A Dry Phase of Life

Freeze-drying and storage stability of
Lactobacillus coryniformis Si3 in sucrose-based formulations

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Cover: A scanning electron microscopy image of a freeze-dried *Lactobacillus*-in-sucrose product, principal thermogram and vials with freeze-dried *Lactobacillus coryniformis* Si3 formulations. Photos and design: Åsa Schoug

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A Dry Phase of Life. Freeze-drying and storage stability of *Lactobacillus coryniformis* Si3 in sucrose-based formulations

Abstract

Freeze-drying is a commonly used drying technique for sensitive biologicals, such as lactic acid bacteria. Freeze-drying survival and storage viability of freeze-dried lactic acid bacteria have been shown to depend upon many factors including species, fermentation and formulation procedure, freeze-drying process, storage conditions and rehydration conditions. *Lactobacillus (Lb.) coryniformis* strain Si3 was selected as the model strain for this thesis work mainly due to its low freeze-drying survival but also potential usefulness as biopreservative, *i.e.* broad antifungal activity.

Preconditioning *Lb. coryniformis* Si3 with mild stress during fermentation induced changes in the lipid membrane (increased the degree of unsaturation) or increased the uptake of the solute betaine. However, freeze-drying survival was significantly lowered after preconditioning *Lb. coryniformis* Si3 with cold, base, acid or salt stress prior to freeze-drying. After either optimal growth or preconditioning by mild heat stress, *Lb. coryniformis* Si3 survived to approximately 70% in a skim milk and sucrose formulation. Betaine was shown to be a poor cryoprotective and lyoprotective agent compared to sucrose. Betaine crystallised upon drying resulting in survival rates of below 3%. By adding small amounts of sucrose to the betaine, crystallisation was inhibited and a 10-fold increase in survival was achieved. Betaine was an effective plasticiser for sucrose, lowering the T_g both in the freeze-concentrate and the freeze-dried product. Our design of experiments approach revealed interactions between the formulation (cell density and sucrose concentration) and freeze-drying process (*i.e.* cooling rate) with regard to survival of *Lb. coryniformis* Si3. It was possible to vary the survival rate from <6% to ~70% by varying the different parameters.

Storage stability was dependent on the formulation, humidity and temperature. At low temperatures, the matrix stability and cell viability of freeze-dried *Lb. coryniformis* Si3 in sucrose could be increased by addition of polymers. The suggested 'rule of thumb' of keeping the product 50 degrees below the T_g for retained stability of the amorphous matrix was applicable in our system to maintain high cell viability and technical quality of the products. By combining this 'rule' with the effect of moisture on the T_g, it was possible to determine product-specific storage conditions to ensure high viability and good technical product quality.

Keywords: Formulation, freeze-drying, lactic acid bacteria, storage stability, drying, amorphous, *Lactobacillus coryniformis*, experimental design.

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Till Isac

"Aim for success, not perfection. Never give up your right to be wrong, because then you will lose the ability to learn new things and move forward with your life"

David Burns

Svensk sammanfattning

Mjölksyrabakterier används flitigt av människan för att konservera mat och foder, ge smak och textur till yoghurt, ost, vin och andra livsmedel och även för att främja vår hälsa genom s.k. probiotiska effekter. *Lactobacillus (Lb.) coryniformis* Si3 är en mjölksyrabakteriestam som har god förmåga att hämma mögelsvamp genom att producera svamphämmande ämnen och skulle kunna användas som biologiskt konserveringsmedel. *Lb. coryniformis* Si3s svamphämmande egenskaper medförde att den var intressant för industrin, men pga. dess dåliga överlevnad vid frystorkning gick den inte att kommersialisera. Den valdes istället som modellstam för mitt avhandlingsarbete, i vilket jag studerat vilka faktorer som påverkar överlevnad under frystorkningsprocessen och vid förvaring av *Lb. coryniformis* Si3 i frystorkad form.

Mjölksyrabakterier förvaras och säljs ofta i nedfryst eller frystorkat format. Att frystorka bakterier och förvara dem torrt, för att sedan återfukta dem och uppnå 100 % överlevnad är svårt. Kunskapen om hur man på ett effektivt sätt skall förvara mikroorganismer i frystorkad form fortfarande begränsad. Man har dock identifierat vissa viktiga faktorer för att få celler att överleva torkning såsom betydelsen av tillsats av olika sockerarter. Olika mjölksyrabakteriestammar uppvisar väldigt olika frystorkningsöverlevnad, vilket i praktiken innebär att det industriella valet av stam inte enbart baseras på bakteriens applikationsegenskaper utan även deras förmåga att överleva torkning. Varför olika stammar uppvisar så pass olika överlevnad är det ingen som vet. Hittills har produktionen av mjölksyrabakterier drivits av mycket ”trial and error”, men det börjar nu förändras i takt med att mer kunskap uppnås.

Jag har studerat hur olika faktorer under produktionsprocessen påverkar både överlevnad och produktkvaliteten av frystorkad *Lb. coryniformis* Si3. Processen inleds med produktion av cellmassa (fermentering), tillsättande av ingredienser som skall ha en hjälpfunktion under frystorkningen och vid långtidsförvaringen av cellerna i torr form, följt av frystorkning, förvaring och slutligen återupplivande (återfuktande) till en aktiv produkt. Utöver själva överlevnaden är det viktigt för många applikationer att slutprodukten håller god teknisk kvalitet, t.ex. att pulvret är lättupplöst i vatten. Sockerarter har visat sig vara viktiga för att uppnå god cellöverlevnad vid frystorkning pga. en förmåga att bilda en oordnad struktur, så kallad amorfstruktur i motsats till kristallin, samt att de kan ersätta vatten som tas bort från cellen. Bildandet av en amorf matris under torkningen är en förutsättning för god

överlevnad, men också en anledning till instabilitet under lagring. Jag har visat att det är möjligt att höja frystorknings överlevnad av *Lb. coryniformis* Si3 från ett par procent till cirka 70 % genom att förändra olika betingelser vid fermentering (pH, temperatur, salthalt), formulering (tillsatts av aminosyraderivat, socker, polymerer eller skummjölkspulver) och vid själva frystorkningsprocessen. Jag har dessutom studerat hur *Lb. coryniformis* Si3 förändrar sin cellmembransammansättning och intracellulära miljö vid olika tillväxtförhållanden och hur dessa förändringar påverkar frystorkningsöverlevnad. För att uppnå hög frystorkningsöverlevnad och god förvaringsstabilitet av frystorkade *Lb. coryniformis* Si3 produkter visade det sig att själva formuleringen i kombination med frystorkningsprocessen var vikigare än att försöka förbereda cellen på den stress som nästa processteg innebär genom att låta cellen aktivera sina stressvarsmekanismer. Hur produkten sedan förvaras i torr form, t.ex. vid vilken temperatur och relativ luftfuktighet, påverkar till stor del både cellöverlevnad och produktkvaliteten. Genom att kombinera kalorimetriska analysmetoder med mikrobiologiska överlevnadsstudier kan man fastställa hur produkten skall lagerhållas för god cellöverlevnad och hög teknisk produktkvalitet.

Contents

List of publications

Papers I - IV.

This thesis is based on the following publications, which are referred to in the text by Roman numerals (**Papers I – IV)**.

I. **Åsa Schoug**, Janett Fischer, Hermann J. Heipieper, Johan Schnürer, and Sebastian Håkansson. 2008. Impact of fermentation pH and temperature on freeze-drying survival and membrane lipid composition of *Lactobacillus coryniformis* Si3. *Journal of Industrial Microbiology and Biotechnology* 35, 175-181.

II. **Åsa Schoug**, Johan Olsson, Johan Carlfors, Johan Schnürer, and Sebastian Håkansson. 2006. Freeze-drying of *Lactobacillus coryniformis* Si3 – effects of sucrose concentration, cell density, and freezing rate on cell survival and thermophysical properties. *Cryobiology* 53, 119-127.

III. **Åsa Schoug**, Anne Wuttke, and Sebastian Håkansson. 2009. Physical solid state behavior of the protective agents betaine and sucrose influence the survival of freeze-dried *Lactobacillus coryniformis* Si3. Manuscript.

IV. **Åsa Schoug**, Denny Mahlin, Mirela Jonson, and Sebastian Håkansson. 2009. Differential effects of polymers PVP90 and Ficoll400 on storage stability and viability of *Lactobacillus coryniformis* Si3 freeze-dried in sucrose. Submitted.

Abbreviations

CFU	Colony forming unit
DSC	Differential scanning calorimetry
FAME	Fatty acid methyl esters
FTIR	Fourier transform infrared (spectroscopy)
GB	Glycine-betaine also named betaine
GC-FID	Gas chromatography flame ionisation detector
IMC	Isothermal micro-calorimetry
KFT	Karl-Fisher titration
LEA	Late embryogenesis proteins
MAS-NMR	Magic angle spinning nuclear magnetic resonance
MRS	De Man, Rogosa and Sharpe (growth medium for *lactobacilli*)
NEB	Non-enzymatic browning
PVP	Polyvinylpyrrolidone
RH	Relative humidity
RSM	Response surface methodology
SEM	Scanning electron microscope
T_c	Crystallisation temperature
t_{cr}	Time to crystallisation
T_{coll}	Collapse temperature
T_{eu}	Eutectic temperature
T_g	Glass transition temperature
T_g'	Maximally freeze-concentrated glass transition temperature

1 Introduction

Life as we know it relies on the presence of water. Water is needed for metabolic processes and to maintain the structure of living cells. Removal of water from cells can have detrimental effects (Potts, 1994). However, some microorganisms have developed strategies to withstand periods of extreme lack of water. Several taxonomic groups are considered desiccation-tolerant, *i.e.* they can survive periods of desiccation with only 0.02 g H_2O per g dry mass by entering into a state referred to as anhydrobiosis (Potts, 1994). Anhydrobiosis is Greek and translates into *life without water*, a particular state of latent life[1]. Back in 1702, Anthony van Leuwenhoek described organisms, so-called animalcules that were later identified as rotifers, which could withstand drying. His findings did not seem to excite his fellow scientists greatly and it was not until 40 years later that John Needham and Henry Baker continued his work and described different states of latent life in other organisms. Baker announced the following observations to the Royal Society in 1743 (Kehlin, 1959):

"We find an Instance here, that Life may be suspended and seemingly destroyed; that /…/ all the organs and vessels of the body may be shrunk up, dried, and hardened; and yet, after a long while, Life may begin anew /…/ all the animal Motions and Facilities may be restored, merely by replenishing the Organs and Vessels by a fresh supply of fluid."

The French scientist Alfred Giard coined the term anhydrobiosis in 1894 (Kehlin, 1959). Since the beginning of the 20th century, there have been important discoveries on how cells can survive without water but the

[1] Latent life can be defined as different states where organisms show no visible or measurable signs of life. Examples other than anhydrobiosis are cryobiosis, anoxybiosis, and osmobiosis. From Kehlin (1959).

complete biochemistry of anhydrobiosis is only now starting to unfold (Iturriaga, 2008).

Lactic acid bacteria are not desiccation tolerant by nature. However, these bacteria are usually handled, stored and sold as freeze-concentrates or as freeze-dried products (Champagne *et al.*, 1991). To succeed in freeze-drying lactic acid bacteria, the physiological status of the cells, the drying matrix and drying procedure need to be carefully planned (Meng *et al.*, 2008). There is still a fundamental lack of understanding of the mechanisms of life and death for lactic acid bacteria during the freeze-drying process and in the freeze-dried state during storage. There is a growing interest in the production of dry lactic acid bacterial cultures, and developments are moving towards being driven by design rather than, as previously, by trial and error (Cogan *et al.*, 2007). The ability to generate and maintain high viable cell numbers throughout the freeze-drying process and subsequent storage is a difficult task and understanding the underlying biological and chemical processes involved is even more complex.

1.1 Aims and thesis outline

The overall aim of this thesis work was to identify intrinsic and extrinsic factors that influence freeze-drying survival and product quality of the model lactic acid bacteria *Lactobacillus coryniformis subsp. coryniformis* strain Si3 (*Lb. coryniformis* Si3) in sucrose-based formulations. It was preordained that this thesis work would be carried out without genetic modification of the *Lactobacillus* species. The outline of the thesis, based on the process steps of production joined with the different factors examined, corresponding papers and techniques used, is presented in Figure 1.

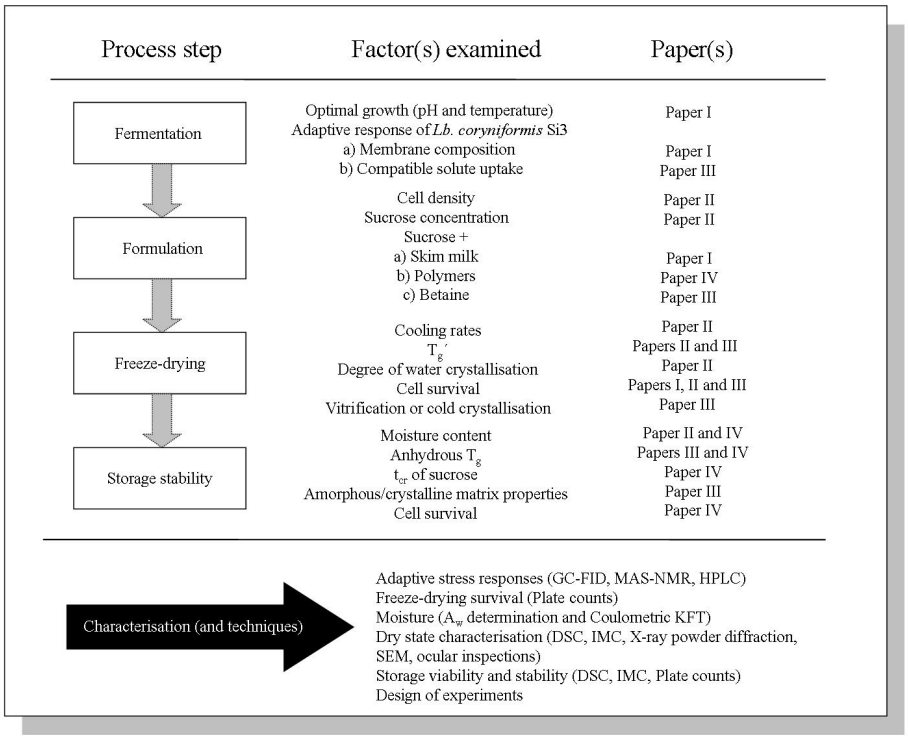

Figure 1. Factors studied in this thesis in the context of the different process steps of production, with corresponding papers and techniques used indicated.

2 Lactic acid bacteria

Man has used lactic acid bacteria since ancient times, mainly to preserve food, but also to add flavour and texture (Driehuis *et al.*, 2000; Salminen, 2004; Shortt, 1999; Stiles, 1996). These bacteria have been used due to their ability to lower pH, but it was not until the mid-19th century that the microbial processes in food fermentation were discovered (Caplice *et al.*, 1999). Lactic acid bacteria are a group of bacteria related mainly through their function in food and feed (Klaenhammer *et al.*, 2005). The core group of lactic acid bacteria consists of the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* (Salminen, 2004). Within the genus *Lactobacillus* there are currently 80 species and subspecies and the genus is expanding (Salminen, 2004). Only in the period 2000-2002, 20 new species were described (Hammes *et al.*, 2006; Salminen, 2004). The lactic acid bacteria group share some typical features, *e.g.* they are Gram-positive, non-sporulating, acid-tolerant, catalase-negative, devoid of cytochromes, non-aerobic but aerotolerant, fastidious, and strictly fermentative with lactic acid as the major end product during sugar fermentation (Van de Guchte *et al.*, 2002). The practical use of a lactic acid bacterial species relies on an established effect and safe use, including minimal spread of antibiotic resistance plus the possibility to produce high numbers of viable cells in a suitable form. Many lactic acid bacteria have been granted 'generally regarded as safe' (GRAS) status (FDA, 2002) and the long history of human use makes them a safe option for food and feed preservation.

2.1 Choice of model strain

The model strain *Lactobacillus coryniformis* Si3 was originally isolated from grass silage (Thylin, 2000). In a screen of 1200 naturally occurring isolates, the ability to inhibit moulds was further investigated in 37 selected strains

and the majority of the strains with high or moderate activity were identified as *Lb. coryniformis* species (Magnusson *et al.*, 2003). Several *Lactobacillus plantarum* and *Pediococcus pentosaceus* strains were also identified (Magnusson *et al.*, 2003). *Lb. coryniformis* Si3 and *Lactobacillus (Lb.) plantarum* strain MiLab 393 were selected for further studies on their mode of antifungal action (Magnusson, 2003; Ström, 2005). *Lb. coryniformis* Si3 produces lactic acid, phenyl lactic acid, cyclic-dipeptides, and a proteinaceous compound with broad antifungal activity (Figure 2) (Magnusson *et al.*, 2001; Magnusson *et al.*, 2003) while *Lb. plantarum* MiLab 393 produces lactic acid, phenyl lactic acid, and cyclic dipeptides (Ström *et al.*, 2002).

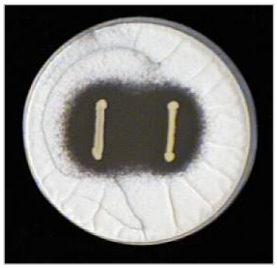

Figure 2. Lb coryniformis Si3 grown in streaks on MRS agar and overlaid with soft agar containing *Aspergillus fumigatus* spores. The overlay method for determination of antifungal activity was developed by Magnusson *et al.* (2003). Photo taken by Dr. Katrin Ström.

The antifungal activity of both *Lb. coryniformis* Si3 and *Lb. plantarum* MiLab 393 makes these strains candidates as biopreservation additives in food and feed. *Lb. coryniformis* Si3 was considered for commercialisation by the Swedish starter culture company Medipharm AB, but due to the low fermentation and freeze-drying yields obtained with this strain, it was not commercialised (Kerstin Holmgren, Medipharm AB, personal communication, 2003). However, the more robust strain *Lb. plantarum* MiLab 393 is currently sold in combination with three other lactic acid bacteria as a silage additive (Feedtech® Silage F3000, DeLaval AB).

The use of lactic acid bacteria requires the cells to survive the stressful conditions during production. Freeze-drying encompasses several stressful conditions such as low temperature, high osmolarity and drying. The general stress tolerance of *Lb. coryniformis* Si3 was found to be low compared with that of *Lb. plantarum* MiLab 393, so the different tolerance between these strains to low temperatures and high osmolarity were studied (unpublished results). The survival after freeze-thaw cycles was considerably higher for *Lb.*

plantarum MiLab 393 than for *Lb. coryniformis* Si3 (Figure 3, unpublished results).

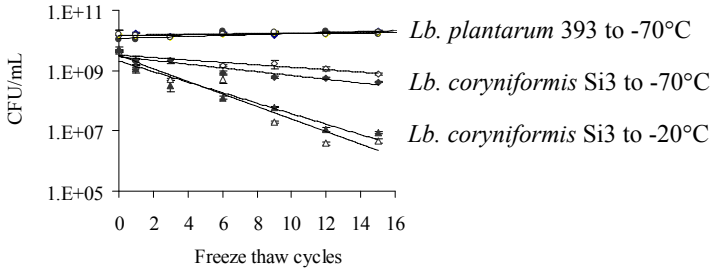

Figure 3. Freeze-thaw tolerance of *Lb. coryniformis* Si3 and *Lb. plantarum* MiLab 393, frozen in 15% disaccharide solution, to different final temperatures. Open symbols denote samples in 15% trehalose and filled symbols samples in 15% sucrose. Thawing was performed in a 37 °C water bath. Error bars show standard deviation of triplicate samples.

Apart from species differences, the freeze-thaw survival of *Lb. coryniformis* Si3 was influenced by the final cooling temperature (Figure 3), which is in good agreement with previous findings on cryopreservation of lactic acid bacteria (Fonseca *et al.*, 2001).

To further assess differences in general stress tolerance between *Lb. coryniformis* Si3 and *Lb. plantarum* MiLab 393, the strains were grown in the presence of increasing amounts of sucrose. The *Lb. plantarum* strain was shown to adapt faster to the increased osmolarity than *Lb. coryniformis* Si3 (Figure 4, unpublished results).

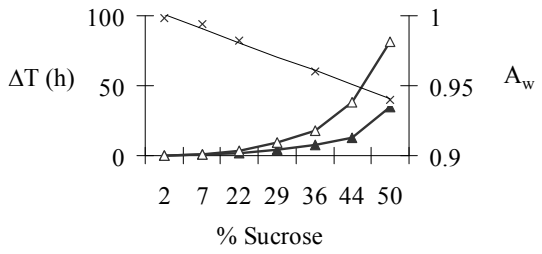

Figure 4. Graph showing the growth in hours ΔT (h) of *Lb. plantarum* MiLab 393 (closed triangles) and *Lb. coryniformis* Si3 (open triangles) in MRS, with sucrose as the only carbon source. The water activity (A_w) of the growth media was determined (crosses) in an Aqua Lab CX-2. Growth is expressed as ΔT = (time for the species to reach exponential phase under growth in 2% sucrose in MRS) – (time for the species to reach exponential phase under growth in MRS with increasing amounts of sucrose). Each ΔT point represents at least one growth experiment, determined by OD_{600} in 200 mL E-flask cultures at 30 °C (unpublished results).

In accordance with the difference in osmotolerance examined by growth at increased sucrose concentrations (Figure 4), the tolerance of *Lb. coryniformis* Si3 to growth on 10% KCl supplemented agar MRS was lower than that of other lactic acid bacteria with antifungal properties (Ström, 2005). In the literature, the mean freeze-drying survival of 25 different *Lb. plantarum* strains is reported to be 90 ± 9% (Alegria *et al.*, 2004), which is in agreement with our preliminary results on *Lb. plantarum* strain MiLab 393 (unpublished results). The freeze-drying survival of *Lb. coryniformis* Si3 varied from a few percent to a maximum of approximately 70% in our studies (**Papers I and II**). *Lb. coryniformis* Si3 is thus less stress-tolerant than *Lb. plantarum* MiLab 393 and was selected as model strain.

2.2 Lactic acid bacteria in silage

Improving the quality of silage by adding lactic acid bacteria alone or in combination with chemicals has been well researched and is applied in practice by farmers (Kung *et al.*, 2004; Lindgren *et al.*, 1988; Rooke *et al.*, 1990; Weinberg *et al.*, 1993). The principal mechanisms by which lactic acid bacteria preserve silage are by lowering the pH through acid production and by inhibition of hazardous microorganisms (Brul *et al.*, 1999). Many lactic acid bacteria also compete by producing specific antimicrobial or antifungal

compounds (Caplice *et al.*, 1999; Lindgren *et al.*, 1990; Magnusson, 2003; Manzanera *et al.*, 2002; Schnürer *et al.*, 2005). Inoculation of silage with *Lb. coryniformis* strain Si3 has been shown to lower the pH levels compared with the controls, indicating that this strain improves the quality of silage *in situ* (Ström, 2005).

3 Fermentation

To succeed in the production of lactic acid bacteria cultures, the first step is to generate a large number of cells by fermentation. Fermentation[2] is defined as the use of internal electron acceptors to produce energy by substrate level phosphorylation. It can also be more loosely defined as the conversion of carbohydrates into end products such as acids or ethanol, or in an even broader sense as the process of growing microorganisms (Stanbury *et al.*, 1995). Lactic acid bacteria are fastidious microorganisms that require complex substrates with vitamins and carbohydrates for growth. Carbohydrates and other necessary ingredients are consumed by the lactic acid bacteria, resulting in an increase in cell mass, generation of energy in the form of ATP and the production of lactic acid (Salminen, 2004). Most lactic acid bacteria convert over 50% of the sugar carbon to lactic acid and they are either homofermentative or heterofermentative with regard to hexose fermentation (Salminen, 2004). The species *Lactobacillus coryniformis* is a facultative heterofermentative lactic acid bacteria (Hammes *et al.*, 2006).

Homo-lactic fermentation:
$$\text{Hexose} + \text{ATP} + 2\ \text{ADP} + 2\ P_i \rightarrow 2\ \text{Lactate} + 2\ \text{ATP} + \text{ADP} + H_2O$$

Hetero-lactic fermentation:
$$\text{Hexose} + \text{ADP} + P_i \rightarrow \text{Lactate} + \text{Ethanol} + CO_2 + \text{ATP} + H_2O$$

Freeze-drying survival is dependent upon the fermentation process, such as the growth medium and the time of harvest (Corcoran *et al.*, 2004; Doleyres *et al.*, 2004; Meng *et al.*, 2008). The carbohydrate components in the medium influence the drying survival (Carvalho *et al.*, 2004). It has been

[2] The word fermentation derives from the Greek word *fevere*, meaning *to boil*. From Stanbury, P.F., Withaker, A. & Hall, S.J. (1995). *Principles of fermentation technology*. Elsevier Science.

shown that the bacteria do not need to be able to metabolise the protective agent, *i.e.* the carbohydrate, in order for it to protect during freeze-drying (Carvalho, 2004; Zayed *et al.*, 2004). Intracellular accumulation of disaccharides has been shown to increase the freeze-drying survival of *Lactococcus lactis* (Termont *et al.*, 2006).

The stationary phase is reached when the cells lack a specific nutrient, carbon source or when they are inhibited by the production of end products (Brock, 1997). In this phase, the cells enter into a general stress-adapted state (Van de Guchte *et al.*, 2002). Cell yields also reach a maximum, which is beneficial for overall process productivity. In addition to being physiologically adapted, several studies have suggested that the enhanced tolerance to downstream processing is related to morphological characteristics, *i.e.* shorter rods are more tolerant than elongated forms (Wright *et al.*, 1981; 1983), whereas others could find no such correlation (Palmfeldt *et al.*, 2000).

Mesophilic lactic acid bacteria such as *Lb. coryniformis* have their optimal growth temperature between 30 and 40 °C and optimal pH range between 5.4 and 5.8. Our design of experiments approach showed that the optimal growth temperature of *Lb. coryniformis* Si3 after fermentation in commercial MRS media was 34 °C and the optimal pH was 5.5 (**Paper I**).

4 Bacterial stress responses

During production of freeze-dried lactic acid bacterial cultures, the cells encounter many different stress conditions. An environmental change resulting in a physiological adaptive response is known as stress (Van de Guchte *et al.*, 2002). Drying is considered an extreme form of stress for microorganisms (Potts, 2001). The bacterial response when faced with the removal of all surrounding water is recognised as being quite different from the bacterial response to low water availability such as increased osmolarity or freezing (Crowe *et al.*, 1990; Potts, 1994; 2001; Welsh *et al.*, 1999).

Freeze-drying is a well-used method to dry cells and is a rather complex drying method, inducing several stress conditions. For example, freeze-drying exposes cells to low temperature (chill and cold stress), freezing of water and thus solute concentration (osmotic stress) and finally desiccation (temperature, oxidation). The cellular injury sites can be several, such as the cell wall, cell membrane, DNA or RNA or proteins (Potts, 1994; Santivarangkna *et al.*, 2008a; Wolfe *et al.*, 1999). This multitude of potential damage sites and the lack of understanding of the underlying mechanisms make it difficult to predict freeze-drying tolerance amongst different species and even strains (Santivarangkna *et al.*, 2008a).

The adaptive responses in lactic acid bacteria consist of altering the lipid membrane, changing the synthesis of stress proteins, and the uptake of compatible solutes. The responses differ among strains (Van de Guchte *et al.*, 2002) but some general features exist and these are discussed in the following sections. Prior to our studies (**Papers I and III**), there was no available information on the adaptive responses in *Lb. coryniformis* strains.

4.1 Membrane adjustments

The cell wall and membrane are primary targets for severe damage in bacteria (Santivarangkna *et al.*, 2008a). Lactic acid bacteria are Gram-positive and thus their cell envelope consists of a lipid membrane and a peptidoglycan layer (Figure 5). In general, Gram-negative bacteria show lower freeze-drying survival than Gram-positive bacteria, which has been suggested to be due to differences in cell envelope structure (Miyamoto-Shinohara *et al.*, 2008).

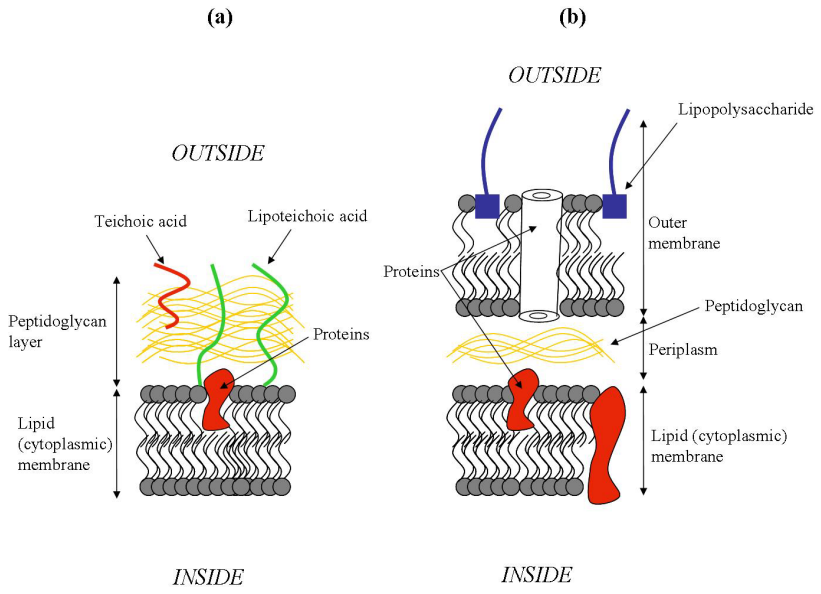

Figure 5. Cell envelope structure of (a) Gram-positive and (b) Gram-negative bacterial cell. Modified from Brock (1997). Different proteins are found in the membranes.

The membrane of active cells is in a liquid crystalline state and bacterial cells control the membrane fluidity in response to environmental factors in order to avoid loss of structure (Sinensky, 1974). Changes in the membrane fluidity and leakage of cytoplasmic components are suggested to be one important reason for cellular death during drying and subsequent rehydration (Crowe *et al.*, 1998; Santivarangkna *et al.*, 2008b). In general, a lower temperature rigidifies the membrane, while a higher temperature fluidises the membrane (Los *et al.*, 2004).

Changes in the membrane or cell wall can be promoted by changing the growth conditions (Piuri *et al.*, 2005; Teixeira *et al.*, 2002; Wang *et al.*,

2005). Changes in the membrane can also be achieved by adding components during fermentation, such as cholesterol (Spector *et al.*, 1985), amino acids (Martos *et al.*, 2007) or Tween 80 (Gomez Zavaglia *et al.*, 2000). The fatty acids identified in *Lb. coryniformis* Si3 (**Paper I**) correlate well with those found in other lactic acid bacteria (Beal *et al.*, 2001; Coulibaly *et al.*, 2008; Gomez Zavaglia *et al.*, 2000; Wang *et al.*, 2005). Inducing an unfavourable temperature or pH during fermentation of lactic acid bacteria has been shown to increase the degree of fatty acid unsaturation (Beal *et al.*, 2001; Guerzoni *et al.*, 2001; Wang *et al.*, 2005). We also found that there was an increase in membrane lipid unsaturation as a response to unfavourable fermentation in *Lb. coryniformis* Si3 (**Paper I**). Correlations have been described between membrane adjustments during unfavourable growth and improved cryotolerance of several lactic acid bacteria (Beal *et al.*, 2001; Wang *et al.*, 2005). However, we did not find any correlations between the changes in lipid membrane composition and the subsequent freeze-drying survival (**Paper I**). The observations in (**Paper I**) can be interpreted in several ways. (i) There is no correlation between the degree of saturation (U/S ratio) of the membrane and freeze-drying damage. If the discrepancy of heat stress lay in other cell adjustments (protein response) counteracting otherwise detrimental membrane adjustments, the results could be interpreted as (ii) there is a correlation between a higher degree of unsaturation and lower freeze-drying survival of *Lb. coryniformis* Si3. It is known the membrane permeability increase with an increased degree of lipid unsaturation (Huster *et al.*, 1997) which might affect the cells negatively by leakage of solutes (Heipieper *et al.*, 1992) as well as by increasing the potential sites for oxidation damage (Borst *et al.*, 2000). Alternatively, (iii) the correlation is not between the fatty acid saturation *per se*, but rather the fluidity that is influenced by other things in the membrane such as proteins (Quinn, 1981).

4.2 Stress proteins

Apart from regulation of the membrane, bacterial cells change their protein expressions when stressed. The role of stress proteins in lactic acid bacteria with regard to freeze-drying tolerance is unclear since there are few studies available. The regulation of stress-related genes is numerous, complex and species-dependent (Van de Guchte *et al.*, 2002). Overproducing heat chaperones GroESL increases freeze-drying survival of *Lactobacillus paracasei* NFBC 338 from 22 to 37% (Corcoran *et al.*, 2006), which show that some

correlations in-between freeze-drying survival and protein expression are to be found.

4.3 Compatible solutes

Increasing the osmolarity of the solution causes a net outflow of water from cells. Cells counteract this effect by either importing or producing so-called compatible solutes (Kempf *et al.*, 1998). Compatible solutes are low-molecular weight solutes that can be accumulated in high concentrations without toxic effects (Brown, 1976). These solutes protect against loss of turgor pressure by their colligative[3] properties, for example they prevent the intracellular volume falling below a critical minimum. Compatible solutes can also function by preferential hydration, by maintaining the lipid bilayer in a crystalline state by water replacement, or by vitrification (Storey, 1997) dependent upon the nature of the solute. These mechanisms are discussed in section 5.

As a response to increased osmolarity, bacteria have been shown to accumulate K^+, amino acids (proline, glutamate), derivates of amino acids (peptides, N-acetylated amino acids) quaternary amines (betaine, carnitine), sugars (trehalose and sucrose) and tetrahydropyrimidines (ectoine) (Csonka, 1989). Lactic acid bacteria mainly accumulate amino acids and derivates thereof, such as betaine, proline and ectoine (Glaasker *et al.*, 1996; Kets *et al.*, 1994; Le Marrec *et al.*, 2007). *Lb. coryniformis* Si3 was shown to import betaine as compatible solute (**Paper III**). Many lactic acid bacteria prefer betaine, a substance they cannot metabolise or synthesise (Glaasker *et al.*, 1998; Glaasker *et al.*, 1996) and that requires energy to import (Hutkins *et al.*, 1987). *Lb. coryniformis* Si3 was shown to import higher concentrations of betaine when grown at increased osmolarity, but also during logarithmic growth independent of growth conditions (**Paper III**) which agrees with the fact that energy is required for uptake.

4.4 Anhydrobiotic engineering

Organisms that are anhydrobiotic by nature, such as *Saccharomyces cerevisiae* and tardigrades, have been found to accumulate up to 20% of their dry weight of trehalose and undergo so-called vitrification (see section 5.2.1) upon drying (Crowe *et al.*, 1998). Desiccation-tolerant plants use the disaccharide sucrose for this purpose (Crowe *et al.*, 1998; Muller *et al.*,

[3] Colligative effects are those that are dependent upon the *collection* (number) of solutes in a solution, rather than the nature of the solute.

1997). Anhydrobiotic engineering involves manipulation of cells by either introducing transgenic genes or over-expressing intrinsic genes to enhance their tolerance to drying (De Castro *et al.*, 2000). Anhydrobiotic engineering without genetic manipulation entails preconditioning or natural selection with the aim of enhancing the tolerance to drying. Inserting (and expressing) the genes for trehalose synthesis has proven to be quite a successful method to improve desiccation tolerance but is not sufficient for complete anhydrobiosis (De Castro *et al.*, 2000). Lately, a group of proteins called late embryogenesis abundant proteins (LEA proteins) have been correlated with desiccation tolerance (Iturriaga, 2008; Wise *et al.*, 2004). The functions of LEA proteins have not been fully elucidated, but they have been suggested to act by stabilising the amorphous matrix of sugars or as molecular chaperones maintaining protein and DNA structure (Goyal *et al.*, 2005; Shih *et al.*, 2008; Wise *et al.*, 2004; Wolkers *et al.*, 2001). A combination of sucrose and LEA protein results in an increased glass transition temperature (T_g) compared with sucrose alone (Wolkers *et al.*, 2001). As of 2008, there were no reported *in vivo* studies combining trehalose and LEA protein expression to engineer desiccation tolerance (Iturriaga, 2008). Anhydrobiotic engineering is a plausible option to make desiccation-sensitive cells such as lactic acid bacteria more tolerant to freeze-drying.

5 Formulation

Formulation is defined herein as the active ingredient (*i.e.* the cells) combined with all other ingredients necessary to form a suitable preparation (*i.e.* a dry product). Formulation and the formulation process (*i.e.* freeze-drying) are closely connected and need to be planned together (Franks *et al.*, 2007; Jennings, 1999).

To successfully preserve cells by either freeze-concentration or freeze-drying, the addition of suitable protective agents is necessary (Carvalho, 2004; Palmfeldt *et al.*, 2003). Freeze-drying lactic acid bacteria without a proper formulation procedure results in very low survival rates (Stadhoud *et al.*, 1969). Protective agents can be divided into cryoprotectants when used for freeze-stabilisation, lyoprotectants if they act during drying and osmolytes if they act mainly to protect structures at high osmolarity. Mono- and disaccharides (trehalose, sucrose, glucose, lactose), sugar alcohols (glycerol, sorbitol), skim milk, peptones, polymers (polyethylene glycol, polyvinylpyrrolidone), and different amino acids and derivates thereof are commonly used protective agents for microorganisms (Conrad *et al.*, 2000; Hubalek, 2003). We studied the effects of using formulations of *Lb. coryniformis* Si3 in sucrose alone or in combination with betaine, skimmed milk or different polymers (**Papers I to IV**). In all studies, we used 0.2% peptone water with 0.01 mg/mL Tween 80 in water as a formulation base.

The molecular mechanisms behind solutes differ depending on the nature of the solute and amount of water available (Crowe *et al.*, 1990; Potts, 1994). A specific solute might have different modes of action and be able to act as a cryoprotectant, lyoprotectant and osmolyte. Hence, the molecular mechanisms of protection discussed below are not as categorically separated as here, and in some cases the mechanisms of protection are not completely understood. Furthermore, apart from the use of protective agents, other formulation ingredients might be necessary to fulfil the formulation criteria,

such as buffers to adjust pH, bulking agents, or other stabilisers (Wang, 2000) depending on the type of active ingredient and application.

5.1 Cryoprotectants

The main effect of cryoprotective[4] agents is to minimise the amount of ice formed (Pegg, 2002). All non-toxic, low-molecular-weight solutes can be used as protection for cells by minimising, via colligative effects, the percentage of water converted to extracellular ice and the extent of cell volume reduction. The detrimental effect on cells of cooling a water-based formulation is considered to lie in both the formation of ice and toxic effects due to the increase in solute concentration (Pegg, 2002). The potentially toxic effects must be balanced against the increased ability to vitrify (mechanism discussed in section 5.2.1) at higher concentrations, resulting in a optimal concentration (Pegg, 2002).

Protective agents that function via their colligative effects are usually accumulated in high concentrations (0.2-2 M), while protective agents with other mechanisms of action are accumulated in lower amounts (Storey, 1997). Glycerol is both a well-used and suitable cryoprotectant for cells (Pegg, 2007). However, a high concentration of glycerol is not a successful lyoprotectant due to its low T_g' (discussed in sections 5.2.1 and 6.1.2). High concentrations of betaine have been shown to depress the freezing point more that would be expected based on only colligative effects (Komai *et al.*, 2006). We determined that betaine was a more suitable cryoprotectant than lyoprotectant for *Lb. coryniformis* Si3, but the freeze-thaw survival rates were still lower than with using sucrose (**Paper III**).

5.2 Lyoprotectants

Disaccharides are considered to be well-functioning lyoprotectants[5]. Anhydrobiotic organisms accumulate high amounts of different disaccharides, which have been related to their ability to tolerate desiccation (Carpenter *et al.*, 1992; Crowe, 2002; Muller *et al.*, 1997). The excellence of disaccharides has been proposed to be due to a combination of two mechanisms; by depressing the T_m of the lipid membranes, *i.e.* by the water

[4] The prefix *cryo-* derives from the Greek word *krýos*, which translates into *cold* or *icy*.

[5] *Lyo-* derives from Greek word *luein*, which means *to loosen* or *to dissolve*. The prefix *Lyo-* is also found in the word lyophilisation relating to the product characteristics, "*Lyophilic = loves to dissolve*".

replacement mechanism, and by the ability to vitrify and form an amorphous matrix upon drying (Crowe *et al.*, 1998; Crowe *et al.*, 1992; Crowe, 2002). The ability to vitrify has many potentially beneficial effects on cells. For example, vitrification leads to increased viscosity and thus a strong delay in chemical reactions requiring diffusion, increases the volume compared with crystalline material and thus prevents cellular collapse, entraps toxic solutes and makes hydrogen bonding possible at interfaces (Koster, 1991).

Both the intracellular and extracellular concentrations of lyoprotective agents are important for optimal desiccation tolerance (Billi *et al.*, 2002; Welsh *et al.*, 1999). It has been shown that complex mixtures of lyoprotective agents can stabilise better than one agent alone (Zayed *et al.*, 2004). Combinations of protective agents can interact to create more advantageous matrices with increased T_g (**Paper IV**). We showed that sucrose alone was a rather successful lyoprotectant for *Lb. coryniformis* Si3, with a maximal survival of 70% depending upon concentration, cell density and cooling rate (**Paper II**). However, the storage stability was in need for improvement when sucrose was used alone. By incorporation of polymers into the cell-sucrose formulation, a higher resistance of the dry formulation to sucrose crystallisation and an increased T_g were achieved and storage stability enhanced at low temperatures (**Paper IV**). On the other hand, including the preferred compatible solute betaine to the sucrose decreased the stability of the glassy sucrose matrix (**Paper III**). Table 1 lists a selection of commonly used lyoprotectants for different lactic acid bacterial species found in the literature (including unpublished **Papers III** and **IV**) from 2000-2008.

Table 1. *Overview of commonly used lyoprotective agents for lactic acid bacteria found in the literature from 2000–2008. LAB = Lactic acid bacterium used, as denoted by the authors, with no regard taken to changes in nomenclature and Lb.* = Lactobacillus. *The species (and strain if available), concentrations of the lyoprotectant, freeze-drying survival directly after drying are given where possible, n.a. = information not available*

Formulation	LAB	Concentration	Survival	Reference	Formulation	LAB	Concentration	Survival	Reference
Skim milk and sodium glutamate	84 strains of 20 different *Lactobacillus* species.	10% and 1%	59 ± 27%	(Miyamoto-Shinohara *et al.*, 2008)	Skim milk + sucrose	*Lb. corynifornis* Si3	10 % + 5%	18 –72%	**(Paper I)**
Skim milk w/ either glucose, fructose, lactose, mannose, or sorbitol	*Lb. delbruckeii* subsp. *bulgaricus*	1% additive in skim milk solution	n.a.	(Carvalho *et al.*, 2004)	Sucrose, glycerol, sorbitol, and skim milk.	*Lb. delbruckeii* subsp. *bulgaricus* LB14	n.a.	87%	(Huang *et al.*, 2005)
Skim milk w/ sucrose or lactose.	*Lb. gasseri* CRL1412 and CRL1421 and *Lb. delbruckeii subsp. delbruckeii* CRL1461	6 % + 6 %	n.a.	(Otero *et al.*, 2007)	Skim milk + intracellular accumulation of trehalose	*Lactococcus lactis* MG1363 and *Lactococcus lactis* NZ9000	10 %	100 %	(Termont *et al.*, 2006)
Skim milk w/ or w/o additives	*Lb. sakei* CTC 494	Up to 20%	n.a.	(Ferreira *et al.*, 2005)	Skim milk	*Lb. reuteri* ATCC 55730	10 %	20–80%	(Palmfeldt *et al.*, 2000)
Skim milk or disaccharides	*Lb. paracasei* NFBC 338 and *Lb. rhamnosus* GG	15%	88-99%	(Miao *et al.*, 2008)	Trehalose and trehalose-phosphate	*Lb. acidophilus*	20%	~ 85%	(Ekdawi-Sever *et al.*, 2003)
Skim milk	*Lb. plantarum* and *Lb. oeni*	n.a.	44 - 100%, *and* 33-100%	(Alegria *et al.*, 2004)	Trehalose (t) + borate (b)	*Lb. acidophilus*	2.5 to 30 and 0.1 or 0.3 mol b/mol t	64-90%	(Conrad *et al.*, 2000)

Formulation	LAB	Concentration	Survival	Reference	Formulation	LAB	Concentration	Survival	Reference
Yoghurt, sucrose and blueberries	*Streptococcus. thermophilus* and *Lb. delbrueckeii subsp. bulgaricus*	10 % + 10 %	n.a.	(Venir *et al.*, 2007)	Lactose	*Lb. paracasei ssp. paracasei* F19	25% (w/v)	n.a.	(Higl *et al.*, 2007)
Sucrose, inulin, skim milk, and fructooligosaccharides	*Lb. reuteri*	5 - 7.5%	n.a.	(Schwab *et al.*, 2007)	Sucrose and betaine or skim milk	*Bifidobacterium animalis* subsp. *lactis* VTT E-012010	5% (w/w)	n.a.	(Saarela *et al.*, 2005)
Sucrose and different fibre preparations	*Lb. rhamnosus* E-97800	10% (w/vol.)	n.a.	(Saarela *et al.*, 2006)	Sucrose	*Lb. coryniformis* Si3	2 to 20%	7-70%	**(Paper II)**
Banana, soybean, barley and nonfat milk powder	*Lb. acidophilus*	12% solid content	<1%	(Trachoo *et al.*, 2008)	Lactose, trehalose or combinations of lactose and trehalose	*Lb. rhamnosus* GG	20%	n.a.	(Pehkonen *et al.*, 2008)
Betine and/or sucrose	*Lb. coryniformis* Si3	Different ratios between 0 to 15%	<3% to 48%	**Paper III**	Sucrose + PVP90 or Ficoll400	*Lb. coryniformis* Si3	17% polymer to sucrose (dry %)	n.a	**Paper IV**
Amino acids and sugars	*Lb. delbrueckeii subsp. bulgaricus* CRL 494	1.25 – 10%	>90%	(Martos *et al.*, 2007)					

Skim milk and different carbohydrates are among the well-studied lyoprotectants for lactic acid bacteria, but there are also studies on the effects of different amino acids, betaine, polyols and polymers, as well as some food-related matrices such as banana and yoghurt with blueberries (Table 1). It is difficult to compare results or draw any conclusions, since freeze-drying survival depends on many factors apart from the formulation, such as species, fermentation, cooling rate, and drying process.

5.2.1 The ability to vitrify

The ability to form an amorphous glassy matrix, *i.e.* vitrify upon drying, is very important for successful dry stabilisation of cells (Crowe *et al.*, 1992; Crowe *et al.*, 1997). We believe that the absence of vitrification when freeze-drying in betaine was one reason for the very low survival rates obtained (**Paper III**). During freeze-drying the amorphous structure is obtained by rapid precipitation of the solute from solution (Hancock, 1997). The term amorphous is used to denote a disordered system compared with a crystalline solid with a high degree of order (Liu *et al.*, 2006). Glass is an amorphous material below the glass transition temperature, while a rubbery or super-cooled liquid is an amorphous material above the glass transition temperature (Hancock, 1997). An amorphous glass has liquid-like properties and a viscosity[6] of approximately 10^{12} Pa · s, resembling a solid (Hancock, 1997). The material has a glass transition temperature (T_g) where it transforms from a liquid rubbery state to a glass. A state diagram of a glass-forming solute is shown in Figure 6.

[6] The viscosity of water at 20°C is 1.00 x 10^{-3} Pa · s (as reference). From Weast, R.C. (Ed.) (1974). *Handbook of Chemistry and Physics*. Cleveland: CRC Press.

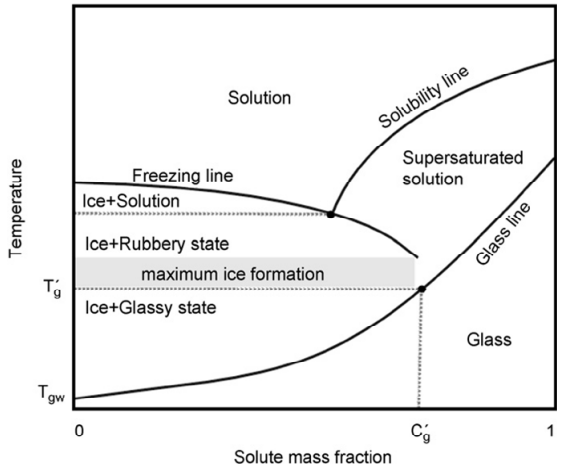

Figure 6. State diagram of a solute, such as sucrose, and water (Santivarangkna *et al.*, 2008b). The glass transition temperature is dependent upon many things, *e.g.* the nature of the solute and water content, and can be shifted by additions of ions, polymers and other additives. T_{gw} = glass transition temperature of water, C'_g = concentration of the solute in the freeze-concentrate. The diagram is reprinted with permission.

The T_g is a dynamic property that is strongly influenced by the nature of the solute, presence of water, and by additives such as ions and polymers (Ekdawi-Sever *et al.*, 2003; Imamura *et al.*, 2002; Kets *et al.*, 2004; Österberg *et al.*, 1999; Zeng *et al.*, 2001). In our studies, T_g was found to be affected by the presence of the plasticisers betaine and water and by anti-plasticiser polymers, but was not affected by the presence of *Lb. coryniformis* Si3 cells *per se* (**Papers II – IV**). Loss of the amorphous glassy state can lead to stickiness and collapse, or transformation to the more thermodynamically stable crystalline form (Foster *et al.*, 2006; Roos, 2002; Roos *et al.*, 1990).

5.2.2 Water replacement theory

Apart from the ability to vitrify, well-functioning lyoprotective agents are considered to act by water replacement. Drying of lipid bilayers results in an increase in T_m of the membrane, resulting in the membrane undergoing detrimental phase transitions and leakage upon rehydration (Crowe *et al.*, 1998), which is considered to be accompanied by cellular death. When a water-replacing compound is present, it depresses T_m by hydrogen bonding

and the membrane remains in the liquid crystalline form and does not pass through the detrimental phase transitions (Figure 7) (Crowe *et al.*, 1998). The increase in T_m is detectable when the water content is below 0.2 g H_2O per g dry weight (Bryant *et al.*, 2001).

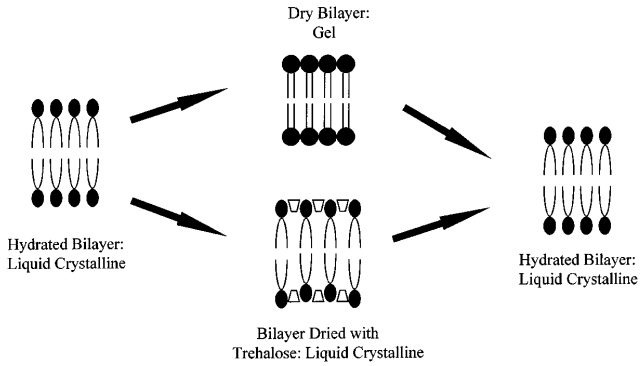

Figure 7. Graphical illustration of water replacement theory on the effects of lyoprotective agent trehalose on lipid membranes. Reprinted here with permission from Annual Review of Physiology, Volume 60 ©1998 by Annual Reviews www.annualreviews.org. (Crowe *et al.*, 1998).

The water replacement mechanism by different solutes has been studied in artificial lipid membranes and living cells, *e.g. Saccharomyces cerevisiae* 131, *Escherichia coli* DH5α, *Bacillus thuringiensis* HD-1 by FTIR spectroscopy (Crowe *et al.*, 1997; Leslie *et al.*, 1995; Leslie *et al.*, 1994a; Leslie *et al.*, 1994b; Tsvetkova *et al.*, 1998). However, when drying *Lactobacillus plantarum* P743, added maltose, trehalose or sorbitol did not change T_m *in vivo* and it was suggested that the function of carbohydrates was not by water replacement but as free radical scavengers (Linders *et al.*, 1997). Inconsistencies among the results have been suggested to depend upon whether the sugars are located intracellularly and can also be affected by sample preparation for FTIR (Oldenhof *et al.*, 2005; Wolkers *et al.*, 2005).

5.3 Compatible solute betaine

Even though the mechanisms behind osmolytes and lyoprotectants might differ, some authors consider the uptake of any compatible solute a natural step in making lactic acid bacteria starter cultures more robust to industrial processing (Sleator *et al.*, 2007). However, this is a sweeping generalisation. Many bacteria, including several lactic acid bacteria, naturally prefer betaine,

a tri-methylated glycine, as the compatible solute (Glaasker *et al.*, 1998; Glaasker *et al.*, 1996). The literature on the beneficial effects of betaine as a lyoprotectant is not conclusive. There are studies in which betaine uptake has been shown to increase the tolerance to drying (Kets *et al.*, 1996; Kets *et al.*, 1994; Koch *et al.*, 2008; Sheehan *et al.*, 2006). But, some reports on positive effects have later on been revised and suggested to be due to other factors (Linders *et al.*, 1998). There is also evidence that betaine is not a suitable lyoprotective agent (Hincha, 2006; Linders *et al.*, 1998; Saarela *et al.*, 2005). The observed negative effects of betaine have been attributed to the fact that betaine destabilises membranes during drying (Hincha, 2006). We showed that betaine was not a successful lyoprotective agent for *Lb. coryniformis* Si3 (**Paper III**). Betaine crystallised upon drying (**Paper III**), leading to very low survival rates of approximately 3%. By examination of the state diagram of betaine in water, some conclusions can be drawn with regard to the success of betaine as a lyoprotectant (Figure 8).

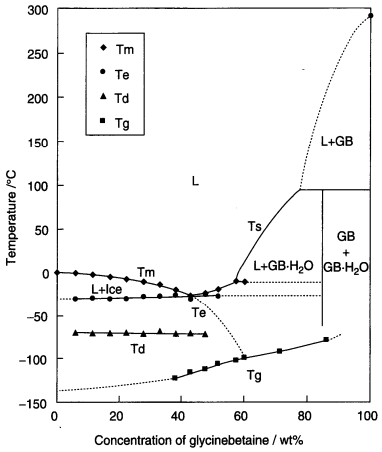

Figure 8. State diagram of the compatible solute betaine in water (Komai *et al.*, 2006). L = liquid phase, T_e = eutectic temperature, T_d = devitrification temperature, T_s = solubility line, T_m = melting line, and T_g = glass transition temperature. The diagram is reprinted here with permission.

In accordance with the state diagram (Figure 8), we determined the T_g' temperature for betaine to be -101 °C, and showed that it had a strong plasticising effect on sucrose, both in the freeze-concentrate and in the dry material (**Paper III**), affecting both the processing costs and storage conditions negatively.

5.3.1 Preferential hydration

Osmolytes can also act through a thermodynamic stabilisation mechanism, *i.e.* by their ability to preferentially hydrate macromolecules. The theory of preferential hydration was developed by Timasheff and colleagues (Inoue *et al.*, 1968; Timasheff, 2002). Exclusion of the solute from macromolecular structures occurs since it is thermodynamically favourable (energy driven), which results in structures being maintained in a native, hydrated and active state.

Methylamines such as betaine are strongly excluded solutes (Auton *et al.*, 2008), but glucose, sucrose and trehalose are also preferentially excluded from hydrated lipid membranes (Westh, 2008) if water availability allows. This mechanism requires more water than is generally available during

drying (*i.e.* 0.3 g H_2O per g dry weight), and is thus not considered a lyoprotective mechanism (Hoekstra *et al.*, 2001).

5.4 Cell density

Increasing cell density in the liquid formulation prior to freeze-drying do increase the survival of *Lactobacillus delbruckeii* subsp. *bulgaricus*, *Streptococcus thermophilus* and *Pantoea agglomerans* CPA-2 (Bozoglu *et al.*, 1987; Costa *et al.*, 2000). Our data also show that a high cell density and high sucrose concentration of formulations with *Lb. coryniformis* Si3 are correlated with high freeze-drying survival (**Paper II**) and it is likely that there is a minimum ratio between the protective agent and number of cells to obtain a high survival during drying.

6 Freeze-drying

Freeze-drying is an old drying method. It was used as early as in the 16th century by South American Indians living at high altitudes in the Andes. They produced a freeze-dried *chuño* (potato product) with extended shelf-life by freezing the potatoes overnight and drying them on the hillside (Franks *et al.*, 2007). Today, freeze-drying is a well-used method for high value foods, pharmaceuticals and sensitive biological material. The knowledge of rational formulation design for freeze-drying have increased with the expansion of the pharmaceutical industry (Franks *et al.*, 2007).

The freeze-drying process can be divided into three steps; a freezing step followed by primary and secondary drying (Figure 9). Freezing of water can be considered the onset of drying, since it effectively decreases the available liquid water. The frozen water is then removed by sublimation, which is achieved by lowering the pressure. During primary drying, the unfrozen water present in the products is not removed and a secondary drying step where the water is desorbed is needed for storage stability. The metabolism and viability of the cells are considered to be relatively unaffected until removal of water reaches a critical point of approximately 0.25 g H_2O per g dry weight, approximately the same as the amount of unfrozen water in the sample (Crowe *et al.*, 1990) and where the water replacement mechanism become critical (Bryant *et al.*, 2001).

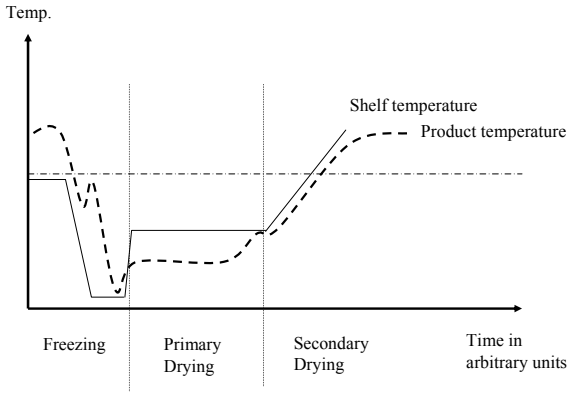

Figure 9. Illustration of a freeze-drying cycle divided into three steps, cooling (freezing step), primary drying and secondary drying. The product temperature (dotted line) during primary drying is determined by the chamber pressure and shelf temperature.

6.1 Freezing

Formation of ice crystals available for sublimation is a precondition for freeze-drying. However, further cooling of the material to a solid state (below T_g, T_{coll} or T_{eu}) has been shown to be necessary both for bacterial survival and to obtain a self-supportive and dry cake (Jennings, 1999; Pehkonen *et al.*, 2008). As ice crystals form there is freeze-concentration of solutes and a maximal freeze-concentrate of the lyoprotectants is reached. The increase in solute concentration can be significant. It has been shown that cooling a 0.9% NaCl solution to -21 °C (T_{eu}) increases the concentration 24-fold (Franks, 1990) and small carbohydrates can reach concentrations of up to 80% (Roos, 1993). The maximal freeze-concentrated amorphous phase transition temperature (T_g') is the approximate temperature at which the amorphous solute solidifies by vitrification.

The degree of ice crystallisation (D-value) can be used to assess the proportion of unfrozen water trapped in the freeze-concentrated phase (Jennings, 1999).

$$Degree\ of\ crystallization = \frac{\Delta H_{f,\ formulation}}{\Delta H_{f,\ total\ water\ in\ formulation}}$$

where the ΔH_f is the heat of fusion in J/g. The phenomenon of loss of structure during freeze-drying formulations with high sucrose concentrations has been mentioned to occur (Palmfeldt *et al.*, 2003). We showed that in cell-sucrose formulations, the sucrose concentration affects the degree of water crystallisation by increasing the amount of unfrozen water with higher sucrose concentrations (**Paper II**). In addition, a high concentration of lactic acid bacteria increased the amount of unfrozen water, which was confirmed by a slight decrease in T_g' (**Paper II**). A high amount of unfrozen water in the amorphous phase leads to many small, entrapped ice crystals that are difficult to remove by sublimation and thus can lead to a loss of structure.

6.1.1 Freeze injury in cells

The freezing of water has long been considered harmful for living cells (Mazur *et al.*, 1972) and there are many reports on the effect of different cooling rates on bacteria (Baati *et al.*, 2000; Dumont *et al.*, 2004; Fonseca *et al.*, 2006; Morozov *et al.*, 2007). A two-factor hypothesis of freezing injury in cells has been developed by Mazur and colleagues (Mazur *et al.*, 1972). The hypothesis is that two mechanisms of damage exist that are dependent upon the cooling rate. A slow cooling rate induces a high degree of osmotic stress by solute concentration, while a fast cooling induces lethal ice formation inside the cells (Park *et al.*, 2002). In accordance with this theory, rapid cooling (100 to 1000 °C/min) induces intracellular ice crystal formation in yeast cells (Nei, 1983). However, the response to different cooling regimes depends on the cell type, in that small cells such as bacteria are more resistant to freezing than larger yeast and mammalian cells (Dumont *et al.*, 2004). There is no evidence for intracellular ice formation in smaller bacterial cells when frozen in the presence of protective agents. In *Lactobacillus delbrueckeii* subsp. *bulgaricus* CFL1, the cause of cellular death after rapid cooling has been determined to be osmotic imbalance upon re-warming rather than ice formation (Fonseca *et al.*, 2006). We showed by a design of experiments approach that the formulation and cooling rate were co-dependent and that the cooling rate was an important factor for the survival of *Lb. coryniformis* Si3 (**Paper II**). The difference in cooling rates in **Paper II**, in addition to influencing ice formation, had a major effect on the time the cells spent in the freeze-concentrate, which is a likely factor influencing differences in survival rate after different cooling regimes.

6.1.2 The significance of T_g'

The significance and interpretation of the maximal freeze-concentrated amorphous phase transition temperature (T_g') is under debate. When a carbohydrate solution is cooled, the T_g' of the freeze-concentrated phase is close in temperature (1-5 °C) to the structural collapse temperature (T_{coll}) (Oetjen, 1999). Collapse during drying should be avoided since it reduces the drying rate and greatly reduce the quality of the product. Interestingly, the T_g' and T_{coll} phenomena in formulations with lactic acid bacteria are separated by as much as 14-26 °C (Fonseca *et al.*, 2004). *Lactobacillus delbruckeii* subsp. *bulgaricus* CFL1 increase the collapse temperature more than *Streptococcus thermophilus* CFS2, which was ascribed to their different shapes and sizes (Fonseca *et al.*, 2004). However, cooling to below the T_g' is important for the survival and storage stability of *Lactobacillus rhamnosus* GG (Pehkonen *et al.*, 2008), indicating that the T_g' transition is very important in determining appropriate cooling temperatures. In (**Paper II**), the sublimation was found to occur at temperatures above the T_g' of the formulation, as determined by DSC (visualised in Figure 19). However, the cakes showed no signs of shrinkage or collapse, confirming the increased robustness indicated by Fonseca *et al.* (2004). The formulation was cooled to -50 °C, which was well below the T_g' determined (**Paper II**) which later on has been shown to correlate with high survival (Pehkonen *et al.*, 2008).

6.2 Primary and secondary drying

After ice formation and solidification of the sucrose-based formulation below T_g', the actual removal of water from the product starts. When cells are freeze-dried in an amorphous surrounding they are not visible and are probably entrapped in the matrix (Figure 10, unpublished results).

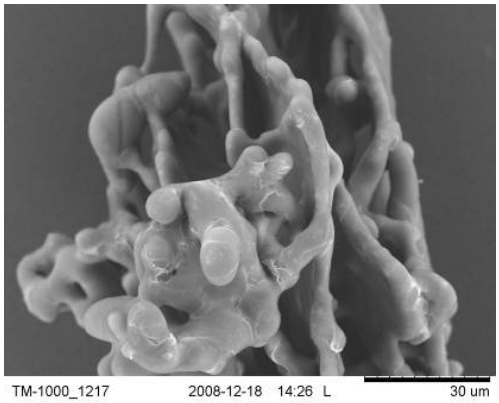

Figure 10. SEM image of a crushed freeze-dried cake of *Lb. coryniformis* Si3 in 15% sucrose at 2000x magnification. Freezing was done by quenching the samples in liquid nitrogen.

The heat transfer, necessary for sublimation to occur, comes from different sources; radiation, conduction (solid/liquid interfaces) and convection (gas phase) (Franks *et al.*, 2007; Jennings, 1999; Oetjen, 1999). Mass transfer of water depends on the cake thickness, total solid content, ice crystal distribution and surface area (Franks *et al.*, 2007). After primary drying, there is still too much water to ensure high product stability (Jennings, 1999). A moisture content of approximately 2-5% is considered optimal for storage stability (De Valdez *et al.*, 1985a; Gardiner *et al.*, 2000) and the bound water needs to be removed by desorption during secondary drying. Secondary drying need to be preformed with the product temperature below the collapse of the material, which usually correlates to the glassy line (some degrees above) showed in Figure 6. It is debatable whether over-drying by removing too much water is a problem, or whether possible negative effects on survival or activity are instead an effect of temperature (Franks *et al.*, 2007). The final water content of the product is influenced not only by drying time and temperature, but also by the formulation and annealing during the process (Ekdawi-Sever *et al.*, 2003; Franks *et al.*, 2007). We determined the effect on the final water activity of the freeze-dried products by changing the sucrose concentration, cooling rate and cell density (**Paper II;** Figure 11). The higher the sucrose concentration, the more water was left in the products, which could be linked to the decrease in degree of water crystallisation, *i.e.* more trapped unfrozen water in the amorphous phase (**Paper II;** Figure 11a). Higher cooling rates result in smaller ice crystals (Oetjen, 1999) and when we increased the cooling rates of our formulation more water was found in the products (Figure 11b).

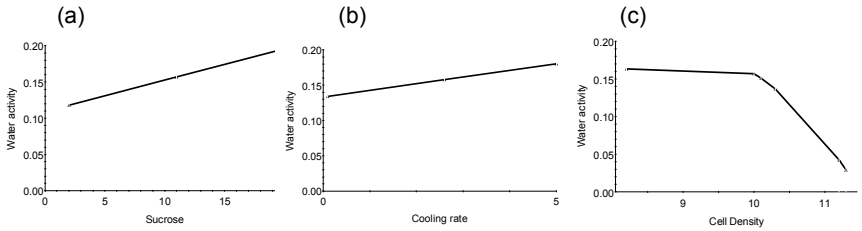

Figure 11. Effects on product water activity of changing (a) sucrose concentration, (b) cooling rate and (c) cell density in the formulation. All experiments were run with the same primary and secondary drying process, resulting in different water activities of the products.

Interestingly, higher cell densities led to lower final moisture content of the products (Figure 11c). This was surprising, since the degree of water crystallisation as well as the T_g' value suggested that more water was entrapped in the sucrose and cell phase, which would lead to higher moisture levels (**Paper II**).

Product properties, such as a high surface area, can be a reason for choosing the more expensive freeze-drying over other drying techniques and should be included as one criterion for a successful freeze-drying process. Figure 12 shows successfully freeze-dried cakes of *Lb. coryniformis* Si3 in sucrose-based formulations examined in **Paper IV**.

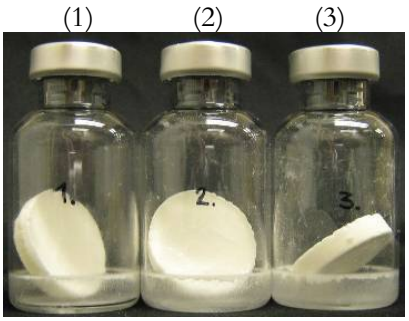

Figure 12. Freeze-dried cakes stored under appropriate storage conditions (*i.e.* low moisture levels and low temperature) for three months. The samples are 10^{10} CFU/mL *Lb coryniformis* Si3 with, from left (1) 15% sucrose; (2) 15% sucrose and 2.5% PVP90; and (3) 15% sucrose and 2.5% Ficoll400. The cakes are of approximately the same volume as the liquid formulation prior to drying.

6.3 Economic aspects of freeze-drying

Stabilising sensitive biological material in a dry form is often a more economically feasible and user-friendly alternative than commercialising its

liquid counterpart. Freeze-concentrates have the disadvantage of requiring very low temperatures in shipping and handling, whereas liquid formulations are bulky and tend to lose cell viability fast when handled at ambient temperatures.

Lactic acid bacteria have been dried by other drying techniques such as spray-drying (Ananta *et al.*, 2005; Chavez *et al.*, 2007; Desmond *et al.*, 2002; Fu *et al.*, 1995; Lian *et al.*, 2002), fluidised bed drying (Kets *et al.*, 1996; Strasser *et al.*, 2007), and vacuum-drying (Conrad *et al.*, 2000). Amongst the different drying techniques, freeze-drying is considered expensive (Roser, 1991). But cost analyses lack in that that no consideration is taken to overall product quality such as cell survival or product characteristics. Combining the cost analysis of different drying methods reported by Roser (1991) with survival rates of *Lactobacillus acidophilus* strains, freeze-drying appears to be an economically competitive technique (Table 2).

Table 2. *Comparison of survival of* Lactobacillus acidophilus *dried with different drying techniques. As a reference for survival rates,* Lactobacillus acidophilus *is used since data are available. Note: not the same strain. The manufacturing cost is taken from Roser (1991) and is based on the cost per kilogram of water evaporated per hour*

Drying technique	Manufacturing cost	Survival (%)	Survival *per* manufacturing cost	Reference (to survival)
Freeze-drying	100	91	0.9	(Conrad *et al.*, 2000)
Spray-drying	20	8	0.4	(Espina *et al.*, 1979)
Fluidised bed drying	18	24	1.3	(Roelans *et al.*, 1990)
Vacuum-drying	52	36	0.7	(Conrad *et al.*, 2000)

Freeze-drying is being successfully used for high value products, *e.g.* pharmaceuticals and cell products, where retained activity is an essential product characteristic and mass production is not the only consideration. The manufacturing cost of freeze-drying has been shown to depend largely upon scale of production and by taking the transportation into account into

the production cost it was determined that freeze-drying was a profitable investment for a kefir product (Kourkoutas *et al.*, 2007).

7 Storage stability

One of the main reasons for drying a liquid formulation is to enhance the storage stability. However, a successful freeze-drying process does not *per se* guarantee a long shelf-life at ambient conditions. Even if the lactic acid bacteria survive the drying process well, they can rapidly lose viability during storage. Temperature and humidity are important factors that affect the storage stability and a controlled storage environment that excludes moisture, heat and oxidation processes is needed for long-term stability of bacteria (Bozoglu *et al.*, 1987; Carvalho, 2004; Santivarangkna *et al.*, 2008a). If kept at low temperatures and low moisture levels, freeze-dried lactic acid bacteria remain active for long periods of time (Champagne *et al.*, 1991).

Short-term loss of cellular viability during storage has been ascribed to membrane lipid oxidation (Santivarangkna *et al.*, 2008b). But with increased water content, a decrease in the T_g follow, with increased rates of loss of product quality and viability (Higl *et al.*, 2007). For many applications, maintained technical quality of the product and thus the maintenance of the amorphous glassy matrix (no collapse) with fast rehydration characteristics are of equal importance to high and maintained viability. However, crystallisation of the amorphous disaccharide enhances the thermodynamic stability of the matrix, and the crystallisation event itself is probably not very harmful for cells, while the absence of vitrification during drying is detrimental. For example, trehalose has a somewhat superior status as lyoprotectant compared with other non-reducing disaccharides, which has been related to both its high T_g and its ability to form crystalline dihydrate, which traps water thorough what can be called a positive phase separation phenomena (Crowe *et al.*, 1996; Kilburn *et al.*, 2006). By the removal of water, which otherwise would plasticise the amorphous phase, in the crystalline dihydrate form the amorphous phase remains dry and more stable during storage. The loss of amorphous structure seems not to be directly

correlated with survival rates of cells (**Paper IV**). However, for several reasons such as an increased loss of viability and loss of technical quality the loss of structure by collapse should be avoided.

7.1 The significance of the glassy amorphous matrix

The glassy amorphous state is only kinetically stable and relaxation, collapse and crystallisation events are likely to occur (Sun, 1997; Yoshioka, 2006). Any new additive in the formulation may affect T_g and an approximation of the T_g in binary systems can be calculated from the Gordon-Taylor equation;

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2}$$

where the k is a constant and w the weight fraction of the solutes. If the ΔC_p of the components are used as k-values, the equation is called the Couchman-Karasz equation (Couchman *et al.*, 1978) and this equation was used in **Paper III** to fit the experimental data obtained. The molecular mobility is related to both the residual moisture content and temperature and there is significant molecular mobility below the T_g temperature (Le Meste *et al.*, 2002), which has been shown to affect the viability of lactic acid bacteria (Higl *et al.*, 2007; Pehkonen *et al.*, 2008). We determined loss of cell viability of freeze-dried *Lb. coryniformis* Si3 in sucrose (Figure 13) and sucrose with polymer formulations when stored at temperatures below T_g (**Paper IV)**.

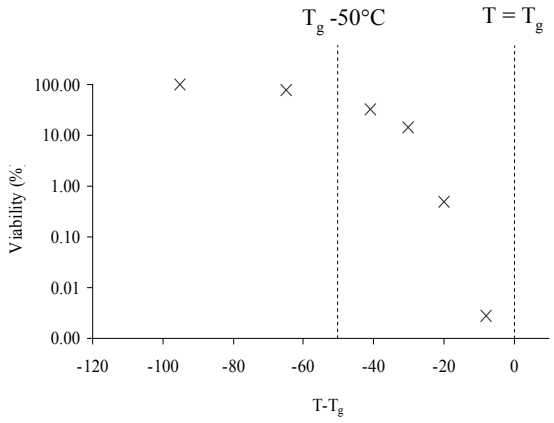

Figure 13. Loss of viability of *Lb. coryniformis* Si3 stored for 2 months in 15% sucrose at different storage temperatures denoted by the storage temperature (T) below the T_g of the formulation. No signs of loss of structure or collapse were observed in any products.

There was significant loss of viability of *Lb. coryniformis* Si3 well below the T_g of the products (**Paper IV**, Figure 14). The loss of cell survival has previously been shown to depend upon the formulation ingredients (Pehkonen *et al.*, 2008) and, in agreement with this, we showed that sucrose or sucrose with Ficoll400 was superior in maintaining the survival compared with sucrose with PVP90 (**Paper IV**). It has been suggested as a rule of thumb that amorphous materials should be kept 50 degrees below the T_g for maintained stability (Craig *et al.*, 1999; Hancock *et al.*, 1995). This rule seems to apply in maintaining the stability of *Lb. coryniformis* Si3 in sucrose-based formulations (**Paper IV,** Figure 13). By combining the information given in Figure 13 with data on how the T_g is affected by different relative humidity (Figure 14), a formulation-specific critical moisture content can be determined for any selected storage temperature (**Paper IV;** Figure 14).

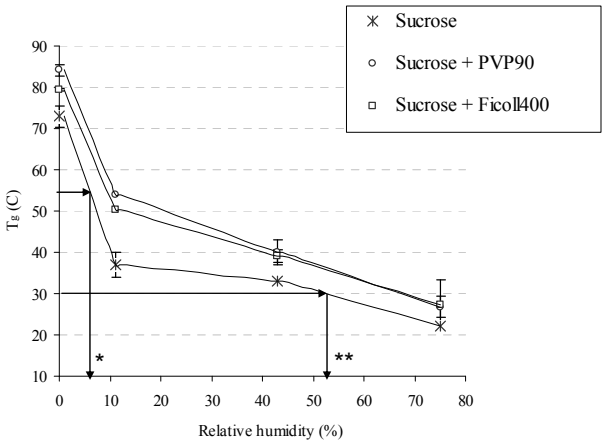

Figure 14. Relationship between T_g and RH% of the sucrose and polymer formulations. By combining this with the knowledge (Figure 12) that the storage temperature should be 50 degrees below the T_g for high microbial stability, product-specific critical moisture content can be determined. Examples are given in the diagram on how to use this correlation together with Figure 12 to determine the critical relative humidity at either a selected temperature of 4°C (*) or -20°C (**) in a sucrose-based product.

The use of this type of data is shown by the arrows in Figure 14. For example, the arrow denoted by ** show the critical relative humidity for the sucrose-based product, if the storage temperature was determined to be -20°C. To assure that the storage temperature is 50 degrees below the T_g, the critical T_g would be 30°C, which corresponds to the critical relative humidity of approximately 53% (Figure 14). The arrow denoted by * show if the storage temperature was determined to be +4°C. This will allow for a rational thinking when selecting storage conditions for freeze-dried lactic acid bacteria products.

7.1.1 Polymer additives

Addition of polymers is an alternative to enhance the stability of amorphous products (Abdelwahed *et al.*, 2006; Berggren *et al.*, 2003; Nasirpour *et al.*, 2007). Polymers usually have high anhydrous T_g and can act as anti-plasticisers, but the stability of an amorphous matrix can be enhanced without changing the T_g and therefore it is likely that other mechanisms are involved, such as a delay in crystallisation of the amorphous component (Shamblin *et al.*, 1996). Not all polymers are well functioning with regard to increasing amorphous stability, and incorporation of a polymer with low T_g

can even promote crystallisation of the sugar component (Chidavaenzi *et al.*, 2001; Corrigan *et al.*, 2002). Also, small amounts of other sugars (Leinen *et al.*, 2006) or electrolytes can also be used to delay sucrose crystallisation or affect the T_g (Österberg *et al.*, 1999; Santagapita *et al.*, 2008).

Polymers alone do not improve the storage stability of different lactic acid bacteria (De Valdez *et al.*, 1983), but when combined with sugars the stability and cell survival is increased, most likely due to interactions between the sucrose matrix and the polymer (Champagne *et al.*, 1996; Chyi *et al.*, 2004; Lodato *et al.*, 1999; Oldenhof *et al.*, 2005). We showed that the addition of either PVP90 or Ficoll400 to a sucrose matrix stabilised the glassy matrix. T_g was raised by either polymer and the time to crystallisation (t_{cr}) of sucrose when exposed to moisture was delayed (**Paper IV**), and PVP90 was superior in increasing amorphous stability then Ficoll400. There was a negative effect on cell viability of adding PVP90 when the products were kept dry (at low relative humidity), suggesting that the correlation between the matrix stability and survival of *Lb. coryniformis* Si3 was complex. We hypothesised that the reason for this lies in the competition for available sucrose. On one hand, the sucrose and PVP90 interact by strong hydrogen bonding (Shamblin *et al.*, 1998), thereby enhancing the amorphous stability of the matrix. On the other hand, sucrose is needed for water replacement to maintain viability in the dry state and cannot function if hindered (bound in the matrix surroundings to the bulky PVP90). Thus, the loss of viable cells could be due to the unavailability of sucrose in the system and not to the PVP90 *per se*. This hypothesis needs to be tested.

7.2 Oxidation

Storing freeze-dried lactic acid bacterial products under vacuum or nitrogen gas is superior to storage in air (Bozoglu *et al.*, 1987) and oxidation of cellular lipids affects the storage survival of bacteria (Santivarangkna *et al.*, 2008b; Yao *et al.*, 2008). By increasing the degree of unsaturation, especially polyunsaturated fatty acids, in *Weisella paramesenteroides* LC11, the susceptibility to oxidation is increased (Yao *et al.*, 2008).

Several recent studies, including **Paper IV**, have shown that there is a substantial loss of lactic acid bacterial viability in products stored well below T_g (Higl *et al.*, 2007; Pehkonen *et al.*, 2008) where the product is dry and molecular mobility quite low. By using a formulation with antioxidant ascorbic acid or gelatine, the loss of viability has been determined to be impaired to approximately 20 degrees below T_g (Selma *et al.*, 2007), which

indicates that by inhibition of oxidation critical storage temperature can be raised by 30°C.

7.3 Non-enzymatic browning

Non-enzymatic browning (NEB) reactions, or Maillard reactions, are known to occur in heated, dried and stored matrices and in organisms (Fay *et al.*, 2005; Kaanane *et al.*, 1989) and these reactions can be used as an indicator of other diffusion-controlled chemical reactions can occur. NEB reactions are chemical condensation reactions between amines and carbonyl compounds, resulting in several different end products with brown colour (Fay *et al.*, 2005). The kinetics of NEB reactions are affected by the concentration of reactants, chemical nature of the reactants (type of amine and carbonyl groups involved), pH, relative humidity, temperature and time of heating (Kaanane *et al.*, 1989).

Sucrose is a non-reducing disaccharide. Hydrolysis into glucose and fructose is needed prior to participation in NEB reactions. However, even in low moisture systems the hydrolysis of sucrose can occur at higher temperatures, which may result in non-enzymatic browning (Schebor *et al.*, 1999). NEB reactions have been shown to be correlated with the T_g temperatures of various glassy matrices and are also directly related to sugar crystallisation (Kawai *et al.*, 2004; Song *et al.*, 2006). In polymeric matrix systems, non-enzymatic browning has been shown to occur even below T_g (Schebor *et al.*, 1999). Since water is a product of the reaction (Fay *et al.*, 2005; Kaanane *et al.*, 1989), the water increases the detrimental breakdown further. PVP90 has been shown to have higher NEB rate than expected from its glass transition temperature (Kawai *et al.*, 2004). No NEB rates were detected in our polymer and sucrose products (Figure 15, unpublished results). We estimated NEB rates by measuring production of the compound furfural at OD_{280}.

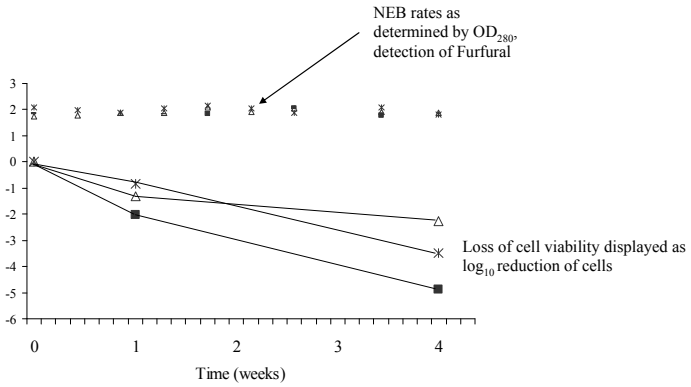

Figure 15. Illustration of some events taking place over time in the freeze-dried sucrose and polymer products (unpublished data). Formulation with sucrose is denoted by filled squares, sucrose with Ficoll4000 by open triangles and sucrose with PVP90 by crosses. The loss of cell viability over time is shown as $\log_{10}$ cell reductions. NEB rates were estimated by reconstitution and determination of furfural compounds by spectrophotometry, at OD_{280}. Sucrose crystallisation (t_{cr}) occurred after 7 hours in sucrose, 28h (approximately 1 day) in sucrose and Ficoll400, and after 90 hours (almost 4 days) for sucrose and PVP90.

8 Reconstitution

Freeze-dried bacterial silage additives are commonly reconstituted in tapwater (rehydration) on farms. It is experimentally difficult to separate damage during drying from that during rehydration, since the latter is a necessary step to assess cellular survival. It has been shown that the rehydration volume, temperature and type of solution (osmolarity, pH) can affect the survival of dried lactic acid bacteria (Champagne *et al.*, 1991; De Valdez *et al.*, 1985a; Selmer-Olsen *et al.*, 1999). The osmotic imbalance during rehydration is considered to be a reason for cellular death (De Valdez *et al.*, 1985a; De Valdez *et al.*, 1985c). Survival rates have been shown to be increased by the use of small volumes of rehydration media (De Valdez *et al.*, 1985a), and as a 'rule of thumb' it has been suggested that rehydration should be done to the same volume as prior to drying (Champagne *et al.*, 1991).

9 Methods

This section provides an introduction to the statistical method used in **Papers I and II**. Some statistical descriptors of importance for the interpretation and evaluation of the models are included. This is followed by a section describing the experimental techniques used.

"All models are wrong, but some models are useful"
George Box, 1979

9.1 Design of experiments

Design of experiments is an efficient way of understanding and optimising complex systems (Eriksson *et al.*, 2000). Experimental design involves choosing a set of experiments that are representative with regard to a given question. Basic knowledge about the system or a screening design is required in order to choose appropriate intervals of the different factors to be studied. It is important to understand that a mathematical model is always an approximation of reality, and it is possible to find false correlations. However, if there are reasons to suspect interactions between parameters, which is often the case in complex systems, a experimental design approach is a better strategy than the commonly used one-variable-at-a-time (OVAT) approach (Eriksson *et al.*, 2000). The reduced number of experiments needed to answer a specific question is another reason for using an experimental design approach (Eriksson *et al.*, 2000).

Response surface methodology (RSM) is an established statistical method to investigate fermentation, formulation and drying procedures (Chauhan *et*

al., 2006; Huang *et al.*, 2005; King *et al.*, 1995). RSM can be used to optimise and understand interactions in a complex system.

Two experimental design approaches were used in this thesis work. A 3-level full-factorial study was used to determine the optimal pH and temperature during fermentation of *Lb. coryniformis* Si3 in order to set non-optimal growth conditions (preconditioning) (**Paper I**); and central composite face-centred (CCF) designs were used to study the impact of formulation and cooling rates with regard to freeze-drying survival and final water content after drying (**Paper II**). All models obtained were significant according to ANOVA and there was no lack of fit. The models were evaluated together with raw data to certify their quality (Eriksson *et al.*, 2000).

9.1.1 Fermentation optimisation model

In order to determine the stress conditions to be used for preconditioning cells with regard to pH and temperature for *Lb. coryniformis* Si3, we evaluated a 'control non-stress' setting where the cell population grew fast to high cell density (**Paper I**). A full factorial 3-level study was performed to determine the maximal growth rate (μ_{max}, h^{-1}) to high cell densities (OD_{600}) of *Lb. coryniformis* Si3 in commercial MRS media. Cell density was determined spectrophotometrically (by OD_{600}) and the maximal growth rate (μ_{max}, h^{-1}) was determined from the linear regression line of $\Delta ln\ OD_{600}/\Delta t$ (in hours) during logarithmic growth. The statistical descriptors of the models are shown in Table 3. There is a species-, medium- and fermentor-dependent optimum value for maximal growth speed and high cell yields.

Table 3. *Statistical descriptors of the model to describe the effects of pH and temperature on growth rate and maximal cell yield (density) of* Lb. coryniformis *Si3*

	r^2	q^2	N	Model validity	Reproducibility
Maximal growth rate	0.96	0.72	12	0.64	0.95
Maximal density	0.99	0.91	12	0.50	0.99

Growth rate and maximal cell yield were dependent upon the factors studied, *i.e.* pH and temperature. Furthermore, it was determined that the temperature and pH parameters interacted. The scaled and centred

coefficient plot for maximal growth and maximal density in the stationary phase is shown in Figure 16.

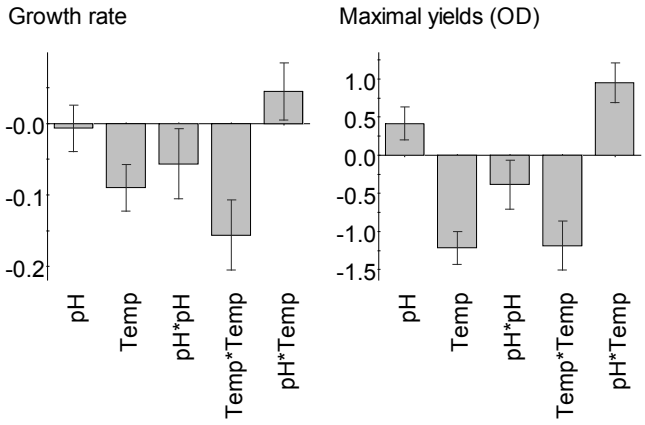

Figure 16. Scaled and centred coefficient plot for maximal growth and maximal density for *Lb. coryniformis* Si3 in MRS. Temp = temperature.

In addition, we showed that after fermentation at optimal pH and temperatures a high survival (70%) of *Lb. coryniformis* Si3 after freeze-drying was achieved (**Paper I**), *i.e.* high process productivity was obtained.

9.1.2 Formulation and freeze-drying model

The CCF design was used to study the effects on freeze-drying survival of varying sucrose concentration between 2 and 20% weight to volume, cell concentration between 10^8 and 10^{12} CFU/mL, and cooling rate determined by shelf temperature between 0.1 °C/min and 5 °C/min (**Paper II**). The statistical descriptors for the model are shown in Table 4.

Table 4. *Statistical descriptors of the model to describe the effects of sucrose concentration, cell concentration and cooling rate on survival of* Lb. coryniformis *Si3*

	r^2	q^2	N	Model validity	Reproducibility
Survival (%)	0.94	0.78	20	0.93	0.84

Due to the experimental setup of mixing the formulation with cell concentrate in a volume ratio of 1:1, reaching 10^{12} CFU/mL was not possible and lower cell numbers were used. There were interactions between

the factors studied and cell survival increased with increasing sucrose concentration, cooling rate and cell density (**Paper II)**.

The effects of changing the factors cell density, sucrose concentration and cooling rate on final water activity (A_w) of the products were also evaluated with a CCF design (**Paper II**) and the statistical descriptors of the model are given in Table 5.

Table 5. *Statistical descriptors of the model to describe the effects of sucrose concentration, cell concentration on water activity (A_w) of the freeze-dried products*

	r^2	q^2	N	Model validity	Reproducibility
Water activity (A_w)	0.74	0.41	17	0.44	0.95

9.2 Experimental methods

The characterisation and understanding of phenomena during formulation, drying and storage of freeze-dried lactic acid bacteria involve the use of set of different experimental methods. Some techniques such as solid-state characterisation methods are well-established in food, pharmaceutical and material sciences. However, when I started these thesis studies in 2003, the number of published studies in which differential scanning calorimetry (DSC) had been used in combination with microbiological techniques to study different aspects of freeze-drying of lactic acid bacteria was low. Since then, solid state characterisation techniques have been integrated into microbiology and now several groups are working with these aspects. This section provides a brief introduction to the methods used in the present study, the reason for selecting them and some examples the (raw) data output. Some experimental setups of special interest are also discussed.

9.2.1 Identification and uptake of betaine

Identification of intracellular solutes is important, both in understanding the intracellular milieu and in assessing the bacterial response to an increased osmolarity. To determine the intracellular pool of solutes during fermentation at increased salt levels or in MRS, high-resolution magic angle spinning nuclear magnetic resonance (HR-MAS-NMR) was used (**Paper III**). ^{1}H NMR spectroscopy is an analytical technique that involves measurement of energy between electron spin of hydrogen atoms when placed under a magnetic field. The proton has a specific spin which give rise to an NMR signal (*i.e.* chemical shift in the spectrum). The shift is

dependant upon the neighbouring environment in the molecule. The magic angle and spinning of the sample create the ability to use high resolution spectra to identify low-molecular weight compounds in intact cells. NMR can be used to determine unknown structures, but also to find compounds where the chemical shifts are known.

The uptake profiles of the main compatible solute as identified by HR-MAS-NMR during different growth conditions were examined by extraction of intracellular material and analysed by high performance liquid chromatography (HPLC) (**Paper III**). HPLC is a liquid chromatography technique where a sample is injected into a column in a liquid phase and the compounds are retained to different degrees depending on their different affinity for the column. After optimisation, we decided on 75% acetonitrile–water mobile phase, a temperature of 35 °C and a flow rate of 1 mL/min. A Zorbax carbohydrate analysis column gave good separation and the possibility to quantify betaine. By simultaneous determinations of number of cells by plate counts on MRS agar, the intracellular concentration of betaine was estimated (**Paper III**).

9.2.2 Determination of the lipid membrane composition

To assess changes in the lipid membrane composition of *Lb. coryniformis* Si3 grown under different pH or temperatures, fatty acid methyl esters (FAME's) were produced and analysed by gas chromatography (GC). Cell suspension samples from the fermentor were extracted in methanol-chloroform, with esters produced by incubation in boron trifluoride/methanol (Morrison *et al.*, 1964) and further analysed by GC-FID (**Paper I**). The separation of compounds in the gas phase occurs since they are distributed differently to the stationary phase (column phase), which results in different retention times. GC is often combined with mass spectroscopy (MS) to identify lipids. In our experiments the lipids were identified by co-injection of reference compounds and detection was performed by a flame ionisation detector (FID). Identification and quantification of the different fatty acids in the membrane of *Lb. coryniformis* Si3 were performed by retention times and calculation of peak area in the chromatogram (**Paper I**; Figure 17).

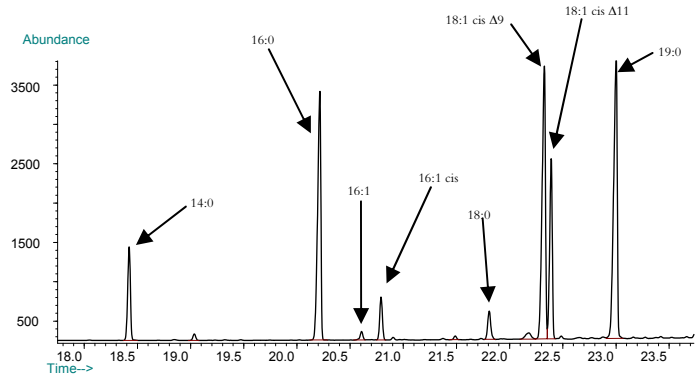

Figure 17. Representative GC-FID chromatogram from the FAME analysis with peaks representing different fatty acid methyl esters extracted from *Lb. coryniformis* Si3 grown at pH 5.5 and 34 °C in MRS broth.

9.2.3 Freeze-drying and storage stability setup

The freeze-drying technique has already been thoroughly discussed in section 6. The reasons for selecting freeze-drying as the drying method in this thesis work were several. Freeze-drying is a well-used drying technique that is known to produce high quality dry bacterial products with high survival rates and can therefore be used to understand the mechanisms needed for desiccation survival. Also, the reason that strain Si3 has not been commercialised was due to low freeze-drying yields compared with other strains, suggesting that the survival of this strain was in need of improvement. A state-of-the-art, pilot plant freeze-drier unit (Figure 18) with the possibility to monitor the process was used.

Figure 18 Lyostar II freeze-drier unit (FTS Systems, Stone Ridge, NY, USA) used in this thesis work. The dryer is equipped with 16 thermocouples for product temperature measurement, and has a Pirani gauge and a conductivity manometer for pressure measurements.

By plotting data collected during freeze-drying, such as thermocouple temperatures, shelf temperatures and pressures from the two vacuum gauges, the process can be visualised (Figure 19).

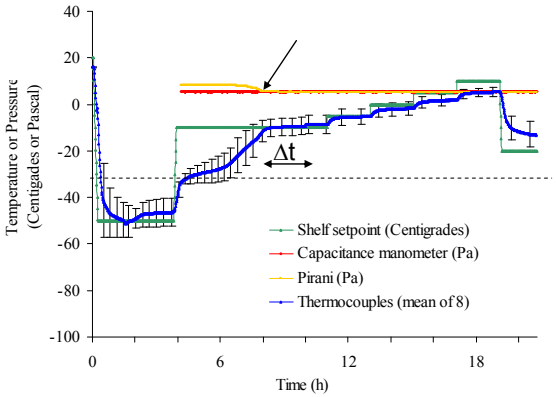

Figure 19. Diagram showing combined data collected from one representative freeze-drying process as performed in **Paper II**. The time between primary and secondary drying, Δt, was shortened for visualisation, *i.e.* optimisation of the process was not carried out (Paper II). The T_g' was determined to be approximately -32 °C (marked by dotted line), and the end of sublimation is marked by the arrow.

The end of primary drying can be determined either by the intercept between the conductivity and capacitance values as shown by the arrow in Figure 20 (Roy *et al.*, 1989).

The storage stability experiments described in **Paper IV** were carried out by equilibration of the freeze-dried cakes at different relative humidity values by including saturated salt solutions in micro-hygrostats placed on the cakes (Figure 20). The relative humidity (RH) was set to 11%, 43% or 75% by oversaturated solutions of NaCl, K_2CO_3, and LiCl. The temperature was controlled in a constant temperature room and samples kept in the dark (**Paper IV**).

Figure 20. Illustration of experimental setup for the storage stability experiments in **Paper IV**. Vial with freeze-dried cake and a hygrostat to set a selected RH environment. The septum was used to inject dry methanol without breaking the RH conditions when determining water content by KFT.

9.2.4 Determination of cell viability

Populations of lactic acid bacteria are known to contain viable but non-culturable cells (Kell *et al.*, 1998; Quiros *et al.*, 2009) and there is some debate on whether the method of culturing bacteria on agar plates to assess CFU/mL is an accurate method to assess viability or survival. For practical reasons and in the absence of good alternative methods, we used the ability to form colonies on MRS agar as the method to calculate viability and survival. The freeze-dried cakes were reconstituted with water to the same volume as prior to drying at room temperature. Dilution series were made and cell suspensions plated on MRS agar kept at 30 °C in a microaerophilic environment. After 48 h, the colonies were calculated within the interval of 10-200 colonies per plate (**Papers I – IV**). The number of colony forming units (CFU) was assessed in the formulation prior to drying and after drying to calculate the percentage survival.

9.2.5 Solid state characterisation

Several important formulation parameters, such as the T_g', T_g, T_c, T_{eu}, or T_{coll}, can be obtained by differential scanning calorimetry (DSC). In our studies, we used both DSC and isothermal micro-calorimetry (IMC) to study the sucrose and *Lb. coryniformis* Si3 formulations (**Paper II – IV**). DSC is the most widely used thermo-analytical technique (Craig *et al.*, 2007). Heat-flow DSC, such as the Seiko 220DSC used in **Paper II,** measures the difference in heat flow between a reference and a sample placed in a single furnace, while power compensation DSC, such as the Perkin Elmer Diamond DSC used in **Papers III and IV**, uses two separate furnaces and measures the power required to eliminate the difference

between the reference and sample (Craig *et al.*, 2007). The data obtained from a DSC thermogram are dependent upon factors such as the heating rate and the use of cups with or without holes. Pyris software was used to analyse the thermograms from the Perkin Elmer DSC (**Papers III and IV**). DSC is an experimentally simple method but interpretation of the thermogram requires experience. In **Paper II**, we determined the T_g' and calculated the degree of water crystallisation from ΔH_f–values obtained by DSC.

Isothermal micro-calorimetry (IMC) has its roots in biological sciences such as the study of cell activity, metabolism and enzyme-substrate interactions (Craig *et al.*, 2007). It is a more sensitive technique than DSC and can be used to study all processes involving heat change. IMC and DSC are complementary techniques that answer different questions on the stability of a freeze-dried product. We used IMC to determine the rate of sucrose crystallisation (t_{cr}) as a result of moisture uptake (**Paper IV**).

Apart from thermal analysis, solid-state characterisation was carried out by scanning electron microscopy (SEM), powder X-ray diffraction and ocular inspections of the material to detect shrinkage or collapse. SEM is a microscopy technique where a beam of electrons creates an image of a sample. We used a Hitachi TM-1000 tabletop microscope where magnifications up to 10,000 times could be achieved. Sample preparation influences the image to a large extent and the technique requires familiarisation. The cells were not visible in most dry formulations as they were entrapped in the amorphous matrix (Figure 21b), but some cells could be seen when betaine was added to the sucrose (**Paper III**). SEM analysis revealed that after freeze-drying of *Lb. coryniformis* Si3 in 0.2% peptone water without sugar, the cells had an average size of 2 x 0.5 μm (Figure 21a).

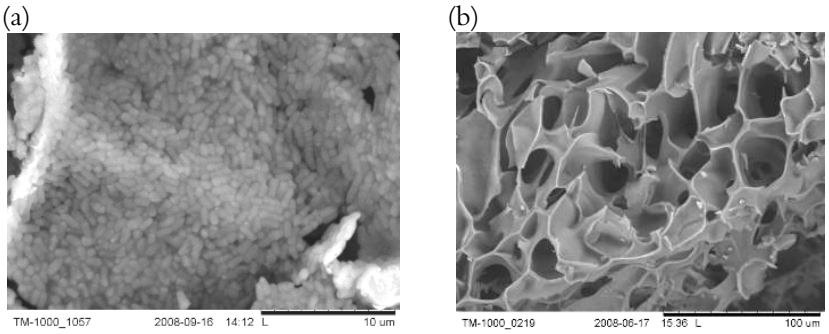

Figure 21. SEM photographs of freeze-dried *Lb. coryniformis* Si3 in (a) 0.2% peptone water at 8000x magnification and (b) sucrose at 1000x magnification. The freeze-dried *Lb. coryniformis* Si3 cells in (a) have a size of approximately 2 x 0.5μm. The image in (b) shows the amorphous structure after freeze-drying in a well-preserved piece.

Since the discovery of X-rays by Wilhelm Röntgen in the 19th century, many different applications for X-ray techniques have been developed. X-ray powder diffraction was used here in combination with DSC to determine whether the dry products were crystalline or amorphous (**Paper III**). When the X-rays interact with the solid sample, a diffraction pattern is obtained that is specific for a crystal structure. The powder data were collected by recording the rotation pattern of a milled powder sealed within a 1.0 mm inner diameter capillary of amorphous quartz. Raw data were obtained by applying SMART software and the exposure time was 300 s. The diffraction patterns of betaine and sucrose are shown in Figure 22.

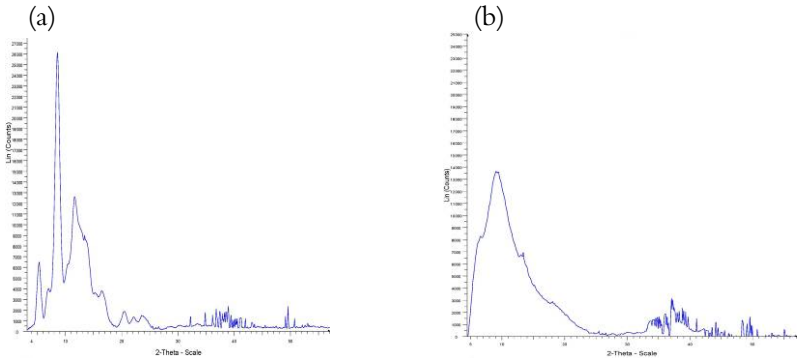

Figure 22. Diffractograms of freeze-dried (a) betaine and (b) sucrose.

The diffraction pattern shows that freeze-dried betaine was crystalline while freeze-dried sucrose was highly amorphous, possibly with some small crystals as a result of storage or sample preparation for the analysis.

9.2.6 Moisture content determination

The moisture content of a dry product is very important for storage stability and an optimal range of 2-5% water content has been suggested for optimal storage stability (De Valdez *et al.*, 1985b; Gardiner *et al.*, 2000). We determined the water activity of the dry products in an Aqualab CX-2 (**Paper II**) and the water content by coulometric Karl-Fisher titration (KFT) (**Paper IV**). Water activity A_w (or equilibrium relative humidity, RH) is a measure of the vapour pressure generated by the moisture present in a hygroscopic product. Standards of saturated LiCl and water were always included as controls. The relationship between A_w and RH is:

$$A_w = 100 \times RH\%$$

The coulometric Karl-Fisher titration to determine the water content is based on a two-step reaction (shown below), where I_2 is generated electrochemically and is consumed in a 1:1 ratio with water, thus allowing the content of water to be calculated.

Step 1. $CH_3OH + SO_2 + \text{base (B)} \rightarrow CH_3SO_3^- + HB^+$

Step 2. $CH_3SO_3^- + H_2O + I_2 + 2B \rightarrow CH_3SO_4^- + 2HB^+ + 2I^-$

To determine the water content of freeze-dried *Lb. coryniformis* Si3 products, they were reconstituted in dry methanol by injecting the methanol through the septum of closed vials (Figure 20) and subjected to shaking for 1h at room temperature to extract all water. After the methanol extraction the cell debris precipitated and the supernatant was used for water determination. We also used KFT to examine whether the known interaction between the PVP-sucrose materials could be seen.

When physical mixtures were created and compared with co-lyophilised (co-freeze-dried) products, the water content in the co-lyophilised PVP90 and sucrose was lower than expected, indicating an interaction between components (**Paper IV**).

10 Main findings and future perspectives

Lb. coryniformis Si3 was found to be more sensitive to high osmolarity, freeze-thaw and freeze-drying than the commercialised antifungal *Lb. plantarum* MiLab 393. However, by optimising the fermentation and freeze-drying processes with sucrose as single protective agent, it was possible to reach a freeze-drying survival of approximately 70% for *Lb. coryniformis* Si3. The average freeze-drying survival of *Lactobacillus* sp. is 59 ± 27% (Miyamoto-Shinohara *et al.*, 2008) showing that after optimisation, the survival of *Lb. coryniformis* Si3 could be in the upper interval of survival rates within the species. We showed that by using fermentation procedures to produce a high yield of cells and then taking the T_g' and the cell: sugar ratio into consideration, it was possible to increase the survival from only a few percent to 70% for *Lb. coryniformis* Si3.

Many parameters were shown to affect the freeze-drying survival, including the fermentation, formulation and freeze-drying process. Preconditioning *Lb. coryniformis* Si3 with osmotic, cold, acid or base stress during growth induced adaptive responses in the cells, *e.g.* changes in the fatty acid profile or increased intracellular concentrations of the compatible solute betaine. However, the preconditioning did not render the cells more tolerant to freeze-drying. The reasons for this were not fully determined, but after increased osmolarity the negative results were suggested to lie in the physical solid state behaviour of betaine, *i.e.* no vitrification upon drying. The rather specific mechanisms needed for survival in a dry state make conclusions obtained about cross-protection of other 'water' stresses somewhat uncertain. One important finding was that adding a mild cold stress prior to freeze-drying lowered the survival of *Lb. coryniformis* Si3 to 18%, showing that the common practice of keeping cells on ice between processing steps should be reconsidered. Growing *Lb. coryniformis* Si3 at optimal pH and temperatures for fast growth to high cell densities or

preconditioning with a mild heat stress (42 °C for 6 h) resulted in high survival rates (72%). The discrepancy produced by heat stress, even though the membrane adjustments were similar, could be due to expression of heat stress genes counteracting the negative effects. However, the effects of preconditioning on the proteome or genome were beyond the scope of this thesis.

Storage stability was a troublesome issue and future work should focus on resolving this. The cellular viability of *Lb. coryniformis* Si3 during storage was shown to largely depend on storage conditions, but also on polymer additives to the sucrose-based formulations. Polymers (PVP90 and Ficoll400) increased the storage viability of the freeze-dried *Lb. coryniformis* Si3 formulation at low temperatures. The T_g transformation of the amorphous sucrose was increased and t_{cr} was delayed by addition of polymers. PVP90 had slightly better stabilising effects on the amorphous matrix than Ficoll400 at 4°C and increasing moisture levels. However, the increased resistance of the amorphous matrix brought about by PVP90 was not directly correlated to increased storage viability of *Lb. coryniformis* Si3 when kept at low humidity (11%) and increased temperatures (25 and 37°C). As expected, keeping freeze-dried cakes below the T_g was necessary for stability but was not a guarantee of stability. Rather, the general rule of thumb that an amorphous product should be stored 50 degrees below its T_g seemed to apply also to the storage stability of *Lb. coryniformis* Si3 in sucrose. By combining the relationship between viability and T_g with the relationship between T_g and moisture, it is possible to determine critical storage conditions where the quality (high viability and high matrix stability) is guaranteed. Rational thinking on appropriate process settings and storage conditions is possible, but storage at high temperature and humidity remain a problem.

Many questions remain unanswered. Primarily, it seems very unlikely that the otherwise well-established mechanism of water replacement is not present in lactic acid bacteria, but since there are no available *in vivo* studies proving this in this cell type, future studies would be highly interesting. Questions remain on whether over-drying by removing too much water is a problem *per se,* or whether the problem is due to temperature or oxidation reactions. Since oxidation events seem to be an important factor determining storage stability, this needs to be further evaluated. Preconditioning by oxidative stress could be an option to study. Further studies should determine the natural mechanisms behind anhydrobiosis and whether they can be used by genetic approaches to enhance the stability of sensitive cells, such as lactic acid bacteria during freeze-drying. Another future topic for

research is how the stability of highly amorphous matrices can be improved so that these products can remain stable at ambient conditions. This is a problem for many food, pharmaceutical and cell products. More mechanistic studies on the function of tertiary systems consisting of cells: sugars: polymers are needed in order to understand interactions and design suitable formulations.

Lactic acid bacteria are widely used and several strains are considered safe for human use. Future applications of lactic acid bacteria can be as live vaccines. It remains difficult to maintain high cell numbers with retained activity during freeze-drying, but the main difficulty lies in keeping high survival rates during storage of the dry products at ambient conditions.

To conclude, in this thesis work I have shown that it is possible to increase the survival rates of *Lb. coryniformis* Si3 from a few percent to approximately 70%. Many factors influenced the survival rates and the fermentation, formulation, freeze-drying procedures and storage conditions should be planned together. By combining solid state characterisation techniques such as calorimetry with microbiological viability assays, it is possible to perform a rational formulation and freeze-drying process and to assess suitable storage conditions where the viability and technical quality is maintained.

"There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened."

Douglas Adams
(1980: *The Restaurant at the End of the Universe*)

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