Drying of *Lactobacillus plantarum*

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Drying of *Lactobacillus plantarum*

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Stellingen

1. De conclusie van Teixeira *et al.* dat sproeidrogen en vriesdrogen van *Lactobacillus bulgaricus* geen verschil in restactiviteit opleveren wordt tegengesproken door de door hen gepresenteerde data.

   Teixeira, P., Castro, H., Kirby, H., 1995. Spray drying as a method for preparing concentrated cultures of *Lactobacillus bulgaricus*, *J. of Appl. Bacteriol. 78*; 456-462.

2. Bij drogen op basis van contact-sorptie is het moeilijk om het droogproces van het bewaarproces te onderscheiden.

   Dit proefschrift.
   Klapwijk, P.M., Klempp, J., Van Rhee, R., 1988. Stable bacterial composition and process for breadmaking using this composition, EP 0 298 605 A1.

3. Het ontbreken van vochtgehaltes of wateractiviteiten in veel literatuur over de overleving van bacteriën na drogen kan leiden tot onjuiste interpretatie van de resultaten.

4. De rangschikking van beschermende suikers door Crowe *et al.* is gebaseerd op een slechte fit, die verbloemd wordt door het gebruik van een dubbellogaritmische schaal.

   Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A., Womersley, C., 1984. Effects of carbohydrates on membrane stability at low water activities, *BBA 769*; 141-150.

5. De "Round Table on Glasses" tijdens ISOPOW 6 gaf aan dat de meningen van een aantal prominente onderzoekers zich in een glastoestand bevinden.

   ISOPOW 6: The roles of water in foods: Applying fundamental knowledge to the design and production of foods, March 2-8, 1996, Santa Rosa, California.

6. De ongebruikelijke weergave van gemeten en voorspeld restvochtgehalte van gesproeidroogde druppels door Etzel *et al.* geeft weinig vertrouwen in de geschiktheid van het gebruikte model.

   Etzel, M.R., Suen, S.Y., Halverson, S.L., Budijono, S., 1996. Enzyme inactivation in a droplet forming a bubble during drying, *J. Food Eng. 27*; 17-34.

7. Ingrijpen in de natuur wordt door velen veroordeeld, totdat het hen persoonlijk raakt, getuige de problemen met de processierups.

8. De populariteit van het instituut kerk is geen graadmeter voor de geloofsbeleving.

9. Een belangrijk argument voor het doorgeven van het dialect aan kinderen is behoud van cultuur.

10. Indien in de komende paar jaar de start van de Tour de France weer in Noord-Brabant wordt gehouden zullen er aanzienlijk minder mensen op de been komen omdat de wachttijd van het publiek in geen verhouding staat tot de tijd waarin de renners passeren.

11. De geloofwaardigheid van milieu-organisaties die bezwaar maken tegen de bouw van windmolens staat op het spel.

12. De Belgen hebben de inwoners van de huidige Zuid-Nederlandse provinciën in 1830 danig in de steek gelaten.

    Hoffman, E.R.M., 1974. Noord-Brabant en de opstand van 1830. Stichting Zuidelijk Historisch Contact, Tilburg.

13. Op filegevoelige plaatsen kan de term snelweg beter vervangen worden door wegsnel(heid).

14. Carnaval is alleen bestemd voor mensen die er op de juiste manier voor openstaan, anderen dienen zich eraan te onttrekken.

Stellingen behorende bij het proefschrift "Drying of *Lactobacillus plantarum*"

Leonie Linders, 22 november 1996.

Voor mijn ouders

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1 Introduction

Use of lactic acid bacteria

Lactic acid bacteria are widely used as starter cultures in the production of food and feed. Fermentation of milk, vegetables, sausages, beverages and bakery products results in products with changed composition and taste, and with prolonged shelf life (10). Lactic acid bacteria are also used as silage inoculates to enhance the fermentation and reduce the risk of spoilage (31). Lactic acid bacteria are also important for human and animal health. Some bile resistant strains survive the passage through the intestinal tract (29) and have beneficial properties when used as probiotics in human and animal nutrition (11).

Freezing and freeze drying are commonly used methods for the preservation of starter cultures (3). The disadvantages of freezing are the high transport and storage costs. Freeze drying is a too costly process for the production of bulk starter cultures. Costs of freeze drying (including fixed capital costs and manufacturing costs) can be 6 times higher per kg of removed water as compared to convective drying methods like spray drying and fluidized bed drying (30). Convective drying methods that have been applied for the drying of starter cultures are spray drying (13, 33), spray granulation (12, 15,35), fluidized bed drying (20, 23, 28), contact sorption drying (19, 34). Vacuum desiccation has also been used for the drying of bacteria (18, 26).

Thermal and dehydration inactivation

During drying of bacteria inactivation occurs. Two main mechanisms of inactivation can be distinguished: thermal and dehydration inactivation, respectively. Thermal inactivation is a function of temperature and moisture content (23, 35). Thermal inactivation can be minimised using low temperatures or short times (15, 24). Thermal inactivation is mainly due to denaturation of DNA, RNA and enzymes (21). Several researchers modelled the thermal inactivation during drying of lactic acid bacteria (13, 23, 35).

Dehydration inactivation occurs during drying, even at conditions where thermal inactivation is negligible (15, 23). Cell membrane damage is an important mechanism of dehydration inactivation (25). This was demonstrated by measuring the leakage of hydrolysed DNA after the incubation of rehydrated *Lactobacillus plantarum* with DNase. Others (15) found that dehydration inactivation was due to the increase in intracellular lactic acid concentration during drying.

Objective

The first objective of this thesis is to determine the importance of several process parameters in relation to each other for the production of active dried *L. plantarum* starter cultures. The second objective is to determine physical and physiological mechanisms explaining the influence of these parameters. In this work we have focused on the dehydration inactivation as the main problem, since the occurrence of thermal inactivation can be prevented by using low temperature drying methods. A process oriented approach was applied, using the process scheme shown in Figure 1. The main research questions at each process step are:

Growth/Harvest: What is the influence of growth parameters like medium composition, osmotic stress during growth, pH-control and reactor concept, and what is the influence of harvesting time on the residual activity of dried *L. plantarum*?

Additives: Do carbohydrates protect *L. plantarum* during drying and what are the physical and physiological mechanisms of protection?

Carrier: What is the influence of the use of carrier material on the residual activity of dried *L. plantarum*?

Drying: What is the influence of the parameters drying temperature, drying time and initial cell concentration on the residual activity of dried *L. plantarum*?

Finally, the activity of the *L. plantarum* during the complete drying process can be followed, including storage. Which are the most important process steps?

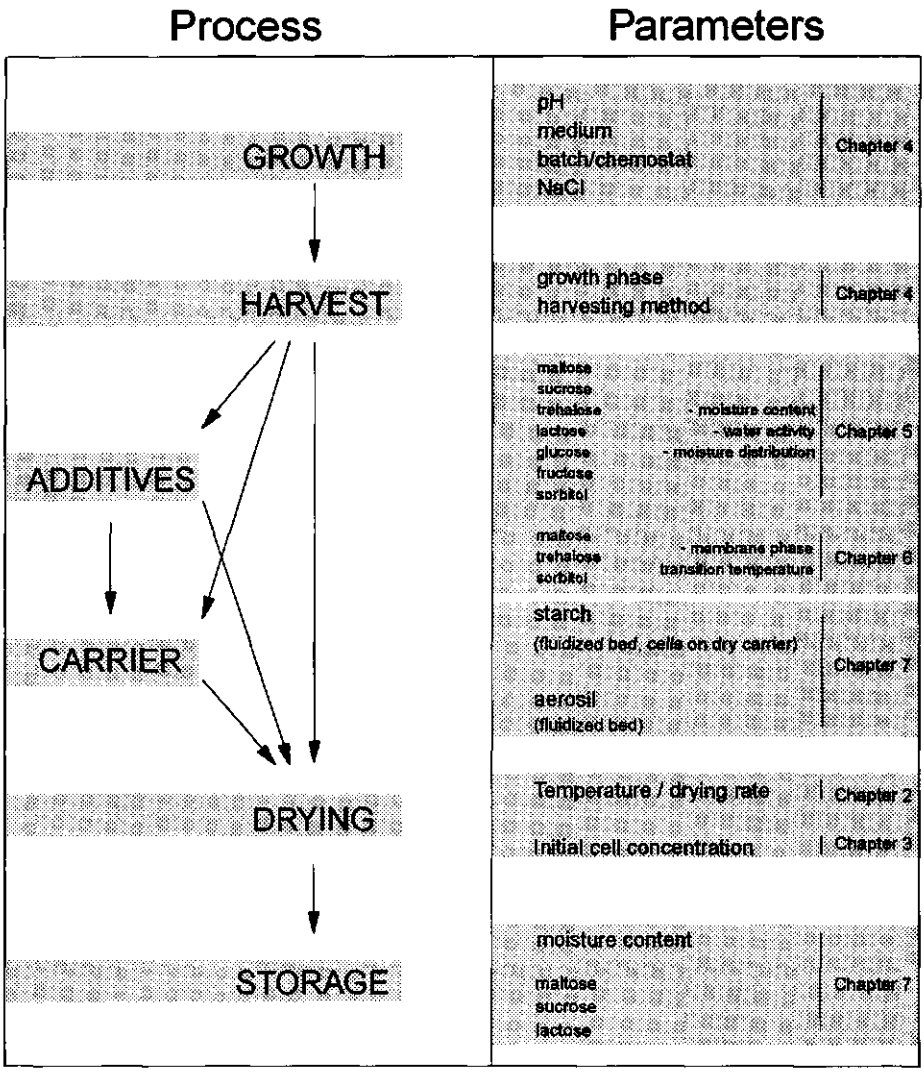

Figure 1 Process scheme for the production of dried *L. plantarum* and overview of the topics covered in this thesis.

The process oriented approach

Growth

The first step in the production of dried lactic acid bacteria is the growth procedure. Growth conditions may influence the residual activity after drying. According to Champagne (3) cells will survive freeze drying best when grown with optimal growth conditions. Others applied osmotic stress during growth, which resulted in an improved survival of *L. plantarum* after drying (16).

The survival after drying was also dependent on the bacterial strain. *L. plantarum* had a higher survival after drying than *Lactobacillus bulgaricus* (17). In our drying experiments we even observed a difference in residual activity after drying between two *L.plantarum*-strains from two different culture collections. The present study, however, is dedicated to one bacterial strain: *L. plantarum* (P743, Netherlands Institute of Dairy Research (NIZO)).

In this thesis a number of growth conditions will be discussed in relation to the residual activity of *L. plantarum* after drying. The influence of osmotic stress during growth of *L. plantarum* was studied (Chapter 3, 4). Other growth parameters studied are: growth medium, pH-control during growth and reactor concept (Chapter 4).

Harvest

After growth the bacterial cells will have to be separated from the growth medium and concentrated during the harvest. The harvesting time has a significant influence on the residual activity of lactic acid bacteria after drying (3, 15, 21, 27). In Chapter 4 the influence of harvesting time will be discussed.

The concentration method will influence the viability and activity of the cells (3), moreover, this process step will determine the overall process cost (30). Centrifugation is applicable on laboratory scale as well as on large industrial scale. For some strains that are sensitive to the centrifugation pressure ultrafiltration or microfiltration may be considered (3). In this thesis, a standard harvesting method was applied: centrifugation on a laboratory scale. *L. plantarum* was not sensitive to centrifugation as controlled by plate counts.

Additives

Carbohydrates have often been used to protect lactic acid bacteria against freeze drying (3, 7, 14) and convective drying (25). Carbohydrates protect model membranes against leakage after drying (4). Membrane damage is an important mechanism of dehydration inactivation of *L. plantarum*, which can be minimised by the addition of sorbitol (25). Accordingly, the influence was studied of a number of disaccharides, monosaccharides

and sorbitol on the residual activity of *L. plantarum* after drying (Chapter 5). In order to explain the difference in protection of the carbohydrates, a physical and a physiological approach were chosen.

For the physical approach it was assumed that the residual activity of the cells depends on the moisture content in the local environment of the cell. The water distribution between the components of a mixture is related to the water activity of the total mixture and depends on the desorption isotherms of the separate components. The presence of carbohydrates in the dried product will influence the desorption isotherm of the product. It is likely that the local moisture content of the cell differs from the overall moisture content depending on the carbohydrate present. The influence of carbohydrates on the moisture distribution and water activity of the dried product will be discussed in Chapter 5 in relation to their protective effect during drying.

In the physiological study the influence of carbohydrates on the phase transition temperature of the *L. plantarum* cell membrane was investigated. During drying and rehydration the membrane may undergo a phase transition from the liquid crystalline phase to the gel phase and vice versa (5). Leakage of components from the cell may occur during these phase transitions. The phase transition temperature may be depressed by interaction of carbohydrates with the membrane phospholipids (5). Chapter 6 describes the influence of carbohydrates on the phase transition temperature of the membrane and the relation with the residual activity of *L. plantarum* after drying.

Carrier

Several drying methods involve the use of carrier materials. In fluidized bed drying carrier material is mixed with the bacteria in order to get a paste that can be extruded to granules. In contact sorption drying a carrier is present to take up the moisture. Those carrier materials may influence the activity of the cells just after mixing and after drying. In order to make an objective comparison between drying methods it is important to know the influence of the carrier material on the residual activity after drying. In Chapter 7 two drying methods requiring the use of a carrier will be discussed: fluidized bed drying and contact sorption drying. Furthermore the influence of some unusual carriers on the residual activity after fluidized bed drying will be studied.

Drying

The drying process, during which water is withdrawn from the sample, involves a number of process parameters. The discussion about the influence of the drying process on the residual activity of dried bacteria in literature often involves the comparison of different drying methods like spray drying, fluidized bed drying and freeze drying.

However, a better comparison of these methods is only possible when the influence of separate parameters involved in the drying process is known.

Drying temperature is an important parameter in thermal inactivation during drying (13, 15, 24). The importance of drying temperature in relation to the dehydration inactivation has never been studied. The drying rate is a parameter on which conflicting opinions exist in relation to the dehydration inactivation. A low drying rate may enhance the residual activity after drying, since it permits the cell to adapt itself to the increased osmotic pressure in the cell (26, 27). Others found no significant influence of the drying rate on the dehydration inactivation of *L. plantarum* (23). In Chapter 2 the drying temperature and the drying rate will be discussed in relation to the residual activity of *L. plantarum* after drying.

The initial cell concentration before drying (C_0) had a considerable influence on the survival of bacteria after freeze drying (2, 3). Accordingly, the influence of C_0 was studied on the dehydration inactivation of *L. plantarum* after convective drying (Chapter 3). Moreover, the combined influence of C_0 and of osmotic stress during growth will be discussed (Chapter 3).

Storage

In many studies no distinction is made between the drying and storage step. Yet, these process steps should be considered separately. The drying parameters may have different importance. For example, the residual activity after drying is proportional to the residual moisture content (23). However, the storage stability is inversely proportional to the residual moisture content (21). The addition of carbohydrates may result in a higher residual activity after drying, but the storage stability does not have to be improved as well. In Chapter 7 the storage stability will be discussed as a function of residual moisture content and in relation to the addition of carbohydrates.

Rehydration and activity measurement

Rehydration is a process step that is associated to drying. In order to asses the activity after drying the cells have to be rehydrated. Activity retention at rehydration can be improved by delaying the rehydration process (14) or by using protectants in the rehydration medium (7, 32). The rehydration of freeze dried lactic streptococcus strains in phosphate buffer resulted in higher viabilities than rehydration in distilled water or a lactose medium (10% w/v), whereas rehydration media composed of sucrose (10% w/v) or reconstituted skim mild resulted in higher recoveries (32). Variations in recovery were found between strains and species (7). In this thesis the rehydration step has not been object of study. The standard rehydration procedure in 0.01 M potassium

phosphate buffer (0.15 M NaCl, pH 7) was used in all experiments, as part of the activity measurement.

The damage incurred with the drying process could be assessed using a test for the metabolic activity or a viability test, or both. The choice between measuring the viability or metabolic activity involves factors like reliability, accuracy and practical aspects like time and convenience. Sometimes the measurement is chosen according to the application of the starter culture (8). Several researchers used various acidification tests to determine the metabolic activity of lactic acid bacteria (1, 8, 9, 22). The metabolic activity measurement is time saving and convenient compared to the viability measurement. The disadvantage of the viability test is that it may result in higher activity numbers, since the repair time for injured cells is higher (21). Furthermore, pairs or chains of cells may occur in starter cultures. Dissociation of the chains during the drying and rehydration process may lead to an overestimation of the viability (6, 21). In this thesis mainly the glucose fermenting activity (22) will be used to determine the influence of the drying process, because of the above mentioned advantages. In Chapter 4 this activity measurement will be discussed in relation to the viability measurement.

Outline of this thesis

In Figure 1 an overview of the topics that are covered in this thesis is shown. Drying is the central process step in the production of dried starter cultures, therefore the drying parameters are discussed first in Chapter 2 (drying temperature and drying rate) and 3 (initial cell concentration). Subsequently, the influence of growth parameters will be covered in Chapter 4. Furthermore, the protective effect of carbohydrate addition will be discussed in Chapter 5, as well as a physical approach to elucidate the mechanism behind the protection. In Chapter 6 the physiological relation between the carbohydrate protection and the membrane phase transition will be discussed. Finally, all aspects involved in the production of active dried *L. plantarum* (including storage stability) will be covered in Chapter 7.

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2 Temperature and drying rate

Abstract

The objective of this work was to study the influence of drying temperature and drying rate on the dehydration inactivation of *Lactobacillus plantarum*. Drying methods with different temperatures and different characteristic drying times were used. Residual activities of 70-85% were realized after convective or vacuum drying of a layer at 30°C as opposed to 30-50% at 4°C. Spray drying (fast drying) and vacuum drying at higher a_w-values (slow drying) both resulted in lower residual activities. These experiments agreed with the following hypothesis: during drying at 30°C a physiological adaptation of the cell takes place which is time dependent. This work provides a tool for improvement of the production of dried *L.plantarum*.

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Introduction

Starter cultures of lactic acid bacteria are applied in the production of food and animal feed. Convective drying is an economical alternative for the conservation of starter cultures compared to freezing and freeze drying. Unfortunately inactivation of the bacteria occurs during drying. Two types of inactivation have been demonstrated for *Lactobacillus plantarum*: thermal and dehydration inactivation (14).

Thermal inactivation can be minimized by choosing a low drying temperature. Dehydration inactivation is the key problem. Important parameters are known to be residual moisture content and water activity (15). Less known is the importance of drying temperature and drying rate on the dehydration inactivation.

Conflicting opinions exist about the influence of drying rate on the dehydration inactivation (10). Several authors mention the importance of drying rate (1, 6, 9, 10, 14, 17, 19) but only a few of them specifically studied the influence of this parameter (1, 14). Slow drying may be beneficial, since it permits physiological adaptation to the increased osmotic pressure in the cell (17, 19). In order to avoid plasmolysis the cells may accumulate compatible solutes through membrane transport or *de novo* synthesis (3, 17). Others (1) showed that residual activity directly after drying was similar after fast and slow drying (20 min versus 24 h, 25°C) for a number of bacterial strains, whereas the storage stability was higher for slow compared to fast dried bacteria.

Lievense *et al.* (14) found no significant influence of the drying rate on the dehydration inactivation of *L.plantarum*. Characteristic drying times varied from seconds (spray drying) to several hours (drying above saturated LiCl-solutions, 5°C). According to Lievense *et al.* the final moisture content is the main parameter determining the dehydration inactivation.

Other authors state that fast drying is beneficial for the residual activity (6, 9). According to Kessler (6) the time period to pass water activity (a_w) = 0.84 should be as short as possible in order to obtain the highest residual activity for dried *Lactococcus diacetylactis*. The high water mobility above this water activity is supposed to accelerate detrimental reactions. Kessler also points to the importance of low temperature. However, these ideas were not confirmed by experiments with varying drying rates and temperatures.

The objective of this study is to elucidate the combined influence of the drying temperature and drying rate on the dehydration inactivation of *L.plantarum*. The influence of temperature was studied using convective and vacuum drying of a layer. Spray drying was used as a fast drying method. Slow drying was performed in vacuum dryers with saturated salt solutions of high relative humidities.

Materials and methods

Micro-organism and growth conditions

Lactobacillus plantarum (P743, Netherlands Institute of Dairy Research (NIZO)) was grown batch-wise, in a 3.5 l (Applikon) or a 2.5 l fermentor (Biotech) in 55 g/l MRS-medium (Difco) enriched with 1 g/l yeast extract (Oxoid) and 10 g/l glucose (Merck). The fermentation took place under microaerophilic conditions with pH adjusted to 6.3 (using 3 M NaOH). Total consumption of glucose, indicated by glucose test sticks (diastix, Ames), marked the start of the stationary phase and occurred between 17.5 and 18.5 hours after inoculation. Four hours after the beginning of the stationary phase, the cells were harvested by two consecutive centrifugation (10 minutes at 10000 rpm, Sorvall) and washing steps with 0.01 M potassium phosphate buffer (0.01 M KH_2PO_4/K_2HPO_4-buffer in 0.15 M NaCl, pH 7). The cells were stored in 0.01 M potassium phosphate buffer at 4 °C for one to three nights without loss of activity. Before sample preparation and drying, the cells were concentrated by centrifugation (10 minutes at 10000 rpm, Sorvall), resulting in a cell pellet ($X \approx 3.2$ g H_2O/g dry weight) containing $\approx 10^{12}$ colony forming units/g dry weight. The *L.plantarum*-cell suspensions were prepared by diluting cell pellet with 0.01 M potassium phosphate buffer.

Spray drying

A *L.plantarum*-cell suspension was prepared by diluting cell pellet to an initial moisture content (X_0) between 10 and 15 g H_2O/g dry weight with 0.01 M potassium phosphate buffer. This cell suspension was spray dried in a laboratory spray-dryer (Büchi 190, Flawill, CH). The process temperatures were kept low in order to minimise thermal inactivation. The inlet temperature and outlet temperature varied from 60 to 80°C and from 42 to 53°C, respectively. The air flow through the column and the suspension flow to the nozzle were set at 45 m^3/h (at 20 °C) and 0.2 l/h, respectively. The air flow through the nozzle was set at 0.4 m^3/h (at 20 °C). The estimated residence time of spray droplets in the column was 0.45 s.

Convective drying

A petri dish (∅ 50 mm) with a layer of 2-3 g of *L.plantarum*-cells (X_0 = 3.2 or 7 g H_2O/g dry weight) was placed in a drying tunnel (100×100×500 mm). The samples were dried with ambient air at 30°C (Relative humidity (RH) = 0.3) or 4°C (RH = 0.5) for 2.5 hours. Afterwards the samples were placed in a glass vacuum desiccator with a saturated LiCl-solution ($a_w = 0.11$) for 2 or 3 days at 4°C in order to obtain an equilibrium

moisture distribution in the sample. In this work, water activity stands for the relative humidity of the air that is in equilibrium with the sample. After drying, the desiccator was aerated with ambient air that had passed a silica gel column.

Vacuum drying

A weighing flask (∅ 20×40 mm) with 2-3 g *L.plantarum*-cells (X_0 = 3.2 g H_2O/g dry weight) or a petri dish (∅ 50×10 mm) with a layer of 2-3 g *L.plantarum*-cells (X_0 = 7 g H_2O/g dry weight) was placed in a glass vacuum desiccator with a saturated LiCl-solution (a_w = 0.11) for 3 days at 4°C or 30°C.

Slow drying at 30°C was performed in a vacuum desiccator: 6 hours above saturated K_2SO_4 (a_w = 0.97) followed by 18 hours above saturated $Mg(NO_3)_2$ (a_w = 0.53) followed by 2 days above saturated LiCl (a_w = 0.11), or 6 hours above saturated NaCl (a_w = 0.75) followed by 2 days above saturated LiCl (a_w = 0.11). After drying, the desiccator was aerated with ambient air that had passed through a silica gel column.

Activity of L.plantarum

The glucose fermenting activity test (11) was used to determine the activity of *L.plantarum*. All samples (0.5-1 g) were resuspended in 33 ml 0.01 M potassium phosphate buffer, in which the decrease in pH was measured at 35 °C after the addition of 1 ml glucose buffer (0.35 g glucose/g potassium phosphate buffer). The activity (A) was defined as the maximal rate of pH-decrease per gram dry cells (ΔpH/min/g dry cells). The activity of the dry sample was determined directly after drying. The residual activity after drying was defined as the ratio of the activity after drying (A) and the activity before drying (A_0).

Thermal inactivation

Thermal inactivation during spray drying was estimated using the combined drying and inactivation kinetics models described by Meerdink (18). The thermal inactivation kinetics for *L.plantarum* were incorporated in the model (12). Lievense described this model for *L.plantarum*-starch paste with moisture contents between 0.1 and 0.85 g H_2O/g dry weight. Thermal inactivation was measured for a high moisture *L.plantarum*-cell suspension (X_0 = 7 g H_2O/g dry weight). A stainless steel inactivation cell (16) (∅ 70×2 mm) was filled with 7 g of cell suspension. The activity of the cell suspension was measured in time at 52.5°C (water bath, Haake). Linear regression of $\ln(A/A_0)$ versus time yielded the specific inactivation rate k_i (1/s). The value of k_i at 52.5°C was $1.8\ 10^{-3}$ /s. This was equal to the one measured for *L.plantarum*-starch paste at X = 0.85 g H_2O/g dry weight (12), thus the inactivation constants (12) were assumed to be

independent of X for $X \geq 0.85$g H_2O/g dry weight. Besides that there was assumed to be no influence of the starch.

Results and discussion

Drying temperature

Drying experiments have been performed at 30°C and 4°C using two drying methods and two initial moisture contents. Figure 1 shows that drying at 30°C resulted in higher residual activities than drying at 4°C, also for convective drying followed by vacuum drying above a saturated LiCl-solution at 4°C (Figure 1a) and for vacuum drying above a saturated LiCl-solution (Figure 1b). The drying temperature of 30°C also gave better residual activity for *L.plantarum*-cells with a lower initial moisture content ($X_0 = 3.2$ g H_2O/g dry weight) vacuum dried above saturated LiCl (Figure 1c). In Figure 1a a hatched triangle is drawn covering intriguing data with respect to high residual activity and low moisture content. This range should be interpreted as a target without statistical significance.

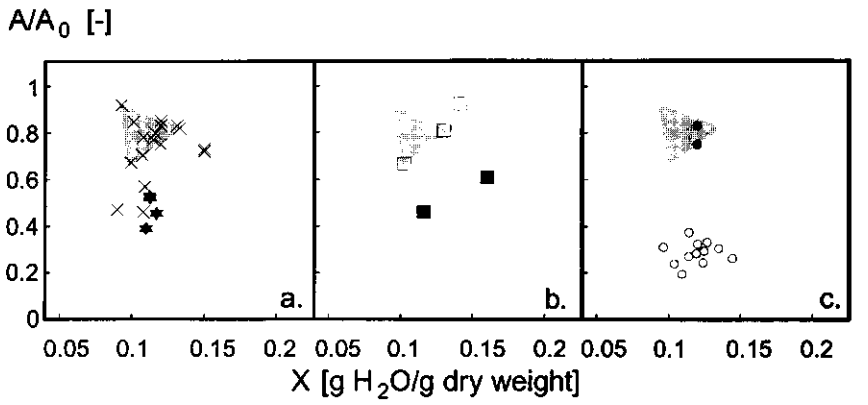

Figure 1 Residual activity of *L. plantarum* after: (a) 2.5 h convective drying , ×: at 30°C, or ✱ : at 4°C, both followed by 3 days vacuum drying above LiCl at 4°C ($X_0 \approx 7$ g H_2O/g dry weight); (b) 3 days vacuum drying above LiCl, □: at 30°C, or ■: at 4°C, ($X_0 \approx 7$ g H_2O/g dry weight); (c) ●: 2.5 h convective drying at 30°C followed by 3 days vacuum drying above LiCl at 4°C ($X_0 \approx 3.2$ g H_2O/g dry weight) or ○: 3 days vacuum drying above LiCl at 4°C ($X_0 \approx 3.2$ g H_2O/g dry weight). The hatched triangle covers data with high residual activity at low moisture content. (This range should be interpreted as a target without statistical significance)

The positive influence of the drying temperature of 30°C seemed to point to a temperature dependent reaction occurring during the drying procedure which did not occur at 4°C. Since *L.plantarum* can grow at 30°C and not at 4°C (22) it is suggested that this reaction was of a physiological nature. The following hypothesis was formulated: during drying at 30°C a physiological adaptation of the cell takes place which results in higher residual activities after drying and this adaptation is time dependent. Physiological adaptations imply that the drying rate should be particularly important in the range of water activities from 1 to 0.92, the value below which growth does not occur (21). The drying rate could be expressed as the characteristic drying time (τ_d), which was defined as the time needed to reach X = 1.18 g H_2O/g dry weight, which is an estimate for the moisture content at a_w = 0.92 (15). In the drying experiments at 30°C, as shown in Figure 1, τ_d varied between 1.1 and 5.5 hours (Figure 2, Table 1). In the following drying experiments drying rates were chosen that were both much higher (spray drying) and much lower (vacuum drying at higher values of a_w).

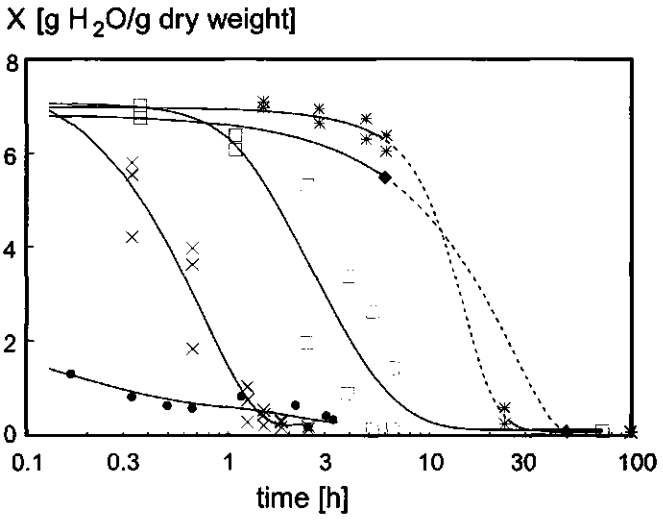

Figure 2 Drying curve of *L.plantarum* dried at 30°C ($X_0 \approx 7$ g H_2O/g dry weight): ×: convective drying; □: vacuum drying above LiCl; ✳: vacuum drying: 6 h above K_2SO_4, followed by 18 h above $Mg(NO_3)_2$, followed by 2 days above LiCl; ◆: vacuum drying: 6 h above NaCl, followed by 2 days above LiCl;●: convective drying with $X_0 \approx 3.2$ g H_2O/g dry weight; —— fit, - - - interpolation.

Table 1 Characteristic drying times for spray drying, convective drying and vacuum drying. In order to calculate the characteristic drying time, τ_d, arbitrary functions were fitted to measured drying curves (Figure 2). τ_d was the time that was needed to reach X = 1.18 g H_2O/g dry weight (a_w = 0.92).

drying method	T [°C]	X_0 [g H_2O/g dry solids]	τ_d [h]
spray drying	50	11.5	$4\ 10^{-6}$ *
convective drying	30	7.7	1.1
convective drying	30	3.3	0.16
vacuum drying	30	7.2	5.5
vacuum drying (a_w 0.97-0.53-0.11)	30	6.9	19
vacuum drying (a_w 0.75-0.11)	30	6.8	31

* τ_d for spray drying was estimated from model calculation

Fast drying

A fast drying method that is often used for the drying of lactic acid bacteria is spray drying (4, 5, 20). Spray drying (Figure 3) resulted in lower residual activities than some of the drying methods at 30°C shown in Figure 1. Higher residual activities could be expected, since the wet bulb temperature (37°C) was close to the temperature at which adaptations were expected to occur. There are two possible reasons for the low residual activity: thermal inactivation or the short drying time. Calculations showed that thermal inactivation was negligible given the low outlet temperatures and short drying times of these experiments. It is likely that the drying time of spray drying ($\tau_d = 4\ 10^{-6}$ h) was too short to permit time for adaptations. Consequently, the spray drying results were close to the results achieved after convective and vacuum drying at 4°C where the hypothesis was also not valid because of the low temperature.

Slow drying

Long drying times in vacuum drying were realized by decreasing the driving force for the water removal in time. Two separate drying regimes were in effect at 30°C with τ_d = 19 and 31 h (Table 2). Slow drying resulted in lower residual activities relative to the hatched triangle area (Figure 4). The long residence time at high water activities had a negative influence on the residual activity after drying. In the presence of substrate the cells would have been able to grow (21) at 30°C and a_w above 0.92. However, biomass

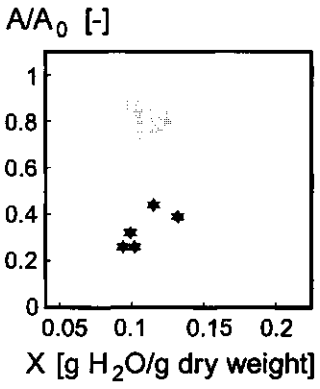

Figure 3 Residual activity of *Lactobacillus plantarum* after * : spray drying ($X_0 \approx 11.5$ g H_2O/g dry weight, T_{outlet} = 43-53°C). see Figure 1.

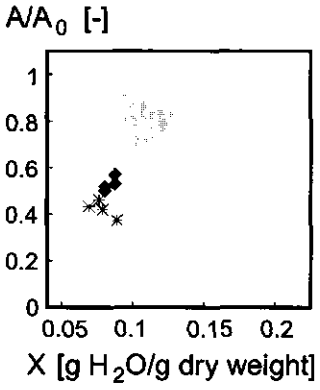

Figure 4 Residual activity of *Lactobacillus plantarum* after vacuum drying at 30°C ($X_0 \approx 7$ g H_2O/g dry weight): ◆: 6 h above NaCl followed by 2 days above LiCl; ✳: 6 h above K_2SO_4, followed by 18 h above $Mg(NO_3)_2$, followed by 2 days above LiCl. see Figure 1.

degradation occurs when the maintenance requirements of the bacteria can not be fulfilled because of the lack of substrate (2). In the present situation substrate was absent, so biomass degradation could occur. However, a cell suspension that was kept in a vacuum desiccator at $a_w = 1$ retained 100% residual activity (or even more) for more than 48 hours. This indicated that inactivation did not take place during the long residence time at high water activities, but occurred during the drying step after this long residence time. Therefore, it is concluded that too long residence times at high values of a_w at 30°C should be avoided, since this reduces the residual activity after drying.

Variable time-temperature

In order to determine at which water content (or water content range) the postulated physiological adaptations occur, a vacuum drying experiment (with saturated LiCl) was performed. Desiccators were first used at 30°C and after 2.5 or 5 hours they were used at 4°C for two days depriving the cells of the possibility for further adaptations. The final residual activity was in the target range (hatched triangle area) for the sample stored for 5 hours at 30°C and not for the sample that was stored only for 2.5 hours at 30°C (Figure 5). The moisture content after 2.5 and 5 hours was 1.5 and 0.5 g H_2O/g dry

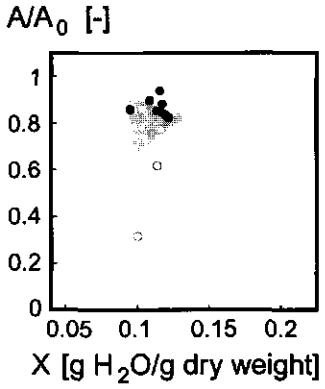

Figure 5 Residual Activity of *L.plantarum* after vacuum drying above LiCl ($X_0 \approx 7$ g H_2O/g dry weight): o: 2.5 h at 30°C followed by 2 days at 4°C or ●: 5 h at 30°C followed by 2 days at 4°C. see Figure 1.

weight, respectively. This indicated that the adaptations occurred when the moisture content decreased from 1.5 to 0.5 g H_2O/g dry weight*, corresponding to a water activity range from 0.94 to 0.83 (15). In accordance with the hypothesis of physiological adaptation it seems logical that there is a specific a_w value or range where the cells become aware of the fact that moisture is being withdrawn, inducing adaptations.

Discussion

The reason why Lievense *et al.* (14) found no significant influence of the drying rate is that they performed slow drying at low temperatures. No increase in residual activity was found, which is in agreement with the present results of drying at 4°C (Figure 1).

The nature of the adaptations can only be guessed. Compatible solutes like betaine and carnitine have a protective effect on the survival of *L.plantarum* after drying (7, 8). However, lactic acid bacteria can only acquire them from the surrounding medium since they are unable to synthesise these components themselves. The washed cells were surrounded by phosphate buffer lacking compatible solutes. An energy source would be required as well (19). In order to verify this the betaine and carnitine content was followed during the drying procedure at 30°C above a saturated LiCl-solution (7). As expected there were no changes in the concentration of these osmolites. Therefore, this mechanism can be rejected. Other adaptations that can be considered are changes in the cell membrane. This is in line with the proposed mechanism of dehydration inactivation: membrane damage (13).

Conclusions

This work shows that it is possible to minimize the dehydration inactivation of *L.plantarum* at temperatures when thermal inactivation is negligible. The following hypothesis was confirmed by several experiments: during drying at 30°C physiological

* A remark should be made about the reproducibility of the experiments. There was a minor experimental error of 5% within one drying experiment. However, the difference between the different drying experiments was larger, especially for vacuum drying, what can be seen in the drying curve (□, Figure 2). This partly accounts for the difference between the two data after 2.5 h at 30°C (Figure 5). The moisture content after 2.5 hours was still high ($X_{2.5\,h}$ = 6.4 g H_2O/g dry weight) for the sample with a final residual activity of 30 %. For the other sample this was much lower ($X_{2.5\,h}$ = 1.5 g H_2O/g dry weight). Perhaps adaptation had already taken place in this sample.

adaptation of the cell takes place, probably in the a_w range between 1 and 0.92, and this adaptation is time-dependent.

Acknowledgements

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3 Initial cell concentration

Abstract

The initial cell concentration before drying influenced the drying tolerance of *Lactobacillus plantarum*. Residual activities ranging from 10% to 83% were achieved using initial cell concentrations between 0.025 and 0.23 g cell/g sample, respectively. There was no influence of initial cell concentration with cells grown with osmotic stress of 1 M NaCl.

This chapter has been submitted as:
L.J.M. Linders, E.P.W. Kets, J.A.M. De Bont, K. Van 't Riet. The influence of initial cell concentration on the residual activity of dried *Lactobacillus plantarum*.

Introduction

Convective drying is an economical alternative for the preservation of bacterial starter cultures. The residual activity of *Lactobacillus plantarum* after convective drying can be influenced in several ways. Previously, a number of process conditions have been studied in relation to the dehydration inactivation of *L. plantarum* during convective drying. Drying conditions such as drying temperature, drying rate, water activity and residual moisture content were of major importance (8, 10, 11). The influence of growth conditions could be considerable. Extreme growth conditions like osmotic stress resulted in physiological adaptations improving the drying tolerance (5, 6). The presence of NaCl and betaine in the growth medium resulted in an increased survival of *L. plantarum* after drying (5). Addition of several carbohydrates significantly improved the residual activity of lactic acid bacteria after drying (9, 10).

Survival of lactic acid bacteria after freeze drying is enhanced by increasing the cell density prior to freezing (1, 2, 13). An increased initial cell concentration decreased the inactivation of bacteria during spray drying (3). The objective of this work was to demonstrate the influence of initial cell concentration on the dehydration inactivation of *L. plantarum* during convective drying. Furthermore, the combined influence of osmotic stress during growth and initial cell concentration on the residual activity after drying was studied.

Drying of *L. plantarum* grown at standard conditions

Lactobacillus plantarum (P743, Netherlands Institute of Dairy Research (NIZO)) was grown in 55 g/l MRS-medium (Difco) enriched with 1 g/l yeast extract (Oxoid) and 10 g/l glucose (Merck), as described before (11). Cells were harvested after 22 h (4 hours after the beginning of the stationary phase). Cells were washed in 0.01 M KH_2PO_4/K_2HPO_4 buffer in 0.15 M NaCl (pH 7). Cells were concentrated by centrifugation (10 minutes at 10000 rpm, Sorvall), resulting in a cell pellet (moisture content (X) of 3.25 g H_2O/g dry weight). *L. plantarum*-cell suspensions were prepared by dilution of the cell pellet with the washing buffer (1 to 10 times), resulting in cell concentrations (C_0) ranging from 0.025 to 0.23 g dry cell/g sample. A layer (1.5 mm) of cell suspension in a petri dish (∅ 50 mm) was dried for 2.5 hours with ambient air of 30°C (11) and afterwards placed in a glass vacuum desiccator with a saturated LiCl-solution ($a_w = 0.11$) for 2 days at 4°C in order to obtain an equilibrium moisture distribution in the sample.

In figure 1 the residual glucose fermenting activity (A/A_0) (7) and the final moisture content are drawn versus the initial cell concentration. The initial cell concentration had an important influence on the residual activity of normally grown cells that were air dried at 30°C (Figure 1). The residual activity varied from 83% for non diluted pellet ($C_0 = 0.23$ g dry cell/g sample) to 10% for 10 times diluted pellet ($C_0 = 0.025$ g dry cell/g sample). The influence of initial cell concentration could not be explained by differences in moisture content. In a control experiment the same trend was observed when the viability of *L. plantarum* after drying was measured using plate counts.

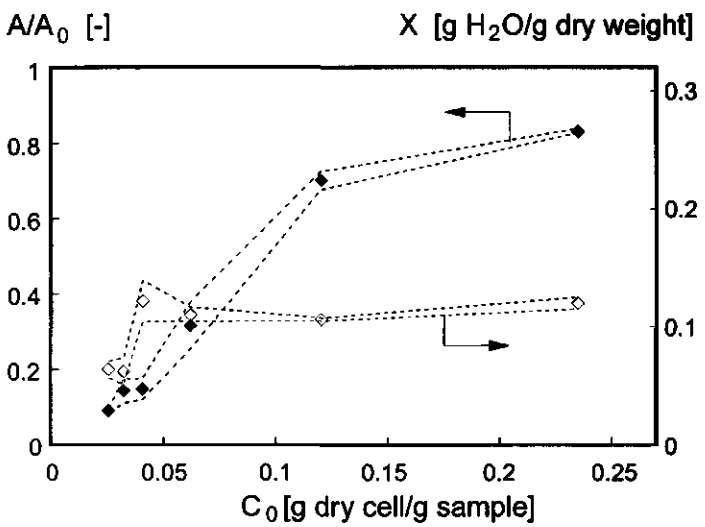

Figure 1 ◆: Residual glucose fermenting activity (A/A_0); ◇ : moisture content (X); of *L. plantarum* after drying versus initial cell concentration (C_0); ····· : standard deviation from average (4 drying experiments).

Drying of *L. plantarum* grown with osmotic stress

In a separate experiment the influence was studied of initial cell concentration on the residual activity of *L. plantarum*-cells grown with osmotic stress. 1 M NaCl was added to the above described growth medium and growth was performed applying the same conditions. Cells were harvested after 66 hours, which was 4 hours after the beginning of the stationary phase. The cells were washed with 0.01 M KH_2PO_4/K_2HPO_4 buffer in 1 M NaCl (pH 7). Cells were concentrated by centrifugation (10 minutes at 10000 rpm,

Sorvall), resulting in a cell pellet (X = 2.8 g H_2O/g dry weight). *L. plantarum*-cell suspensions were prepared by dilution of the cell pellet with the washing buffer (1 to 10 times), resulting in cell concentrations ranging from 0.025 to 0.11 g dry cell/g sample. Drying of these suspensions as described above revealed that there was no influence of initial cell concentration on the residual activity of cells grown with osmotic stress (Figure 2). The residual activity was lower than 10% irrespective of the initial cell concentration. In a control experiment there was also no influence of C_0 on the viability (measured using plate counts) of cells grown with osmotic stress after drying.

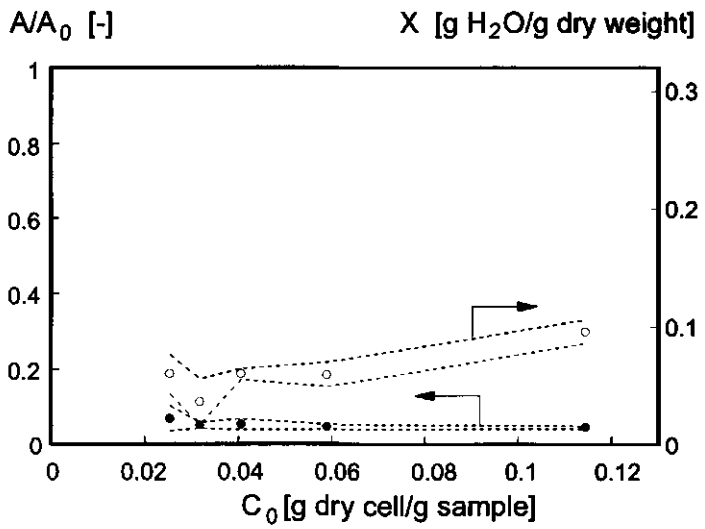

Figure 2 ●: Residual glucose fermenting activity (A/A_0); ○: moisture content (X); of *L. plantarum* grown with 1 M NaCl after drying versus initial cell concentration (C_0). ⋯⋯⋯: standard deviation from average (4 drying experiments).

Comparison of Figure 1 and 2 shows that the application of osmotic stress during growth resulted in a decreased residual activity after drying when high C_0-values were used. The present works shows that the results achieved with low C_0-values can not be extrapolated to higher C_0-values, since C_0 has a considerable influence on the residual activity of cells grown without osmotic stress. Kets and de Bont (5) found that growth of *L. plantarum* in the presence of NaCl and betaine improved the drying tolerance of the cells. This can be explained because in their drying experiments a low C_0 (<0.02 g dry cell/g sample) was used.

Discussion

For spray drying of bacteria the observed positive influence of increased initial cell concentration could be explained by the increased thermostability (3). In the present situation though, no thermal inactivation could occur since the drying temperature was 30°C (8). According to Bozoglu et al. (1) the influence of C_0 is related to the area of the cells exposed to the environment. At increasing C_0 the interfacial area decreases since the cells are shielding each other from the detrimental effect of the external medium (chemical reactions). In recent work (11) it was shown that during drying at 30°C physiological adaptations may occur, which are time dependent. Possibly the influence of high C_0 values on the drying curve was favourable for the occurrence of physiological adaptations.

Furthermore, the appearance of the samples with high and low C_0 was different. The high C_0-samples were transparent (glass-like) after drying whereas the low C_0-samples were white and non-transparent. When the cells indeed were in a glass phase it would be beneficial because of: a) absence of oxygen in neighbourhood of cells; b) protein stabilisation (4); c) reduced reaction rate of deleterious reactions (12).

Acknowledgements

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4 Growth parameters

Abstract

In order to select optimal conditions for the production of dry and active starter cultures it is important to determine the influence of growth conditions on the residual activity of dried bacteria. The influence of medium composition, pH-control during growth, reactor concept and growth phase was studied on the residual activity of *Lactobacillus plantarum* after drying. The effect of osmotic stress during growth on the residual activity of *L. plantarum* after drying was measured. The drying methods used were convective drying of a layer and fluidized bed drying. Bacteria with the highest residual activity after drying were produced in batch or chemostat with pH-controlled growth using enriched or diluted MRS medium. Osmotic stress during growth resulted in a decreased residual activity after drying. Variations in growth conditions generally did not result in improved residual activities after drying.

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L.J.M. Linders, G. Meerdink, K. Van 't Riet. The effect of growth parameters on the residual activity of *Lactobacillus plantarum* after drying.

Introduction

The residual activity of dried *Lactobacillus plantarum* can be improved by choosing the appropriate drying conditions (8, 9). Growth conditions may also influence the production of dry active starter cultures (2, 4, 5, 6, 10), although literature on this topic is not abundant. Growth conditions that have been studied are the influence of osmotic stress during growth, accumulation of compatible solutes, pH-control and growth phase. Addition of NaCl (osmotic stress) and betaine to the growth medium resulted in betaine uptake by *L.plantarum* cells, which reduced the dehydration inactivation of the cells (5). pH-control during growth had no significant influence on the residual activity of dried *Lactococcus diacetylactis* (4). There is much evidence that bacteria harvested in the stationary growth phase possess a higher residual activity after drying compared to bacteria harvested in the exponential growth phase (2, 4, 6, 10, 11). According to Champagne *et al.* (2) cells will survive freeze drying best when grown with optimal growth conditions.

In the present work the following growth conditions will be discussed: medium composition, pH-controlled growth, reactor concept and growth phase. The influence of these variables will be studied for *L.plantarum* cells grown both without and with osmotic stress induced by the addition of NaCl to the growth medium.

Materials and methods

Micro-organism and growth conditions

Lactobacillus plantarum (P743, Netherlands Institute of Dairy Research (NIZO)) was grown in enriched MRS medium (EMRS) containing 55 g/l MRS-medium (Difco), 1 g/l yeast extract (Oxoid) and 10 g/l glucose (Merck) without or with 1 M NaCl or 1.25 M NaCl. The batch fermentation (3.5 l, Applikon) took place under microaerophilic conditions with or without pH-control (pH 6.3). Cells were harvested after 22 h (without extra NaCl) or after 66 hours (1 M NaCl, 1.25 M NaCl), which was 4 hours after the beginning of the stationary phase. The influence of a diluted MRS medium (DMRS) was studied using 2.75 g/l MRS with or without 1 M NaCl. A chemostat culture was grown in 27.5 g/l MRS (Difco) at 30°C, pH-control (pH 6.3), 300 rpm, dilution rate 0.1 /h.

Cells grown without extra NaCl were washed in 0.01 M potassium phosphate buffer with 0.15 M NaCl (0.01 M KH_2PO_4/K_2HPO_4 buffer in 0.15 M NaCl, pH 7), whereas the cells grown with 1 M NaCl were washed in 0.01 M potassium phosphate buffer with 1 M or 1.25 M NaCl (0.01 M KH_2PO_4/K_2HPO_4 buffer in 1 M or 1.25 M NaCl, pH 7).

Before sample preparation and drying, cells were concentrated (cell pellet) by centrifugation (10 minutes at 10000 rpm, Sorvall).

Drying method

Fluidized bed drying: A homogeneous *L.plantarum*-starch paste was prepared by mixing 1 g of cell pellet with 0.65 g native potato starch (Perfectamyl P-6, Avebe). This paste was extruded to granules (∅ 1 mm×10 mm) and dried in a laboratory fluidized bed dryer with dehumidified compressed air of 30°C.

Convective drying of a layer: A *L.plantarum* cell suspension was prepared by mixing 1 g of cell pellet with 1 g of potassium phosphate buffer with the NaCl concentration of the growth medium. A petri dish (∅ 50 mm) with two grams of *L.plantarum* cell suspension was placed in a drying tunnel (100×100×500 mm) and dried with ambient air of 30°C (relative humidity (RH) ≈ 0.4) for 2.5 hours. Afterwards the samples were placed in a vacuum desiccator above a saturated LiCl-solution (water activity (a_w) = 0.11) for 2 days at 4°C in order to obtain an equilibrium moisture distribution in the sample (8).

Activity measurement

Glucose fermenting activity (7)*:* Samples (0.2-0.5 g) were resuspended in 33 ml potassium phosphate buffer with 0.15 M NaCl. The pH-decrease was measured at 35°C after the addition of 1 ml glucose buffer (0.35 g glucose/g potassium phosphate buffer). The activity (A) was defined as the maximal velocity of pH-decrease per gram dry cells (ΔpH/min/g dry cells). The residual activity after drying was defined as the ratio of the activity immediately after drying (A) and the activity before drying (A_0).

Viability: Samples (0.5 g) were resuspended in 9 ml peptone physiological salt solution (0.85 % NaCl, 0.1 % peptone). Decimal dilutions were prepared and plate-counted on MRS agar plates (2% agar). Plates were incubated at 30°C for 48 h. Survival was defined as the ratio of the counts after (N) and before drying (N_0).

HPLC analysis

Directly after harvesting, the cell pellet was freeze dried. Compatible solutes were extracted using a modified Bligh and Dyer (1) technique as described earlier (3). HPLC analysis of compatible solutes was performed on an Aminex HPX-87C column (Bio-Rad, The Netherlands) using 5 mM $Ca(NO_3)_2$ as eluant.

Results

Osmotic stress

Osmotic stress was applied during growth of *L.plantarum* by the addition of NaCl to the growth medium. Figure 1 shows that growth of *L.plantarum,* 18 hours after inoculation, was inhibited by NaCl at increasing concentrations and above 1.4 M NaCl hardly any growth could be seen. In following experiments NaCl concentrations of 1 M and 1.25 M were applied in order to subject the cells to considerable osmotic stress.

The residual activity after fluidized bed drying and convective drying of a layer was higher for cells grown without NaCl compared to cells grown with 1.25 M NaCl (Figure 2). The same trend can be seen when viability measurements are used (Table 1). The results obtained by the measurement of the glucose fermenting activity were

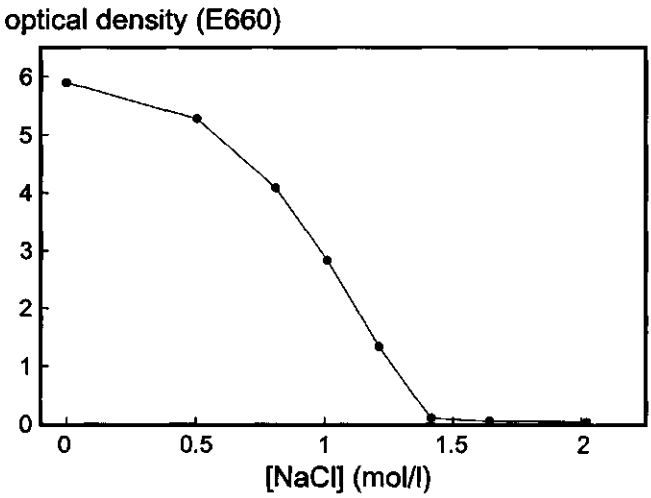

Figure 1 Optical density after 18 hours of *L.plantarum* cells grown with different concentrations of NaCl in batches of 10 ml with 55g/l MRS-medium enriched with 10 g/l glucose and 1 g/l yeast extract.

consistent with the results of the viability measurements. The glucose fermenting activity had an experimental error of 2% within experiments, compared to 28% for colony counts. Therefore, the glucose fermenting activity test is prevailed in demonstrating small differences in residual activities. The experimental error between experiments was similar for both activity tests, although dependent on the drying method (Table 1). Figure 3 shows that within a considerable scatter there was a linear relation between the two tests.

Table 1 also shows that the residual activity for cells grown without NaCl was dependent on the drying method. Convective drying of a layer resulted in higher activities compared to fluidized bed drying. At convective drying of a layer physiological adaptations were assumed to account for the high residual activities (8). Probably, the rate of fluidized bed drying was too high, so adaptations were not possible.

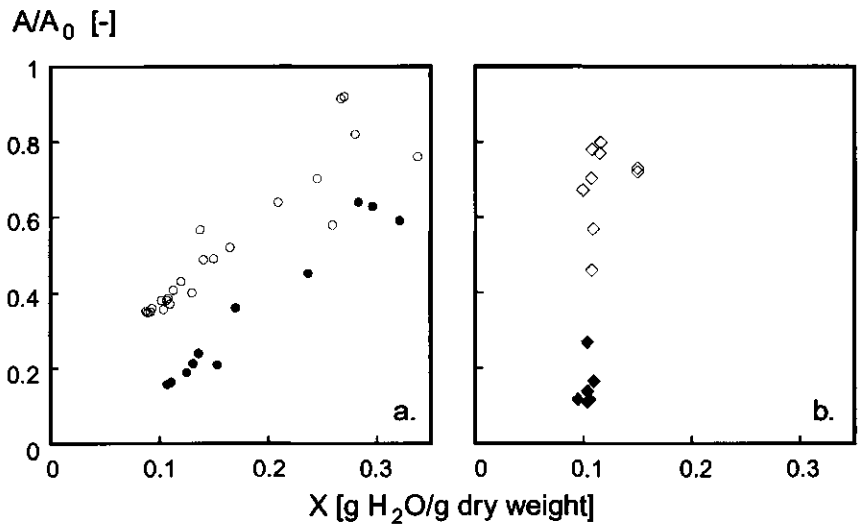

Figure 2 Residual activity (based on glucose fermenting activity) after drying of *L.plantarum*-starch paste: open symbols: *L.plantarum* cells grown without extra NaCl; closed symbols: cells grown in the presence of 1.25 M NaCl; (a) ○, ●: Fluidized bed drying at 30°C; (b) ◇,◆: Convective drying of a layer at 30°C followed by vacuum drying at 4°C.

Table 1 Influence of the presence of 1.25 M NaCl during growth and of the drying method on the activity of dried *L. plantarum*.

	Convective drying of a layer		Fluidized bed drying	
Addition of 1. 25 M NaCl	-[a]	+[b]	-[c]	+[d]
X (g H_2O/g dry weight)	0.12 ± 0.02	0.10 ± 0.01	0.10 ± 0.01	0.13 ± 0.01
A_0 (ΔpH/min/g dry cells)	2.12	1.68	2.14	1.66
A (ΔpH/min/g dry cells)	1.60	0.27	0.69	0.29
Residual Activity (%)	76 ± 11	22 ± 9	37 ± 1	18 ± 2
N_0 (CFU/g dry cells)	$4.1 \cdot 10^{12}$	$2.2 \cdot 10^{12}$	$6 \cdot 10^{12}$	$2.1 \cdot 10^{12}$
N (CFU/g dry cells)	$2.4 \cdot 10^{12}$	$3.7 \cdot 10^{11}$	$2 \cdot 10^{12}$	$6.1 \cdot 10^{11}$
Survival (%)	63 ± 22	19 ± 5	32 ± 8	32 ± 6

± standard deviation from average ; [a] 7 experiments from 4 batches; [b] 4 experiments from 3 batches; [c] 4 experiments from 4 batches; [d] 3 experiments from 3 batches

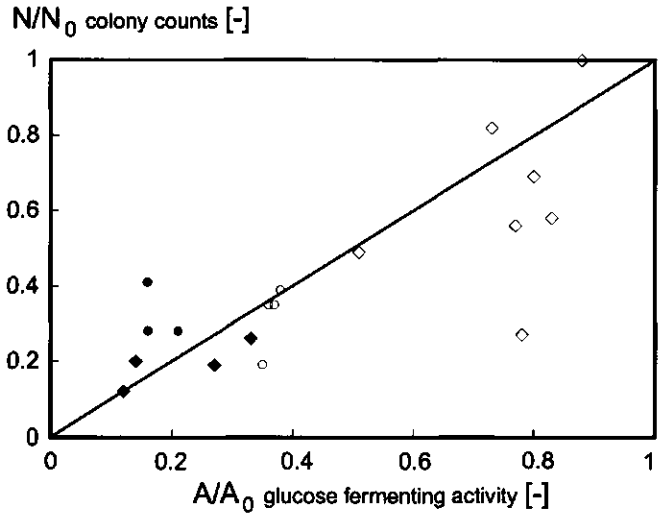

Figure 3 Survival (colony counts) versus residual activity (glucose fermenting activity) after drying: o: growth without extra NaCl, fluidized bed; ●: growth with 1.25 M NaCl, fluidized bed; ◇: growth without extra NaCl, convective drying; ◆: growth with 1.25 M NaCl, convective drying. The line describes the function Y = X.

Medium concentration

Two initial values of medium concentration have been used: enriched MRS (EMRS) medium and diluted MRS medium (DMRS). The influence of the presence of 1 M NaCl in EMRS and DMRS during growth on the residual activity after drying is shown in Table 2. In both cases the residual activity of cells grown without osmotic stress was higher than of cells grown with 1 M NaCl. Using EMRS, betaine and carnitine were accumulated in the cells with and without NaCl in the medium. Betaine and carnitine could be accumulated because they were present in the complex MRS medium and the yeast extract. In DMRS the accumulation of carnitine and betaine only occurred in the presence of 1 M NaCl. There was no positive correlation between the residual activity after drying and the accumulation of betaine and carnitine.

Table 2 Influence of medium composition on the accumulation of compatible solutes and on the residual activity after drying.

	EMRS		DMRS	
1M NaCl	-	+	-	+
betaine (μmole/g dry weight)	127	213	5	44
carnitine (μmole/g dry weight)	91	173	3	164
X (g H_2O/g dry weight)	0.12	0.08	0.14	0.10
Residual Activity (%)	$76 \pm 29^{a)}$	$3 \pm 0.1^{b)}$	$99 \pm 6^{b)}$	$4 \pm 0.5^{b)}$

Drying method: convective drying of a layer at 30°C followed by vacuum desiccation above a saturated LiCl-solution at 4°C; *Activity measurement:* glucose fermenting activity; ± standard deviation from average; [a)] average from 7 experiments from 4 batches; [b)] average from 2 experiments from 2 batches

pH-control

Table 3 shows fluidized bed drying results of cells grown in batch culture without pH-control. The residual activity was equal for cells grown with and without NaCl. However, the absolute activity of cells grown with NaCl was much lower compared to the cells grown without NaCl. The influence of pH-control on the activity of dried *L. plantarum* cells grown without NaCl can be seen comparing the third column from Table 1 and the first column from Table 3.

Table 3 Influence of the presence of 1 M NaCl during non pH-controlled growth on the residual activity of *L. plantarum* after fluidized bed drying

	Fluidized bed drying	
Addition of 1M NaCl	-	+
X (g H_2O/g dry weight)	0.11	0.13
A_0 (ΔpH/min/g dry cells)	2.82	0.77
A (ΔpH/min/g dry cells)	0.53	0.16
Residual Activity (%)	19	21

Activity measurement: glucose fermenting activity

Reactor concept

The time to reach the stationary phase in batch culture was extended when cells were grown in the presence of 1 M NaCl. In order to avoid comparison of cells in different growth phases chemostat cultures were grown without and with extra NaCl. The results are shown in Table 4. The residual activity after convective drying of a layer of cells with initial cell concentration of 0.25 g dry cells/g dry sample was 61% and 40% for cells grown in a chemostat without and with NaCl, respectively. This can be compared to 76 and 3 % for cells grown in batch without and with NaCl, respectively.

Table 4 Influence of reactor concept on residual activity of *L. plantarum* grown without and with 1 M NaCl.

	Residual Activity (%)	
1 M NaCl	-	+
Batch (EMRS)	76 ± 29	3 ± 0.1
Chemostat	61 ± 3	40

Medium: batch (EMRS), chemostat (27.5 g/l MRS); *Drying method:* convective drying of a layer at 30°C followed by vacuum desiccation above a saturated LiCl-solution at 4°C; *Activity measurement:* glucose fermenting activity

Discussion

Osmotic stress

Growth with osmotic stress resulted in a reduced residual activity after drying (Table 1, 2 and 4, Figure 2) irrespective of the applied drying method. The increased accumulation of betaine and carnitine after growth with NaCl did not result in higher residual activity after drying. The results agree well with conclusions of Champagne *et al.* (2), saying that optimal growth conditions will result in cells that will be prepared best to survive a freeze drying process. Stress inducing actions add to the detrimental influences of freezing or drying.

The present results did not correspond with the results of Kets and De Bont (5) which showed an increased residual activity after drying of *L.plantarum* as a result of the accumulation of betaine after growth with osmotic stress (5). The initial cell concentration has an important influence on the residual activity after drying of cells grown without NaCl (9). Kets and de Bont (5) used low initial cell concentrations (C_0 < 0.02 g dry cell/g sample) which explaines their results. An extra chemostat experiment was performed to confirm this. In this experiment the viability was measured after drying of cells with low initial cell concentration (C_0 < 0.02 g dry cell/g sample). This was compared to the chemostat experiments from Table 4 ($C_{0\ (no\ NaCl)}$ = 0.25 g dry cell/g sample, $C_{0\ (1\ M\ NaCl)}$ = 0.11 g dry cell/g sample). The survival of cells grown without NaCl was 3 % after drying with a low C_0 compared to a residual activity of 61 % after drying with a high C_0. The initial cell concentration had much less influence on cells grown with NaCl (survival 26 % and residual activity 40 %, for respectively low and high initial cell concentration, which is not significant in view of the experimental error), which is in agreement with earlier results (9).

Medium concentration

The residual activity after drying was higher for cells grown in DMRS had than for cells grown in EMRS, despite the difference in betaine and carnitine accumulation (Table 2). Yet, this effect is not significant in view of the experimental error. The absolute activity of cells grown in DMRS was in the same range as of those grown in EMRS (data not shown). The influence of NaCl during growth on the residual activity of *L.plantarum* after drying did not change with the medium concentration. Furthermore, process cost will be an important argument in the judgement of the medium, as process cost is related with reactor volume. Above that, downstream processing will be higher for cells grown in a poor medium.

pH-control

Kessler (4) found no significant influence of pH-control during growth on the residual activity of *Lactococcus diacetylactis* after drying. Our results, however, show that pH-control during growth of *L.plantarum*-cells (without osmotic stress) results in a higher residual activity after fluidized bed drying (37%, Table 1) compared to growth without pH control (19%, Table 3). This is a significant difference, since the experimental error in fluidized bed drying is low (Table 1). During growth without pH-control there was no influence of osmotic stress on the residual activity after drying.

Reactor concept

The residual activity of *L.plantarum* was higher for cells in the stationary growth phase (data not shown) which is in agreement with reports in literature (2, 4, 6, 10, 11). The presence of NaCl influenced the growth significantly, which resulted in triplication of the time to reach the stationary phase. Also for cells grown with NaCl the residual activity after drying was higher when the cells were harvested in the stationary phase.

The chemostat cultures allowed comparison of cells grown with and without osmotic stress at an equal growth rate. The influence of NaCl during growth on the residual activity of *L.plantarum* after drying did not change with the reactor concept. Both in batch and chemostat the residual activity was depressed as a result of growth in the presence of NaCl. However, the reactor concept did have an effect on cells grown with osmotic stress. The residual activity of cells grown with osmotic stress after drying was considerably higher when grown in chemostat (40%) compared to when grown in batch (3%) (Table 4).

This paper shows that the use of optimal growth conditions results in *L. plantarum* cells with the highest residual activity after drying. The application of osmotic stress, and the absence of pH-control depressed the residual activity after drying. Improvement of the drying procedure is more effective in achieving higher residual activities after drying.

Acknowledgements

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5 Carbohydrate addition - physical effects

Abstract

Sucrose, maltose, lactose, trehalose, glucose, fructose and sorbitol were tested for their ability to minimise the dehydration inactivation of *Lactobacillus plantarum* during fluidized bed drying. Desorption isotherms were measured of starch and *L.plantarum*, of binary mixtures containing starch and a carbohydrate, and of ternary mixtures composed of *L.plantarum*, starch and a carbohydrate. The moisture distribution inside the drying particle was calculated. Sorbitol, maltose, and to a lesser extent, sucrose protected *L.plantarum* during fluidized bed drying. It was shown that moisture distribution and water activity changes as a result of the addition of carbohydrates could not explain the protection.

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L.J.M. Linders, G.I.W. de Jong, G. Meerdink, K. Van 't Riet. Carbohydrates and the dehydration inactivation of *Lactobacillus plantarum* - The role of moisture distribution and water activity.

Introduction

Starter cultures of lactic acid bacteria are applied in the production of various types of fermented foods or as silage additives in agriculture. Conservation of bacteria by convective drying is advantageous compared to freeze drying, considering the cost of producing large quantities. Moreover, the higher transport and storage cost of frozen starters are to the credit of dried products. A disadvantage of drying is the inactivation of bacteria during drying. Two inactivation mechanisms are known for *Lactobacillus plantarum*: thermal and dehydration inactivation (11). Thermal inactivation is caused by denaturation of critical cell components, mainly DNA and RNA. Cytoplasmatic membrane damage is generally considered as the main mechanism of dehydration inactivation (12, 13). Thermal inactivation can be minimised by optimising process conditions. Therefore, dehydration inactivation becomes the main problem in convective drying.

The use of additives like (low molecular weight) carbohydrates might prevent membrane damage during dehydration. Crowe *et al.* (2) studied the effects of several carbohydrates in preserving the structural and functional integrity of model membranes (microsomes) at low water activities. The protection against leakage increased at increasing carbohydrate contents until a maximum protection level was reached. The effectiveness, which was defined as the amount of carbohydrate necessary to obtain maximal protection, was highest for trehalose, maltose, and lactose, followed by sucrose, glucose, fructose and sorbitol. In literature, the use of carbohydrates to protect lactic acid bacteria against drying is restricted to freeze drying and vacuum desiccation. With lactose, sucrose and adonitol an increase in survival rate from <1% to values ranging from 34% to 86% was achieved during freeze drying of several species of *Lactobacillus* (1, 4, 7, 8, 15). Vacuum desiccation of *L.plantarum* with sorbitol resulted in a residual activity of 100% (13).

Although several authors suggest that the protection by carbohydrates is related to carbohydrate-membrane interactions (2, 9, 13), the exact mechanism of protection is yet unknown. Moreover, the relation between the protection by carbohydrates and physical parameters like moisture distribution and water activity has never been considered. In heterogeneous systems, the water is distributed among the components according to their sorption isotherms. This implies that the moisture content in the cell can differ from the overall moisture content. The influence of this unequal distribution of water on the apparent protective effect of carbohydrates is not known.

The objective of this work was to study the effect of a number of carbohydrates on the dehydration inactivation of *L.plantarum* during fluidized bed drying. The choice of carbohydrates was based on the results of Crowe *et al.* (2) with microsomes. The

additives studied were trehalose, sucrose, maltose, lactose, glucose, fructose and sorbitol. The importance of moisture content, moisture distribution, and water activity was studied in relation to the dehydration inactivation of *L.plantarum* and the protective effect of the above mentioned carbohydrates. Fluidized bed drying experiments were conducted, desorption isotherms were determined of single components and mixtures, and the moisture distribution was calculated using the measured desorption isotherms and a mass balance equation.

Materials and Methods

Micro-organism and growth conditions

Lactobacillus plantarum (P743, Netherlands Institute for Dairy Research (NIZO)) was grown batch-wise, in a 3.5 l fermentor (Applikon) in 55 g/l MRS-medium (Difco) enriched with 1 g/l yeast extract (Oxoid) and 10 g/l glucose (Merck). The fermentation took place under microaerophilic conditions with pH adjusted to 6.3 (using 3 M NaOH). Total consumption of glucose, indicated by glucose test sticks (diastix, Ames), marked the start of the stationary phase and occurred between 17.5 and 18.5 hours after inoculation. Four hours after the beginning of the stationary phase, the cells were harvested by two consecutive centrifugation (10 minutes at 10000 rpm, Sorvall) and washing steps with 0.01 M potassium phosphate buffer (0.01 M KH_2PO_4/K_2HPO_4-buffer in 0.15 M NaCl, pH 7). The cells were stored in 0.01 M potassium phosphate buffer at 4 °C for one to three nights without loss of activity. Before sample preparation and drying, the cells were concentrated by centrifugation (10 minutes at 10000 rpm, Sorvall), resulting in a cell pellet containing 1.4 10^{12} colony forming units/g dry pellet.

Granulation

Carbohydrates were mixed with the *L.plantarum*-cell pellet during 10 minutes. This cell suspension with dissolved sugar or the cell pellet (control) was manually mixed with native potato starch (Perfectamyl P-6, Avebe) to form a homogeneous paste. This paste was extruded to granules (Ø1mm×10mm). The amount of starch added in order to obtain a well extrudable paste varied, since each carbohydrate caused a varying degree of osmotic withdrawal of water from the cells. Table 1 shows the quantitative data of the paste preparation.

Desorption isotherms

Desorption isotherms were determined for cell pellet, native potato starch, starch-carbohydrate mixtures, and *L.plantarum*-carbohydrate-starch-paste. The native potato

Table 1 Preparation of *L.plantarum*-sugar-starch paste: quantitative data.

	dry weight composition (dw_{cell} : dw_{starch} : dw_{sugar})	X_0 (g H_2O/g dry weight)
L.plantarum ('pellet')	-	3.25
starch	-	0.07
L.plantarum-starch paste with: control	1 : 2.4 : 0	0.90
maltose	1 : 4.5 : 1.1	0.47
sucrose	1 : 4.5 : 1.2	0.48
lactose	1 : 2.6 : 1.2	0.65
trehalose	1 : 5.2 : 1.1	0.45
glucose	1 : 4.1 : 1.1	0.60
fructose	1 : 4.2 : 1.1	0.60
sorbitol	1 : 3.5 : 0.8	0.57

starch, whether mixed with carbohydrate or not, was first wetted with de-ionised water for one week at 4 °C (Initial moisture content (X_0) = 4 g H_2O/g dry weight). Weighing flasks, containing about 1.5 g sample, were placed above saturated salt solutions in a glass vacuum desiccator at 25 °C. In this work, water activity (a_w) stands for the relative humidity (RH) of the air that is in equilibrium with the sample. (a_w-values of the saturated salt solutions at 25 °C (6): KOH, a_w = 0.08; LiCl, a_w = 0.11; CH_3COOK, a_w = 0.22; $MgCl_2$, a_w = 0.33; K_2CO_3, a_w = 0.43; $Mg(NO_3)_2$, a_w = 0.53; NH_4NO_3, a_w = 0.62; $CoCl_2$, a_w = 0.64; NaCl, a_w = 0.75). After 14 days the moisture content of the samples (X, g H_2O/g dry weight) was determined by weighing before and after drying in an atmospheric oven at 115 °C for 2 days. The samples were visually checked for mould growth.

Fluidized bed drying

L.plantarum-starch granules (10-20 g) with or without carbohydrate were dried in a laboratory fluidized bed dryer with dehumidified compressed air of 30 °C. The influence of thermal inactivation is negligible at this temperature (11). The drying time varied from 10 to 240 minutes. After drying the glucose fermenting activity was measured; the moisture content was determined as described for the desorption isotherms. Each drying experiment was performed at least 3 times from at least 2 fermentations.

Activity of L.plantarum

The glucose fermenting activity test (10) was used to determine the activity of *L.plantarum*. Samples of granulated *L.plantarum*-paste (0.5-1 g) were resuspended in 33 ml 0.01 M potassium phosphate buffer, in which the pH-decrease was measured at 35 °C after the addition of 1 ml glucose buffer (0.35 g glucose/g potassium phosphate buffer). The time to complete the activity test varied from 1 to 3 hours. No growth of cells occurred during the activity test. The activity (A) was defined as the maximal velocity of pH-decrease per gram dry cells (ΔpH/min/g dry cells). The residual activity after drying (RA) was defined as the ratio of the activity immediately after drying (A) and the activity before drying (A_0).

X-ray diffraction

X-ray diffraction analysis (Philips PW 1820/PW 1710) with CoK_α-radiation (λ = 1.7889 Å) was carried out to detect the presence of sugar crystals in dry *L.plantarum*-starch paste with maltose, sucrose, and lactose.

Estimation of moisture distribution and it's effect on the residual activity

The moisture distribution of a mixture was estimated using desorption isotherms of the components. The desorption isotherms were described with the Gugenheim Andersen and de Boer (GAB) Equation (16):

$$X = \frac{X_{w1} \cdot c_g \cdot k \cdot a_w}{(1 - k \cdot a_w)(1 - k \cdot a_w + c_g \cdot k \cdot a_w)} \quad (1)$$

The GAB-constants X_{w1} (g H_2O/g dry weight), c_g (-) and k (-) were estimated by fitting Equation 1 to measured data sets. The overall moisture content of a mixture of components ($X_{overall}$) was calculated as a function of a_w (Equation 2), from the dry weight fractions (d_i) of the components i and their desorption isotherms ($X_i(a_w)$, Equation 1), assuming no thermodynamic interaction between components.

$$X_{overall} = \sum_{i=1}^{n} d_i * X_i(a_w) \quad (2)$$

The moisture content of component i in the mixture at equilibrium was calculated using a_w of the mixture and Equation 1. The paste with carbohydrates was assumed to be a pseudo-binary system existing of a *L.plantarum*-cells component and a 'starch-carbohydrate' component.

The time scale of the drying process is too short to reach thermodynamic equilibrium. These calculations are therefore used as an approximation of the moisture distribution.

The effect was estimated of the difference between $X_{overall}$ and X_{cell} on the residual activity. The data (RA versus $X_{overall}$) of *L.plantarum*-starch paste were used, since no data of pure *L.plantarum*-cells were available (pure cells could not be fluidized bed dried). For each $X_{overall}$, X_{cell} was estimated assuming no interaction between *L.plantarum* and starch. The measured residual activity versus the estimated X_{cell} was described by Equation 3 in the region $0.05 < X_{overall} < 0.2$. This range was chosen because this is the region where the differences between the carbohydrates were most pronounced. In this range the change in RA with decreasing X could be well described by a linear function.

$$RA(X_{cell}) = aX_{cell} + b \qquad (3)$$

The parameters a and b were fitted by applying linear regression. For the paste with carbohydrates X_{cell} and $RA(X_{cell})$ were calculated using Equations 1 to 3.

Results

Protection by carbohydrates

Figure 1 shows the influence of carbohydrates on the drying tolerance of *L.plantarum* as a function of overall moisture content. From the disaccharides, maltose increased the residual activity to the highest extent, followed by sucrose, whereas lactose and trehalose were not protective. The sugar alcohol sorbitol also had a protective effect, contrary to the monosaccharides glucose and fructose. The differences in residual activity (varying from 0.45 to 0.76 at $X_{overall} = 0.1$ g H_2O/g dry solids) were most pronounced in the low moisture content region ($0.05 < X < 0.2$). The scatter in residual activity varied per paste (average ± 10%) and was highest for the paste with glucose (± 20%). Considering the scatter, maltose, sucrose and sorbitol were clearly protective.

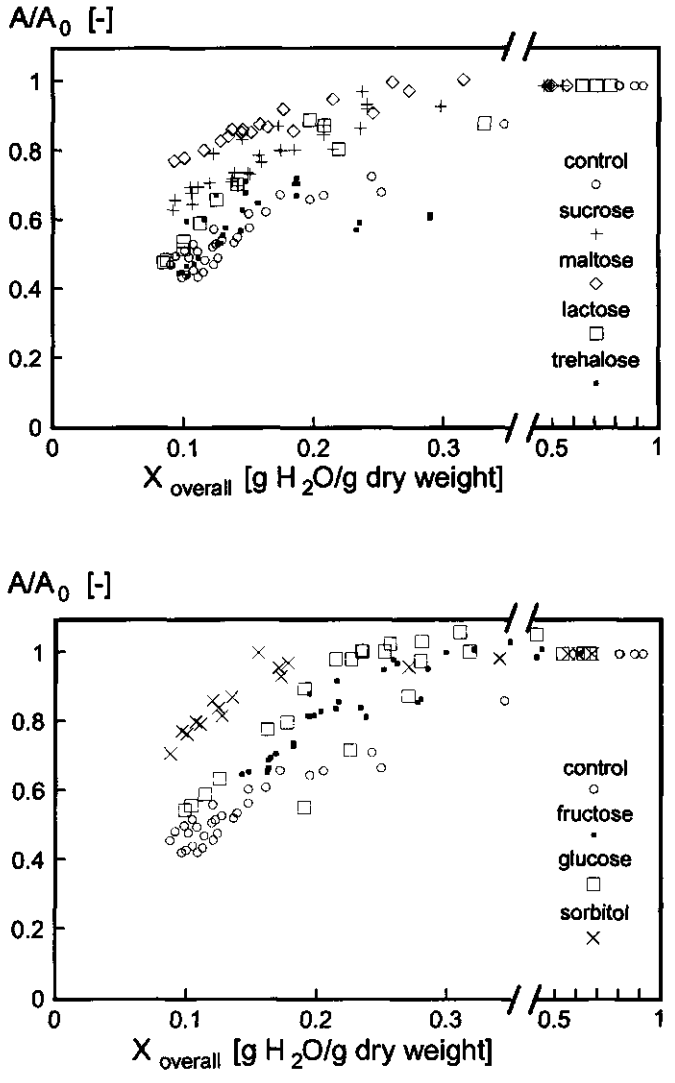

Figure 1 Residual activities of *L.plantarum*-starch paste (control) and *L.plantarum*-starch paste with carbohydrates after fluidized bed drying at 30 °C as function of overall moisture content.

Moisture distribution

L.plantarum-starch paste was used as a model system to demonstrate the presence of an unequal moisture distribution. The desorption isotherms of *L.plantarum* and starch (Figure 2) were not equal in the moisture content region of our interest ($0.05 < X < 0.2$), meaning that an unequal moisture distribution was likely to occur in a mixture of the two components. Before estimating the moisture distribution with Equation 2, this equation was tested using the model system *L.plantarum*-starch paste. The calculated isotherm did not agree with the measured one (Figure 3), indicating thermodynamic interaction between components. This meant that the moisture distribution calculated with Equation 2 was not the real (thermodynamic) moisture distribution. However, since the agreement in the region of interest was satisfactory and since this is the best method available, this equation was used to approximate the moisture content in the cell. Another, indirect, approximation of the moisture in the cell is the water activity of the paste. Both approximations have been carried out and the results are now discussed separately in relation to the protective effect of carbohydrates.

Table 2 Estimated parameter values in the GAB-equation (Equation 1)

	carbohydrate	X_{w1} (g H_2O/g dry weight)	k (-)	c_g (-)
L.plantarum	-	0.084	1.01	21.3
starch	-	0.146	0.64	12.4
starch with:	sucrose	0.092	0.74	22.5
	maltose	0.097	0.68	24.7
	lactose	0.087	0.66	28.3
L.plantarum-starch-paste	-	0.098	0.77	18.7
L.plantarum-starch-paste with:	sucrose	0.078	0.85	25.2
	maltose	0.087	0.81	58.3
	lactose	0.090	0.85	126
	trehalose	0.085	0.91	91.0
	glucose	0.072	0.89	32.3
	fructose	0.077	0.77	18.4
	sorbitol	0.061	0.95	32.4

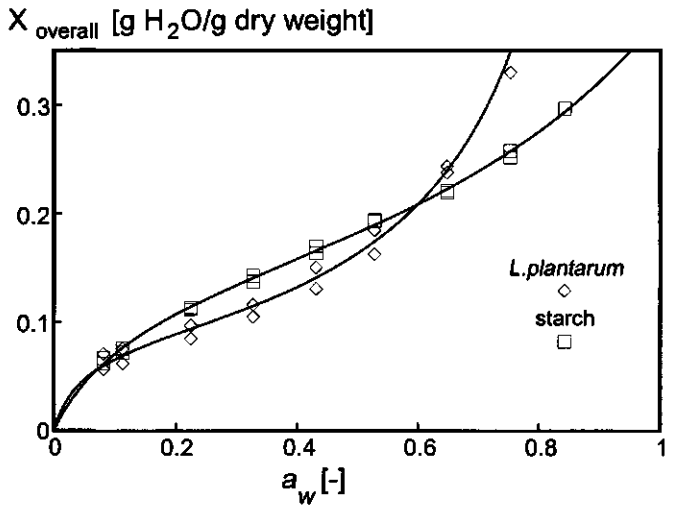

Figure 2 Desorption isotherms of *L.plantarum* and starch measured at 25 °C. The lines represent the fit with Equation 1 (estimated parameter values Table 2).

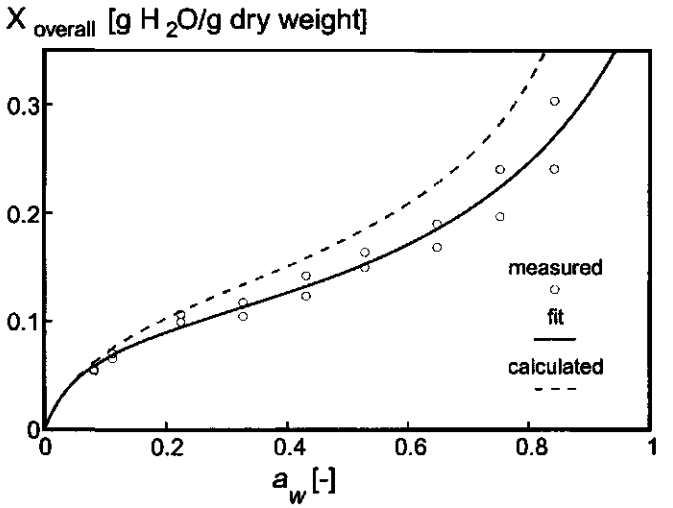

Figure 3 Desorption isotherms of *L.plantarum*-starch: measured at 25 °C; fit (estimated parameter values Table 2); calculated with Equation 1 and 2.

Moisture content in the cell

Figure 4 shows the desorption isotherms of starch-carbohydrate mixtures. The comparison of these isotherms with the one for starch (Figure 2) shows that the carbohydrate addition resulted in a decrease in moisture content at the same water activity. The desorption isotherms of the starch-carbohydrate mixtures were even below the one for pure *L. plantarum*-cells. Consequently, X_{cell} should be smaller than $X_{overall}$ in paste with starch and bigger than $X_{overall}$ in paste with starch and carbohydrates. Calculation of X_{cell} in paste with a carbohydrate using these isotherms in Equation 2 is shown in Figure 5. The question was whether the relative difference between X_{cell} and $X_{overall}$, that can be more than 50%, could have a significant effect on the residual activity. Therefore the change in residual activity was calculated that could be expected considering the change in X_{cell} by the addition of carbohydrate. The residual activity was based on the activity of a cell in the *L.plantarum*-starch paste (Equation 3). In Figure 6 the difference between measured and calculated residual activity is given. This graph shows that the measured residual activity is (significantly) higher than calculated for the paste with maltose and sucrose. Consequently, the difference between X_{cell} and $X_{overall}$ could not explain the increase in residual activity as a result of the addition of carbohydrates.

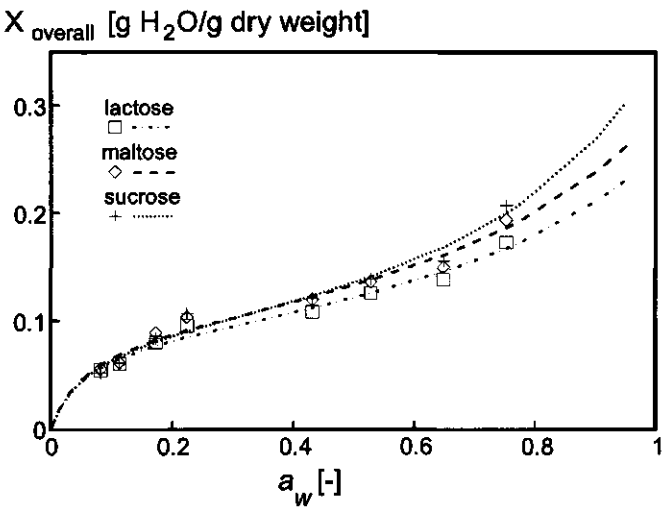

Figure 4 Desorption isotherms of mixtures of starch and carbohydrates: measured at 25 °C. The lines represent the fit with Equation 1 (estimated parameter values Table 2).

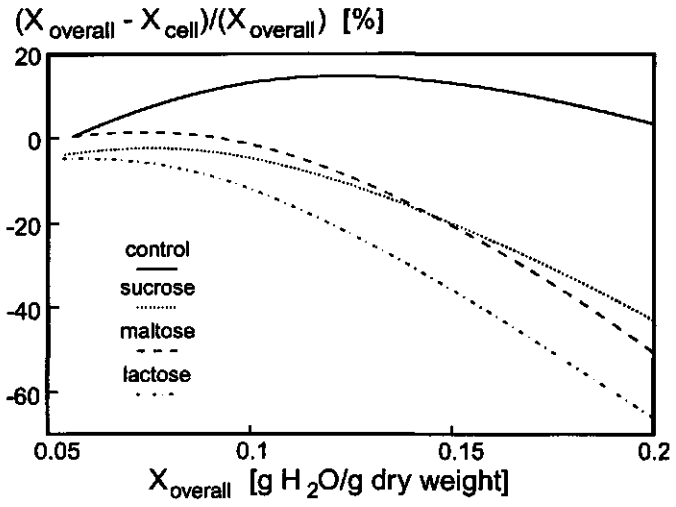

Figure 5 Relative difference between the overall moisture content and the calculated moisture content in the cell versus the overall moisture content. Control is *L.plantarum*-starch paste without carbohydrates.

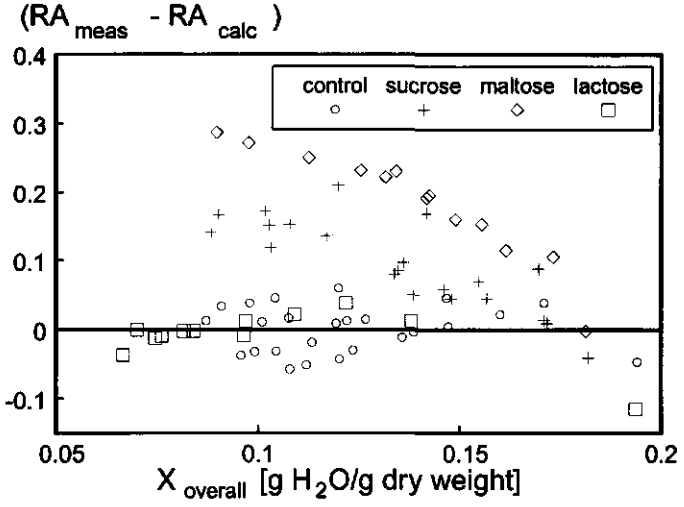

Figure 6 Difference between measured and calculated residual activity (Equation 3, a=2.3; b=0.27) of paste with and without carbohydrates based on the calculated moisture content in the cell. Control is *L.plantarum*-starch paste without carbohydrates.

Water activity

The desorption isotherms of *L.plantarum*-starch paste with carbohydrates are shown in Figure 7. The fit of the isotherms was almost the same for paste with glucose and fructose. For clarity only the one for glucose was displayed. Each isotherm, except for the one with lactose, was below or in the vicinity of the control paste, indicating that the water activity was higher at the same moisture content for samples with carbohydrates. X-ray diffraction analysis was carried out on the fluidized bed dried pastes with sucrose, maltose, and lactose. In paste with maltose and sucrose no crystals were detected, indicating that these sugars were amorphous in the paste. Lactose crystals were present in the lactose paste.

To obtain the residual activity as a function of water activity the data from Figure 1 were used. $X_{overall}$ was converted to a_w using the desorption isotherms from Figure 7 (estimated parameter values in Table 2), assuming no a_w-profile in the fluidized bed dried particle. The residual activity drawn versus the water activity (Figure 8) shows a protective effect for maltose, sucrose, and sorbitol. In Figure 8 even lactose is protective.

Discussion

The dehydration inactivation of *L.plantarum* was studied, especially the effect of the addition of carbohydrates. Significant differences were found in the extent of protection provided by different carbohydrates, differences that occasionally conflicted with the results of other researchers. The effectiveness of sorbitol corresponded to the results of Lievense *et al.* (13) for *L.plantarum*, but not with those of Crowe *et al.* (2) for model membranes. Remarkably, trehalose did not protect *L.plantarum* (Figure 7), whereas this additive is highly successful in protecting model membranes and *Saccharomyces cerevisiae* (2, 3). Comparison of the present work with data from literature is difficult for a few reasons. The definitions of maximal protectiveness are not the same. In this work the protectiveness is based on the increase in residual activity after drying as a result of the addition of carbohydrates (applying a nearly constant sugar-cell ratio). The protectiveness of Crowe *et al.* (2) was based on the concentration of carbohydrate (g carbohydrate/g dry microsomes) necessary to obtain maximal protection against leakage. However, even if our definition (protection at one sugar-cell ratio) was applied to their data the results still did not agree. Most probably, the reason for the discrepancy

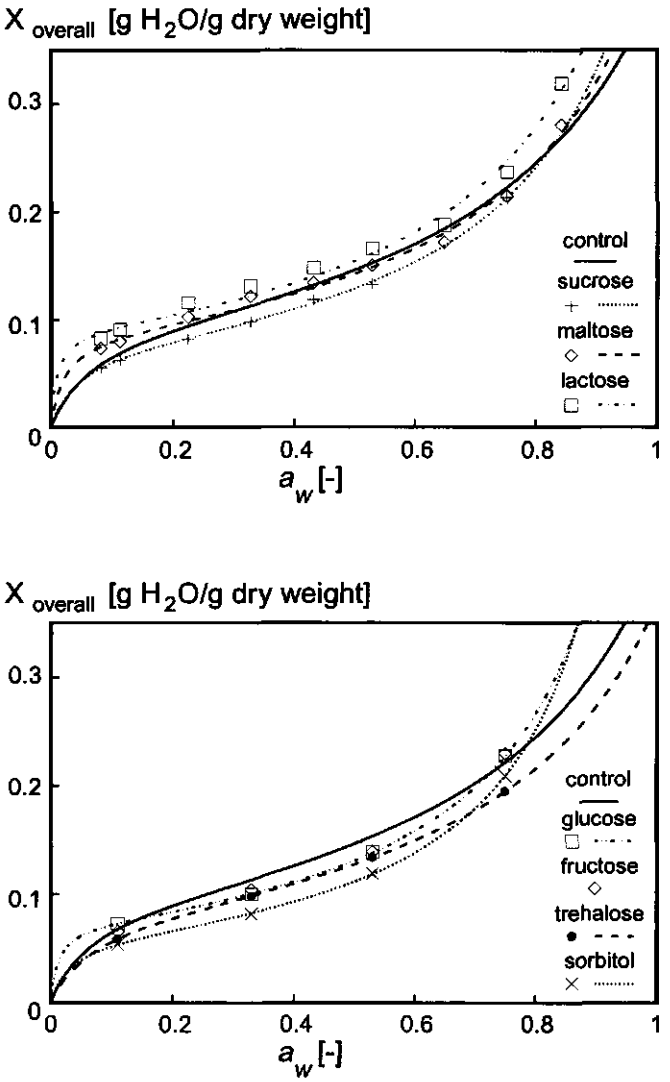

Figure 7 Desorption isotherms of *L.plantarum*-starch with carbohydrates measured at 25 °C. The lines represent the fit with Equation 1 (estimated parameter values Table 2). Control is *L.plantarum*-starch paste without carbohydrates.

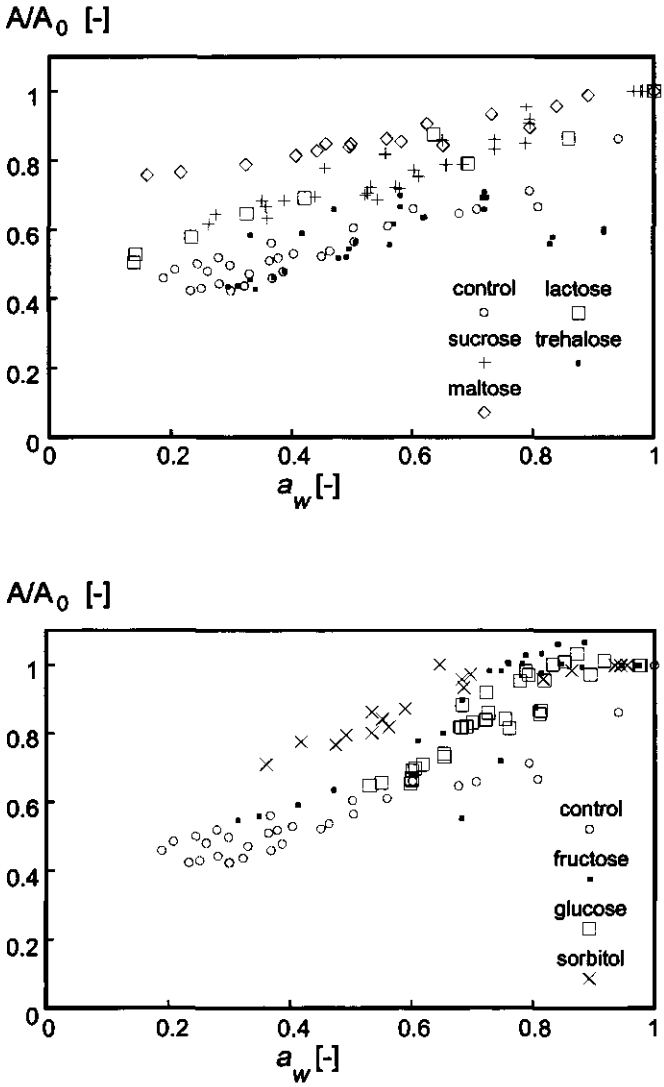

Figure 8 Residual activities of *L.plantarum*-starch paste (control) and *L.plantarum*-starch paste with carbohydrates after fluidized bed drying at 30 °C as function of water activity. The water activity is calculated using the measured overall moisture content in Equation 1 (estimated parameter values Table 2).

of our results with those from literature is the use of different micro-organisms or model systems (microsomes) and the use of another drying method (14).

A direct conclusion about the protective effect of carbohydrates based on residual activity as a function of overall moisture content is not allowed, since the moisture distribution in the sample can be inhomogeneous with respect to the different constituents of the samples. In this work the protective effect of carbohydrates was studied in relation to the presence of an inhomogeneous moisture distribution. The moisture distribution in *L.plantarum*-starch paste was influenced by the addition of carbohydrates. To estimate the moisture distribution X_{cell} and a_w were used, two approximations of the moisture in the cell. The calculation of X_{cell} showed that the carbohydrate addition had a significant effect on the moisture distribution. This change in X_{cell} could theoretically result in a 30 to 45 % increased residual activity at $X_{overall}$ = 0.2 g H_2O/g dry solids, a significant effect taking into account an experimental scatter of 10%. However, the difference between measured and estimated residual activity (Figure 6) showed that the increase in X_{cell} as a result of the addition of maltose or sucrose could not account for the increased residual activity. Therefore, the calculated X_{cell} does not seem to be the primary parameter determining the residual activity. Water activity was used as an alternative measure of the moisture in the cell (Figure 7). The protective effect of the carbohydrate addition was still visible, meaning that the change in water activity could not explain for the change in residual activity. This confirmed the results obtained using X_{cell}, namely that the effects of carbohydrates were not restricted to changes in moisture distribution.

Following these results, $X_{overall}$ and a_w as parameters in modelling biological activity do not explain the changes in measured residual activity. This means that a direct physical explanation is absent and another, protective, mechanism should be present. The use of moisture content versus water activity in modelling the dehydration inactivation of *L.plantarum* has been discussed previously by Lievense *et al.* (11). Moisture content is claimed to be the key parameter, since a change in water activity by changing the temperature did not influence the residual activity at one moisture content. The use of water activity as a measure of biological activity has been critically commented by Franks (5). According to Franks it is a misrepresentation to believe that biological activity, involving many coupled processes associated with survival, depends solely on water activity. Our results confirm this statement. $X_{overall}$ is not the best parameter, because an inhomogeneous moisture distribution is not taken into account. Furthermore, it is important to realise that the apparent relative protectiveness of different carbohydrates can be dependent on the parameter chosen, $X_{overall}$ or a_w.

We have shown that the effects of carbohydrates were not restricted to physical changes in water distribution. This indicates that physiological interactions have to be taken into

account. Probably, carbohydrates have a function in preventing membrane damage by interaction with the membrane. In literature there is some evidence to this, although exact mechanisms are not known. Therefore, further work is necessary to reveal the mechanism of physiological interaction between carbohydrates and cell membrane.

Acknowledgements

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6 Carbohydrate addition - physiological effects

Abstract

The relation between the protective effect of externally added carbohydrates on *Lactobacillus plantarum* cells during air drying and the phase behaviour of cell membranes was studied. The residual activity after drying could be improved from 44% in the control to 79% and 66% after the addition of sorbitol and maltose, respectively, whereas trehalose addition resulted in a residual activity of 30%. Membrane phase transition temperatures (T_m) were determined in intact hydrated and dry cells, using Fourier transform infrared spectroscopy. T_m of hydrated cells was 4°C, to increase to only 20°C after drying. Because endogenous soluble sugars were absent, this phase behaviour is attributed to the structure of the phospholipids, PG and lysyl-PG. The restricted increase of T_m is held responsible for the survival of part of the cells. The added maltose, trehalose and sorbitol did not influence T_m *in vivo*. We suggest that the effective carbohydrates act through their free radical scavenging activity and not by direct interaction with the polar lipid headgroups.

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L.J.M. Linders, W.F. Wolkers, F.A. Hoekstra, K. van 't Riet. Effect of carbohydrate addition on membrane phase behaviour and survival of dried *Lactobacillus plantarum*.

Introduction

Compared to freezing and freeze-drying, convective drying is an economical alternative for the preservation of starter cultures of bacteria. A prerequisite is that inactivation during drying is kept at a minimum. At low drying temperatures thermal inactivation is negligible but dehydration inactivation may impose serious problems (30). Cell membrane damage is associated with this dehydration inactivation (29). This was demonstrated by measuring the leakage of hydrolysed DNA from rehydrated *Lactobacillus plantarum* cells after incubating them with DNase.

Added carbohydrates decrease dehydration inactivation of dried *L. plantarum* cells (31). The residual activity of these fluidized bed-dried cells improves when the bacteria are dried in the presence of maltose, sucrose or sorbitol. Protection of sorbitol was associated with reduction of leakage of hydrolysed DNA from the rehydrated bacteria after incubation with DNase (29). Therefore, carbohydrates may decrease the dehydration inactivation by protection of the cell membrane.

Endogenous trehalose is known as an effective protectant for bacteria and yeast cells in the absence of water (16, 22, 27, 33, 37, 39, 46, 47). Also, the accumulation with drying of trehalose in fungal spores, nematodes, and cysts of the brine shrimp *Artemia* (9, 5) or of sucrose in pollen and seeds of higher plants (8, 20) improves the tolerance to desiccation. It has previously been described for anhydrobiotic organisms that the protective effect of carbohydrates particularly concerns cell membranes (9, 14).

Experiments with model membrane systems (liposomes, vesicles) showed that leakage from them is prevented as a result of interaction of disaccharides with the polar headgroups of the membrane phospholipids (7, 9, 14, 18). Leakage occurs when the phospholipids undergo a phase transition from the liquid crystalline to the gel phase or vice versa (5, 11). The phase transition temperature (T_m) increases with decreasing moisture content. Interaction of the carbohydrates with the polar headgroups depresses T_m to such an extent that a phase transition during drying and rehydration is largely prevented (8, 20, 45).

Using Fourier transform infrared spectroscopy (FTIR) it is possible to measure phase transitions in intact cells (12). Leslie *et al.* (27) recently used this technique to show that T_m of freeze-dried *E. coli* and *B. thuringiensis* was depressed when exogenous trehalose or sucrose was allowed to penetrate into the cells. These sugars also improved survival of the bacteria after drying.

In the present paper the importance of membrane phase transitions was studied in relation to the desiccation tolerance of *L. plantarum* cells after adding maltose, trehalose and sorbitol. Phase transition temperatures were measured in intact cells and in liposomes made from purified phospholipids extracted from *L. plantarum* cells, using FTIR.

Materials and methods

Micro organism and growth conditions

Lactobacillus plantarum (P743, Netherlands Institute of Dairy Research (NIZO)) was grown in enriched MRS-broth at 30°C and pH 6.3, as described previously (32). The cells were harvested 4 hours after the start of the stationary phase and washed in 0.01 M potassium phosphate buffer in 0.15 M NaCl (pH 7). A concentrated cell pellet ($1.4\ 10^{12}$ colony forming units/g dry weight) was obtained by centrifugation of the washed cells. For the carbohydrate addition experiments the cell pellet was mixed with carbohydrate at 0.3 g/g fresh cell pellet. This corresponds to at least a mass ratio of 5 g carbohydrate per g phospholipid.

Drying

A petri dish (∅ 50×10 mm) with a layer (1 mm) of cell pellet or cell-carbohydrate mixture was placed in a container that was continuously flushed with dry air (relative humidity (RH) = 3%) at 22°C. The samples were dried for 18-22 hours.

Activity measurement

The glucose fermenting activity test according to Lievense *et al.* (28) was used to determine the activity of *L. plantarum* cells. After drying, the samples were rehydrated in 0.01 M potassium phosphate buffer at 20°C. The maximum rate of pH-decrease at 35°C after the addition of 1 ml potassium phosphate buffer containing glucose (0.35 g glucose/g buffer) was used as a measure for the activity (ΔpH/min/g dry cells). The residual activity was defined as the ratio of the activity after drying (A) to the activity before drying (A_0).

Phospholipid extraction

Directly after harvesting, the cell pellet was freeze-dried. Phospholipids were extracted with chloroform/methanol/water as described earlier (2, 24). For further purification of the phospholipids, the extract was loaded on a BioSil A column (18 cm length, 16 mm diameter) that was pre-washed with 110 ml of $CHCl_3$. Possible neutral lipids were removed by washing the column with 150 ml of $CHCl_3$ and 100 ml of acetone. The polar lipids were subfractionated with MeOH (200 ml) and analysed on TLC (15). Those containing phosphatidylglycerol (PG) and lysyl-phosphatidylglycerol (lysyl-PG) were combined. The MeOH was evaporated, and the isolated phospholipids were stored in $CHCl_3$ at -80°C until further use.

Vesicle preparation

Multilamellar vesicles (MLV) were prepared from the phospholipids extracted from *L. plantarum* and from palmitoyl-oleoyl-phosphatidylglycerol (POPG). Five mg of dry phospholipids were hydrated in 0.5 ml water or in sugar solution and sonicated for 5 min. Subsequently, the samples were frozen in liquid nitrogen and thawed five times. The resulting multilamellar vesicles were pelleted by centrifugation in an Eppendorf centrifuge.

Fatty acid analysis

Fatty acid methyl esters were prepared from the extracted phospholipids by a 15 minutes incubation at 95°C in boron trifluoride/methanol using the method of Morrison and Smith (35). The fatty acid methyl esters were extracted with hexane. Fatty acid analysis was performed using gas chromatography (Chrompack-Packard CP-9000 GC; CP-Sil 88 capillary column, 50 m; temperature program from 160 to 220°C; flame ionisation detector). The fatty acids were identified with the aid of standards. The relative amounts of the fatty acids were determined from the peak areas of the methyl esters with a chromatopac C-R6A integrator (Shimadsu, Kyoto, Japan) (19).

Fourier Transform Infrared measurements

FTIR spectra were recorded on a Perkin-Elmer 1725 Fourier transform infrared spectrometer equipped with an external beam facility to which a Perkin-Elmer IR-microscope was attached. The microscope was equipped with a LN_2-cooled narrow band Mercury/Cadmium/Telluride IR-detector. Data were acquired with a MS-DOS computer using Perkin Elmer software (versions 2.5 and 3.5). The samples were sandwiched between two CaF_2 windows (∅ 13 mm) that were hermetically closed and then loaded in a temperature-controlled cell. This cell was placed in the microscope of the instrument. After cooling the samples to -50°C the samples were slowly heated (1 °C/min), and every 2-3 °C a spectrum was recorded. Each spectrum was the average of 64 scans at each temperature in the IR region 3500-900 cm^{-1}.

The position of the CH_2 stretching vibration bands [at approximately 2853 cm^{-1} and 2926 cm^{-1} for the symmetrical and asymmetrical bands, respectively (1)] was determined in each spectrum. The plot of the wave number (cm^{-1}) against temperature resulted in a curve from which the phase transition temperature was determined by fitting a non linear equation to the curve and determining the second derivative.

Results

FTIR spectra of intact cells

In the FTIR spectrum of dry *L. plantarum* cells at 20°C (Figure 1a) the absorption bands associated with the CH_2 symmetrical (2853 cm^{-1}) and asymmetrical (2926 cm^{-1}) stretching vibration can be observed. These bands are typical of lipids. Two other bands can be distinguished in this region associated with the CH_3-asymmetrical vibration band (2959 cm^{-1}) and the CH_3-symmetrical vibration band (2876 cm^{-1}) (1). Comparison of the peak-heights of the CH_3 and CH_2 bands indicate that the CH_3 band was relatively high, which may be due to the high protein content of the sample (6). Other bands that can be recognised are the OH-stretching vibration band (3291 cm^{-1}), and, in the protein region, the C=O stretching vibration band (amide I; 1652 cm^{-1}) and the N-H bending vibration band (amide II; 1547 cm^{-1}) (1).

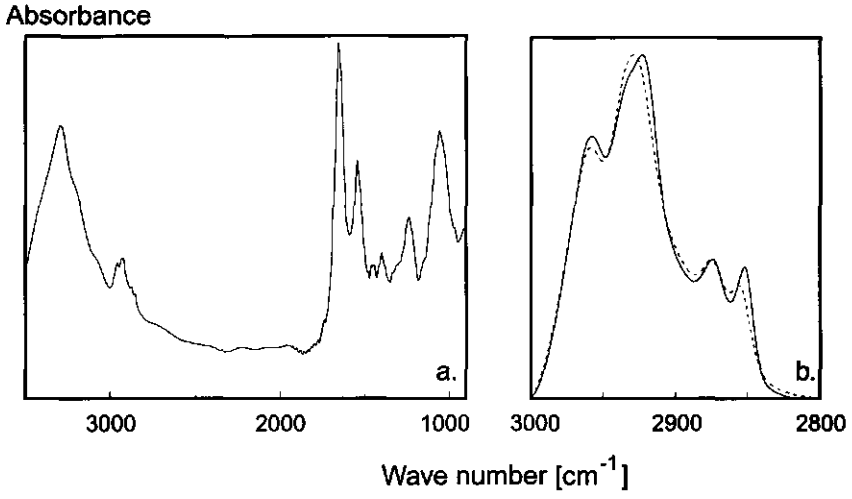

Figure 1 FTIR-absorption spectrum of dry *Lactobacillus plantarum* cells: (a) complete spectrum; (b) Flatted, normalized CH_2-vibration region at -50°C (————) and at 50 °C (- - - - - - - -)

Figure 1b shows a magnification of the normalized, flatted CH_2 and CH_3 vibration bands in dried cells at -50°C and at 50°C. We assume that the CH_2 bands here are derived from the acyl chains of phospholipids and not from oils, because the latter compounds are absent in *L. plantarum* (40, own observation). The relatively narrow band width of the CH_2 symmetrical vibration band at 2851 cm^{-1} indicates that membranes were in gel phase at -50°C. At 50°C membranes were in liquid crystalline phase (at 2853.5 cm^{-1}). By plotting the wave number of the CH_2 vibration band maximum against temperature, T_m can be determined (see also Figure 2).

Membrane phase transitions in intact cells

T_m of hydrated *L. plantarum* cells was approximately 4°C (Figure 2). The presence of added maltose or sorbitol hardly had an effect on T_m of hydrated cells. This is in agreement with results of hydrated liposomes where only a slight increase in T_m (2 to 3°C) was found after the addition of carbohydrates (17).

After air drying, T_m of dry *L. plantarum* cells had increased to 20°C (Figure 3), which means that T_m increased approximately 16°C upon drying at 3 % RH. The presence of maltose during drying had no influence on T_m of the dried *L. plantarum* cells (Figure 3). Data of similar experiments using sorbitol and trehalose are shown in Table 1. Because T_m values were around 20°C in all the dry spectra, it can be concluded that the effect of added carbohydrates on T_m in dry cells was small, and not significant in view of the accuracy of the T_m determination.

Table 1 also shows the influence of maltose, trehalose and sorbitol on the residual metabolic activity after air drying. Maltose and sorbitol both improved the residual activity significantly. However, trehalose was not protective and even lowered the residual activity of the cells.

Table 1 Phase transition temperatures (T_m) of hydrated and dry *L. plantarum*-cells with and without carbohydrates. A/A_0 is the residual activity of *L. plantarum*-cells after convective drying at ambient temperature (22°C).

		T_m [°C]	
Carbohydrate addition	A/A_0 [%]	hydrated	dry
no additive	44	4	20
maltose	66	4	20
trehalose	30	n.d.	23
sorbitol	79	9	19

n.d. no data

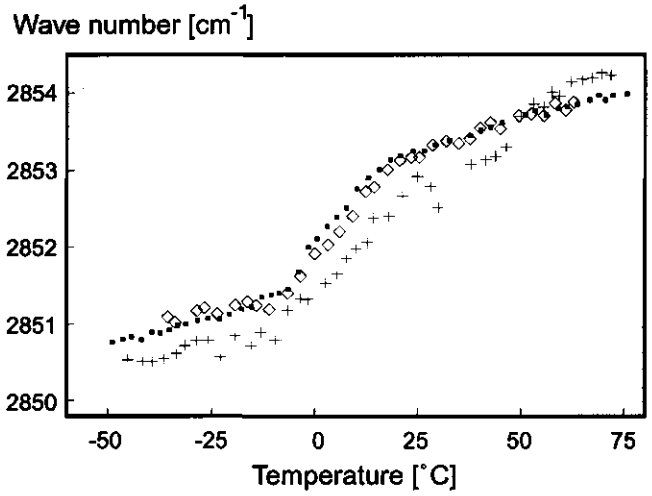

Figure 2 Vibrational frequencies for CH_2 symmetrical stretch versus temperature in: hydrated L.plantarum cell pellet (◇), in hydrated *L.plantarum* cell pellet with maltose (▪) or with sorbitol (+).

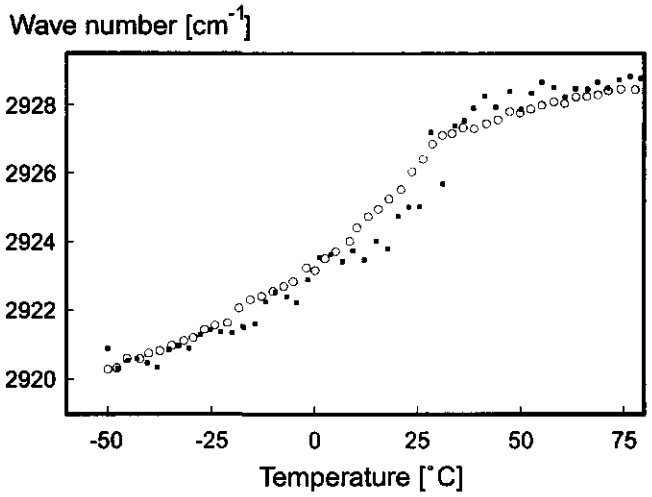

Figure 3 Vibrational frequencies for CH_2 asymmetrical stretch versus temperature in dried *L. plantarum* cell pellet (o) and in dried *L. plantarum* cell pellet + maltose (▪)

Phase transitions in liposomes

Table 2 shows that the phospholipids of *L. plantarum* membranes consisted mainly of palmitic (16:0) and oleic (18:1) acid. PG and lysyl-PG were the predominant phospholipids (15, Table 2), which was confirmed using TLC. Lysyl-PG is the lysine ester of phosphatidyl glycerol (23). Assuming the saturated, palmitic acid to be located at the side-chain 1 position and the unsaturated fatty acids at the side-chain 2 position (4), it is expected that a large proportion of the PG is POPG. Thus, phase behaviour will be largely determined by this phospholipid species.

Table 2 Membrane composition of *L. plantarum* cells

	16:0	16:1	18:0	18:1	19:cyc
Fatty acid composition	32	4	4	55	5
	PG	lysyl-PG	DPG	other	
Head group composition[1]	68	23	4	5	

[1] Eksterkate 1971 (15)

MLV were prepared from pure POPG. Temperature versus wave number plots of POPG-vesicles showed sharp discrete shifts from which T_m could be accurately determined (Figure 4). T_m of hydrated and dry POPG was 11 and 40°C, respectively.

T_m of MLV prepared from a purified phospholipid mixture extracted from *L. plantarum* was calculated at -5°C in the hydrated specimens and at 13°C in the dry ones (Figure 5a). T_m values of these vesicles dried in the presence of excess maltose, sorbitol or trehalose were far below 0°C (Figure 5b). The phospholipids did not completely reach gel phase upon cooling because at -60°C the wavenumber position was still above 2852 cm^{-1}. T_m was estimated at approximately -45°C in these vesicles (assuming the midpoint of the phase transition curve to be between 2852 cm^{-1} and 2852.5 cm^{-1}).

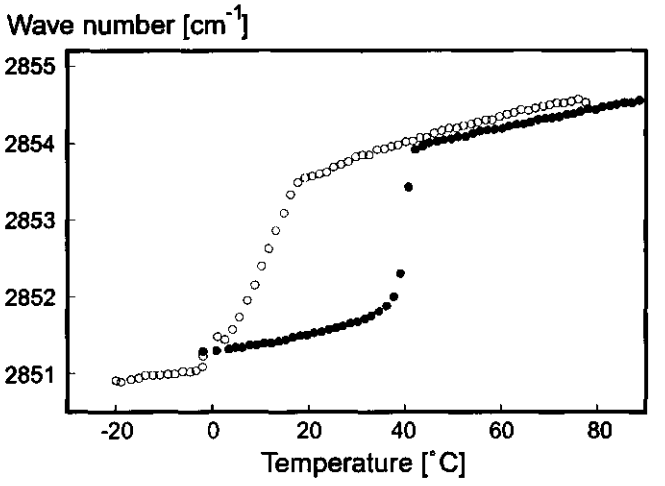

Figure 4 Vibrational frequencies for CH_2 asymmetrical stretch versus temperature: hydrated (o) and dried (•) MLV from POPG.

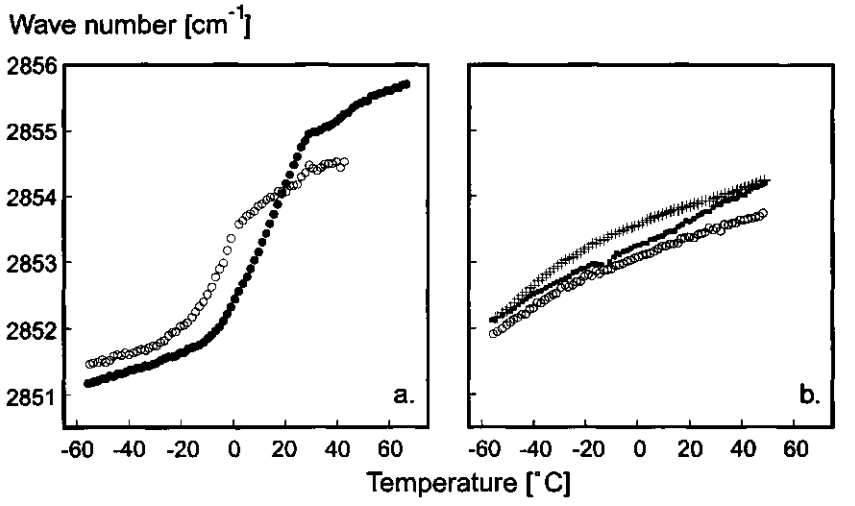

Figure 5 Vibrational frequencies for CH_2 asymmetrical stretch versus temperature of MLV of purified phospholipids extracted from *L. plantarum*: (a) hydrated (o) and dried (•); (b) dried with maltose (o), dried with sorbitol (▪) and dried with trehalose (+).

Figure 6 shows the phosphate region of FTIR spectra of POPG liposomes. The asymmetric P=O-stretching vibration band in hydrated POPG liposomes was at approximately 1215 cm^{-1}. After drying this band had increased to approximately 1227 cm^{-1}. The presence of maltose and trehalose during drying resulted in a small depression of this band to approximately 1222 cm^{-1}. Sorbitol addition depressed this band further to approximately 1208 cm^{-1}. In intact *L. plantarum* cells the asymmetric P=O-stretch was not a reliable measure of the state of the phospholipid headgroup because this stretch was also influenced by P=O-bonds in proteins and other phosphates. In vesicles made from purified phospholipids extracted from *L. plantarum* the phosphate bands were masked by a band at 1270 cm^{-1}.

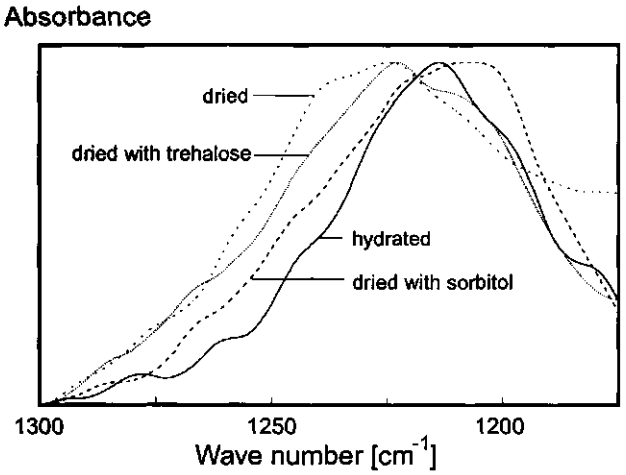

Figure 6 FTIR absorption spectra (normalized) of POPG vesicles in the phosphate region. The curve of POPG dried with maltose was identical to the curve of POPG dried with trehalose. For clarity only the one of POPG dried with trehalose was shown.

Discussion

In our hands, *L. plantarum* cells are reasonably desiccation tolerant (44% survival), which can be improved by the exogenous addition of sorbitol or maltose. Because desiccation

sensitivity coincides with considerably increased membrane permeability (29), we assumed membrane phase behaviour to be involved in the beneficial effects of these compounds. In liposome systems sugars were found to depress T_m of the membrane lipids with drying and, thus, to prevent formation of gel phase and leakage (9, 10). External sugar additions were expected to also bring about these effects in dried *L. plantarum* cells.

T_m values could be very well measured in intact *L. plantarum* cells using FTIR. In hydrated cells they were in the same range (4°C) as those of other hydrated bacteria (27). After drying, T_m increased to 20°C, which is a small upward shift compared to those of 40°C and 30°C in bacteria (27) and pollen (20), respectively. Larger shifts of 70°C have been observed in membranes isolated from pollen (45) and in liposomes made from several phosphatidylcholines (PC) (7). Because the average T_m in a sample of dried *L. plantarum* cells did not exceed room temperature, it is expected that at least part of the membranes remained in the liquid crystalline state. If liquid crystalline membranes are abundant within certain dried cells, it would explain the relatively high degree of desiccation tolerance. Passage of the phospholipids through T_m during drying and rehydration is assumed to result in leakage and loss of viability (10).

Our measurements indicate that the added sugars did not depress T_m in dry cells, irrespective of their beneficial effect on desiccation tolerance. The lack of effect on T_m can have two causes. Firstly, the sugars may have entered, but do not interact with the polar headgroups. This is not likely because T_m of dried vesicles made from purified phospholipids extracted from *L. plantarum* cells, was depressed to far below T_m of the hydrated vesicles. Such depression is evidence of interaction of the added sugars with the headgroups (9). Furthermore, the mass ratio of the added carbohydrates to phospholipids (> 5) would have been sufficiently high to realise such interaction. Secondly, the sugars may not get access to the cytoplasm. The latter possibility is likely because the cells were grown on glucose and fructose and, thus, were not adapted to the uptake of other carbohydrates (38). Leslie *et al.* (27) were able to achieve penetration of trehalose and sucrose only after incubation at T_m, when membranes have a higher permeability.

The improved desiccation tolerance has therefore to originate from other effects of the sugars. Sugars are likely to form highly viscous glasses at room temperature when they are dehydrated from solution (42). The improved storage stability of anhydrobiotes (26) and liposomes (44) has been associated with the presence of a glassy state. However, because the glass transition temperature (T_g) of dried sorbitol is below room temperature (42), the protective effect of sorbitol cannot be explained by the formation of glasses around *L. plantarum* cells. In contrast, the superior glass former, trehalose, was not an effective protectant. It is more likely that the effect of sorbitol (Table 1) is due to its anti-oxidant properties (43).

The comparatively small increase of T_m with drying in intact cells without added sugars would suggest that other endogenous soluble carbohydrates interact with the polar headgroups. However, no endogenous sugars were detected in any large quantity (Dionex HPLC analysis; data not shown). This would be the first example of a desiccation tolerant organism that does not contain endogenous sugars.

The small increase in T_m with drying of intact cells and liposomes made from extracted phospholipids may stem from the phospholipid composition of *L. plantarum* membranes. The major phospholipids are PG and lysyl-PG (91% of the total; 15). No earlier reports are known to us on phase behaviour of PG-liposomes during drying. For POPG the increase in T_m after drying was 30°C (Figure 4), which is small compared to the 60-70°C as generally observed for PCs, abundant in eukaryotic membranes. This difference is probably due to the position of the headgroup with respect to the membrane bilayer. In PCs the headgroup is positioned parallel to the membrane surface (3). Conversely, the PG headgroup is sticking out of the bilayer with an angle of 30° (3, 34). Removal of the hydration layer from PC headgroups will decrease the electrostatic interactions between the phospholipid headgroups and will therefore favour the formation of gel phase. Withdrawal of the hydration layer from the PG headgroup probably will have relatively less influence on the phase behaviour of the membrane, because of the position of this headgroup outside of the membrane. This is reflected in the smaller increase of T_m after drying of POPG vesicles.

The P=O asymmetric stretch can be used to follow the loss of the hydration shell of the headgroup and to detect interaction of sugars with the dry phospholipid (8, 21). In hydrated and dry POPG the P=O band occurred at a significantly lower (30 cm^{-1}) wavenumber position than usually found in PC. This is probably due to the molecular conformation of the PG-head group, as shown in Figure 7, where a molecular model is drawn using the program Alchemy. Occurrence of internal H-bridges is likely there, because the distance between the last H-atom of the glycerol head and the O-atoms in the phosphate group is small (2.57 Å). This may explain the low energy for the P=O asymmetric stretching vibration. After drying, this stretch moves to a higher value. Also in hydrated vesicles hydrogen bonds (between water and the phosphate headgroups) are responsible for the lower band position. Sorbitol addition resulted in a depressed P=O-stretching vibration in dry vesicles, even below the hydrated ones. This was earlier observed for dry DPPC and POPC vesicles in the presence of trehalose (13, 36), indicative of a strong interaction between the carbohydrate and the polar headgroups. We may conclude, therefore, that in liposomes made from POPG and from extracted *L. plantarum* phospholipids, strong interaction of the headgroups with sorbitol had occurred.

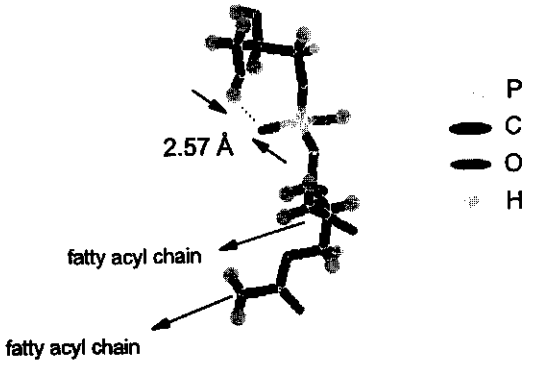

Figure 7 Schematic representation of the structure of the PG headgroup. The estimated distance between the H-atom in the glycerol part and the O-atom in the phosphate group was 2.57 Å.

The special structure of the phospholipids in *L. plantarum* may be the reason for its considerable intrinsic desiccation tolerance. However, desiccation tolerance of *Lactobacillus bulgaricus* is less good (25, 41), while having an identical phospholipid composition (40). We may conclude, therefore, that control of T_m in membranes of dry cells is only one of the factors determining desiccation tolerance and that control of free radical activity, - a putative role for the added sorbitol - is also important.

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7 General discussion: Activity of dried starter cultures

Introduction

The objective of this thesis was firstly, to identify the process conditions which favour the production of active dried starter cultures and secondly, to determine the physical and physiological mechanisms explaining the influence of these process parameters. The influence of several process parameters on the dehydration inactivation of *L. plantarum* has been studied, using a process oriented approach (Figure 1). In this chapter most aspects involved in the production of active dried *L. plantarum* are reviewed in relation to each other. Furthermore, new results will be considered concerning drying methods that have not (contact sorption drying) or that have only scarcely (fluidized bed drying) been discussed in the other chapters. The influence of carrier material will come up for discussion. The process parameters studied in this thesis will be used to predict and discuss the results of these additional drying methods. Furthermore, the influence of carbohydrates on the overall process will be discussed.

The integrated approach has led to a better understanding of the importance of several drying parameters. We have found that important process parameters are moisture content, water activity, water distribution, temperature, drying time, initial cell concentration, and the presence of carbohydrates. Under certain conditions physiological adaptations occur during drying, which lead to increased residual activities after drying. In order to initiate these adaptations the drying temperature is important. Furthermore, the drying time should be such that the residence time at a

critical water activity range is long enough to allow these adaptations to occur and at the same time short enough to prevent decrease of the residual activity. High initial cell concentrations improve the residual activity after drying of cells grown with standard conditions. However, the initial cell concentration (C_0) does not influence the residual activity of dried cells that were grown with osmotic stress. Variation in growth conditions did not lead to improved residual activities.

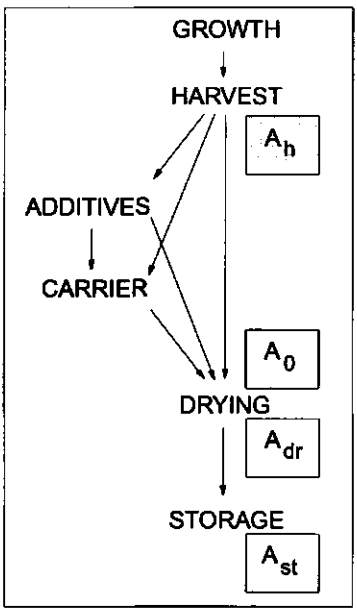

Figure 1
Process scheme: absolute activities

For the production of dried starter cultures, the absolute activity is important. Each process step will affect the absolute activity of the dried starter cultures. Therefore, a set of absolute activities can be defined (Figure 1). The starting point is the cell pellet after harvesting with the activity A_h. The influence of the rehydration procedure is incorporated in all activities. One rehydration procedure was used in all studies of this thesis. The addition of carbohydrates, or the addition of a carrier may further influence the activity, which leads to the activity before drying A_0. After drying the activity A_{dr} is reached. The residual activity after drying was defined as A_{dr}/A_0, and was used in chapter 2 to 5 as a measure of the dehydration inactivation. Storage leads to a further decrease in activity to A_{st}. The residual activity after storage can therefore be defined as A_{st}/A_{dr}. The overall residual activity covering the whole process is A_{st}/A_h. In the design of a process for the production of dried starter cultures, A_{st} will finally be the major parameter.

Growth/Harvest

The influence of growth conditions has been elaborated in Chapter 4. Variations in growth conditions (like the application of stress during growth) did not result in improvement of the residual activity of *L. plantarum* after drying. The influence of process parameters (like initial cell concentration) on cells grown without stress

conditions can not always be extrapolated to cells grown with stress conditions (like osmotic stress) (Chapter 3). The activity after harvest (A_h) of the *L. plantarum* cell pellet, grown in enriched MRS without stress conditions, was 2.42 ± 0.25 dpH/min/g dry cells.

Additives/Carrier

Carrier

In several drying methods the use of carrier material is necessary. Before fluidized bed drying, the cell pellet must be mixed with a carrier in order to be able to produce fluidizable particles. The addition of a carrier may result in a change of A_0 with respect to A_h. Native potato starch was used as a carrier in the fluidized bed drying experiments reported in chapter 5. The addition of starch resulted in an average A_0 of 2.14 ± 0.24, which was a decrease of 12 % compared to the activity of fresh cell pellet (A_h). The influence of carrier addition should be incorporated in the design of a drying process.

Additives

In Chapter 5 the protective effect was demonstrated of several carbohydrates during fluidized bed drying. The influence of carbohydrate addition on A_0, however, has not been considered in that chapter. A cell-carbohydrate-starch mixture was extruded to granules (as described in Chapter 5) before fluidized bed drying. A_0 was the activity of *L. plantarum* in the granules before drying. Remarkably, the absolute glucose fermenting activity of *L. plantarum* was significantly increased after the addition of some carbohydrates. The A_0 of paste with fructose, glucose and trehalose (not protective during drying based on A_{dr}/A_0) was 3.41, 2.92 and 2.64 dpH/min/g dry cell, respectively compared to 2.14 ± 0.24 dpH/min/g dry cell for *L. plantarum*-starch paste without carbohydrates (Table 1). A_0/A_h shows that the preparation of the paste with fructose, glucose and trehalose resulted in an increase in activity compared to the activity after harvest. The addition of sucrose resulted in a decrease in A_0 after paste preparation (1.77) compared to the blank. When the degree of protection by the different sugars was compared using the absolute activity after drying A_{dr} the most protective carbohydrates were maltose, glucose and sorbitol (Table 1). Comparison of these results with the results of Chapter 5, where glucose is not protective, illustrates that the order of protective carbohydrates depends on the parameter that is chosen to measure the degree of protection.

Table 1 Influence of carbohydrates on the absolute activity of *L. plantarum* in a *L. plantarum*-sugar-starch paste before and after fluidized bed drying

Carbohydrate	A_0 [dpH/min/g dry cell]	A_0/A_h * [-]	A_{dr} * [dpH/min/g dry cell]	A_{dr}/A_0 * [-]
no sugar	2.14	0.88	1.00	0.47
sucrose	1.77	0.73	1.17	0.66
lactose	2.00	0.83	1.15	0.58
maltose	2.17	0.90	1.65	0.76
trehalose	2.64	1.09	1.28	0.49
glucose	2.92	1.21	1.52	0.52
fructose	3.41	1.41	0.98	0.29
sorbitol	1.96	0.81	1.52	0.78

* X = 0.1 g H_2O/g dry weight

It is not possible that the observed increased activity after the addition of some carbohydrates was caused by an increased number of cells, because the mixing time (10 min) was too short to allow for substantial growth of the cells. It was verified whether the presence of these carbohydrates in the activity test could explain the effect of the carbohydrates on the absolute activity, because *L. plantarum* is capable of converting all carbohydrates mentioned in Table 1 (12). Experiments with carbohydrates added to the test liquid showed that the amount of added carbohydrates did not influence the activity measurement. This can be expected, because glucose is present in excess as a result of the addition of glucose in the buffer medium (6). Furthermore, it is not likely that the bacteria convert other carbohydrates than those present in the growth medium, because the transport proteins are adapted to the carbohydrates that are the main substrate in the medium (10) (glucose and fructose in enriched MRS). The time period of the activity measurement is too short for the transport proteins to adapt to the other sugars present in the medium. The importance of the absolute activity for the overall process will be discussed in relation to the addition of carbohydrates later in this chapter.

Drying

The drying parameters play an important role in the residual activity of *L. plantarum* after drying. The dehydration inactivation depends on the residual moisture content. However, systems with different compositions may have an unequal moisture

distribution and therefore the water activity rather than the overall moisture content is the parameter to use in the comparison of these systems (Chapter 5). The drying temperature and drying time were important for the occurrence of physiological adaptations (Chapter 2). Furthermore, an increased initial cell concentration could improve the residual activity after drying (Chapter 3). In this paragraph two drying methods will be discussed: fluidized bed drying and contact sorption drying. Both drying methods involve the use of a carrier. First the influence of the carrier in fluidized bed drying will come up for discussion. Second, contact sorption drying will be introduced. The results of the drying methods will be discussed in the light of the hypotheses postulated in this thesis.

Carrier in fluidized bed drying

The influence of the carrier material on the residual activity after fluidized bed drying has hardly been described in literature. Starch was used as a carrier in fluidized bed drying (7, Chapter 5). In this paragraph the influence was studied of two completely different carriers in fluidized bed drying: a hydrophilic and a hydrophobic non-biopolymer carrier. Two Aerosil® (Degussa) types were applied: Aerosil 200 (Degussa), a hydrophilic silicon oxide with Si-OH-groups on the surface and Aerosil R972 (Degussa), a hydrophobic silicon oxide with Si-CH_3-groups on the surface.

L. plantarum was mixed with 0.09g Aerosil R972/g cell pellet or with 0.07 g Aerosil 200/g cell pellet to form a well extrudable paste. Fluidized bed drying experiments were performed (30°C) of which the results are presented in Figure 2 together with the data with starch as carrier. The results with the hydrophilic Aerosil 200 and starch as a carrier were similar, opposite to the results with the hydrophobic Aerosil R972. With Aerosil R972 as a carrier residual activities increased significantly to ranges from 70 to 80 % at a residual moisture content of 0.07 g H_2O/g dry weight. The hatched triangle covers optimal data obtained from convective drying of layer experiments from Chapter 2. In these experiments physiological adaptations were assumed to occur.

In order to elucidate this phenomenon, first, the influence of the Aerosil carriers on the desorption isotherms was determined. Desorption isotherms of both Aerosil types and of the pastes were measured as described in Chapter 5. The hydrophobic Aerosil R972 absorbs hardly any water (9) what can be seen from the desorption isotherm (Figure 3). The desorption isotherms of the pastes with both Aerosil types were slightly below the isotherm of *L. plantarum* (Figure 3). The Aerosil addition did not influence the desorption isotherm of *L. plantarum* to a large extent, as could be expected because of the small amount of Aerosil added. Therefore, it may be assumed, that both the

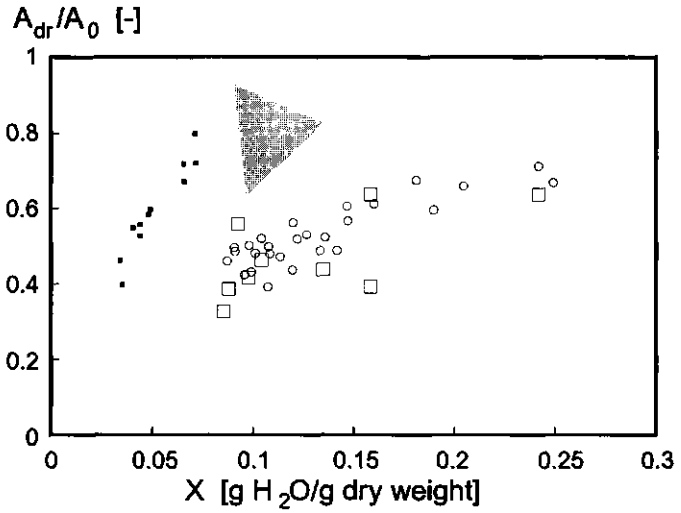

Figure 2 Residual activity of fluidized bed dried *L. plantarum* (30°C) versus moisture content with different carriers: Aerosil 200 (□); Aerosil R972 (•); Starch (o). The hatched triangle ( ) covers optimal data from drying experiments from Chapter 2.

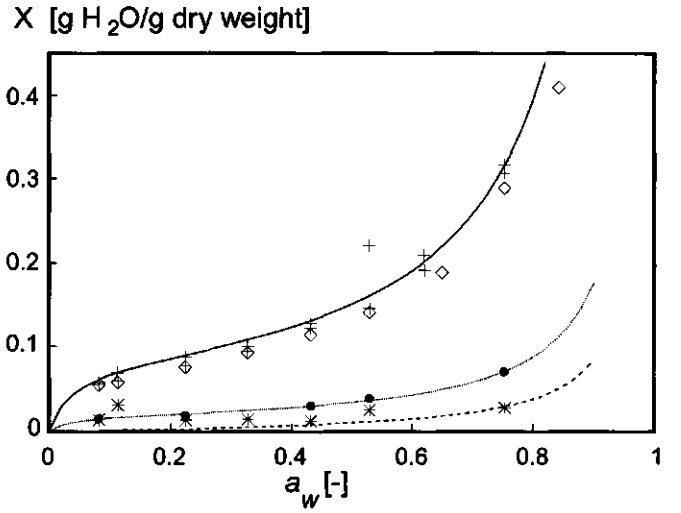

Figure 3 Desorption isotherms of *L. plantarum*-Aerosil 200 paste (+), *L. plantarum*-Aerosil R972 paste (◇), Aerosil 200 (•) and Aerosil R972 (✳) measured at 25°C above saturated salt solutions and fit of desorption isotherm of *L. plantarum* (————), Aerosil 200 (·····) and Aerosil R972 (- - - - - -).

overall moisture content and the water activity well represent the degree of dehydration of the individual *L. plantarum* cells. Furthermore it means that the water activity and moisture distribution can not explain the high residual activity of cells dried with Aerosil R972.

Second, the possibility for physiological adaptations was considered. The drying temperature was 30°C, a temperature at which adaptations should be possible (Chapter 2). Furthermore the drying rate is of importance. The characteristic drying time (τ_d) was defined as the time to reach $a_w = 0.92$, since this is the region where the adaptations were expected to occur. τ_d was in the order of a few minutes for fluidized bed drying of *L. plantarum*-starch paste as well as of paste with the hydrophilic Aerosil 200. Compared to this τ_d fluidized bed drying of paste with the hydrophobic Aerosil R972 was slightly faster. Since the estimated τ_d values were all in the same order of magnitude the difference in drying rate can not explain the high residual activity with the hydrophobic Aerosil R972.

Aerosil 200 and Aerosil R972 have completely different characteristics. The hydrophilic Aerosil 200 can form H-bridges with water molecules. In a dispersion of Aerosil R972 and water Aerosil forms a layer around water droplets, thus keeping a stable dispersion (9). Possibly, Aerosil R972 forms a layer around the cells in the *L. plantarum*-starch paste. Therefore, the influence of Aerosil R972 on the water structure around the cells should be considered, in order to understand why the use of Aerosil R972 as a carrier for *L. plantarum* resulted in high residual activities at low water activities. This interesting phenomenon asks for further investigation.

Contact sorption drying

A completely different method of dehydrating bacterial cells is contact sorption drying. The principle of this method is mixing of the cells with a dry carrier, resulting in water transport from the cells to the dry carrier material. This method has been used as a method of preserving starter cultures (3, 4).

In our experiments *L. plantarum* cells were grown and harvested as described in Chapter 2. Droplets (∅ 1.5 mm) of a *L. plantarum*-cell suspension (C_0 = 0.19 g dry cell/g sample), produced using a syringe, were mixed at ambient temperature (23°C) with native potato starch (Perfectamyl P-6, Avebe, The Netherlands) that was previously dried ($X_0 = 0.008 \pm 0.006$ g H_2O/g dry weight). The resulting mixture should be considered as a starch matrix containing *L. plantarum*-cell droplets. The mixing ratio was chosen such that the overall moisture content of the mixture had a value between 0.04 and 0.2 g H_2O/g dry weight. In a number of experiments the size of the cell droplets was decreased from the original 1.5 mm to about 0.2 mm by pressing the complete mixture through a sieve plate, 5 minutes after mixing. The glucose fermenting

activity test (6) was used to determine the activity of *L. plantarum*. Five grams of the *L. plantarum*-starch mixture was resuspended in 33 ml 0.01 M potassium phosphate buffer (0.01 M KH_2PO_4/K_2HPO_4 in 0.15 M NaCl, pH = 7), in which the pH-decrease was measured at 35 °C after the addition of 1 ml glucose buffer (0.35 g glucose/g 0.01 M potassium phosphate buffer). For the A_0-measurement 5 g of starch was added to 33 ml 0.01 M potassium phosphate buffer before the addition of the *L. plantarum*-cells, to correct for the presence of starch. Starch affected the titration curve of lactic acid.

Figure 4 shows the residual activity after contact sorption drying of *L. plantarum* after 1 hour versus the overall moisture content of the mixture. It is worth mentioning that this moisture content is the average moisture content of the mixture containing dry starch and (initially) wet cell droplets. The residual activity of the mixture with droplets of 1.5 mm was around 100%, whereas the activity of the mixture with small droplets was around 50%, which was similar to fluidized bed drying results (Figure 2). A possible explanation for this remarkable difference was that the small droplets were further dehydrated than the big droplets. The decreased droplet size resulted in an increased surface area and thus the moisture transport from the cells to the starch was enhanced. To illustrate this the characteristic drying time of the droplet was estimated using the Fourier time, assuming that the mass transfer is internally controlled. The

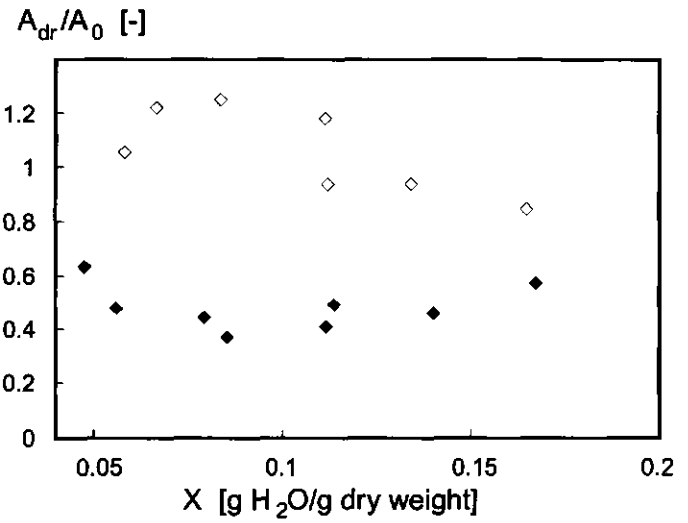

Figure 4 Residual activity of contact sorption dried *L. plantarum* after 1 hour (C_0 = 0.19 g dry cells/g sample) versus overall moisture content: droplet size: (◇) about 1.5 mm; (◆) about 0.2 mm.

characteristic drying time of a droplet with a diameter of 1.5 mm is 63 hours, assuming a Fourier time of 0.1 and a diffusion coefficient of 10^{-12} m^2/s. A 10-fold decrease in the droplet size results in a 100-fold decrease in drying time. The estimated drying time of the 0.2 mm droplet was 1.1 hour. Consequently, the 1.5 mm droplets have been far from equilibrated after 1 hour, whereas the 0.2 mm droplets were dried to some extent. The difference in residual activity between droplets with different sizes, dehydrated by contact sorption drying, can therefore be explained by the difference in local moisture content in the droplet. The high residual activities with a scatter around 100% for the sample with the 1.5 mm droplets were in agreement with results of another experiment, where a suspension of cells was kept at 30°C for several hours, without drying. The residual activity of the suspension was 100% or more for over 6 hours (data not shown).

Following the results of Chapter 3, where the initial cell concentration C_0 proved to be of significant influence on the residual activity of *L. plantarum* after convective drying of a layer, the influence of C_0 on the residual activity after contact sorption drying was studied. The drying procedure was performed as described above using cell suspensions with C_0 values of 0.19, 0.12 and 0.06 g dry cell/g sample. The droplet size was reduced as described above, 5 minutes after mixing. There was no significant influence of C_0 after 1 hour drying (Figure 5). A high C_0 was expected to be

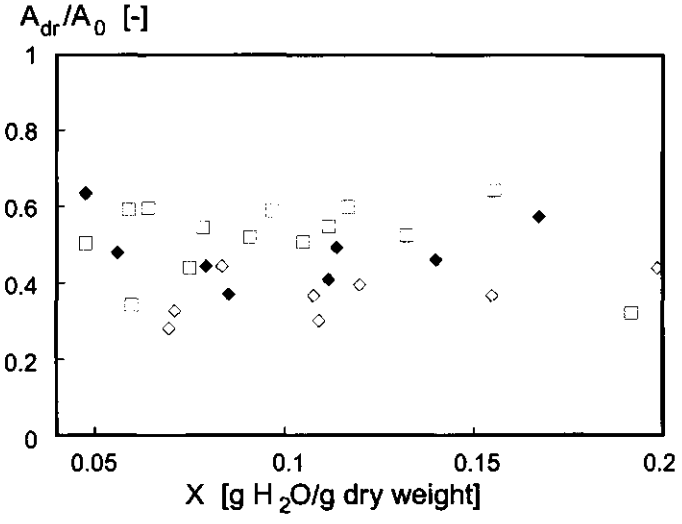

Figure 5 Residual activity of contact sorption dried *L. plantarum* after 1 hour (droplet size 0.2 mm) versus overall moisture content: Influence of initial cell concentration: (◆) C_0 = 0.19 g dry cell/g sample; (◇) C_0 = 0.12 g dry cell/g sample; (□) C_0 = 0.06 g dry cell/g sample.

advantageous (Chapter 3). The cells may protect each other from the deleterious influences from the environment (1). A possible explanation for the absence of a significant influence of C_0 in contact sorption drying could be that the need of protection is eliminated as a result of the abundant presence of starch, diminishing the influences of the environment.

Each experiment shows a flat profile of residual activity versus moisture content (Figure 5). The residual activity was dependent on the local moisture content in the cell droplets rather than on the overall moisture content. The overall moisture content was fixed by the mixing ratio of cell suspension and starch. The individual droplets were only aware of their local environment, which did not depend on the amount of starch present in the total mixture. When complete equilibrium is established over the mixture, it can be expected that the flat profile will be gone. After one hour, and even after one day (data not shown) the flat profile was still there. When the time to monitor the residual activity after the mixing is increased to several days, the drying step will overtaken by the storage step. In contact sorption drying it is hard to distinguish between the drying and storage step.

In general contact sorption drying did not result in higher residual activities compared to convective drying of a layer or fluidized bed drying. In contact sorption drying the overall moisture content was no decisive parameter, because there was no uniform moisture distribution. The initial cell concentration of the cell suspension had no influence on the residual activity after contact sorption drying.

Storage

In the preservation of starter cultures it is important to retain activity after prolonged storage. The beneficial influence of process parameters during drying may have an adverse effect during storage. In this paragraph two aspects in this respect will be discussed: the influence of residual moisture content and the influence of protectants.

Moisture content

Whereas the residual activity after drying decreases with decreasing moisture content (7, 8), the residual activity after storage is enhanced at low residual moisture contents (2). In order to demonstrate this effect experiments have been performed with samples of contact sorption dried and fluidized bed dried *L. plantarum*. The samples were stored at

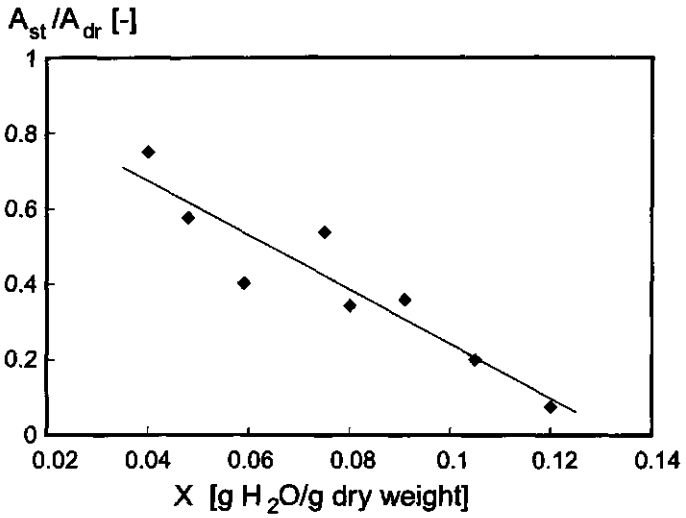

Figure 6 Residual activity after storage of contact sorption dried *L. plantarum* (droplet diameter 2 mm) versus overall moisture content after 28 days storage at 30°C. The line is a linear regression.

30°C in vacuum sealed bags (Allvac-PIE), which were impervious to light and air. After a storage time varying from 1 to 5 weeks the glucose fermenting activity after storage (A_{st}) was determined as described elsewhere in this thesis.

Figure 6 demonstrates that the residual activity after storage (A_{st}/A_{dr}) of contact sorption dried *L. plantarum* (droplet diameter 2 mm) after 28 days storage was inversely proportional to the overall moisture content. Where the residual activity after contact sorption drying (A_{dr}/A_0) showed a flat profile versus overall moisture content (Figure 5) the storage stability decreased at increasing moisture content, as expected (2). Figure 7 shows the decrease of the residual activity during storage, with a stronger decrease at higher moisture contents. Also the absolute A_{st} was lower for the samples with the higher moisture contents. Figure 7 shows that, at equal moisture content, the storage stability of the fluidized bed dried cells was to some extent higher than of cells dried using contact sorption drying. Probably, the local moisture content in the cell droplets (contact sorption drying) was still higher than the overall moisture content.

The residual activity after drying decreased with decreasing moisture contents, whereas the storage stability increased at decreasing moisture contents. This opens a possibility for optimisation of the production of active starter cultures.

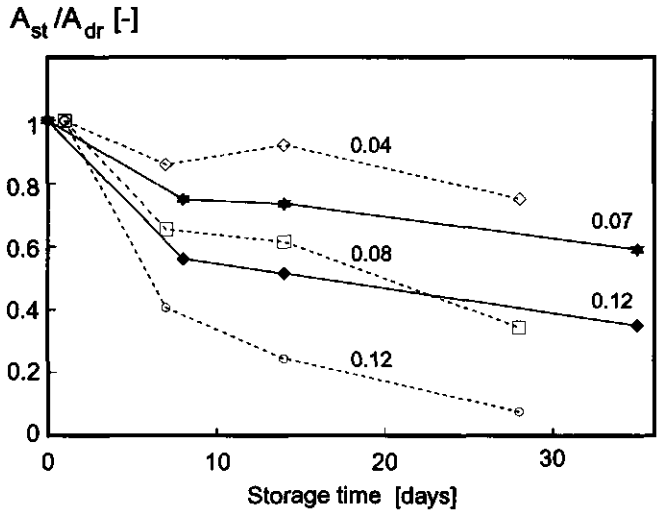

Figure 7 Residual activity after storage of dried *L. plantarum* at 30°C in vacuum sealed bags (Allvac PlE): Contact sorption dried *L. plantarum* (C_0 = 0.24 g dry cells/g sample) (open symbols, - - - - - -) and fluidized bed dried *L. plantarum* (closed symbols, ————). The numbers show the overall moisture content (g H_2O/g dry weight).

Additives

The different properties of carbohydrates all together determine the effectiveness of carbohydrate addition on the residual activity after drying and after storage. However, advantageous properties should not be counterbalanced by the disadvantageous properties. Sorbitol and sucrose are known as a radical scavengers (11), protecting enzyme systems against oxidative damage. The disadvantage of sorbitol during storage is its low glass transition temperature (T_g) of -2 °C (5). When a sample is in the glassy state, the diffusion rate has diminished to very low values, which is advantageous during storage, since the reaction rate of deleterious reactions will decrease. The disadvantage of maltose and lactose in storage is their being a reducing sugar, which may cause Maillard reactions. The reducing activity of glucose and fructose is even higher which makes these sugars less appropriate as storage stabilisers.

The influence of lactose, maltose and sucrose on the activity of fluidized bed dried *L. plantarum* during the complete process was monitored (Figure 8). The first decrease in activity after growth was during the addition of sugars and starch (between A_h and A_0). After drying the activity of the paste with maltose was highest, followed by the

paste with sucrose and lactose and the paste without sugars. During storage the activity of the paste with maltose decreased firmly. After 2 weeks of storage the activity of the paste without sugars and of the paste with sucrose was even higher than that of maltose. The presence of these carbohydrates does not seem to be profitable for activity preservation after storage as can be seen after 3 weeks, where the activity of the paste without additives is equal to the activity of the paste with sucrose. The bad storage stability of maltose might probably be related to its reducing sugar properties. Lactose had always the lowest activity, which was probably due to the formation of lactose crystals, which were identified using X-ray diffraction (Chapter 5). Monitoring of the activity during the complete process, as performed in Figure 8 gives more insight in the mode of action of the carbohydrates. Maltose was protective during drying, whereas none of the sugars tested improved the residual activity after storage. This type of knowledge can be used to design protective mixtures of carbohydrates containing drying protectants, as well as storage stabilisers.

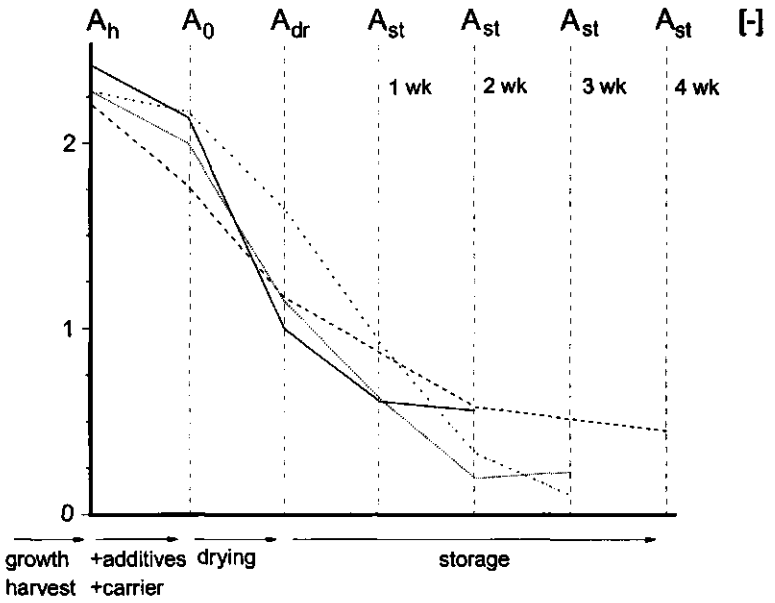

Figure 8 The influence of the addition of carbohydrates on the absolute activity (A) of *L. plantarum* during the production process: no additives (X_{dr} = 0.12 g H_2O/g dry weight, ——————); lactose (X_{dr} = 0.08 g H_2O/g dry weight, ·····); maltose (X_{dr} = 0.14 g H_2O/g dry weight, - · - · - · - · - ·); sucrose (X_{dr} = 0.12 g H_2O/g dry weight, - - - - - -).

Concluding remarks

This thesis gives an overview of the influence of several process parameters on the residual activity of *L. plantarum*. The drying procedure has proved to be an important process step in reducing the dehydration inactivation. The drying procedure can be optimised if the mechanism of the physiological adaptation is known. Therefore, further research on this topic is necessary. The addition of protective carbohydrates was the other process step that improved the activity of *L. plantarum* after drying. In the choice of protective additives it is important to consider the influence on each process step. Some carbohydrates may protect during drying, where others may protect during storage. Physical effects should be considered, like the change in moisture distribution as a result of carbohydrate addition, as well as physiological mechanisms, like membrane protection. The physical and physiological approach have given us a better understanding of the mode of action of carbohydrates, despite the fact that the exact mechanism of protection has not been found. Variation in growth conditions did not lead to significant improvement of the drying tolerance of *L. plantarum*. Research on growth conditions will lead to a better understanding of the physiology of bacteria, but until now it did not provide tools for the improvement of the production of dried *L. plantarum*.

With this thesis a comprehensive approach to the drying of *L. plantarum* is presented. This work can be used as a tool in the development of a production process for dried starter cultures. Furthermore, the presented work has led to a new research question: the mechanism of physiological adaptation during drying.

Acknowledgements

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List of symbols and abbreviations

A	glucose fermenting activity	ΔpH/min/g dry cells
a_w	water activity	-
C_0	initial cell concentration	g dry cells/g sample
c_g	GAB-constant	-
d	dry weight fraction	-
DMRS	diluted MRS medium	
DPG	diphosphatidylglycerol	
DPPC	dipalmitoylphosphatidylcholine	
dw	dry weight	kg
EMRS	enriched MRS medium	
FTIR	Fourier transform infrared spectroscopy	
k	GAB-constant	-
k_i	specific inactivation rate	s^{-1}
lysyl-PG	lysyl-phosphatidylglycerol	
MLV	multilamellar vesicles	
N	cell counts	
PC	phosphatidylcholine	
PG	phosphatidylglycerol	
POPC	palmitoyl-oleoyl-phosphatidylcholine	
POPG	palmitoyl-oleoyl-phosphatidylglycerol	
RA	residual activity	-
RH	relative humidity	-
T	drying temperature	°C
T_g	glass transition temperature	°C
TLC	thin layer chromatography	
T_m	membrane phase transition temperature	°C
X	moisture content	g H_2O/g dry solids
X_{w1}	GAB-constant	g H_2O/g dry solids

greek

λ	wavelength	Å
τ_d	characteristic drying time	h

subscript	
0	before drying, initial
calc	calculated
cell	in cell
dr	after drying
h	after harvest
i	component i
meas	measured
overall	overall
st	after storage

Summary

Starter cultures of lactic acid bacteria are widely used in the production of food and feed. Lactic acid bacteria are also used as probiotics in human and animal nutrition, because of a supposed beneficial influence on the intestinal flora. Preservation of large quantities of bacteria by convective drying is an economical alternative compared to freezing and freeze drying. Unfortunately, inactivation of bacteria occurs during drying. Two types of inactivation can be distinguished: thermal and dehydration inactivation. Thermal inactivation can be minimised using low temperatures or short times. Dehydration inactivation becomes the main problem in convective drying. The objective of this study was first to identify process conditions which favour the production of active dried *Lactobacillus plantarum* and second to determine the physical and physiological mechanisms explaining the influence of the process parameters on the dehydration inactivation.

The production of dried starter cultures involves the following process steps: growth, harvest, any addition of protectant or carrier material, drying and storage. Each step involves parameters that may influence the activity of the microorganism.

The drying parameters studied were drying temperature and drying time. A number of drying methods with varying drying rates were used. The drying procedure proved to have an important influence on the residual activity of the dried *L. plantarum*. The highest residual activity was achieved after convective drying of a layer at 30°C. The residual activities were depressed using lower drying temperatures or using drying methods with a very short or a very long drying time. These experiments confirmed the following hypothesis: during drying at 30°C a physiological adaptation of the micro-organism occurs which is time dependent.

The influence of cell concentration before drying was studied for cells grown under standard growth conditions as well as for cells grown with osmotic stress. The residual activity of cells grown under standard conditions was higher when high initial cell concentrations were used. Variation in initial cell concentration did not influence the residual activity of cells grown with osmotic stress.

Growth of micro-organisms under stress situations can result in adaptations, which might improve the residual activity after drying. Therefore, the influence of the following growth conditions was studied on the dehydration inactivation of *L. plantarum*: composition of the growth medium, osmotic stress, pH-control during growth, growth phase and reactor concept. The highest activities were achieved applying optimal growth conditions. Stress during growth did not result in an improvement of the residual activity after drying.

Carbohydrate addition could improve the residual activity after fluidized bed drying. There was a difference in effectivity of the carbohydrates. Sorbitol, maltose and sucrose addition resulted in increase in residual activity, whereas lactose, trehalose, glucose and fructose were not protective. In order to get a better understanding of the mechanism of protection, a physical and a physiological approach were chosen.

Water activity and moisture distribution at a given overall moisture content are influenced by the addition of carbohydrates. Desorption isotherms have been measured in order to determine the moisture distribution in a mixture. Calculations showed that significant shifts may occur in water activity and moisture distribution as a result of the presence of carbohydrates. These shifts could not explain the protection. Former research showed that dehydration inactivation is associated with cell membrane damage. This damage may occur when the cell membrane undergoes a phase transition from the liquid crystalline phase to the gel phase and vice versa during drying and rehydration. Membrane phase transition temperatures were determined using Fourier transform infrared spectroscopy in order to study whether carbohydrates influence the phase transition behaviour of the membrane by interaction with the phospholipid head groups of the membrane. Surprisingly, there was only a small increase in phase transition temperature during drying, which was independent of the presence of carbohydrates. This phase behaviour could be attributed tot the molecular structure of phosphatidylglycerol and lysyl-phosphatidylglycerol, the predominant phospholipids in *L. plantarum*. The relatively high activity of dried *L.plantarum* can be attributed to its low membrane phase transition temperature.

Finally the complete process was considered. The drying procedure and the use of additives were the most effective process steps in improving the activity of dried *L. plantarum*. The applied process oriented approach has led to a better understanding of the importance of several parameters.

Samenvatting

Melkzuurbacteriën worden gebruikt als startercultures in de productie van levensmiddelen en veevoer. In de humane en dierlijke voeding worden melkzuurbacteriën ook wel gebruikt als probiotica vanwege een veronderstelde positieve werking op de darmflora. Convectief drogen (= drogen met lucht) is een goedkope conserveringsmethode voor startercultures in vergelijking met vriezen en vriesdrogen. Een belangrijk nadeel van drogen is dat niet alle bacteriën dit proces overleven. Twee inactiveringsmechanismen zijn te onderscheiden: thermische en ontwaterings-inactivering. Door het gebruik van een lage droogtemperatuur of een korte droogtijd kan de thermische inactivering tot een minimum terug gebracht worden. Ontwaterings-inactivering het grootste probleem bij het drogen van bacteriën. Het doel van dit onderzoek is: (1) het bepalen van de factoren in het productieproces die leiden tot een verbetering in de productie van actieve gedroogde starterculturen; (2) het ophelderen van de fysische en fysiologische mechanismen die de invloed van de gevonden factoren kunnen verklaren. In dit onderzoek is de invloed van verschillende procesvariabelen op de ontwateringsinactivering van *Lactobacillus plantarum* onderzocht door gebruik te maken van een procesgerichte aanpak.

Het productieproces voor de bereiding van gedroogde starterculturen bestaat uit de volgende stappen: het kweken van de micro-organismen; het wassen en concentreren; het (eventueel) toevoegen van beschermende stoffen of een dragermateriaal; het drogen; en het bewaren. Elke processtap zijn er variabelen die de activiteit van het micro-organisme kunnen beïnvloeden.

Bij het droogproces zijn de variabelen droogtemperatuur en droogtijd onderzocht. Een aantal droogmethoden met verschillende droogsnelheden werden met elkaar vergeleken. Het is gebleken dat de droogprocedure van bijzonder belang was voor de activiteit van het gedroogde product. De hoogste restactiviteit werd behaald bij het convectief drogen van een laagje cellen bij een droogtemperatuur van 30°C. Drogen bij 4°C of het gebruik van droogmethoden met een zeer korte of met een lange droogtijd leverden lagere restactiviteiten op. Deze experimenten bevestigden de volgende hypothese: Tijdens het drogen bij 30°C ondergaat het micro-organisme een tijdafhankelijke fysiologische aanpassing.

De invloed van de celconcentratie voor drogen is onderzocht bij cellen die gekweekt zijn onder standaard omstandigheden en bij cellen die gekweekt zijn onder osmotische stress. Bij standaard gekweekte cellen was de restactiviteit na drogen het hoogst als een hoge celconcentratie voor drogen werd gebruikt. De restactiviteit van

cellen die onder osmotische stress gekweekt zijn was lager dan van standaard gekweekte cellen en werd niet beïnvloed door de celconcentratie voor drogen.

Micro-organismen kunnen zich aanpassen aan stress omstandigheden tijdens het kweken. Mogelijk leiden deze aanpassingen eveneens tot een hogere restactiviteit na drogen. Om dit te onderzoeken is de invloed van de volgende kweekcondities op de restactiviteit van *L. plantarum* na drogen bekeken: samenstelling van het kweekmedium, osmotische stress, pH-controle, groeifase van de micro-organismen en reactor concept. De beste resultaten werden bereikt bij het gebruik van optimale kweekcondities. Toepassing van stress omstandigheden tijdens het kweken leidde niet tot verbetering van de restactiviteit na drogen.

Het toevoegen van suikers kan leiden tot een verhoogde restactiviteit na drogen. Sorbitol, maltose en sucrose zorgden voor een aanzienlijke activiteitsverhoging, terwijl lactose, trehalose, glucose en fructose niet effectief waren. Om een beter begrip te krijgen van de werking van deze suikers is zowel naar een fysische als een fysiologische verklaring gezocht.

Vochtgehalte, wateractiviteit en vochtverdeling worden beïnvloed door de toevoeging van suikers. De vochtverdeling in een mengsel werd berekend met behulp van gemeten desorptie isothermen. Uit deze berekeningen bleek dat er duidelijke verschuivingen in wateractiviteit en vochtverdeling optraden door de suikertoevoeging. Deze verschuiving kon echter de beschermende werking van de suikers niet verklaren. Uit eerder onderzoek was gebleken dat ontwaterings-inactivering samen gaat met schade aan het celmembraan. Deze schade kan optreden als het membraan tijdens drogen overgaat van de vloeibare naar de vaste (gel) fase. Er werd onderzocht of de suikers de fasenovergang van het membraan kunnen beïnvloeden door interactie met de fosfolipide kopgroepen in het membraan. De fasenovergangstemperaturen werden bepaald met Fourier transform infrared spectroscopy. Het bleek dat de stijging van de fasenovergangstemperatuur tijdens drogen minimaal was en dat deze onafhankelijk was van de aanwezigheid van suikers. Dit fase gedrag kan toegeschreven worden aan de structuur van phosphatidylglycerol en lysylphosphatidylglycerol, de fosfolipiden in *L. plantarum*. De lage fasenovergangstemperatuur is een van de redenen voor de relatief hoge restactiviteit van gedroogde *L. plantarum*.

Tenslotte werd het proces in zijn geheel bekeken. Het bleek dat de processtappen drogen en het doen van toevoegingen het meeste effect hadden in de verbetering van de activiteit van gedroogde *L. plantarum*. Het bekijken van het gehele proces heeft geleid tot een beter inzicht in het belang van de verschillende procesvariabelen.

Nawoord

Viereneenhalf jaar ben ik bijna dagelijks van Bakel op en neer naar Wageningen gereden om promotie-onderzoek te doen. Tijdens deze ritten was er volop tijd om de gebeurtenissen van de dag te overdenken. In dit nawoord wil ik een aantal mensen bedanken omdat zij mijn gedachten bezig hielden, waardoor ik niet in slaap sukkelde achter het stuur, en vooral omdat zij een rol speelden in de totstandkoming van dit proefschrift.

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Bij de keuze voor dit AIO-onderzoek woog het argument dat Klaas van 't Riet promotor was zwaar tegen het nadeel van de reistijd op. Klaas kan met zijn aanstekelijk enthousiasme voor het onderzoek motiveren, stimuleren, het beste uit de mensen halen en door een originele benadering nieuwe impulsen geven aan het onderzoek.

Onderweg naar huis stond ik met Edwin Kets in de file. Hij maakte met Jan de Bont deel uit van het Industriële Microbiologie-team dat deel uit maakte van het totale 'droogproject'. Zij zorgden tijdens de gezamenlijke besprekingen voor de noodzakelijke microbiologische accenten en voor de nodige discussies over drogestofbepalingen. De resultaten van Edwin hebben een aanzienlijke invloed gehad op dit proefschrift.

Na een gesprek met Folkert Hoekstra en Wim Wolkers van plantenfysiologie kwam ik altijd later thuis dan gewoonlijk. Uren hebben we gedelibreerd over resultaten van FTIR-metingen en over T_m's, PG's en PC's. Wim stond altijd klaar om ervoor te zorgen dat mijn studenten betrouwbare FTIR-metingen produceerden.

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De praktische ideëen die ik o.a. tijdens de autorit opdeed werden altijd vakkundig uitgevoerd door werkplaats, fotolocatie, tekenkamer en I&D.

Voordat ik de reis naar Bakel kon ondernemen moest ik eerst kamer 720 verlaten. Op het proceskunde-eiland 720 zorgde Ida Günther voor gezelligheid en voor de plant; Taco Wijtzes voor duikverhalen en koffie; Rob van den Hout voor verrassende gespreksonderwerpen; Dirk Martens voor doorzettingsvermogen en voor de telefoon; Imke Leenen voor thee en activiteit; en Marian Vermuë voor huis- en computerperikelen.

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Maar natuurlijk reisde ik elke dag terug naar Jeu, die in ons prettige huisje in Bakel zorgde dat ik de nodige afstand kon nemen van het onderzoek.

Curriculum Vitae

Leonie Johanna Maria Linders werd op 17 december 1966 geboren in Boxmeer. Zij bezocht het Elzendaalcollege te Boxmeer waar zij in 1985 het VWO diploma behaalde. In datzelfde jaar begon zij haar studie Levensmiddelentechnologie aan de Landbouwuniversiteit te Wageningen. Haar hoofdvak was Proceskunde. Daarnaast deed zij een gecombineerd afstudeervak Chemie/Proceskunde voor Unilever Research Laboratorium te Vlaardingen. Zij deed stages bij Campina Melkunie in Veghel en bij Sanofi Pharma Industrie in Frankrijk. In 1991 rondde zij haar studie af. Van 1992 tot 1996 was zij werkzaam als Assistent in Opleiding bij de sectie Proceskunde van de Landbouwuniversiteit te Wageningen.