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**RESEARCH ARTICLE**

# EXTERNAL AND INTERNAL FACTORS AFFECTING SURVIVAL OF PROBIOTIC LIVING CELLS DURING DESICCATION

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***ABSTRACT***

***P****robiotics are defined as 'live micro-organisms which confer a health benefit on host while being consumed in adequate amounts. This paper discusses drying process of these micro-organisms to minimize cell death and the research done on the improvement of probiotic bacteria survival during drying and subsequent storage. The preferred method for long term storage of microbial cultures with maintained cell viability is drying. The process of dehydration exposures probiotic bacteria to a variety of stresses including extremely high or low temperatures, oxygen and osmotic stresses which lead to the loss of viability during the process and subsequent storage. The aim of this review is to discuss the process of producing dried probiotic cultures in steps, to determine the ways leading to the highest cell viability.*

***Keywords****: probiotic, cell viability, freeze drying, protectants.*

## 1. INTRODUCTION

Probiotics are defined as 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' (Gill. *et al*, 2004). There is clinical evidence indicating that these bacteria can positively affect certain human health conditions and play an important role in the control inflammatory bowel diseases, suppression of endogenous/ exogenous pathogens by normalization of the intestinal microbial composition, lowering serum cholesterol, removing of aflatoxin, improving lactose tolerance, and reducing risk factors for colon cancer by metabolic effects (EL-Nezami H. *et al* 1998; Saarela M. 2002). Table I lists some potentially probiotic cultures used in probiotic foods or probiotic food supplements. Although specific numbers are not mentioned in the definition, for having a health benefit, high levels of viable microorganisms in food are recommended and also many of the clinical studies use daily doses in excess of $1\times10^9$ CFU/day (Knorr D. 1998; Meng X.C. *et al* 2008).

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**TABLE I. POTENTIALLY PROBIOTIC CULTURES USED IN PROBIOTIC FOODS OR PROBIOTIC FOOD SUPPLEMENTS (**SALMINEN S. *et al* 2004**)**

| 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* |

Therefore, the retention of high viability during drying and storage can be regarded as a major objective in commercial probiotic production. Food and pharmaceutical industries have found drying technologies as the preferred methods for preserving of different food and drug preparations in bulk quantities. Despite this worldwide usage of drying technologies, there are still many different methods to desiccate micro-organisms and there is no generic method for all applications (Meng X.C. *et al* 2008).

This paper discusses different steps of drying process of micro-organisms to minimize 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. 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.

## 2. INTRINSIC FACTORS

In order to maintain cell viability, it is essential to keep cellular structures during drying and maintain the functional properties of cells after rehydration. In the case of intrinsic factors there are four distinct characteristics that influence cell resistance to extremely low temperatures, these include (i) species; (ii) strain; and (iii) cell size, form, and stage (Hubalek Z.1996). Distinct species of one given genus may often exhibit rather different behaviors during freezing, drying and subsequent storage (Hubalek Z. 2003; Fonseca F. *et al* 2000; Gardiner G. E. *et al* 2000; Carvalho A. S. *et al* 2002). Freeze drying of two different species of lactobacillus showed that *Lactobacillus gasseri CRL1421* was significantly more resistant than *Lactobacillus gasseri CRL1412* to the process (Otero M. C. *et al* 2007). Previous works (Fonseca F. *et al* 2000; Carvalho A. S. *et al* 2002) 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 (Fonseca F. *et al* 2000), as the surface area of the cell gets higher, the membrane damage caused by extracellular ice crystal formation during freezing, would be more.

## 3. GROWTH PHASE AND CELL CONCENTRATION

The growth of bacterial in batch cultures occurs during four distinct phases which concluded (lag phase, log phases, stationary phases and death phases. The responses of bacterial cultures to stress could vary depending on the growth phase. In stationary phase for example, due to carbon starvation and exhaustion of available food sources, bacteria develop a general stress resistance and therefore they would be more resistant to various types of stresses when compared with bacteria in the log-phase (Brashears M. M. and Gilliland S. E. 1995; Morgan C A. *et al* 2006). These survival responses can protect the cell in other adverse conditions, such as desiccation and extremely high or low temperatures (Morgan C A. *et al* 2006). The optimal growth phase for desiccation survival largely depends on the organism. As an 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 (Corcoran B. M. *et al* 2006 ). However, in earlier studies, late-logarithmic (Corcoran B. M. *et al* 2006) or early-stationary (Champagne C. P. *et al* 1996; Carvalho A. S. *et al* 2004); phase lactic acid bacteria cells were commonly used for freeze-drying. Palmfeldt (Zayed G. and Roos Y. H. 2004) 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 $1\times10^9$ and $1\times10^{10}$ CFU/ml. Rault (Rault A. *et al* 2010) suggested that cell cryotolerance

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increases with fermentation time or when cells are harvested during culture at pH 5, as compared with fermentations at pH 6 or without pH control. Costa *et al*. (2000) found that the type of protective medium can influence the optimum initial cell concentration. When using sucrose, a high initial cell concentration of $10^{10}$ CFU/ml was optimal for the highest freeze dried recovery; but, when non-fat skimmed milk was the protective medium, an initial cell concentration of $10^{8}$ CFU/ ml achieved the highest cell survival (Costa E. *et al* 2000). Palmfeldt *et al*.(2003) showed that there is a correlation between cell concentration and protective media. The initial cell concentration to maximize cell survival during freeze- drying decreases as the concentration of sucrose as a protective agent is increased (Palmfeldt J. *et al* 2003).

## 4. APPLICATION OF MILD STRESS PRIOR TO DEHYDRATION

It is well known that, when probiotics are used in industrial food processing they exposed to a number of stress conditions, such as high or low temperatures, low pH and low water activity, which cause damage to their membrane and cell wall which decreases cell survival caused by morphological changes. Therefor bacteria have developed adaptive strategies to face the challenges of changing environments, and to survive under conditions of stress (Carvalho A. S. *et al* 2003). For instance, the response of bacteria to hyperosmolarity includes two aspects: their ability to develop tolerance towards other environmental stresses, and their ability to accumulate osmoprotective compounds (Abee T. and J.A. 1999). As an example, the glycerol formation is considered to be a protective mechanism for the survival of the algae Dunaliella in its natural habitat which contains high amount of salinity (Wegmann, 1971). It was suggested (Carvalho A. S. *et al* 2003) that addition of NaCl to the growth medium, and also different concentrations of compatible solutes (e.g. peptones, tryptone, and meat and yeast extracts) (Carvalho A. S. *et al* 2003), could increase production/accumulation of compatible solutes, and therefore might advance survival of probiotics throughout storage in the dried state. It is reported (Carvalho A. S. *et al* 2003) that raising 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 observed only when these bacteria were previously grown in MRS supplemented with NaCl.

## 5. GROWTH MEDIA

Although major emphasis is on the effect of the drying medium, the growth medium can also be a critical parameter, which plays 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 has been demonstrated. Tymczyszyn, (Tymczyszyn E. E. *et al* 2007) 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. It has been demonstrated that the preservation of dehydrated bacteria with sucrose, after growing them in a low water activity medium (MRSsucrose), was as efficient as dehydration with trehalose. A research done by Carvalho (Carvalho A. S. et al 2003) 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. “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 by the water activity decreasing would face with an increasing osmotic stress. Production of compatible solutes enables them to keep the osmotic balance between the highly concentrated extracellular environment and the more dilute intracellular environment and the organism will be able to contract this stress. These solutes can also help to stabilize proteins and the cell membrane during osmotic stress conditions because of providing low water activity during drying processes (Hubalek Z. 2003; Morgan C A. *et al* 2006). The reason for this is most likely that the bacterial cells get adapted to the low water activity medium and these compounds may act as water molecules to maintain the structure of cell. Exopolysaccharide includes to two types of polysaccharides; the first type is attached to the cell wall as a capsule, whereas the other is produced as slime (Degeest B. *et al* 2001). It has been suggested (Torino M. I. *et al* 2001) that exopolysaccharide formation is part of a survival strategy under stressful conditions. Variations in the environmental conditions induce variation of the membrane lipid structure, which in turn affect its fluidity; by changing its fatty acid composition bacteria maintain the ideal membrane fluidity (Annous B. A. *et al* 1999). These studies imply that stress induces a number of survival strategies which provide protection to micro-organisms upon drying.

## 6. PROTECTANTS

Protective agents can be in growth media during culturing of the micro-organism, or prior to freezing or drying. The type of protectant used depends on the micro-organism; although, there are a few protective agents 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 (Hubalek Z. 2003; Morgan C A. *et al* 2006). Usage of compatible cryoprotectants in growth media prior to fermentation assists in the adaptation of probiotics to the environment (Capela P. *et al* 2006).

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In low moisture content, compatible solutes and sugars in the drying media can provide protection of the cells by being preferentially excluded from the proteins or membrane surfaces, or in another sense they keep proteins and membranes hydrated. When water is continually removed until very low moisture content is achieved, the cell membrane is the critical site of damage which may cause 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. Therefore, the cell membrane can retain in its liquid crystalline phase under drying or rehydration conditions. The sugar glass reduces molecular mobility and therefore inhibits deteriorative reactions. Therefore, the glass transition temperature of sugars should be considered as a critical point for choosing a sugar as a protectant agent 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 won't be an appropriate agent to protect cells during drying (Corcoran B. M. *et al* 2006; Palmfeldt J. *et al* 2003; Santivarangkna C. *et al* 2008). In a recent work the ability of galacto-oligosaccharides (GOS) to protect *Lactobacillus delbrueckii* subsp. *bulgaricus* upon freeze drying on the basis of their capability to form glass structures was investigated (Tymczyszyn *et al* 2012). Given that glass transition temperatures ($T_g$) of a GOS matrix significantly effects the molecular mobility and subsequent cell loss during the process, the glass transition of GOS at various relative humidities (RH) were determined by DSC. The results showed that the preservation of microorganisms was improved at low molecular mobility and this condition was obtained at low water contents and low storage temperatures. It would worthy to note that in previous research done by Tymczyszyn et al (2011) the higher content of galacto-oligosaccharides in the commercial products was correlated with their higher protective capacity.

## 7. DRYING METHODOLOGY

Different methodologies are used for desiccation of living cells. 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 (Mujumdar A. S. 2003). In recent decades freeze-drying has been used to manufacture probiotic powders and it is based on sublimation, which occurs in three phases; freezing, primary, and secondary drying. Cells are first frozen at -196°C in liquid nitrogen and then are dried by sublimation under high vacuum (Santivarangkna C. *et al* 2007). In the third stage temperature goes up to ambient temperatures to provide the energy needed to sublimation of boundary water. As the processing conditions in freeze-drying are milder than spray-drying, higher probiotic survival rates are typically achieved in freeze-dried powders (Wang Y. C. *et al* 2004).

In commercial scale production of probiotic cultures freeze- drying is an expensive process with low yields, and as such spray-drying offers alternative inexpensive approach yielding higher production rates (Zamora L. M. *et al* 2006). Spray-drying 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, dehydration, oxygen exposure and osmotic stress (Brennan M. *et al* 1986; Teixeira P. *et al* 1995).

Fluidized bed dryers use the moving flow of heated air and mechanical shaking to create a fluidized effect in a solid product. Larena (Larena I. *et al* 2003) showed freeze drying and fluidized bed drying maintained 100% viability of *Penicillium oxalicum conidia.* Stummer *et al* (2012) evaluated the suitability of fluidized-bed technology for the dehydration of probiotic *Enterococcus faecium* M74. The effect of different process conditions including atomizing air pressure, processing temperature and time impact on cell viability was investigated. Changing these variables had an excessive stress on the cells and affected the cell survival. When comparing the cell viability of fluidized-bed drying with that of freeze-drying, the results indicated that Fluidized-bed drying caused more substantial losses of cell viability.

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 produce immobile amorphous, non-crystalline glass foams directly from a liquid. Then the foams are subjected to further drying at elevated temperatures to increase their stability at ambient temperatures (Bronshtein V. 2004). Numerous new drying techniques proposed and tested over the past decade have potential for application to bioproducts. Table Π lists some such emerging technologies (Mujumdar A. S. 2003).

**TABLE II. COMMONLY USED DRYERS AND EMERGING DRYING TECHNOLOGIES SUITABLE FOR BIOTECH PRODUCTS.(** MUJUMDAR A. S. 2003**)**

| Dryers for biotech products | |
|---|---|
| *Conventional dryers* | *Emerging dryers* |
| Spray dryer | Heat-pump dryers (below/above freezing point) |
| Spray/fluid-bed (two-stage) | Intermittent batch dryer |
| Freeze dryer | Vacuum fluid-bed dryer |
| Vacuum tray | Low-pressure spray dryer with ultrasonic atomizer |
| Continues tray dryer | Sorption dryer |

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| Dryers for biotech products | |
|---|---|
| *Conventional dryers* | *Emerging dryers* |
| Drum dryer/vacuum | Pulse combustion dryer |
| Indirect vacuum | Cyclic pressure/vacuum dryer |
| Plate or turbo dryer | High electric field (HEF) dryer |
| | Superheated steam dryer at low pressures |

## 8. PACKAGING AND STORAGE

The method of storage and the packaging material and methodology will influence the shelf life of dried product. Common reactive agents to decrease the shelf life of products include; oxygen, moisture, light, microbial contamination and elevated temperatures. Therefore the packaging materials would be 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 (Hubalek Z. 2003).

Otero (Otero M. C. *et al* 2007) 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 which cannot metabolize them, or transport them out of the cell, may lead to irreversible damaging processes occurring within the cell (Otero M. C. *et al* 2007).

The storage conditions have an important effect on the survival of probiotics in dried powders, and the correct storage conditions are essential to maintain viable populations of dried probiotic bacteria. Costa (Costa E. *et al* 2000) 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 (Foerst P. *et al* 2011) 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 (Foerst P. *et al* 2011).

## 9. REHYDRATION

Rehydration of probiotic powders is the final critical step for the revival of cells after dehydration. The rehydration solution itself, as well as the rehydration conditions (in terms of rehydration temperature and volume) may affect the rate of survival of the viable state, and thus influence survival rates (Carvalhoa A. S. *et al* 2004). For optimum results, it is recommended to dry the cells at the stationary phase of growth and to use slow rehydration procedures (Teixeira P. *et al* 1995).

Costa (Costa E. *et al* 2000) 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 (Costa E. *et al* 2000). 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 (Abadias M. *et al* 2001). The temperature of rehydration could also influence cell recovery after freeze-drying; Ray (Ray B. *et al* 1971) found rehydration at 15 – 25 °C produced the highest numbers of recovered cells, rather than 35 °C and 45 °C where the cell recovery was lower.

Another factor to consider, 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, as compared to immediate rehydration (Poirier I. *et al* 1999).

## 10. CONCLUSION

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 specific probiotic strain, the condition of the culture media, the culture entering the dryer, the use of protectants and desiccation methodology. In addition, knowledge of the impact and the nature of the injury produced by a variety of stressful conditions (e.g. freezing, drying, storage or rehydration), and also information about 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.

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