlling the sporulation mechanism and interaction between them, we refer the reader to published articles [64-65].

2.2.1 Initial dose-dependent response of the sensing system and formation of spores

In order to study the stability and viability of our spore-based bacterial biosensing system, we compared its analytical performance before and after storage in different conditions for various periods of time. Incubation of bacterial cells with the analytes arsenite, arsenate and antimonite results in the release of the ArsR dimer protein from the operator region, and expression of the different proteins required for the arsenic resistance machinery in the bacteria. Along with these proteins, the reporter enzyme *β*-galactosidase is also expressed. The amount of *β*-galactosidase expressed correlates with the concentration of bioavailable analyte present in the sample. This enzyme catalyzes the hydrolysis of *β*-galactosides and its activity can be monitored, for example, by using a luminescent luciferin-galactoside substrate, 6-O-*β*-galactopyranosyl-luciferin present in Bets-Glo reagent. *β*-galactosidase cleaves this substrate into luciferin and galactose [66]. The obtained luciferin serves as the substrate for firefly luciferase, which catalyzes a reaction leading to light emission at 565 nm, whose intensity is directly proportional to the amount of *β*-galactosidase (Figure 2.2). To evaluate the initial whole-cell sensing system response, we incubated the bacterial cells with various concentrations of arsenite,

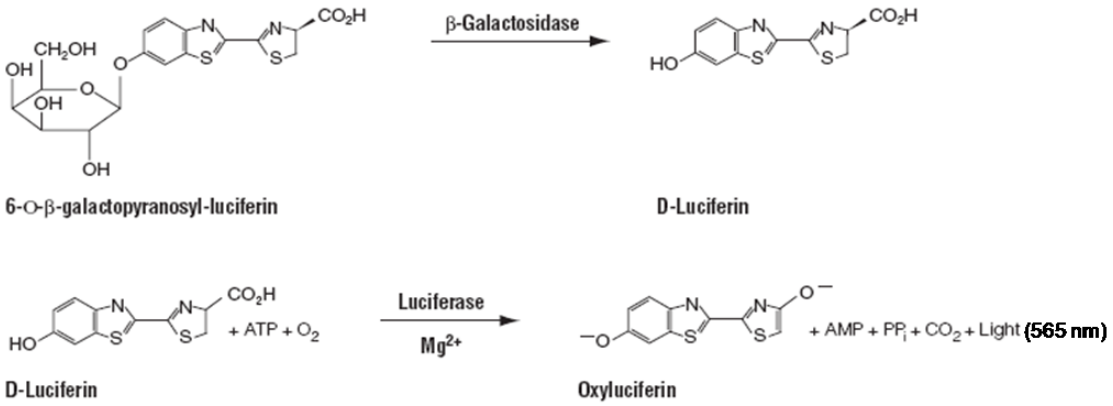

Figure 2.2. Coupled enzyme reaction of the Beta-glo system. 6-O-β-galactopyranosyl-luciferin is cleaved by β-galactosidase to yield luciferin, which is then catalyzed by luciferase in presence of cofactors to yield light at 565 nm.

arsenate and antimonite, respectively, ranging from 1.7 x 10^{-4} to 1.7 x 10^{-10} M and the luminescence signal was recorded after addition of the substrate. Dose-response curves were obtained, which revealed that the bioreporter bacteria could sense all the three analytes in a dose-dependent fashion. For example, in the arsenite assay it was observed that the luminescent signal reached maximum intensity at arsenite concentration of 1.7 x 10^{-5} M. The signal intensity decreased at higher concentrations because of the toxicity associated with the analyte itself. A representative dose-response curve for arsenite is shown in the inset of Figure 2.3. Based on the curves obtained, the detection limits were found to be 1.7 x 10^{-7}, 5 x 10^{-8}, 5 x 10^{-8} M for arsenite, arsenate and antimonite, respectively. The detection limit was defined as the analyte concentration that produced a signal higher than the average signal produced by the blank plus three standard deviations. The % relative standard deviations of the luminescent signals were less than 10%, thus confirming the reproducibility of the system. The cell-based biosensing system assays were performed in 96-well microtiter plates by employing a two-step protocol, which allowed simplified, fast analysis of multiple samples in one analytical run. Figure 2.3 shows the image of the luminescent signal obtained in the microtiter plate. It can be observed that the light intensity is very high for higher concentrations of analyte and diminishes with decreasing analyte concentrations. Interestingly, the light intensity achieved with the 6-O-β-galactopyranosyl-luciferin substrate is so strong that it is visible to the naked eye. This may enable an immediate, rough estimate of the presence of arsenic in biological and environmental samples. After evaluating the initial analytical performance of the bacteria, the remaining sensing cells were sporulated. Scanning electron micrographs were taken to assure that the cells actually turned into

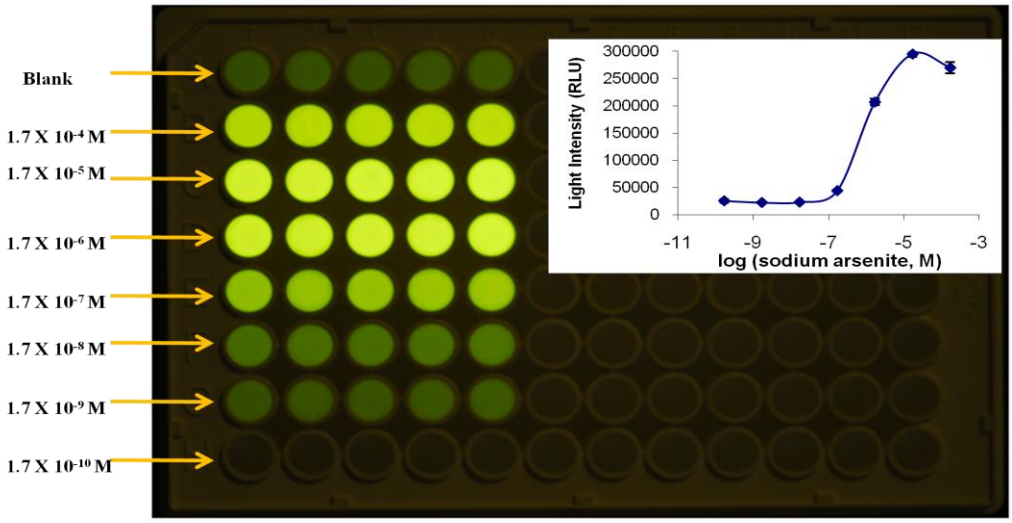

Figure 2.3. Image of a 96-well microtiter plate where the assay was performed. The digital image was taken after 30 minute incubation of 6-O-β-galactopyranosyl-luciferin substrate with the sodium arsenite induced cells. The obtained signal is the light emitted at 565 nm from the firefly luciferin-luciferase reaction. Five replicates were measured for each concentration of sodium arsenite (1.7×10^{-4} M – 1.7×10^{-10} M). The inset shows the initial dose-response curve of the sensing bacteria before sporulation.

spores. Figure 2.4 shows one of such SEM images of the *B. subtilis* ars-23 spores.

2.2.2 Dose-response curves for arsenite, arsenate and antimonite upon storage

Harsh environments include atmospheric conditions, such as, very high or low temperatures and excessive moisture or dryness as well as geochemical conditions, such as, desiccation, high or low pH, and presence of oxidising agents and other toxic compounds. To test whether these extreme environments can affect the stability and sensing capability of man-made biosensing systems when stored as bacterial spores, we exposed the sensing spores to four different harsh conditions of dry heat, wet heat, cold and desiccation for a period of 12 months. The sensing spores were stored in different laboratory conditions that simulated those found in real life harsh environments. Dry heat is the most challenging condition in hot deserts with an average day temperature of 38 °C and this condition was simulated in the lab by storing the spores in an oven maintained at a constant temperature of 37 °C. Wet heat is common in tropical areas with an average temperature of 27 °C and high relative humidity betweeen 77 and 88 %. Such conditions were approximated by using a water bath maintained at 28 °C. The relative humidity was maintained above 90 % during the 12 month storage period. Arctic and polar areas exhibit very low temperatures, from -22 °C to -25 °C, for most of the year and year long, respectively. We used a -20 °C laboratory freezer to mimic these conditions. Desiccation or extreme dryness is another harsh condition. The drying-out process (limitation of water) brings about stressful responses within cells resulting in irreversible phase changes, denaturation of proteins and nucleic acids, and accumulation of reactive oxygen species [67]. The effect of desiccation was evaluated by storing the

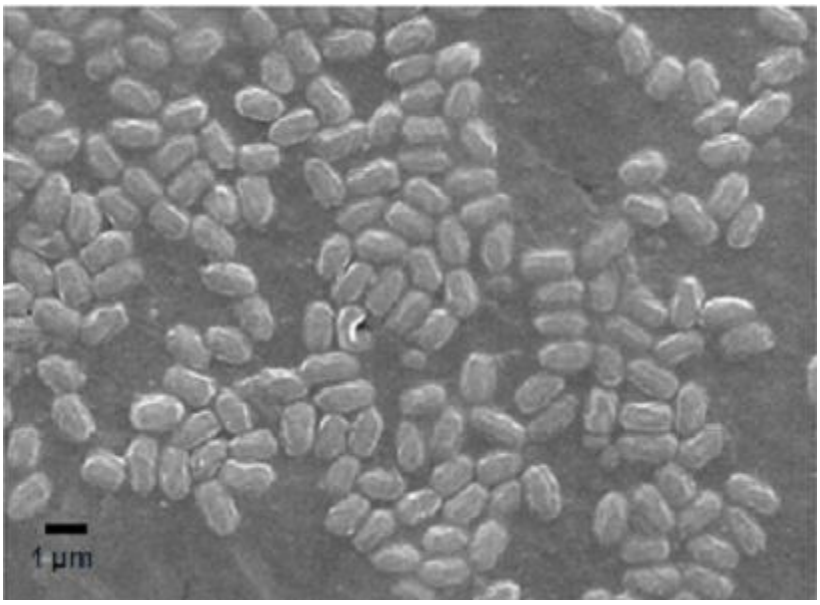

Figure 2.4. Scanning electron micrograph of *B. subtilis* ars-23 spores. Scale bar represents 1 µm. The size of the spores was determined to be approximately 1 μm by 0.5 μm.

spores in a typical laboratory desiccator at room temperature.

On a monthly basis, spore aliquots were transferred to fresh growth media and were allowed to germinate and grow into active vegetative cells. Then, the stability and analytical performance of the sensing bacteria were monitored through bioanalytical assays, as described above. No significant differences were found in the viability and sensing ability of the vegetative cells upon the spore storage, regardless of the different storage conditions tested (Figure 2.5). In fact, all spore aliquots were able to germinate and the obtained vegetative cells were able to multiply and quantitatively respond to the analytes. As shown in Table 2.2 for arsenite, the analytical performance of our biosensing system remained virtually unaffected throughout the storage period of 12 months in different extreme conditions. After one year of storage in dry heat, wet heat and desiccation conditions, the detection limits of the sensing system for arsenite were found to be 2×10^{-8} M; comparable results were observed upon storage in extremely cold condition, with a detection limit of 5×10^{-8} M. Similarly, the system maintained its sensing capability for antimonite, with limits of detection of 5×10^{-8} M in all the four storage conditions. The response towards arsenate showed the same trend with detection limits of 5×10^{-8} M in dry heat and desiccation, and 5×10^{-7} M in cold and wet heat conditions. The dose-response curves showed dynamic ranges of two orders of magnitude, as before storage. The results obtained in the one-year storage study of the arsenite/arsenate/antimonite biosensor are summarized in Table 2.3. Overall, the analytical performance of the spore-based sensing system, in terms of detection limit, dynamic range and reproducibility, did not change significantly during the twelve months of storage in different extreme climatic conditions. Our results prove that spore-based

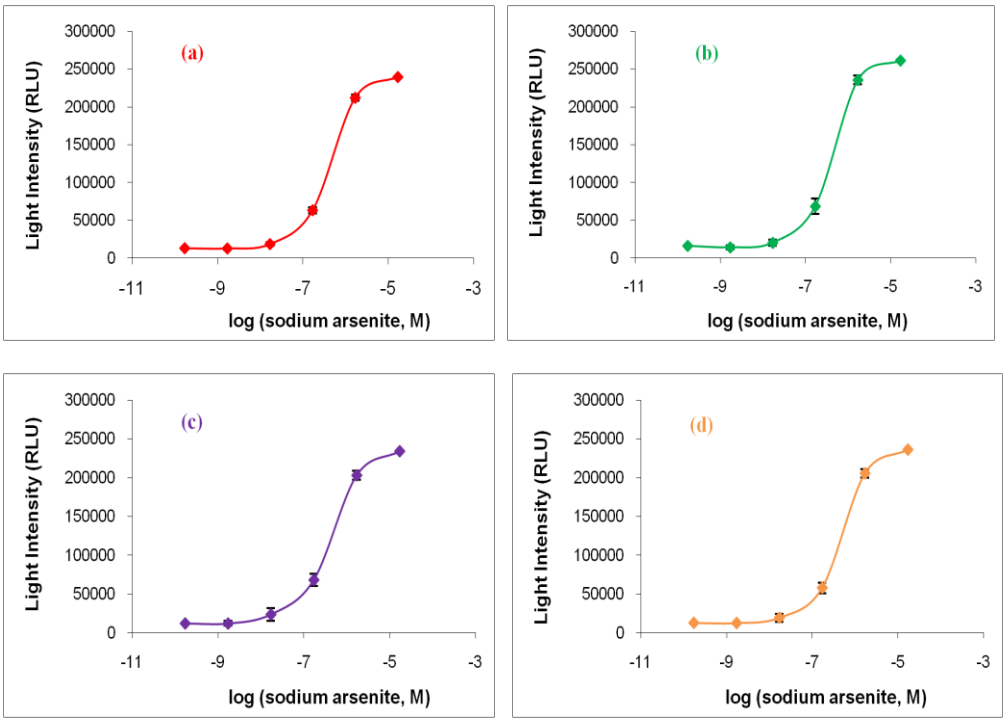

Figure 2.5. Dose-response curves of *B. subtilis* spore-based sensing system after storing the spores for 12 months in different storage conditions of cold (a), dry heat (b), desiccation (c) and wet heat (d), respectively. Data shown are the average ± one standard deviation (n = 5).

Storage time (months)	Cold -20°C	Wet heat 25°C/RH>90%	Dry heat 37°C	Desiccation
1	1.7×10^{-7}	3.2×10^{-7}	1.7×10^{-7}	3.2×10^{-7}
2	1.7×10^{-7}	1.7×10^{-7}	1.7×10^{-7}	3.2×10^{-7}
3	1.7×10^{-7}	3.2×10^{-7}	1.7×10^{-7}	–
4	1.7×10^{-8}	3.2×10^{-7}	3.2×10^{-7}	1.7×10^{-8}
5	5.0×10^{-8}	8.0×10^{-8}	1.0×10^{-7}	1.7×10^{-8}
6	8.0×10^{-8}	5.0×10^{-8}	1.7×10^{-8}	8.0×10^{-9}
7	8.0×10^{-9}	5.0×10^{-9}	5.0×10^{-9}	3.2×10^{-9}
8	2.0×10^{-8}	5.0×10^{-9}	5.0×10^{-9}	2.0×10^{-9}
9	1.7×10^{-8}	1.7×10^{-8}	1.7×10^{-8}	1.7×10^{-8}
10	1.7×10^{-8}	2.0×10^{-8}	5.0×10^{-8}	2.0×10^{-8}
11	2×10^{-8}	1.7×10^{-8}	1.7×10^{-8}	1.7×10^{-8}
12	5×10^{-8}	2×10^{-8}	2×10^{-8}	2×10^{-8}

Table 2.2. Detection limit (M) of the spore-based whole-cell biosensing system for arsenite evaluated every month for a period of 12 months in different storage conditions. RH: relative humidity.

		Cold - 20 °C	Wet heat 28 °C/RH >90%	Dry heat 37 °C	Desiccation
Arsenite	Limit of detection	5×10^{-8}	2×10^{-8}	2×10^{-8}	2×10^{-8}
	Dynamic range	$5 \times 10^{-6} - 5 \times 10^{-8}$	$5 \times 10^{-6} - 2 \times 10^{-8}$	$5 \times 10^{-6} - 2 \times 10^{-8}$	$5 \times 10^{-6} - 2 \times 10^{-8}$
Arsenate	Limit of detection	5×10^{-7}	5×10^{-7}	5×10^{-8}	5×10^{-8}
	Dynamic range	$1.7 \times 10^{-5} - 5 \times 10^{-7}$	$1.7 \times 10^{-5} - 5 \times 10^{-7}$	$1.7 \times 10^{-5} - 5 \times 10^{-8}$	$1.7 \times 10^{-6} - 5 \times 10^{-8}$
Antimonite	Limit of detection	5×10^{-8}	5×10^{-8}	5×10^{-8}	5×10^{-8}
	Dynamic range	$1.7 \times 10^{-6} - 5 \times 10^{-8}$	$1.7 \times 10^{-6} - 5 \times 10^{-8}$	$1.7 \times 10^{-6} - 5 \times 10^{-8}$	$1.7 \times 10^{-6} - 5 \times 10^{-8}$

Table 2.3. Analytical characteristics of the sensing system for arsenite, arsenate and antimonite evaluated after 12 months of storage in different extreme conditions of cold, wet heat, dry heat and desiccation, respectively. RH: relative humidity.

sensors possess all the characteristics required for field analysis. In fact, in addition to being sensitive, selective, reproducible, robust, easy to use, and cost effective, as traditional whole-cell sensing systems are, they also maintain their viability and sensing capability during long-term storage in unfavorable conditions and, as such, they facilitate portability.

CHAPTER THREE

CONCLUSIONS AND FUTURE PERSPECTIVES

We have tested the effect of storage time under different extreme environmental conditions on a spore-based biosensing system. Our results demonstrate that the spore-based concept of packaging the biosensors provide a very good storage approach for long periods of time in challenging conditions. The robustness and intrinsic resistance of spores in harsh environments make them ideal storage vehicles for long-term preservation of the recombinant sensing bacterial DNA and also help maintaining the stability and analytical performance of the biosensing system in natural settings. This is particularly important for on-field applications in geographical locations where there is no easy access to laboratory facilities, and that experience poor storage and transportation facilities. We envision that the long-term stability of such sensing systems in, both, mild and extreme environmental conditions would enable their integration into easily operated analytical devices that will provide a portable solution to environmental field studies. In this regard, work has been performed in our laboratory to incorporate spore-based sensing systems in compact disk (CD) centrifugal microfluidic platforms (submitted manuscript). Bacterial spore-based sensing systems have been integrated into lab-on-a-CD system and it has been demonstrated that the germination and sensing ability of the spore-based sensing systems on these microfluidic platforms is good and is comparable to the conventional bench top protocols. Research is currently underway towards the development of strategies for the effective immobilization of spores on different types of supports, such as, paper, glass, and polymeric materials. We also envision that incorporation of spore-based sensing systems on an appropriate analytical platform would

result in robust and simple to use portable sensing systems, suitable for the analysis of real environmental and biological samples even by minimally trained personnel in the field.

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1. S. K. Jha, A. Sangal, Rama Kant, Diffusion-controlled potentiostatic current transients on realistic fractal electrodes, J. Electroanal. Chem., 615 (2008), 180-190

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