The challenge to desication of probiotic living cells

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***Abstract*—*Preservation of micro-organisms by desiccation has been the preferred method for long term storage of cultures whilst preserving cell viability. These processes result in exposure of the live probiotic bacteria to a variety of stresses, such as heat, cold, oxygen and osmotic stresses, leading to impaired functionality and loss of viability during drying and storage. The aim of this review is to discuss the process of producing anhydrobiotics in steps to obtain the highest cell viability*.

Keywords-component; drying; probiotic; viability; protectants

I. INTRODUCTION

Probiotics are defined as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' [1]. There is rapidly accumulating clinical evidence that these bacteria can positively affect certain human health conditions, playing an important role in the control of irritable bowel syndrome and inflammatory bowel diseases, suppression of endogenous/ exogenous pathogens by normalization of the intestinal microbial composition, alleviation of food allergy symptoms in infants by immunomodulation, lowering serum cholesterol, removing of aflatoxin, improving lactose tolerance, and reducing risk factors for colon cancer by metabolic effects [2,3]. Table I lists some potentially probiotic cultures used in probiotic foods or probiotic food supplements. Although specific numbers are not mentioned in the definition, high levels of viable microorganisms are recommended in probiotic foods for efficacy [4] given that many of the clinical studies use daily doses in excess of 1×10^9 CFU/day [5]. Consequently, the retention of high viability during drying and storage presents particular challenges and can be regarded as a major bottleneck in commercial probiotic production.

Food and pharmaceutical industries have found drying technologies to be the preferred methods for preserving a multitude of different food and drug preparations in bulk quantities. Even with this worldwide usage of drying technologies, it would appear that there are still many varied methods of desiccating micro-organisms and that there is no generic drying method for all applications [6].

This paper discusses drying processes for micro-organisms without causing cell death and the research done on the improvement of probiotic bacteria survival during drying and subsequent storage. In the preparation of this review limited data was found which could give an indication of the stability of the dried micro-organisms over extended periods of time, especially when precise numbers of organisms had been preserved. Areas that the review will focus on include intrinsic factors, the growth phase of the micro-organism, growth conditions, sub-lethal treatments, drying medium, protectants, drying method, storage and rehydration procedures.

II. INTRINSIC FACTORS

In order to maintain cell viability, the fundamental requirement is to keep essential cellular structures intact after drying and fully functional after rehydration. Distinct species of one given genus may often exhibit rather different behaviors during freezing, drying and subsequent storage [7, 8, 9, 10]. Previous works [8, 11] on effects of bacterial cell upon survival during freezing and freeze-drying reported that enterococci (i.e. small spherical cells) are apparently more resistant to freezing and freeze-drying than lactobacilli (rods). According to Fonseca [8], the higher the surface area

of the cell, the higher the membrane damage owing to extracellular ice crystal formation during freezing.

TABLE I. POTENTIALLY PROBIOTIC CULTURES USED IN PROBIOTIC FOODS OR PROBIOTIC FOOD SUPPLEMENTS [12]

Probiotic cultures	Species
Lactobacillus	*acidophilus/johnsonii/gasseri, delbrueckii subsp. Bulgaricus, casei, crispatus, lactis, paracasei, fermentum, plantarum, rhamnosus, reuteri , salivarius*
Bifidobacterium	*adolescentis animalis/lactis , bifidum, breve, essensis, infantis, longum*
Bacillus	*Subtilis, clausii*
Enterococcus	*Faecalis, faecium*
Escherichia	*coli* strain Nissle
Pediococcus	*Acidilacti*
Propionibacterium	*Freudenreichii*
Saccharomyces	*Boulardii*
Streptococcus	*Thermophilus*

III. GROWTH PHASE AND CELL CONCENTRATION

When grown in batch culture, the growth of bacterial cultures occurs during four distinct phases, i.e. lag, log, stationary and death phases. It is known that the stress responses of bacterial cultures vary depending on the growth phase. Indeed, bacteria that enter into stationary phase, due to carbon starvation and exhaustion of available food sources, develop a general stress resistance and are thus more resistant to various types of stresses than bacteria in the log-phase [13, 14]. The survival response also protects the cell in other adverse conditions, such as desiccation and adverse temperatures [14]. The optimal growth phase for desiccation survival has been found to be largely dependent on the organism. For example, it was reported that stationary phase cells of *Lactobacillus. rhamnosus* yielded the highest recovery rates after drying (31–50% survival), whereas early log-phase cells exhibited only 14% survival, and lag phase cells showed the highest susceptibility, with only a 2% cell survival under similar conditions of drying [15]. However, in earlier studies on the freeze-drying of lactic acid bacteria, late-logarithmic [16] or early-stationary [17, 18]; phase cells were commonly used. Palmfeldt [19] optimized initial cell concentration of *Pseudomonas chlororaphis* to enhance the viability after freeze-drying. The highest freeze-drying survival values, 15–25%, were obtained for initial cell concentrations between 1×10^9 and 1×10^10 CFU/ml. For cell concentrations outside this range more than 10 times lower survival values were observed.

Rault [20] suggested that cell cryotolerance increases with fermentation time or when cells are harvested during culture at pH 5 and frozen, as compared with fermentations at pH 6 or without pH control. Long term cell cryotolerance could be predicted by determining an early physiological parameter such as a low initial acidification activity when the cells are harvested.

IV. APPLICATION OF MILD STRESS PRIOR TO DEHYDRATION:

It is widely accepted that, when used in industrial food processing, probiotics are exposed to a number of stress conditions, such as low temperature, low pH and low water activity, which cause membrane and cell wall damage, inhibition of active transport, retention of nutrients, morphological changes and loss of viability. Bacteria have meanwhile developed adaptive strategies to face the challenges of changing environments, and to survive under conditions of stress [21]. For instance, the response of bacteria to hyperosmolarity encompasses two aspects: their ability to develop multi tolerance towards other environmental stresses, and their ability to accumulate osmoprotective compounds [22]. It was suggested [23] that addition of NaCl to the growth medium, as well as different concentrations of undefined components that are sources of compatible solutes (e.g. peptones, tryptone, and meat and yeast extracts) [24], may lead to increased production/accumulation of compatible solutes, and therefore might promote survival of probiotics throughout storage in the dried state. It also has been reported [23] that rising the medium osmolarity through addition of an electrolyte (NaCl) or of a non-electrolyte (sucrose) has distinct consequences upon *Lactobacillus. bulgaricus* survival during storage in the dried state. Higher survival rates during storage in dried form were indeed observed only when these bacteria were previously grown in MRS supplemented with NaCl.

V. GROWTH MEDIA:

Although major emphasis has been placed on the effect of the drying medium, the growth medium is also a critical parameter, which is likely to play a role upon survival subsequent to drying. The composition of the growth media is a contributing factor to the survival rate of probiotic cultures during drying, and in this respect, the importance of the presence of sugars, accumulation of compatible solutes, production of exo-polysaccharides, and altered fatty acid profile of the membrane has been demonstrated.

A. Sugar substrates present in the growth medium:

Tymczyszyn, [25] reported the difference in the effectiveness of lactose, sucrose and trehalose in the recovery of *Lactobacillus. delbrueckii* subsp. *bulgaricus* following drying, when grown at different water activities. Indeed, it has been demonstrated that the preservation of dehydrated bacteria with sucrose, after growing them in a low water activity medium (MRSsucrose), appears to be as efficient as dehydration with trehalose. A research done by Carvalho [26] indicated that *Lactobacillus bulgaricus* showed the lowest decrease in viability after freeze-drying

when grown in the presence of mannose, compared to fructose, lactose or glucose. Other sugar types, such as fructose and sorbitol also provided better protection than the standard growth media carbohydrate glucose [17].

B. Compatible solutes:

Compatible solutes or osmolytes are small organic compounds that do not interfere with cell functions and are used for osmotic adjustment. These include polyols, amino acids and amino derivatives. Micro-organisms undergoing drying are faced with an increasing osmotic stress as the water activity decreases. One of the ways organisms counteract the osmotic stress is to accumulate compatible solutes to maintain the osmotic balance between the highly concentrated extracellular environment and the more dilute intracellular environment. These solutes can also help to stabilize proteins and the cell membrane during osmotic stress conditions brought on by low water activity during drying processes [7, 14]. The reason for this is most likely that the bacterial cells get adapted to the low water activity medium.

C. Exopolysaccharide production:

In general, the term exopolysaccharide refers to two types of secreted polysaccharides; the first type is attached to the cell wall as a capsule (capsular polysaccharides, or CPS), whereas the other is produced as loose, unattached material (slime exopolysaccharide, or exopolysaccharide proper) [27]. It has been suggested [28] that exopolysaccharide formation is part of a survival strategy in harmful environments. Most functions proposed for exopolysaccharide are of a protective nature, e.g. protection against desiccation, phagocytosis, phage attack, antibiotics, toxic compounds and osmotic stress [29].

D. Altered membrane profile:

The cytoplasmic membrane, which provides the boundary between the cytoplasm and the external environment, regulates the flow of nutrients and metabolic products into and out of the cell, thereby permitting homeostasis of the cytoplasmatic environment. Modifications in the cell environment may even alter the composition of the membrane. Variations in the prevailing environmental conditions induce variation of the membrane lipid structure, which in turn affect its fluidity; the major way through which bacteria maintain the ideal membrane fluidity is by changing its fatty acid composition [30].

These studies taken together imply stress induces a number of survival strategies which provide protection to micro-organisms upon drying. The production of stress response proteins during growth of bacterial cells may arm the cells with valuable proteins during the recovery stage after drying [31].

VI. PROTECTANTS:

Protective agents can be added during growth of the micro-organism, or prior to freezing or drying. The type of protectant largely depends on the micro-organism; however, there are a few that appear to work well with many species. These include non-fat milk solids, serum, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose and polymers such as dextran and polyethylene glycol [7, 14]. Usage of compatible cryoprotectants in growth media prior to fermentation assists in the adaptation of probiotics to the environment [32].

When drying to modestly low moisture content, these solutes and sugars in the drying media may protect the cells by being preferentially excluded from the proteins or membrane surfaces, or in another sense they keep proteins and membranes preferentially hydrated. In the case wherein water is continually removed until very low moisture content is achieved, the cell membrane has been proved to be a critical site of damage owing to the leakage of cells due phase transition during drying or rehydration. At this stage, sugars protect cell membrane by depressing the membrane phase transition temperature of cells. Hence, the cell membrane can retain its liquid crystalline phase under given drying or rehydration conditions. The mechanism behind the depression of the membrane phase temperature is anyhow still controversial and it is not clear whether specific interactions between sugars and membrane are required. During drying at a high or subzero temperature, sugars may protect cells against these additional stresses. The sugar glasses reduce molecular mobility and therefore retard deteriorative reactions. Therefore, it can be inferred that the glass transition temperature of sugars should be considered as a criteria for the selection of sugars in the drying media. Nevertheless, polymeric sugars, which easily form glasses, often do not have suitable molecular sizes or structures to be able to depress membrane phase transition. A polymeric sugar with a high molecular weight or with a rigid structure will therefore fail to protect cells during drying [15, 19, 33].

VII. DRYING METHODOLOGY:

Spray drying and freeze drying are some of the most common drying technologies used for drying of bioproducts, although fluid-bed, foam formation, and vacuum dryers are also common [34].

Freeze-drying has been used to manufacture probiotic powders for decades and is based upon sublimation, occurring in three phases; freezing, primary, and secondary drying. Typically, cells are first frozen at -196°C and then dried by sublimation under high vacuum [35]. As the processing conditions associated with freeze-drying are milder than spray-drying, higher probiotic survival rates are typically achieved in freeze-dried powders [36].

Commercial scale production of freeze-dried cultures is an expensive process with low yields, and as such spray-drying offers alternative inexpensive approach yielding higher production rates [37]. The spray-drying process involves the injection of the spray-drying medium at high velocity at temperatures up to 200°C, which then blasts through a nozzle leading to formation of granules. Consequently, this process results in exposure of the drying medium to high temperatures for a short time, which can be detrimental to the integrity of live bacterial cells. During

spray-drying, bacterial cells encounter heat stress, in addition to the other stresses already mentioned during freeze-drying, i.e. dehydration, oxygen exposure and osmotic stress [38, 39].

Fluidised bed dryers use an upward moving flow of heated air and mechanical shaking to create a fluidised effect in a solid product. Larena [40] showed freeze drying and fluidized bed drying maintained 100% viability of *Penicillium oxalicum conidia.*

Foam formation is a new drying technique which uses protective sugar matrices to transform biological suspensions into mechanically stable dry foams. These foams are made by boiling them under vacuum at ambient temperatures to induce a process called vitrification, which produces immobile amorphous, non-crystalline glass foams directly from a liquid. The foams are then subjected to further drying at elevated temperatures to increase their stability at ambient temperatures [41].

Numerous new drying techniques proposed and tested over the past decade have potential for application to biotech products. Table Π lists some such emerging technologies [34].

VIII. Packaging and storage:

The method of storage and the packaging it is stored within will influence the shelf life of any dried product. As with most perishable products the most common reactive agents to avoid are; oxygen, moisture, light, microbial contamination and elevated temperatures. Therefore the packaging materials are different types of barriers to these reactive agents. In general, freeze dried products are stored within ampoules, or glass vials. For dried products there are other options such as high barrier plastic bags and blister packs [7].

Bozoglu [11] compared viability of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* following storage in air compared to nitrogen and under vacuum. Storage within glass vials sealed under vacuum or nitrogen gas were found to be superior compared to storage in air. They concluded the poor cell recovery was due to oxygen diffusion into the dry cells through the interfacial area of the cell, possibly because the cells remain permeable throughout storage. The accumulation of free radicals such as oxygen species within a cell that cannot metabolize them, or actively transport them out of the cell, can result in irreversible damaging processes occurring within the cell [11].

TABLE II. Commonly used dryers and emerging drying technologies suitable for biotech products.[34]

Dryers for biotech products	
Conventional dryers	***Emerging dryers***
Spray dryer Spray/fluid-bed (two-stage) Freeze dryer Vacuum tray Continues tray dryer Drum dryer/vacuum Indirect vacuum Plate or turbo dryer	Heat-pump dryers (below/above freezing point) Intermittent batch dryer Vacuum fluid-bed dryer Low-pressure spray dryer with ultrasonic atomizer Sorption dryer Pulse combustion dryer Cyclic pressure/vacuum dryer High electric field (HEF) dryer Superheated steam dryer at low pressures

The storage conditions have significant influences on the survival of probiotics in dried powders, and the correct storage conditions are essential to maintain viable populations of dried probiotic bacteria. Costa [42] observed that the shelf life of freeze-dried products is highly dependent on the storage temperature. *Pantoea agglomerans* was found to decrease in viability by 0.5 log after 90 days at 4 °C, compared to a decrease of 3 logs after 28 days at 25°C.

Forest [43] demonstrated that vacuum drying, especially when dried with appropriate protectant, can be a promising method to produce dried probiotic cells with high stability for the storage at non-refrigerated temperatures. Furthermore an appropriate protectant against drying inactivation may not effectively stabilize cells during storage and both aspects must be considered together [43].

IX. Rehydration:

Rehydration of probiotic powders is the final critical step for the revival of cells after dehydration. The rehydration solution itself (in terms of osmolarity, pH and nutritional energy source), as well as the rehydration conditions (in terms of rehydration temperature and volume) may significantly affect the rate of recovery to the viable state, and thus influence survival rates [44]. For optimum results, it is recommended to dry the cells at the stationary phase of growth and to use slow rehydration procedures [39].

Costa [42] tested seven different types of rehydration media to revive *Pantoea agglomerans* cells. Complex media such as 10% non fat skimmed milk and PTM medium (1.5% peptone, 1% tryptone and 0.5% meat extract) as well as a 10% sucrose solution were found to produce a significantly higher cell recovery than media such as phosphate buffer, sodium glutamate and water [42]. Abadias has demonstrated a significant increase in viability of *Candida sake* cells when the same solution tested as protectant was used to rehydrate dried samples [45].

The temperature of rehydration could also influence cell recovery after freeze-drying, Ray [46] found rehydration at 15 – 25 °C produced the highest numbers of recovered cells, compared to 35 °C and 45 °C where the cell recovery was lower but the growth more rapid.

Another factor to be taken into account, is the rate of rehydration, Poirier have hypothesized that increased cell recovery of *Saccharomyces cerevisiae* is achieved when the dried cells were rehydrated slowly (7–16 days) under controlled conditions, rather than immediate rehydration. However, this amount of time for rehydration makes the revival of dried microorganisms uneconomical [47].

X. CONCLUSION:

Preservation of micro-organisms by desiccation is a science mostly based on testing rather than facts and tested theories. Due to the lack of generic theories for all bacterial strains, for optimum results, it is important to consider a variety of factors, including the selection of the particular probiotic strain, the condition of the culture entering the dryer, the use of protectants and desiccation methodology. In addition, information on the sites of impact and the nature of the injury produced by a variety of stressful conditions (e.g. freezing, drying, storage or rehydration), together with knowledge of the induction of stress proteins (particularly those which provide resistance during drying and subsequent storage) are definitively important towards production of dried starter cultures, which will be characterized by high survival rates even after extended storage.

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