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Advances in preservation methods: keeping biosensor microorganisms alive and active

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The ability of bacteria to sense their surroundings can be employed to measure the bioavailability and toxicity of pollutants. However, long-term maintenance of both viability and activity of the sensor bacteria is required for the development of cell-based devices for environmental monitoring. To meet these demands, various techniques to conserve such bacteria have been reported, including freeze drying, vacuum drying, continuous cultivation, and immobilisation in biocompatible polymers of organic or inorganic origin. Much effort has been invested in merging these bacterial preservation schemes with the construction of sensor cell arrays on platforms such as biochips or optic fibres, hopefully leading to effective miniaturised whole-cell biosensor systems. These approaches hold much promise for the future. Nevertheless, their eventual implementation in practical devices calls for significant enhancement of current knowledge on formulation of reporter microorganisms.

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Introduction

Bacteria have evolved a repertoire of positive and negative responses to different environmental conditions, such as the presence of nutrients or toxins, respectively. This constant sensing of their immediate environment makes bacteria ideal for determination of the bioavailability and/or toxicity of pollutants. Recently, considerable attention has focused on the development of bacterial whole-cell biosensors in combination with high-throughput, low-cost instrumentation for the analysis of environmental samples. Although this review focuses on the formulation of bacteria, it is important to note that eukaryotic cells can also be used for biosensor applications and each cell type has its own formulation requirements.

Bacterial biosensors rely on the ability of cells to produce a detectable signal that can serve as a reporter of a particular environmental condition, and these can be distinguished into two principle classes [1,2]. Constitutive reporter cells produce a constant measurable signal, and the general toxicity of a sample is estimated from the inhibition of this signal. Inducible reporter microorganisms are usually more specific in their performance, as they are based on a reporter gene fused to an inducible promoter that is activated by a target compound or stress response. Commonly used reporter genes include *lacZ* (encoding β-galactosidase), *luxAB* (encoding bacterial luciferase), *luc* (encoding eukaryotic luciferase), and *gfp* (encoding the *Aequorea victoria* green fluorescent protein) [1].

Although numerous reports describe the genetic engineering of bacterial sensor strains, very few address the largely unresolved concern of the 'shelf-life' of such constructs. Indeed, storage of reporter bacteria at ambient temperatures for prolonged periods, with maintained response characteristics and without the need to regrow the cells, is still a major challenge [3,4]. Among many operational requirements from such a process, it is essential that the cells have sufficient energy for reporter function within the formulated product. A weaker response will be obtained from cells with a compromised energy status, regardless of the environmental condition being reported upon [1]. Here, we highlight some of the approaches currently used to preserve biosensor bacteria and to keep them 'alive and kicking'.

Suspended life: freeze drying and vacuum drying

Life and its reactions are dependent upon water. Nevertheless, dehydration to the point of total desiccation can be a good method to preserve live cells in a state of arrested metabolism (anhydrobiosis), which hopefully can be restarted following rehydration. Several microorganisms, invertebrates and plants naturally use anhydrobiosis for survival during periods of drought [5,6]. A common feature of true anhydrobionts is the accumulation of compatible solutes (e.g. trehalose) that are believed to function as metabolically inert chemical chaperones [7,8]. The molecular functions of compatible solutes are debated, but are generally thought to involve

Table 1

Selected methods for stabilizing biosensor bacteria.

Preservation method	Advantages	Disadvantages
Freeze drying (lyophilization)	Conserves formulation structure Proven industrial performance record Easily rehydrated product	Costly and complex technique Product sensitive to moisture
Vacuum drying	Yields potentially high survival rates and long-term stability Relatively low production costs Possible alternative for freeze-sensitive microorganisms	Less well-proven performance record Harsher drying conditions than freeze drying
Continuous culture	Provides a biocompatible static environment and fresh, active bacteria	Complex and labour-intensive maintenance Risk of genetic drift Risk of contamination
Encapsulation in organic polymers (e.g. hydrogels)	Immobilisation, physical shielding and isolation Allows solute diffusion	Biodegradable Bacterial growth may occur Opacity may hinder optical signal detection
Encapsulation in inorganic polymers (e.g. sol-gel)	Immobilisation, physical shielding and isolation Mechanical rigidity and good optical properties Allows solute diffusion Limits bacterial growth	Less well-proven performance record Tested for a limited variety of microorganisms

the protection of proteins and lipid membranes from desiccation-induced damage.

The current industrial standard for preserving microbes is freeze drying (Table 1). Briefly, freeze drying or lyophilization is a three-step process that produces a structurally intact and easily reconstituted end product [9,10]. Initial freezing is performed in such a fashion that the amounts and sizes of ice crystals are kept to a minimum. This is usually accomplished by the use of cryoprotective substances and carefully controlled freezing rates that cause a glass transition, or vitrification, of the mixture, which in effect stops further ice growth [11]. Importantly, the random molecular orientation of the liquid before the transition is essentially conserved through this phase change of solution into an amorphous solid. The free water of the sample is subsequently removed by sublimation (direct vapourization) of ice under vacuum. During this primary drying step, the temperature must remain below the highly critical collapse temperature to avoid melt-back of the solid material and destruction of the product [12]. The remaining bound water is forced out in the secondary drying step by gradually increasing the process temperature. The resulting anhydrous product can normally be stored, tightly sealed under vacuum or a protective atmosphere, for long time periods with good activity upon rehydration.

Several commercially available toxicity-screening kits are based on freeze-dried bioluminescent bacteria; examples include the 'Microtox®' assay where the *Vibrio fischeri* strain used has a shelf-life of one year when stored at −20 °C, or the 'ToxScreen' test with *Photobacterium leiognathi*, which can be shipped at ambient temperatures without affecting overall luminescence after rehydration [13]. Preservation conditions are still being optimized for several recombinant stress-inducible reporter bacteria, many of which are based on bioluminescent *Escherichia coli* strains for toxicity monitoring. Several have been formulated by lyophilization, with reported storage times from 10 days up to several months at −20 °C [14–16]. Although these results are encouraging, improvements in lyophilization protocols are needed if long-term storage, at ambient temperatures and with preserved sensor activity, is to be realized.

Vacuum drying is another attractive, albeit less well-explored, alternative for preserving microorganisms (Table 1). The name of the technology is misleading; in fact, the principles of vacuum drying are much the same as for lyophilization, except for the use of elevated non-freezing temperatures [17,18]. Thus, the removal of water is more rapid than during sublimation and the microorganisms experience an increasing desiccation stress while still metabolically active. Stocker *et al.* [19•] used vacuum drying for preservation of an *E. coli* reporter strain with inducible β-galactosidase production following exposure to arsenite. The bacteria were formulated and vacuum-dried on paper strips that could be stored for two months at 30 °C without any apparent loss of reporter activity. Our own results have shown that vacuum drying of *Pseudomonas* spp., *Lactobacillus* spp., and the yeast *Pichia anomala* is an effective method for maintaining viable cells. Initial survival rates up to 50% were obtained for *Pseudomonas* spp. After storage for more than two months at ambient temperatures, viability rates above 35% were maintained (S Håkansson, unpublished).

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