Innovative product formulations

applying the fluidised bed technology

Dissertation

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Abstract

Fluidised bed technology offers process options suitable for product preservation and formulation. The first part of this thesis is dedicated to two different drying technologies - lyophilisation and fluidised bed drying - and the influence of protective compounds on process survival and storage stability of two lactic acid bacterial strains. *Lactobacillus plantarum* turned out to be more sensitive to both drying methods than *Enterococcus faecium*, which may be explained by the higher surface area of *Lactobacillus* cells, different fermentation conditions and lower initial bacterial concentration. Dehydration inactivation and oxidative stress made untreated cells of both strains suffer higher losses during fluidised bed drying. Trehalose and sucrose revealed overall best protection for both lactic acid bacteria during processing and storage. Elevated storage temperatures correlate with a higher decline of viable bacterial cells.

Secondly, the release behaviour of coating materials was investigated in order to obtain appropriate formulations for allelochemicals - biologically active substances affecting plants - to be used as biopesticides. Waxes and resins are useful to prolong the release of coated ingredients, while their mechanism of performance is mostly based on slow biological degradation or erosion. The evaluation of sustained release systems was carried out by encapsulating aluminium sulphate in four different coating substances and further by investigating the sustained release kinetics of the encapsulated compound. The study revealed that carnauba wax and tall oil rosin mediate slower release patterns than shellac and soya oil coatings. Depending on the desired release kinetics all four natural coating substances are potential candidates to be used for encapsulating allelochemicals. Besides providing a cost-effective alternative for preserving sensitive compounds, fluidised bed technology offers operational modes to increase the value of basic products.

Keywords: Fluidised bed technology, drying, lyophilisation, lactic acid bacteria, industrial starter cultures, protective additives, shelf life, coating, microencapsulation, allelopathy, biodegradation, controlled/sustained release, agrochemicals

Zusammenfassung

Die Wirbelschichttechnologie bietet Verfahren für Konservierung und Formulierung an. Einerseits wurden die Auswirkungen von Gefrier- und Wirbelschichttrocknung sowie von Schutzstoffen auf Milchsäurebakterien im Prozess und während der Lagerung untersucht. Im Vergleich zu *Enterococcus faecium* reagierte *Lactobacillus plantarum* empfindlicher auf beide Trocknungsmethoden. Erklärungen dafür könnten die größere Zelloberfläche von *Lactobacilli*, die unterschiedlichen Fermentationsbedingungen und die niedrigere Anfangszellkonzentration sein. Inaktivierung durch Austrocknung und oxidativer Stress verursachten höhere Einbußen in den Überlebensraten beider Stämme während der Wirbelschichttrocknung. Trehalose und Saccharose konnten beide Stämme während Trocknung und Lagerung am besten schützen. Erhöhte Lagerungstemperaturen führten zu höheren Abnahmen an lebensfähigen Bakterienzellen.

Andererseits wurde das Freisetzungsverhalten von Coatingsubstanzen untersucht, um geeignete Formulierungen für Allelochemikalien - biologisch aktive Substanzen, die auf Pflanzen einwirken - zu erhalten, die im Pflanzenschutz eingesetzt werden können. Wachse und Harze als Coating eingesetzt, verzögern die Freisetzung von verkapselten Substanzen, wobei der Mechanismus zumeist auf biologischem Abbau und Erosion beruht. Die Evaluierung von solchen Systemen wurde mittels Verkapselung von Aluminiumsulfat in vier verschiedenen Coatings und weiterer Untersuchung der Freisetzungskinetik der verkapselten Substanz durchgeführt. Carnaubawachs- und Tallharzcoating zeigten langsamere Freisetzungsdynamik als die Schellack- und Sojaölverkapselung. In Abhängigkeit von den Freisetzungseigenschaften sind alle vier natürlichen Substanzen geeignet für die Verkapselung von Allelochemikalien. Die Wirbelschichttechnologie bietet eine kostengünstige Alternative für die Konservierung von empfindlichen Stoffen und stellt weitere Verfahren zur Wertsteigerung von Produkten zur Verfügung.

Stichwörter: Wirbelschichttechnologie, Trocknung, Lyophilisation, Milchsäurebakterien, industrielle Starterkulturen, Schutzstoffe, Haltbarkeit, Coating, Mikroverkapselung, Allelopathie, biologischer Abbau, kontrollierte/verzögerte Freisetzung, Agrochemikalien

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1   General Introduction

Fluidised Bed Technology for Drying Processes

The fluidised bed process was primarily developed in the 1950s by the pharmaceutical industry in order to obtain dry, oral delivery systems for drugs applying so-called enteric coatings that enable transit and site directed controlled release of the sensitive active ingredient in the gastrointestinal tract (Dewettinck and Huyghebaert, 1999). In the meanwhile, other process industries, such as food, feed, agrochemical, cosmetics and other chemical industries, have adopted this technology to utilise its advantages in preservation and product formulation (Boerefijn and Hounslow, 2005; Guignon et al., 2003; Saleh et al., 2003). Fluidised bed technology is traditionally used for granulation and the drying and coating of powders, granules and spheres.

Principle

Fluidised bed technology is based on the intense interactions between particles, which are treated almost equally. Filtered and optionally dehumidified inlet air enters the product chamber through a perforated plate, thereby compensating the gravitational force and fluidising the product previously loaded or sucked into the granulator (Figure 1a). The fluidising air stream provides fluid-like or liquid-like properties to the particle bed, hence the terms fluidisation and fluidised bed (Figure 1b). The basic processes dominating fluidised bed technology - dehydration, heating and cooling - are enhanced due to the rapid exchange of heat and mass with air. Small particles carried by the air flow to the cylindrical expansion chamber are held back by filters positioned there (Figure 1c), and then they fall back into the conical product chamber and continue cycling throughout the process (Figure 1b). The process is primarily ruled by variation of product bed temperature and fluidisation air flow.
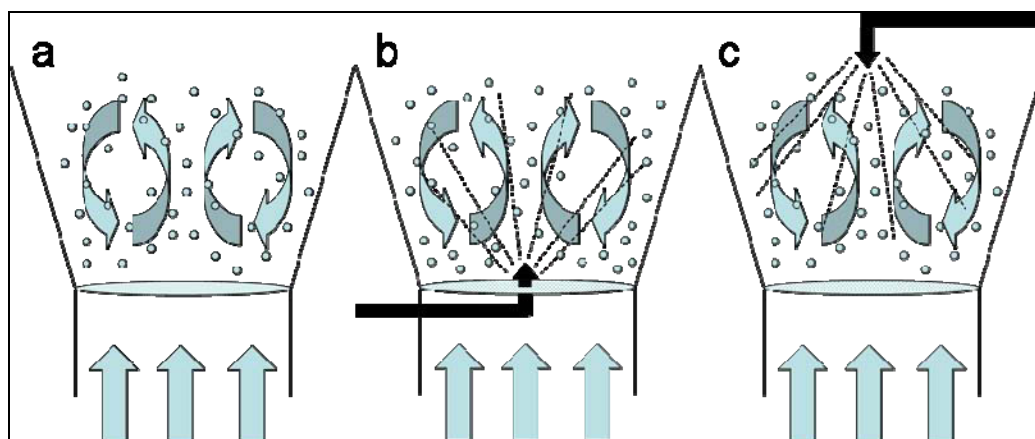
Applications

Depending on the operational mode there are mainly three processes namely drying, coating, and granulation possible, which are described in more detail in the following sections.

Figure 1 Photographs of the fluidised bed granulator at the Institute for Environmental Biotechnology, Department of Agrobiotechnology, IFA-Tulln, Austria, illustrating a. the lower part of the granulator next to a twin screw extruder, b. a detailed picture of the conical product chamber showing fluidised particles and the sampling device, and c. the filter unit located in the upper part of the granulator.

Apart from drying, for coating and granulation processes the application of a nozzle is required as device to spray coating or binder solution onto fluidised particles. Depending on the position of the spray nozzle bottom-spray and top-spray mode are to be distinguished (Figure 2b,c), while the latter is mainly applied in coating processes and the former position is basically used for granulation purposes. The bottom-spray position is further applied in the Wurster process, where a tube positioned above the spray nozzle enables better coating results than achieved with the conventional top-spray technique.

To increase the performance of the single process new systems like vibrating beds, centrifugal chambers or pulsed fluidisation (Barbosa-Canovas and Uliano, 2004) as well as unit modifications, such as circulating fluidised bed coating or tangential-spray in rotary fluidised bed coating are being developed and optimised continually (Dewettinck and Huyghebaert, 1999; Kage et al., 2003; Watano et al., 2004).

Figure 2 Schematic drawings of fluidised bed processes used for different applications: a. simple drying of moist particles, b. bottom-spray position mainly used for agglomeration/granulation applying appropriate binders, and c. top-spray position generally applied for coating of spherical carrier material.

The efficiency of fluidised bed techniques is dependent on process, ambient and thermodynamic variables. Appropriate modifications and combinations of these factors will result in thermal efficiency of the process and improved product quality (e.g. residual moisture content, coating quality, free-flowing powders).

Major process variables are

- batch size, fluidisation air velocity,

- inlet and bed temperature, fluidising air moisture,

- nozzle diameter and position,

- temperature, mass, viscosity, and surface tension of coating fluid or binder,

- spray rate, atomisation air pressure and temperature.

Together with the environmental variables ambient air temperature and relative humidity those parameters govern the thermodynamic variables outlet air temperature and relative humidity. Climatic conditions, i.e. strong variations of relative humidity in particular can play a significant role in the fluidised bed process making dehumidification of the fluidising air despite high costs inevitable. Next to the tackiness of the coating or binder substance applied, the moisture content of the bed determines agglomeration of particles in the case of aqueous solutions, while the accordance of bed and congealing temperature is ruling this effect, when molten substances are sprayed onto fluidised particles during the process called hot melt coating (Dewettinck and Huyghebaert, 1999).

1.1 Drying

Principle

Fluidised bed drying can be accomplished by two different means depending on the composition of the substrate: On one hand a liquid containing solids is sprayed countercurrently into the empty granulator and is dried like in a conventional spray drier (Figure 2c). On the other hand moist particles can be loaded into the granulator, which are subsequently fluidised and the product is gently dried (Figure 2a). As the whole particle surface is available to be dried in the fluidised bed, heat transfer is best and drying time is optimal. Due to the continuous fluidisation of particles a homogeneous product temperature is achieved and the product is evenly dried. Quality losses associated with thermal inactivation are minimised as drying at low temperatures is feasible. It is possible to carry out the process in batch as well as in continuous large scale production, where special loading facilities are employed and the final product is recovered by separation devices like cyclones or screw conveyors. An enormous number of possible variants of fluidised bed driers are in use and numerous innovative designs and operational modifications like two-stage or pulsating fluidised bed driers are being developed (Mujumdar, 2004).

Applications

The most prominent application of fluidised bed drying in food industry is the commercial production of dried baker's yeast as this gentle drying process retains high final cell viability and enhanced shelf life. *Saccharomyces cerevisiae* active dry yeasts (ADY) are employed in the baking, wine and brewing industry as well as in the industrial production of ethanol. Intensive research on factors influencing process survival of ADY were carried out (Bayrock and Ingledew, 1997a). In a series of concentration steps after fermentation - centrifugation, vacuum filtration, twin-screw extrusion and finally fluidised bed drying - residual moisture levels of 5-8% are obtained and no damage due to the removal of structural water is observed. Neither heat nor oxidation but dehydration of ADY was identified to be the mechanism leading to viability losses during fluidised bed drying (Bayrock and Ingledew, 1997b). By using the same drying protocol as for commercial

baker's yeast the percentage of viable biocontrol yeast *Pichia anomala* was only 10%, suggesting the use of osmoprotectants. Furthermore fluidised bed drying together with liquid formulations was associated with low storage survivals at higher storage temperatures compared to freeze and vacuum dried yeast cells (Melin et al., 2007). Fluidised bed drying of matrix-encapsulated biocontrol agents, such as fungi, yeast, or *Bacillus thuringiensis* protein toxin (Brar et al., 2006) produces granules that are dried fast and homogeneously in a cost-effective way. During the process the creation of lumps, risk of abrasion, and uneven heating is minimised (Daigle et al., 1997). Some studies focused on adapting the fluidised bed process as a simple and rapid convective drying method for lactic acid bacterial species and subsequent optimisation of process survival (Linders et al., 1997a; Mille et al., 2004; Selmer-Olsen et al., 1999b).

Compared to three further commonly used drying processes fluidised bed drying generates the lowest costs producing 8.8 and 17.9%, respectively, of the fixed and manufacturing costs of freeze drying (Table 1) (Santivarangkna et al., 2007). A combination of large scale spray driers with fluidised bed driers enables the removal of internal moisture under mild drying conditions while achieving both capital and operating cost improvements (Barbosa-Canovas and Uliano, 2004).

Table 1 Costs of drying processes referenced to that of freeze drying.

Drying process	Fixed costs (%)	Manufacturing costs (%)
Freeze drying	100.0	100.0
Vacuum drying	52.2	51.6
Spray drying	12.0	20.0
Fluidised bed drying	8.8	17.9

Nevertheless the main drawback of fluidised bed drying are the poor survival rates of microorganisms achieved compared to that of freeze drying. Fluidised bed drying time is longer than that of spray drying, but heat inactivation can be minimised by using relatively low air temperatures. Vacuum drying is like fluidised bed drying a suitable process for heat sensitive materials, but in addition the former process is further appropriate to prevent deteriorating oxidation reactions, thereby saving especially oxygen sensitive biological compounds from harm.
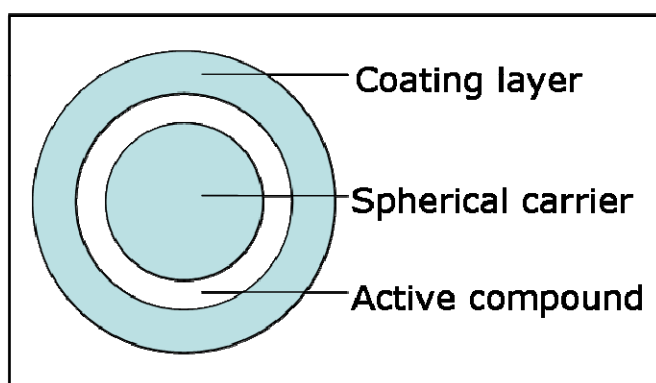
1.2 Coating

Principle

In the fluidised bed coating process one or more liquid layers of coating material are deposited on a solid substance and subsequently dried to form solid films resulting in a homogenous coating layer of uniform thickness that exerts specific functions desired in the final product. Spherical carrier material is either loaded into the product basket of the granulator prior to process start or sucked into the plant when the fluidising air flow causes suction pressure. In both cases the carrier material is fluidised by the air flow applied and thereby the fluidised bed is established.

The coating substance is sprayed on the fluidised carrier particles either as suspension or as hot melt depending on its physical characteristics, such as solubility in water or organic solvents or melting range of a wax, resin or hydrogenated oil. For coating purposes normally the conventional top-spray position of the spray nozzle is employed (Figure 2c) and the coating solution is sprayed countercurrently onto randomly fluidised particles. The liquid is atomised or sheared into droplets by a two-fluid nozzle applying a certain spraying air pressure. In the coating zone of the fluidised bed, the spray droplets are brought into contact with the repetitively passed carrier material and subsequently the droplets dry or solidify on the surface of the particles to be coated. As droplet formation, contact, spreading, coalescence, evaporation, or hardening occur almost simultaneously, the procedure leads after several cycles to a layer of coating (Dewettinck and Huyghebaert, 1999; Strom et al., 2005).

Depending on the amount of coating substance applied a homogenous coating layer of a certain thickness around the particles is achieved giving an encapsulated product. In the top-spray configuration coating imperfections may occur due to premature droplet evaporation as the distance droplets have to travel differs. In order to enable more uniform coating layers the Wurster insert is applied in bottom-spray position (Figure 2b) creating a fluidisation pattern that reduces the travel periods of droplets to a minimum thereby preventing premature droplet evaporation. Additionally, this system allows processing of particles as small 100 µm and coating solutions of high viscosities (Desai and Park, 2005).

The structure of an encapsulated product achieved by fluidised bed coating consists of at least three parts: inside the spherical carrier or core material, uniform coating layer, shell or wall or encapsulant, respectively, on the outside, and in between the coated active ingredient of interest (Figure 3). Sometimes multiple layers of different coating materials are required to obtain the desired product properties. The size of the resulting microcapsules varies between 200 µm and 2 mm (Desai and Park, 2005; Dewettinck and Huyghebaert, 1999; Hemati et al., 2003).

Figure 3 Schematic illustration of a typical fluidised bed coated spherical carrier particle comprising a layer of active ingredient.

Properties and efficiency of coatings are strongly dependent on substance characteristics and chosen process parameters. Next to the main coating component the choice and application of solvents, plasticisers, pore formers etc. is crucial for the success and functionality of the formulation.

The formation of a coating film is a rather complex process that occurs due to continual evaporation of the solvent or solidification of the hot melt. Most prominent water soluble coating substances for edible films are cellulose derivatives, starch derivatives, chitosan, gums and proteins; meltable film formers to mention are hydrogenated soya bean oil, candelilla and carnauba wax. Plasticisation results in a decrease of intermolecular forces between polymer chains, generally causing a decrease in glass transition temperatures and tensile strength (Kramar et al., 2003). Plasticisers, such as glycerol, triacetin, triethyl citrate, and polyethylene glycol are added in order to increase the film forming abilities of certain polymers, whereas the right choice and the appropriate concentration determine the characteristics of the resulting coating film. Especially in the case of aqueous polymer dispersions a thermal after treatment (curing) of the coating may enhance film formation (Bodmeier and Paeratakul, 1997).

To achieve an optimal microencapsulated product a series of factors needs to be taken into account, e.g. amount and diameter of the carrier material applied, surface tension, hygroscopicity, water binding capacity or viscosity of the coating material as well as fluidising air flow and drying temperature. Furthermore the coating structure (crystalline or amorphous) and surface roughness influence the adhesion characteristics of the resulting product.

On the one hand premature drying of particles before striking the carrier material and on the other hand agglomeration of particles need to be avoided during the coating process. The tackiness of films during the coating procedure is a material- and process-related limitation that can result in extensive agglomeration of particles. Side-effect agglomeration was reported to be an "all or nothing" phenomenon, causing complete collapse of the fluidised bed accompanied by a coverage of reactor walls and nozzle with agglomerated particles when a critical point (temperature, moisture content) is exceeded (Dewettinck et al., 1999). Therefore, various coating applications include anti-sticking additives like magnesium stearate, glyceryl monostearate, silicon dioxide or sodium chloride (Yuasa et al., 1997) that decrease tackiness of films and prevent agglomeration.

When dealing with suspensions containing organic solvents, a gas scrubber has to be inserted to clean the exhaust air flow. Even though efficiency of the coating process using organic solvents is enhanced, those coatings are avoided because of environmental, toxicological, and safety reasons. Furthermore the recovery of organic solvents is expensive, hence causing higher manufacturing costs than aqueous coatings.

Hot melt coating is a dry coating approach presenting the following advantages: As there are neither aqueous nor organic solvents applied, there is no need for a drying process or solvent recovery, respectively. Due to the lack of the evaporation step no particle shrinkage occurs and short processing times can be achieved. The fluidising air stream must be kept near to, but lower than the melting range of the molten coating material in order to prevent particle aggregation, while the atomising air temperature is set higher than the melting range to prevent premature congealing of the coating material. As main drawbacks damage of heat sensitive products, poor flow properties and strength, and perhaps undesired odours due to oxidation processes are to be mentioned (Ivanova et al., 2005).

For up-scaling the fluidised bed coating process, approximate scaling parameters based on the geometry of the equipment were described. Among those are Wurster tube diameter and length, maximum volume, maximum batch size, relative spray rate and coating time, if air flow rate, temperature and relative humidity were held constant (Turton and Cheng, 2005).

Fluidised bed coating is usually a batch process, nevertheless continuous processes, such as monocell, multicell or horizontal continuous fluidised beds are being utilised in industries, where large quantities and low prices are required, in order to reduce production costs. Further improvements of the continuous process must be made to overcome its drawbacks, such as incomplete layering and product heterogeneity (Teunou and Poncelet, 2002).

Applications

Microencapsulation is a technology for packing solids, liquids, or gaseous materials in sealed capsules thereby imparting a number of beneficial properties to the active substance being encapsulated. This technique is applied (Desai and Park, 2005; Dewettinck and Huyghebaert, 1999; Hemati et al., 2003)

- to mask unfavourable flavours and odours,

- to protect volatile aromas from evaporation, reaction or migration,

- to protect sensitive or reactive products from heat, moisture, oxygen, light, and further undesirable interactions thereby enhancing the product's shelf life,

- to ensure optimal dosage,

- to make the product easier to handle,

- to separate reactive components within a mixture,

- to simply improve appearance,

- to enhance the mechanical stability of the product and prevent abrasion, or

- to mediate controlled or sustained release that is dependent on pH, degradation, compression, temperature, etc.

In the food industry fluidised bed coating of functional compounds, such as preservatives, enzymes, sweeteners, flavours etc. is mainly used to assist in the development of colour and flavour, to reduce degradation or loss during processing and storage, and to improve flowability of the final product (Desai and Park, 2005). Next to protection against moisture and oxygen the consumers' acceptance concerning product appearance of the coatings employed needs to be evaluated (Lee et al., 2002).

Controlled or sustained release of certain active ingredients at a desired time, site and rate is a main topic in product formulation and applied in many industries: certain coatings protect pharmaceuticals as well as food and feed additives like probiotics from damage caused by the low pH encountered in the gastric juice and furthermore releases the encapsulated ingredient at the specific target site in the intestine. The detergent and agrochemical industries use microencapsulation to continually release a certain amount of active ingredient over time. As a consequence the total amount of active substance in the product is lower than without encapsulation, thereby reducing chemical loads and production costs. The release mechanism may be one or a combination of the following incidents: a change in temperature, moisture or pH, the application of pressure or shear, the addition of surfactants, diffusion, swelling, and enzymatic or microbial degradation (Pothakamury and Barbosa-Canovas, 1995).

The pharmaceutical literature comprises a lot of studies with the focus on biodegradable coatings and controlled release. Chitosan, like other biocompatible polymers, has attracted considerable attention in the pharmaceutical industry as mediators for controlled and site directed delivery of drugs (Agnihotri and Aminabhavi, 2004). Glyceryl behenate is described as an innovative hot melt coating agent for prolonged release drug formulations; the quantity of wax applied is directly related to the release profile (Barthelemy et al., 1999). From the industrial point of view, the outstanding advantage of hot melt coating is that the coating formulation is concentrated, making the process more cost-effective (Desai and Park, 2005). Often polymethacrylates are applied in pharmaceutical dosage forms because comparably exact pH dependent release profiles can be obtained (Bruce et al., 2003; Chan et al., 2001; Huyghebaert et al., 2004; Kramar et al., 2003). In vertebrate pest control a coating combination of shellac and polymethacrylate showed to be capable of masking the flavour and delaying the release of the toxic compound to be absorbed in the gastrointestinal tract (Nadian and Lindblom, 2002). Certain formulations containing either

hydroxypropyl methylcellulose, hydroxypropyl methylcellulose acetate succinate or amylose and/or ethylcellulose were confirmed to exhibit gastric and small intestine resistance and consequently may be used for drug delivery in the colon (Milojevic et al., 1996; Obara et al., 1999; Pearnchob and Bodmeier, 2003a; Sangalli et al., 2004).

Furthermore, bioactive edible coatings of chitosan were reported to inhibit growth of the food spoilage fungi *Aspergillus niger* and additionally, to establish a good moisture barrier for the protection of sensitive foods (Sebti et al., 2005). Moreover, hydroxypropyl methylcellulose coatings containing high levels of appropriate surfactants could be effective moisture barriers in edible films (Villalobos et al., 2006). Last but not least, waxes and solid lipids are commonly used in edible coatings to reduce film water vapour permeability and moisture migration because of their hydrophobicity (Talens and Krochta, 2005).

Next to fluidised bed coating several other microencapsulation techniques, such as spray drying, spray cooling, cocrystallisation, extrusion coating, liposome entrapment, coacervation, inclusion complexation, centrifugal extrusion, and rotational suspension separation may substitute or complement the former mentioned method in order to optimise the quality and characteristics of the product formulation (Desai and Park, 2005).
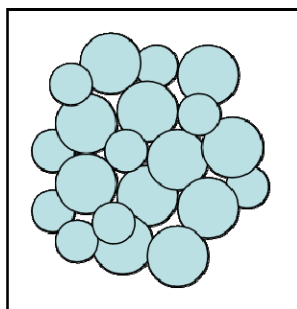
1.3 Granulation

Principle

The fluidised bed granulation method evolved from the fluidised bed driers by additionally using a granulation liquid or binder solution. In the simplest case this may be water, in more sophisticated approaches a dispersion or solution of starch, sugars, cellulose derivatives, chitosan, polyvinylpyrrolidone (PVP) etc. or a hot melt is applied (Hemati et al., 2003; Link and Schlunder, 1997; Planinsek et al., 2000). During the granulation process the growth of particles is caused by spraying the binder solution concurrently with the air flow onto dry fluidised powder material most commonly applying the bottom-spray position of the spray nozzle (Figure 2b).

While agglomeration is a negative side-effect during coating, it is the desired result of the granulation process. Whereas coalescence during the coating process cause the formation of well rounded and uniform particles, agglomeration of largely dried droplets leads to the formation of "raspberry-type" structured granules (Figure 4) (Link and Schlunder, 1997).

The aim is the production of granules by drying or congealing of so-called liquid bridges between small adhering powder particles that cause fine particles to form stable agglomerates. Furthermore, intermolecular attractive forces, van der Waals forces, and electrostatic forces also play an initial role in the agglomeration of particles. The powder enlargement process in the fluidised bed process is affected by the consecutive and competitive steps: particle mixing, liquid spreading, partial evaporation of a solvent from the particle surface or congealing of the binder matrix, agglomeration, abrasion, and fragmentation (Hemati et al., 2003).

Figure 4 Schematic illustration of a typical "raspberry-type" structured granule being formed by drying or congealing of liquid bridges between powdered particles.

Main parameters influencing optimum granulation conditions are spreading of the binder over the substrate, adhesion between the binder and the substrate as well as binder cohesion (Planinsek et al., 2000). Droplet adhesion on the surface is influenced by the droplet momentum, the properties of the liquid binder, and the surface characteristics of droplet and particle. The ability of the binder droplet to wet the particle and to spread over its surface is referred to as "wettability" (Link and Schlunder, 1997).

Generally, it can be stated that the mechanism of agglomerate formation is primarily dependent on the ratio of the size of the atomised binder droplets to the size of the solid particles. When solid particles are larger than the binder droplets, distribution leads to a rather open agglomerate structure, whereas immersion results in a denser structure if the solid particles are smaller (Abberger et al., 2002). If the liquid droplets are too large with respect to the particles, wet agglomerates are formed that are too large to be fluidised causing the bed to defluidise as those particles stick together as large wet clumps. Moreover, the structure of the resulting agglomerates depends on the fluidising air flow: Higher flow rates trigger rapid evaporation of the binder solution and favour the formation of smaller and friable granules while lower flow rates enhance larger, fluffier, and denser granules (Hemati et al., 2003).

Applications

Agglomeration of small particles leads to size enlargement that improves product handling and compression characteristics of the initial powders. The advantage of fluidised bed granulation is the resulting open, coarse, porous, and homogenous product, which is almost dust-free, free flowing, and easily solubilised. Even a desired particle size distribution may be obtained, when critical process parameters, such as fluidising air velocity, relative humidity, and spray rate are optimised accordingly.

Due to larger and more uniform particle size distribution fluidised bed granulation is mainly applied in the pharmaceutical industry in order to improve dosage accuracy and handling during subsequent processing steps like tabletting, powder compression, or briquetting. Moreover, fluidised bed granulation is employed for chemical products, such as fertilisers, pesticides and other powders which tend to produce undesirable, hazardous dusts when being processed in the dry form.

Instant food products are agglomerated to increase their dispersion rate in liquids as they should dissolve as quickly as possible with minimum stirring to satisfy the consumer's demands. Spray drying technology produces very fine powders in the range between 10-100 µm in diameter. In order to obtain a free flowing and easily soluble product further processing like fluidised bed granulation is necessary (Madene et al., 2006). Most of the fluidised bed granulated products require less particle wetting than a high shear granulation or spray drier processed product (Hemati et al., 2003).

2   Preservation of Lactic Acid Bacteria (LAB)

2.1 LAB Starter Cultures

Lactic acid bacteria comprise a heterogeneous group of Gram-positive, non-sporulating and facultative anaerobe bacteria, which are found ubiquitously from the human and animal body to plants. Due to their commercial significance in the fermentation industry the most prominent genera of LAB are *Lactobacillus* (*Lact.*), *Leuconostoc*, *Lactococcus*, *Streptococcus*, and *Pediococcus* (Daly et al., 1998).

The growth of LAB is accompanied by the generation of acidic end products of fermentation that accumulate in the extracellular environment. The pronounced organic acid production of these bacteria creates an environment unfavourable for many spoilage and pathogenic microorganisms. This characteristic is the basis of numerous methods of food preservation by fermentation, so the use of LAB as biopreservatives is a fairly widespread technique showing encouraging results in different areas of the food industry (Zamora et al., 2006).

Lactic acid, as the metabolic end product of carbohydrate fermentation, is their most common specific feature. Homo-fermentative LAB convert glucose almost entirely to lactic acid via glycolysis, while hetero-fermentative LAB produce ethanol and carbon dioxide in addition to lactic acid utilising the pentose-phosphate pathway (Wee et al., 2006).

LAB have very complex growth requirements. They require low oxygen tension, strain specific fermentable carbohydrates, protein and its breakdown products, a number of special growth factors like vitamins of the B-complex, nucleic acid derivatives, unsaturated free fatty acids, and minerals, such as magnesium, manganese and iron for their growth. On laboratory scale they are cultivated in MRS broth, a medium introduced by de Man, Rogosa and Sharpe (De Man et al., 1960), which is recommended for isolation and cultivation of *Lactobacillus* species and meets with all the requirements mentioned above.

Due to their natural occurrence in food as well as in human and animal mucosal surfaces LAB are classified according to the requirements of the United States of America Food and

Drug Administration (FDA) as "generally recognised as safe" (GRAS). Starter cultures have specific characteristics as they are selected, well-defined and viable microorganisms in pure or mixed culture.

Lactic acid bacterial starter cultures are applied in the food and feed industry to ensure more directed, faster, safer, and more standardised production processes. In the food industry LAB are mainly applied for conservation by lowering the pH. The fields of application range from the dairy (yoghurt, cheese) to the bakery (sourdough), meat (salami), wine, and vegetables industries (e.g. sauerkraut, soy sauce). Next to fermentation and transformation of foods from animal or plant origin LAB play a crucial role in the development of the organoleptic and textural properties of fermented products (Carvalho et al., 2004b; Knorr, 1998; van de Guchte et al., 2002). Furthermore, LAB starter cultures are widely used as silage inoculants in order to accelerate the fermentation as well as to reduce the risk of spoilage and thus to improve the resulting silage (Weinberg et al., 2003).

In biotechnology the production of lactic acid by LAB is of economical importance because of its broad range of application in the food, cosmetic, pharmaceutical, and chemical industries. Lactic acid is used as preservative, pH regulator, flavouring agent, and moisturiser to name just a few. Besides the application as feedstock for certain chemicals lactic acid is recently becoming more and more important as polymer precursor for the production of the biodegradable, thermoplastic, aliphatic polyester polylactic acid (PLA) (Wee et al., 2006).

Several LAB strains are capable of producing antimicrobial proteins, so-called bacteriocins, which enhance their antagonistic properties and are often proposed as a beneficial characteristic of probiotic bacteria by increasing the competitiveness of the probiotic strain in the gut (Avonts et al., 2004). In recent years new applications of LAB, such as probiotic dietary food and feed supplements, so-called nutraceuticals have been developed. Probiotic products contain at least one potentially beneficial microbial culture, which is able to stimulate the immune system of humans and animals by improving the properties of the indigenous microflora (Shortt, 1999). Further proposed mechanisms of viable and non-viable probiotic health effects are alleviation of lactose intolerance symptoms, shortening rotavirus diarrhoea, and modulation of cancer development (Salminen et al., 1999).

As probiotic bacteria may not survive in sufficient numbers when incorporated into dairy or feed products and during passage through the gastrointestinal tract, microencapsulation in matrices like calcium alginate, shellac or hydrogenated soya oil protects the bacteria from adverse environmental conditions (Chandramouli et al., 2004). The development of new applications, such as live vaccines reinforces the need and selection for robust LAB, since they may have to exert their specific functions in conditions unfavourable for growth (Termont et al., 2006).

Formerly starter cultures were kept in-house for the individual production line, while nowadays there are specialised producers that distribute a range of certain starter cultures worldwide. In the broad field of industrial applications LAB are confronted with adverse conditions encountered both during processing and storage. In order to meet the requirements of product quality in terms of long-term stability, viability and activity of LAB starter cultures, the focus is on the preservation technology employed. The ability to withstand technological processes and remain viable during a certain shelf life period was mentioned as important key selection criteria for probiotic strains (Shortt, 1999). On industrial settings, LAB may be preserved and distributed in liquid, spray or fluidised bed dried, frozen or lyophilised forms. Liquid forms are the most unstable losing viability within weeks, while frozen cultures keep viabilities over years (Carvalho et al., 2004b).

Microbial cell survival throughout the whole production process (cell bank - pre-culture - fermentation - downstream processing) is dependent on many factors focusing one distinct strain. During fermentation, mode (batch vs. continuous), dissolved oxygen (pO$_2$), pH, temperature, time, media composition, and neutralising agent are influencing survival of starter cultures. Further factors, such as cell concentration, mechanical stresses (centrifugation vs. filtration), osmotic pressure, freezing/drying technology are impacting on vitality of microorganisms during downstream processing (Santivarangkna et al., 2007).

2.2 Drying of LAB

From the industrial standpoint, a good starter culture should maintain high levels of viability during the preservation process and for long periods of storage afterwards. Drying can be accomplished by a number of means including freeze drying, spray drying and fluidised bed drying (Santivarangkna et al., 2007).

For many years LAB have been mainly preserved by freezing or freeze drying to enable their use as industrial starter cultures. Even though a loss of culture viability may be encountered during the freeze-thaw process, frozen cultures are known to retain high percentage of the initial population. The main advantage of dry cultures over the frozen ones are that they are easier to handle as high transport and storage costs are associated with frozen concentrated cultures (Knorr, 1998).

Mostly fairly good survival rates are obtained by freeze drying approach, this preservation method, however, is costly in terms of time and expenses. Materials are first frozen and then dried at low temperature in the lyophilisation process, therefore loss in survival of LAB occur in each one of these steps due to ice crystal formation in the first and due to desiccation in the second case (Santivarangkna et al., 2007).

In comparison, spray drying, one of the predominant processing tools used in the dairy industry, can be used to preserve microbiological material on a large scale relatively inexpensively. Thus, compared to freeze drying it is the cheaper method and has a higher material throughput. Spray dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods (Santivarangkna et al., 2007). However, a reduction of viability generally observed during spray drying is due to thermal stress caused by the high inlet and the resulting outlet temperatures necessary for the process even if the exposure time is relatively short. High temperatures are extremely detrimental to microorganisms and non-thermostable enzymes, so a spray drying apparatus with a high volume would be needed to be able to reduce the inlet air temperature (Johnson and Etzel, 1995).

For protecting heat-labile microorganisms and compounds it is desirable to dry at low air temperature which is facilitated by a fluidised bed drier. Advantages of fluidised bed

drying, such as equal temperature distribution and cost-effectiveness, were already depicted in chapter 1.1.

All these preservation processes are harming cells to a certain extent, leading to losses in bacterial populations. The optimal performance of the strains depends on the stabilisation of their survival potential and metabolic activity which are functions of several factors ranging from culture conditions and cell density to post-drying stresses. According to Baati et al. (2000) those stress conditions are low temperature, low pH, and low water activity (a_w) that cause

- a loss of viability,

- membrane and cell wall damage,

- inhibition of active transport and retention of nutrients, as well as

- morphological changes.

Cell shape and morphology may play a further role in the susceptibility of different strains to certain preservation steps. The higher the surface area of the cell, the higher the membrane damage owing to extracellular ice crystal formation during freezing (Fonseca et al., 2000). The phenomenon that Gram-positive *Cocci* are more resistant than *Bacilli* appeared to be when spray drying was used instead of freeze drying of *Streptococcus thermophilus* and *Lact. acidophilus* (Wang et al., 2004).

2.3 Desiccation Tolerance - Stress Response

Desiccation by drying imposes various threats on cells as the removal of water induces both osmotic and ionic stress. In natural habitats the ability to quickly respond to stress is essential for survival, e.g. some Gram-positive species differentiate to spores, and others enter a stationary phase. It is now well established that LAB, like other bacteria, evolved defence mechanisms referred to as adaptive responses that are species or even subspecies specific. These responses involve a series of genetic up- and down-regulations as well as physiological mechanisms to induce tolerance against moderate levels of stress that allow them to withstand harsh conditions and sudden environmental changes (van de Guchte et al., 2002). Nevertheless, cell damage is not simply an on/off phenomenon and depends strongly on type and intensity of stress the cells are exposed to. Furthermore stress response to elevated salt concentrations, variances in pH or temperature are associated with cross protection which varies between species and may result in cells being more desiccation-tolerant (Abee and Wouters, 1999).

Osmotic Stress

In contrast to water content, water activity (a_w) does not include water bound to matrix components, thus determines the amount of water available for microbial growth. A reduction in the a_w surrounding the cell resulting from desiccation or the addition of solutes causes a hyperosmotic shock to cells triggering a rapid efflux of water and a temporary loss of turgor. To re-establish turgor pressure compatible solutes are accumulated to high levels within the cell by either *de novo* biosynthesis or by uptake from the environment (Beales, 2004). These solutes are called compatible as they are expected not to interfere too seriously with cytoplasmic enzymes. These compounds are small organic molecules with the following properties (Abee and Wouters, 1999):

- neutral or zwitterionic molecules soluble at high concentrations,

- can be accumulated to very high levels in the cytoplasm,

- specific transport systems present in the cytoplasmic membrane to control intracellular concentration,

- protect enzymes against denaturation by salts, freezing or drying.

Most prominent examples are

- the quaternary ammonium compounds glycine betaine and carnitine,

- the disaccharides trehalose and sucrose,

- amino acids like glutamate and proline,

- amino acids derivatives (small peptides, N-acetylated amino acids),

- the sugar alcohols mannitol, sorbitol, and glycerol,

- tetrahydropyrimidines (ectoines).

The initial response after an osmotic upshock is much more rapid if compatible solutes can be taken up from the environment via semiconstitutive transport systems than by being *de novo* synthesised (Poolman and Glaasker, 1998).

Reasons why osmotic stress (lowering a_w) may lead to cell death are surplus concentration of internal solutes, water efflux across the membrane and structural modifications of phospholipids resulting from the removal of water molecules. The addition of virtually dry powder to a bacterial pellet induces osmotic shock that determines bacterial viability. The higher the a_w of the powder, the more water is available for the microorganisms, and the greater their viability (Mille et al., 2004). A further study about the osmotic tolerance of microbial cells revealed that this resistance is species specific as *Lact. bulgaricus* turned out to be the most sensitive and among others *Lact. plantarum* the most resistant (Mille et al., 2005).

A difference in cell viability was observed when osmotic stress was applied to *Lact. plantarum* cells by decreasing the water potential rapidly and slowly with NaCl. During a slow decrease in water potential, the cells seem to be able to respond to the progressive increase of the sodium intracellular concentration to avoid the resulting toxic effect and thus preserving cell viability (Poirier et al., 1998). Other results (Linders et al., 1997a) revealed that *Lact. plantarum* showed higher residual activity after fluidised bed and convective drying when grown without NaCl compared to cells grown with 1.25 M NaCl. A study on *Lact. bulgaricus* grown in low a_w media showed that the protective effect of sugars, in particular of trehalose and sucrose, is enhanced due to osmotic stress adaptation (Tymczyszyn et al., 2007).

pH Stress

The effect of low pH and weak organic acids may provoke an acid tolerance response in cells achieved by the expression of certain acid shock proteins (ASP). Weak acids can diffuse into the cell in their undissociated form, where they dissociate, thereby lowering the intracellular pH resulting in the inhibition of various essential metabolic processes. As strong acids are not able to permeate through the cell membrane, their mode of action is outside the cell by denaturing enzymes on the cell surface and by disrupting cytoplasmic pH homeostasis (Beales, 2004). An important additional aspect of acid tolerance response (ATR) is the induction of cross protection to various stresses like heat, osmolality, oxidation in exponentially grown cells (Abee and Wouters, 1999).

The regulation of pH allows obtaining cultures with high densities and maintains the viability during storage at -80°C, where no decrease of survival rate was observed during three months of storage compared to cultures grown at non-regulated pH. In spite of a pronounced decrease in cell resistance during growth at non-regulated pH, exposure at low pH preserved the survival rate after freezing (Baati et al., 2000). Similar findings were achieved during fluidised bed drying trials of *Lact. plantarum*, where pH control during growth of cells resulted in a higher residual activity compared to growth without pH control (Linders et al., 1997a).

Lowering the pH during cultivation of *Lact. reuteri* from 6 to 5 induced a greater protection of the cells during freeze drying (Palmfeldt and Hahn-Hagerdal, 2000). A further study revealed that *Lact. bulgaricus* cells grown under non-controlled pH showed greater survival during subsequent heating and drying treatments. The increased resistance was suspected to be the result of the cross protection conferred by the low pH encountered during growth under non-controlled conditions (Silva et al., 2005).

Heat Shock

Bacterial heat tolerance increases upon exposure to sublethal heating temperatures. Protection against heat may be achieved by sporulation and by accumulation of compatible solutes that may enhance protein stability and protect enzymes against heat inactivation. Furthermore exposition to heat as much as acid and oxidative stress rapidly induces the expression of a set of heat shock proteins (HSP), which comprise chaperones as well as proteases. These classes of proteins maintain quality control of cellular proteins by

supporting proper protein folding on one hand and by degrading damaged proteins on the other hand (Abee and Wouters, 1999). The functions of chaperones are not limited to heat stress but involve a wider role in cellular processes like growth, mRNA stability, and cytoplasmic protein folding. Studies on probiotic *Lact. paracasei* overproducing the chaperone protein GroESL demonstrated about ten- and about twofold enhanced survival, respectively, after either spray or freeze drying (Corcoran et al., 2006).

Cold Shock

Membrane modifications maintaining fluidity and measures to sustain the structural integrity of ribosomes and proteins are involved to permit bacterial growth at low temperatures. Synthesis of cold shock proteins (CSP), an increase in the proportion of shorter and unsaturated fatty acids in the lipids and the accumulation of compatible solutes contribute to cold adaptation (Abee and Wouters, 1999).

It was reported that cell survival of *Lact. acidophilus* after a freezing treatment depends on growth temperature and composition of suspending buffer. The highest survival rate was obtained with cells grown at 22°C without the addition of any buffer. Cells suspended in phosphate buffered saline (PBS) buffer showed lower viabilities compared to phosphate buffer alone. When *Lact. acidophilus* was frozen directly at -80°C for 24 hours viability decreased considerably (43%), while a slow stepwise decrease in the temperature, i.e. 37, 20, -4, -20, -80°C resulted in a survival rate of 74%, while the maximal rate of death occurred between -4 and -20°C. Frozen cells showed the best survival rate when subjected to a thermal adaptation to 22°C up to 18 hours, i.e. an adaptation at low temperature in broth medium or slow freezing of cell suspensions leads to the development of cryotolerance (Baati et al., 2000).

Oxidative Stress

There are several ways to overcome stress caused by oxygen (van de Guchte et al., 2002): Among those are the prevention of reactive oxygen species formation and the elimination of reactive oxygen species either by the enzymes catalase, pseudocatalase and superoxide dismutase or by extremely high manganese contents serving as efficient radical scavengers. Furthermore it was suggested that zinc(II) might be able to protect thiol groups of proteins from oxidative conversion into disulfide bonds. The ultimate possibilities against oxidative

damage are the repair mechanisms present in the cell. Additionally the general stress response could confer cross protection against oxidative stress.

The deleterious effects of oxygen are attributed to reactive oxygen species like the superoxide and the hydroxyl radical that attack important cellular components. No enzymes are known to degrade the latter radical, pointing out the importance of the availability of compatible solutes - in particular polyols showing hydroxyl radical scavenging activity - to prevent damage from reactive oxygen species (Smirnoff and Cumbes, 1989).

Stationary Phase-Associated Stress

Due to nutrient depletion and/or product inhibition cells enter the stationary phase resulting in decreasing growth rates, modifications of cell morphology, changes in bacterial metabolism, and further up- and down-regulations of genes. This lack of nutrients imposes stress to bacteria that is overcome by the stationary phase-associated stress response that involves the induction of numerous regulons. Due to the acquired general stress resistance bacteria that enter stationary phase are more capable of coping with various types of other stress conditions like heat, oxidative, ethanol, acid, and osmotic stress being more robust during subsequent downstream processing and storage than bacteria in the exponential phase (van de Guchte et al., 2002).

A study on *Lact. acidophilus* revealed that stationary phase cultures were inherently more resistant to exposure to bile and heat than exponential phase cultures (Kim et al., 2001). Adaptive and/or cross-protective responses were detected in three stationary phase *Lact.* strains, while the responses turned out to be strain specific and improvements in viability varied widely (Saarela et al., 2004).

2.4 Protective Compounds

A microorganism that has survived steps of a certain drying method may eventually lose its viability during storage; hence, the storage stability of LAB is a very important factor toward their industrial application. Unlike other bacterial species LAB are multiple amino acid auxotrophs and have limited or no possibilities to synthesise compatible solutes (Poolman and Glaasker, 1998). Therefore incubating cells with compatible solutes favours their intracellular accumulation, which is advisable as this step may minimise adverse effects of stress and improve survival. As the drying process is probably too short for the bacteria to accumulate protective compounds in the cell interior, this step should be considered to take place before the drying process, even during growth (Efiuvwevwere et al., 1999).

Several compounds added to the microbial suspensions before drying were found to affect the stability of the cells during processing and long term storage, so their use is recommended for production of dried cultures that will eventually be used as starters. The protective additives need to stabilise the native state of proteins as well as the cell membrane and to scavenge free radicals. Generally the term protectant or more detailed osmoprotectant refers to compounds that alleviate the inhibitory effect of hyperosmotic stress but often the term is also used for any solute that can overcome osmotic inhibition. Therefore it was suggested to preferably use the term compatible solute for any compound that offers protection to high osmolality by accumulation to high cytoplasmic concentrations either by uptake from the medium or by *de novo* synthesis (Poolman and Glaasker, 1998).

Summing up, the different protectants should have high waterbinding potentials together with other mechanisms for stabilising proteins as well as mechanisms to strengthen membrane structures. When water is removed, the lacking hydrogen bonding partner of the phosphate entity of the membrane's phospholipids needs to be substituted. The proposed mechanism of protection especially exerted by disaccharides is described as the "water replacement theory" (Figure 5) (Crowe et al., 1992):

Cell membranes need to be hydrated in order to be capable of exerting the normal functions. Among those are the actions of proteins embedded in the plasma membrane like

pumping ions, taking up nutrients, and performing respiration. Therefore, it is essential that the membrane lipids are in the liquid crystalline state. The removal of hydrogen-bonded water from the phospholipid bilayer results in the increase in packing of membrane lipids leading to increased van der Waals interaction among the hydrocarbon chains. Therefore, the lipids change from liquid crystalline to gel phase in the dried state. Apart from the gel phase several other deformations of the cell membrane may occur upon freezing and/or dehydration like fluid-fluid separation, lipid-protein separation, and inverse phases some of which are associated with damage of living cells (Wolfe and Bryant, 1999).
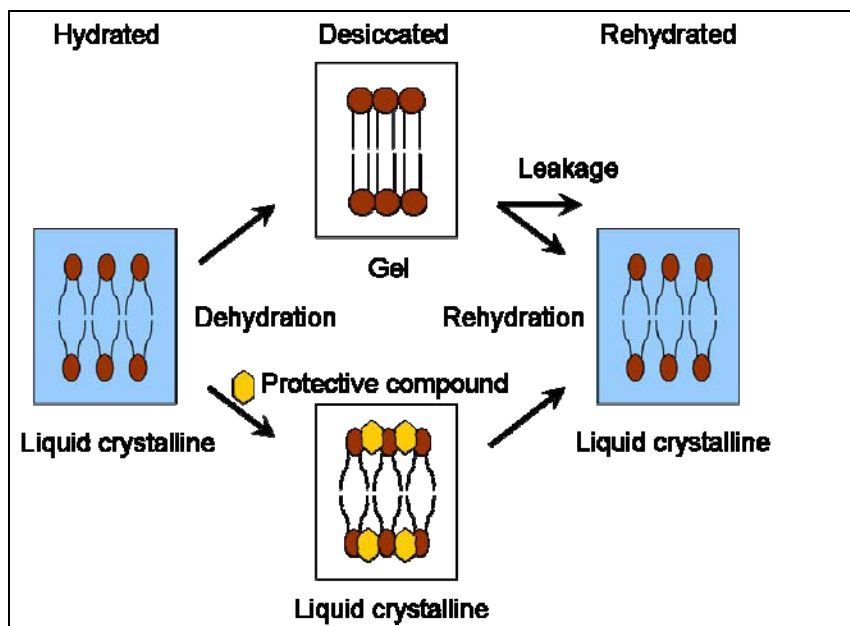
Figure 5 Schematic drawing illustrating the water replacement theory (Crowe et al., 1992).

If lipid bilayers are dehydrated and rehydrated in the absence of any protective additive the membrane undergoes phase transition twice, when being dried from the liquid crystalline into the dry gel state and upon rehydration back into the liquid crystalline phase. Especially the second step is crucial as the membrane gets transiently leaky as abundant water is available for transport of entrapped solutes (Hoekstra et al., 1997).

The addition of a protective compound that depresses the phase transition temperature prevents phase changes by keeping the plasma membrane in the liquid crystalline phase even in the dry state. This is achieved upon the removal of water during the drying process by the direct interaction between the protective agent and the polar headgroups of the cell membrane's phospholipids as well as membrane proteins (Crowe et al., 1992).

Investigating the effects of severe dehydration on *Escherichia coli* revealed that most probably a combined effect causes cell death. Phase separation of membrane lipids from the liquid crystalline into the gel phase and volumetric contraction caused by a certain osmotic pressure applied to the cells mainly contribute to this combined effect (Beney et al., 2004).

At the end of a drying process and during storage a further protective mechanism, vitrification or the formation of the glassy state, is of particular importance with regard to survival and stability of microorganisms. A glass is defined as oversaturated, thermodynamically instable liquid showing high viscosities, thereby "freezing" of chemical and physical processes that are detrimental for bacterial survival. An elevated glass transition temperature (T_g) is advantageous, if the dry matrix should be maintained in the glassy state during storage (Crowe et al., 1998). Vitrification protects membranes by means of limiting further dehydration, lowering the probability of crystallisation of solutes, and keeping the membranes in the fluid lamellar phase (Wolfe and Bryant, 1999).

A simplified state diagram (Figure 6) shows the glass transition curve which relates T_g and moisture content. Due to the high viscosity of the glassy state (amorphous solid) cellular constituents are immobilised, diffusion is inhibited, and changes in the structures and chemical composition are slowed down. With rising temperature and/or water content the glass transition curve is exceeded and molecular mobility as well as deleterious reaction rates, in particular of oxidations, increase in the rubbery state (amorphous liquid) (Santivarangkna et al., 2008).
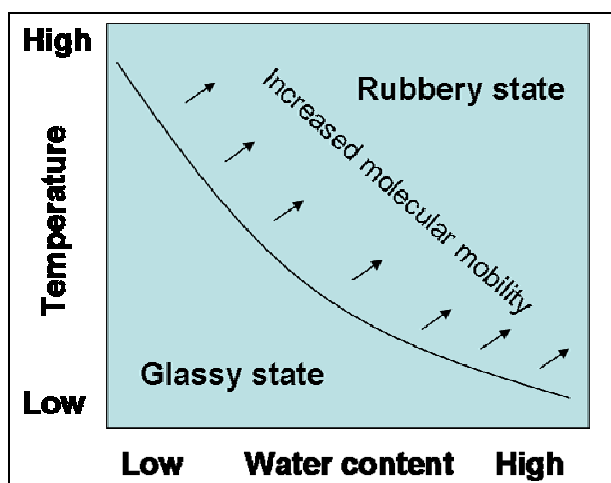
Figure 6 Simplified state diagram of the glass transition curve relating glass transition temperature (T_g) and moisture content (Santivarangkna et al., 2008).

The entrapment of dried bacteria in the glassy state is an effective tool to improve stability and reduce inactivation mechanisms during storage. Thus it is important to know about the glass transition temperatures of the single components of the product to be dried and stabilised. Water has a very low T_g and acts as a plasticiser, thus it is responsible for depressing T_g of dried materials (Bhandari and Howes, 1999). Generally low molecular weight additives like glucose and sucrose in their pure form have lower glass transition temperatures than high molecular weight polymers like maltodextrins. Moreover, in a multicomponent system carbohydrates have the largest effect in influencing the T_g of dried material (Bhandari and Howes, 1999).

Both vitrification and water replacement must not be seen as exclusive mechanisms underlying the protection of biomolecules exerted by certain additives (Crowe et al., 1998). Many studies have been carried out referring to the above mentioned protective mechanisms in order to prevent or reduce the harmful effects of preservation processes like freezing and drying by the addition of numerous protective substances to concentrated cell solutions. A short overview focusing on certain types of protective additives and their effects on LAB is given in the following.

Skim Milk

Due to its protective effect skim milk is defined as a most useful suspending medium for frozen or freeze dried starter culture preparation. Skim milk as a rather complex substance prevents cellular injury by stabilising the cell membrane constituents, it creates a porous structure in the freeze dried product that makes rehydration easier and contains proteins that provide a protective coating for the cells.

As skim milk previously showed to have a protective effect during heat treatment, spray drying of probiotic *Lact. paracasei* and *Lact. salivarius* was evaluated in skim milk medium. No difference in the ability to survive at 55°C could be observed between the two strains, while at temperatures above 58°C *Lact. paracasei* showed higher thermal tolerance than *Lact. salivarius* (Gardiner et al., 2000).

Survival rates of immobilised fluidised bed dried *Lactobacilli* varied with the strain and the protective solute. Adonitol and non-fat milk solids (NFMS) gave the best protection for mesophilic *Lactobacilli*, while NFMS was optimal for thermophilic *Lactobacilli* and glycerol showed the least protective effect (Selmer-Olsen et al., 1999a).

Carbohydrates and Derivatives

Sugars and sugar derivatives were in most cases found to be effective toward protection of various LAB by stabilising cell membrane lipids and proteins, and maintenance of turgor. Sugars act as effective protectors due to the presence of hydroxyl groups preventing intracellular ice formation due to their water binding capacity and avoiding oxidative damage by scavenging of free reactive oxygen radicals. In particular the non-reducing disaccharides trehalose and sucrose are accumulated by anhydrobiotic organisms in large amounts during drying inferring that they might play a role in desiccation tolerance (Hoekstra et al., 1997). Apart from having higher glass transition temperatures trehalose is less prone to crystallisation and less reactive than most other sugars, therefore this disaccharide is considered a protectant of particular importance (Wolfe and Bryant, 1999). Due to these stabilising properties the wide range of applications of trehalose has increased thereby raising the demand for an economically feasible large scale production by the biotech industry (Schiraldi et al., 2002).

De Giulio et al. (2005) compared the survival rates of three strains of LAB, *Lact. acidophilus*, *Lact. delbrueckii* ssp. *bulgaricus*, *Streptococcus salivarius* ssp. *thermophilus*, frozen or freeze dried after treatment with cryoprotective sugars (trehalose, lactose, sucrose, glucose, maltose). All sugars tested exhibited a good preservative effect without significant differences after freezing and freeze drying procedures compared to isotonic solution.

It was discovered that the addition of borate ions can significantly enhance the protective abilities of trehalose for the stabilisation of freeze dried *Lact. acidophilus*. This effect can be explained by the fact that the borate ions crosslink the trehalose molecules, which results in higher glass transition temperatures of the dry matrix (Conrad et al., 2000).

A combination of trehalose, sucrose, and skim milk turned out to be the most efficient protective media for *Lact. salivarius* ssp. *salivarius* since this mixture gave a survival rate of 83-85% immediately after freeze drying in contrast to a survival rate of only 4% obtained without the addition of any protective agent (Zayed and Roos, 2004). Especially if proteins are present in the drying medium non-reducing sugars like sucrose and trehalose are advantageous protectants as they do not undergo Maillard reactions with amino groups (Ferreira et al., 2005).

In contrast, the addition of inositol, sorbitol, fructose, and trehalose to the drying medium did not significantly increase the survival of *Lact. plantarum* and *Lact. rhamnosus* during the freeze drying process (Carvalho et al., 2002). Further studies on *Lact. bulgaricus* revealed that the lowest decrease in viability after freeze drying was obtained when *Lact. bulgaricus* was grown in MRS-mannose, followed by MRS-lactose. Using MRS-fructose as growth medium made *Lact. bulgaricus* even more susceptible to freeze drying than conventional MRS-glucose (Carvalho et al., 2004a).

Carbohydrates have been shown to decrease dehydration inactivation of fluidised bed dried *Lact. plantarum* cells. The residual activity of fluidised bed dried cells improves when the bacteria are dried in the presence of maltose, sucrose, or sorbitol; remarkably, trehalose did not protect *Lact. plantarum* (Linders et al., 1997c).

A study of *Lactococcus lactis* subjected to convective drying revealed that higher survival rates could be obtained when the sugar alcohol mannitol was included in the drying buffer but an extended lag time after drying and rehydration was observed compared to freshly harvested cells (Efiuvwevwere et al., 1999).

There are aims to use prebiotics not only as dietary supplements but also as protective compounds for dried bacterial formulations and in combination with calcium alginate as coating materials for probiotic microencapsulation (Chen et al., 2005). Most potential prebiotics are carbohydrates, such as fructooligosaccharides (FOS), xylooligosaccharides (XOS), and galactooligosaccharides (GOS), but the definition as non-digestible food ingredients stimulating the growth of beneficial bacteria in the colon does not exclude the use of non-carbohydrates as prebiotics (Fooks et al., 1999). However, the presence of the prebiotics polydextrose and inulin in the spray drying solution offered neither elevated process survival nor improved storage viability of three tested probiotic *Lactobacilli* compared with reconstituted skim milk (Corcoran et al., 2004).

Antioxidants

Ascorbic acid is an antioxidant agent and its effectiveness as protector seems to be related to the inhibition of oxidative stress mainly damaging membrane lipids, which in turn affects the survival of cells during drying and subsequent storage in the dried state. For *Lact. acidophilus*, *Lact. paracasei* ssp*. paracasei* and *Lact. salivarius*, the application of ascorbic acid alone and in combination with the protective substances skim milk and/or

lactose led to better survival rates of all three strains than without the antioxidant included (Zarate and Nader-Macias, 2006).

No significant differences were reported in the viability of *Lact. plantarum* and *Lact. rhamnosus* cells during freeze drying in the presence or absence of the antioxidant propyl gallate (Carvalho et al., 2002).

Ammonium Compounds

Like many organisms, LAB accumulate compatible solutes by uptake from the environment, in particular the prominent examples betaine and carnitine. While *Lact. bulgaricus* did not accumulate either of the quaternary ammonium compounds in several growth conditions tested, an uptake of betaine and/or carnitine by *Lact. plantarum, Lact. halotolerans,* and *Enterococcus* (*Ent.*) *faecium* previously exposed to salt stress could be observed. Furthermore betaine included in osmotically stressed medium clearly protected the latter three strains against drying (Kets et al., 1996).

The stabilisation of protein structures by reactions between the amino groups of the protectant and the carboxyl groups of the microorganism's proteins was put forward as tentative explanation of the protective effect exerted by the amino acid monosodium glutamate (MSG) during freeze drying: Furthermore, the ability to retain greater amounts of residual moisture was attributed to MSG. Nevertheless, protection during freeze drying of *Lact. plantarum* and *Lact. rhamnosus* by MSG could not be observed (Carvalho et al., 2002).

Extended cellular survival during storage of spray dried *Lact. sakei* was observed in the presence of MSG and sucrose in the growth medium. Increased glutamate levels were identified as part of the osmoadaptive response, in which glutamate probably acts as counterion for potassium ions thereby balancing the intracellular charge (Ferreira et al., 2005).

In order to achieve an enhanced cryoprotective effect skim milk was supplemented with different substances and the influence on viability and acidifying activity of frozen and freeze dried cells of *Lactococcus lactis* ssp. *lactis* was studied. Despite a negative impact on cells during freezing, it could be observed that alanine protects cells during freeze drying, which could be related to the osmoprotective effect of other aminoacidic

compounds, such as proline and glycine betaine. In consistence with previous studies glutamic acid turned out to be a good provider of viability and acidifying activity after freezing or freeze drying. Due to the reported cryoprotective effects of methionine, asparagine, and threonine two protein digests were further investigated: While meat extract only provided good protection during freezing at -80°C, a pancreatic digest of gelatine increased cryoprotection significantly (Carcoba and Rodriguez, 2000).

To sum up, these diverse and sometimes contradictory findings point out that no standard procedure for the stabilisation of LAB during preservation processes may be formulated and improvements need to be made based on the individual circumstances with regard to growth and drying conditions, properties of the bacterial strain, and formulation additives determining the composition of the final product.

2.5 Storage and Rehydration

Drying of cultures must not be seen independent from storage for longer periods of time because inactivation of bacteria is significantly dependent on the storage conditions, such as temperature, relative humidity, exposure to light, and atmosphere, the absence of oxygen in particular. Thus, dried powders are suggested to be stored under vacuum or inert gases, maintained in controlled a_w, and exposed to darkness in order to be able to recover sufficient numbers of dried cells to be used as starter cultures (Abadias et al., 2001).

Previous studies have shown that temperature is a critical parameter for microbial survival during storage. The stability of dried samples generally decreases during storage, but higher survival rates are recorded at lower storage temperatures. The increased survival of skim milk formulated, freeze dried cultures of *Lact. bulgaricus* at low temperatures was explained by a possible reduction in the rate of fatty acid oxidation (Castro et al., 1995).

Taken together with storage temperature the moisture content should be low enough to maintain the dried culture in the glassy state as depicted previously (Figure 6). High a_w values lower the glass transition temperatures and trigger the dry matrix to enter the rubbery state, thereby increasing mobilisation of components and accelerating oxidation reactions. The optimal moisture range needs to be determined for the individual bacterial strain, taking into account that the residual moisture is directly related to the type of drying medium employed (Zayed and Roos, 2004).

The survival of freeze dried *Lact. plantarum* and *Lact. rhamnosus* throughout storage at room temperature, in the presence of the compounds inositol, sorbitol, fructose, trehalose, MSG, and propyl gallate significantly increased the survival during storage. Sorbitol was the most effective protectant for both LAB (Carvalho et al., 2002). The influence of the drying medium on survival of freeze dried *Lact. bulgaricus* during storage depended on the specific growth medium employed. Maximum protection of cells was achieved with glucose, fructose, and sorbitol (Carvalho et al., 2004a).

Moreover, inactivation of bacterial cells during storage due to the consequences of oxidation is related to the formation of radicals in the presence of oxygen, fatty acid oxidation, and DNA damage (Selmer-Olsen et al., 1999b). Finally, the drying method was observed to impact on storage stability, as the viability reduction of both LAB and

Bifidobacteria differed between freeze dried and spray dried cultures under similar packaging and storage conditions (Wang et al., 2004).

Recovery of dried cultures is furthermore dependent on rehydration conditions employed. First, the rehydration solution must be optimised in terms of osmolality, pH, and nutritional energy source. An environment characterised by a high osmotic pressure could control the rate of hydration, and thus avoid osmotic shock (Costa et al., 2000). The use of the same protective compounds in the growth, the drying and the rehydration medium should be investigated.

Secondly, rehydration conditions with regard to rehydration temperature and volume need to be selected carefully to increase the rate of recovery of the viable state. Optimum rehydration temperatures for the recovery of microorganisms from the dried state were observed to be dependent on the drying method initially applied (Wang et al., 2004). The viability of dried cultures was reported to be influenced by the rehydration rate, being higher after slow rehydration (Selmer-Olsen et al., 1999a).

2.6 Influence of Sugars on the Viability of LAB

In order to meet the requirements of product quality in terms of long-term stability and viability of lactic acid bacteria starter cultures as well as economic feasibility, the focus of the present study is on the stabilisation of two strains of lactic acid bacteria, *Ent. faecium* and *Lact. plantarum*, during pilot scale preservation processes and storage. Furthermore this study comprises a direct comparison of the drying methods lyophilisation and fluidised bed drying in terms of survival of lactic acid bacteria, which is often impossible due to distinct experimental setups and strains applied. In general, literature on fluidised bed drying of lactic acid bacteria - especially on pilot scale as done in this study - is limited (Santivarangkna et al., 2007). The effect taken by protective carbohydrate compounds on bacterial cells was investigated because the preconditioning of bacterial cells prior to the drying process appears to be of particular importance.

Due to their broad range of application lactic acid bacteria are of commercial significance for the fermentation industry. Next to fermentation and transformation of foods the production of lactic acid is of economic importance. In recent years new applications of lactic acid bacteria, such as probiotic dietary food and feed supplements (Gomes and Malcata, 1999; Saxelin et al., 1999) and silage inoculants have increased the needs for research and production focusing on stabilisation of starter cultures. All these industrial applications require the maintenance of high viabilities of bacterial populations during preservation and storage, therefore one of the main obstacles in the commercialisation of lactic acid bacteria is the development of storable formulated products that retain the viability and the activity of the initial population.

For many years lactic acid bacteria have been mainly preserved by freeze drying, which is a gentle, but time-consuming and expensive drying method (Morgan et al., 2006; Ratti, 2001; Santivarangkna et al., 2007). Spray drying would allow inexpensive production of large amounts, as the costs of spray drying can be six times lower per kilogramme water removed than the cost of freeze drying (Knorr, 1998). High operating temperatures, however, result in poor survival of microorganisms subjected to spray drying (Johnson and Etzel, 1995). By contrast the optimal heat and mass transport as well as the equal temperature distribution in a fluidised bed drier allow gentle drying of sensitive

compounds. In addition the fluidised bed process consumes less time and energy than freeze drying (Barbosa-Canovas and Uliano, 2004; Chua and Chou, 2003) and is therefore a cost-effective alternative for preserving bioactive compounds like heat-labile microorganisms (Bucio et al., 2005).

Desiccation brings about different types of cell damage, primarily due to changes in the physical state of membrane lipids and in the structure of sensitive proteins that lead to an often severe loss in bacterial viability (Leslie et al., 1995). In many cases sugars and sugar derivatives were found to be effective toward protection of various lactic acid bacteria: Due to the presence of hydroxyl groups of carbohydrates replace water thereby stabilising cell membrane lipids and proteins; polyols were found to prevent oxidative damage by scavenging of free radicals (Smirnoff and Cumbes, 1989). It was previously demonstrated that the protective effect does not depend on whether the sugars can be metabolised or not and therefore a physicochemical nature of the effect was postulated (Carvalho et al., 2002).

A few reports describe the stabilising effects of carbohydrates or their derivatives (Efiuvwevwere et al., 1999; Linders et al., 1997b; Linders et al., 1997c) and other osmoprotective molecules (Selmer-Olsen et al., 1999a) on survival of lactic acid bacteria subjected to convective drying. Several studies dealt with the influence of cryoprotective sugars that exhibited a good preservative effect on lactic acid bacteria during the well established freeze drying method (Carcoba and Rodriguez, 2000; De Giulio et al., 2005) and during subsequent storage (Carvalho et al., 2002; Zarate and Nader-Macias, 2006). An enhanced stabilisation of freeze dried *Lactobacillus* species could be achieved either by cross linking a carbohydrate compound with salt ions (Conrad et al., 2000) or by applying a mixture of sugars with complex media like skim milk (Zarate and Nader-Macias, 2006; Zayed and Roos, 2004). Further investigations revealed that the survival of freeze dried *Lact. bulgaricus* during storage is dependent on the drying medium and the growth medium (Carvalho et al., 2004a). A study on mutant *Lact. acidophilus* revealed that the internalisation of the compatible solute trehalose plays an important role in cryoprotection (Duong et al., 2006).

2.7 Material and Methods

Bacterial Strains

Two lactic acid bacteria strains, *Ent. faecium* strain IFA No.045 and *Lact. plantarum* strain IFA No.278 (Department of Agrobiotechnology, Institute for Environmental Biotechnology, IFA-Tulln, Austria) were used in this study. The bacteria were grown in de Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) at 37°C overnight and maintained as glycerol (20% v/v, JT Baker, Phillipsburg, USA) stocks at -80°C. For experiments, the bacteria were precultured and grown without aeration in a two-m^3-fermenter. Because of confidentiality arrangements growth conditions, which were kept constant for all production processes, cannot be disclosed in detail. Due to the schedule of pilot scale production processes *Ent. faecium* cells were harvested eight hours after the end of the acidifying activity, whereas *Lact. plantarum* cells were separated at the end of the exponential growth phase. Either bacterial strain was concentrated by separation (Westfalia Separator, Ennigerloh, Germany). The resulting bacterial solutions still contained growth medium components that passed the separation step.

Incubation with Protective Carbohydrates

The carbohydrates and the applied concentrations used in this study were chosen because of their reported protective capacity during freeze drying of lactic acid bacteria (De Giulio et al., 2005). 32% w/v of the following protective carbohydrates: glucose (Avebe, Veendam, The Netherlands), sucrose (Agrana, Tulln, Austria), trehalose (Cargill, Mechelen, Belgium), or maltodextrin (DE 19, Syral, Marckolsheim, France) was directly suspended in the concentrated bacterial solutions. Per trial 600 mL of concentrated bacterial solution and 192 g of protectant were used for the freeze drying experiments, while 2 L of concentrate mixed with 640 g of protectant were applied to fluidised bed drying trials, which were carried out in duplicates, consecutively and randomised. For control purposes, a blank trial without addition of any compound was carried along with each set of trials. An incubation time of one hour was chosen as on one hand being representative for industrial applications (Conrad et al., 2000) and on the other hand to allow for equilibration between the cells and the added protectant (Carvalho et al., 2004a;

Carvalho et al., 2002). After incubation at room temperature the solutions were subjected to either drying method (Conrad et al., 2000). Non-processed bacterial suspension was stored at 4°C for maximum 30 hours before drying.

Fluidised Bed Bottom-Spray Drying

The bacterial solution was atomised by a two-fluid nozzle in bottom-spray position using a peristaltic pump (Watson Marlow, Falmouth, UK) applying a spraying air pressure of 250 kPa. Each trial was sprayed onto 5 kg of powdered cellulose carrier material Arbocel G350 (JRS, Rosenberg, Germany) previously loaded into a pilot scale fluidised bed drier (AMMAG, Gunskirchen, Austria) of a maximum loading capacity of 50 L depending upon powder density. Inlet air temperature was set to 45°C which resulted in a maximal bed temperature of 37°C depending on the spraying rate. The spraying rate ranging between 50 and 100 g min^{-1} was adjusted so that agglomeration of particles was avoided. The fluidising air flow was held constant at 800 m^3 h^{-1}. Drying was conducted at a bed temperature of 37°C; finally the powder was cooled to room temperature.

Lyophilisation

The incubated suspensions were filled into 30 x 40 cm plastic bags, put flattened in a deep freeze and frozen to -80°C. The frozen mixtures were then desiccated under vacuum (31 Pa) in the freeze drier (Martin Christ, Osterode, Germany) for 2 hours while the product temperature was rising from -30 to 0°C and further 9 hours while the product temperature is rising from 0 to 30°C. Finally 17 hours after the generation of vacuum it was raised to 0.1 Pa. Drying took place below glass transition temperature being glucose the protectant having the lowest glass transition temperature (31°C) of all protectants applied in this study (Bhandari and Howes, 1999; Chen et al., 2000).

Storage and Rehydration Conditions

One-g-samples of each experiment were weighed into a 15-mL-plastic tube immediately after drying, retaining residual oxygen and humidity of the packaging environment. As atmosphere, relative humidity (Castro et al., 1995), and exposure to light appear to be important factors for the recovery of freeze dried cells, tubes containing samples that were to be analyzed at the same point of time were hermetically sealed in aluminium sachets, exposed to darkness, retaining residual oxygen and humidity of the packaging

environment. Since previous studies (Champagne et al., 1996; Wang et al., 2004) showed that temperature is a critical parameter for microbial survival during storage, one-g-samples of each experiment were weighed into a 15-mL-plastic tube and stored at 4, 22, and 35°C. The rehydration conditions were held constant for all samples as temperature, volume, and the rehydration solution itself may significantly affect the rate of recovery to the viable state, and thus influence survival rates (Selmer-Olsen et al., 1999a).

Determination of Colony Forming Units (CFU)

CFU were determined by the pour plate method in MRS agar (Oxoid). The CFU assay was carried out in triplicates and after 48 h of incubation at 35°C the plates containing 20 - 200 CFU were taken to determine the relative bacterial viability by the following equation [%]:

$$\frac{\text{Population of the powder} \, (CFU\,g^{-1}) \times \text{amount of powder} \, (g) \times 100}{\text{Population of the suspension} \, (CFU\,mL^{-1}) \times \text{amount of solution} \, (mL)}$$

The resulting bacterial concentrates after separation contained 1.9×10^{11} and 4.2×10^{10} CFU of *Ent. faecium* and *Lact. plantarum*, respectively. To observe the shelf life of the different formulations, cell viability was determined over a period of half a year after drying. The results are given as means $\pm$ standard deviation (SD). Comparisons between means of relative viabilities of either dried bacterial species and at certain storage temperatures were performed by one-way analysis of variance (ANOVA), and by Tukey's Multiple Comparison Test. *p* values of 0.05 or less were considered significant.

Determination of Dry Matter Content

Dry matter contents were determined with an infrared balance (Sartorius MA30, Goettingen, Germany). A certain amount (about 2 g) of sample is dried to equilibrium moisture and then the dry matter content is automatically calculated.

2.8 Results

Effect of Protective Carbohydrates on Drying Methods

In this study the monosaccharide glucose (Glc), the two disaccharides trehalose (Tre) and sucrose (Suc), and the polysaccharide maltodextrin (MD) were tested with respect to their ability to protect bacterial cells against drying. After either lyophilisation or fluidised bed drying, cell viabilities of *Ent. faecium* in the absence of the protective agents were significantly lower (39.74±2.21 and 11.00±6.37%, respectively) than with the addition of any compounds tested (Figure 7a). In general, survival of cells was higher after lyophilisation than after fluidised bed drying being statistically significant ($p < 0.05$) for the untreated freeze and fluidised bed dried cells, and the protective additive glucose. All compounds tested were found to be equally effective in protecting *Ent. faecium* during freeze drying (>83.41%) and fluidised bed drying (>57.27%) compared to the untreated sample (Figure 7a).
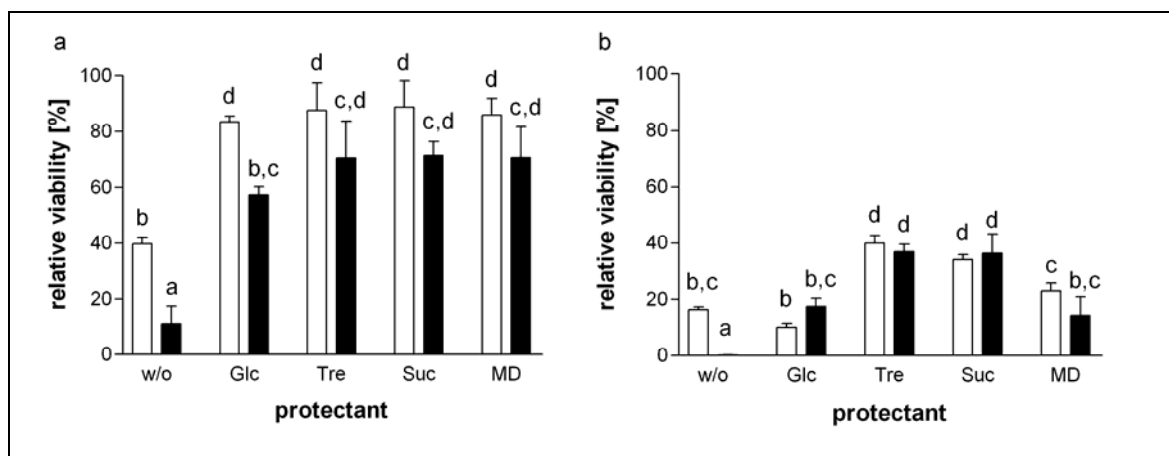
Figure 7 Observed relative viabilities of a. *Ent. faecium* and b. *Lact. plantarum* cells in the absence (w/o) and in the presence of the protective additives glucose (Glc), trehalose (Tre), sucrose (Suc), and maltodextrin (MD) at a concentration of 32% (w/v) either after freeze drying (FD, white bars) or fluidised bed drying (FBD, black bars). Different letters within one figure indicate that means are significantly different ($p < 0.05$).

The highest viability of *Lact. plantarum* cells after freeze drying was obtained by the addition of trehalose (40.05±2.68%) followed by sucrose (34.06±1.84%) as cryoprotectants (Figure 7b). Formulating cells with glucose and maltodextrin before lyophilisation did not enhance the survival rates (10.08±1.34% and 22.95±2.88%, respectively) significantly compared to the untreated sample (16.28±1.06%). The best

results protecting *Lact. plantarum* cells during fluidised bed drying were obtained by the addition of trehalose and sucrose showing relative viabilities of 36.87±2.79 and 36.39±6.78%, respectively. Viabilities of cells formulated with glucose and maltodextrin were both significantly lower (17.49±2.85 and 14.21±6.78%, respectively), but compared to the untreated sample still higher by a factor of 100 (Figure 7b). Without the addition of protectants the fluidised bed process was recognised to cause more damage to the survival rates of *Lact. plantarum* cells than lyophilisation, resulting in relative viabilities of 0.16±0.17 compared to 16.28±1.06%, respectively. No significant differences were observed between the freeze dried and the fluidised bed dried formulations containing the protective additives.

Effect of Protective Carbohydrates on Storage Stability of Fluidised Bed Dried *Ent. faecium*

The shelf life of the different carbohydrate formulations was investigated at refrigerated storage (4°C) and at the temperatures of 22 and 35°C for the time interval of half a year. At 4°C storage temperature no significant loss of initial viability of fluidised bed dried *Ent. faecium* cells without any additive and formulated with the four protective additives was observed throughout the whole period of investigation (Figure 8a). At an elevated storage temperature of 22°C no significant loss of initial viability was demonstrated by the trehalose and sucrose formulations showing relative viabilities of 57.90±6.79 and 56.04±6.63%, respectively, after six months (Figure 8b). Formulations of glucose and maltodextrin decreased in viability after six months of storage declining to 36.92±6.58 and 42.74±5.35% viability. Without any protective compound viability was considerably lower but no statistically significant changes were detected throughout six months of storage. Glucose was found to be ineffective in the protection of *Ent. faecium* at a storage temperature of 35°C as viabilities dropped to <1% after one month, while without any additive this level was reached after six months of storage (Figure 8c). Trehalose and sucrose proved to be the best protectants at 35°C storage temperature losing viability significantly after three months and leading to final viabilities of 35.82±2.52 and 39.34±5.51%, respectively, after six months (Figure 8c). Using maltodextrin as protective agent caused a significant loss in viability already after one month, but still a relative viability of 30.98±5.23% could be preserved after six months of storage at 35°C.
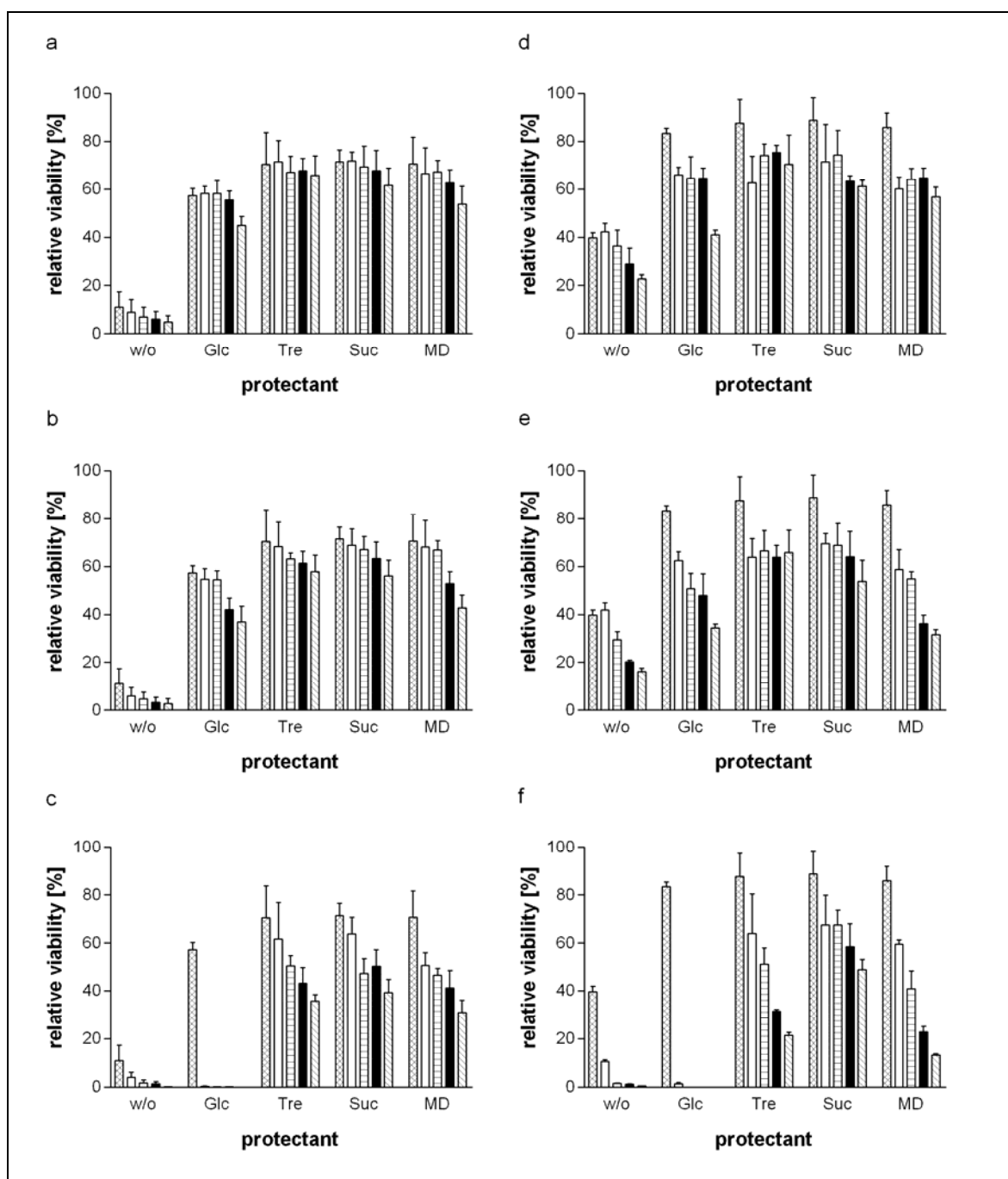
Figure 8 Relative viabilities of fluidised bed dried (a,b,c) and freeze dried (d,e,f) *Ent. faecium* cells in the absence (w/o) and in the presence of the protective additives glucose (Glc), trehalose (Tre), sucrose (Suc), and maltodextrin (MD) after 0 (cross-hatched bars), 1 (white bars), 3 (lined bars), 4 (black bars), and 6 (hatched bars) months of storage at different temperatures. a,d Cells stored at 4°C. b,e Storage at 22°C. c,f Cells stored at 35°C.

Effect of Protective Carbohydrates on Storage Stability of Freeze Dried *Ent. faecium*

The long-term stability of freeze dried samples formulated with trehalose and stored at 4°C showed no significant decrease in viability after half a year of storage (70.33±12.33%), while neither sucrose nor maltodextrin could prevent a drop during storage ending up with relative viabilities of 61.25±2.38 and 56.79±4.12%, respectively (Figure 8d). After six months and with 41.08±1.88% relative viability glucose formulated cells did not differ any longer from untreated cells, whose viabilities did not decrease significantly during the same period of time showing a viability of 22.78±1.76% after half a year. Furthermore non-treated freeze dried *Ent. faecium* cells exhibited a better overall performance than non-treated fluidised bed dried cells (Figure 8a,d). A decline of viability after the first month of storage at 22°C could be observed in all formulated freeze dried cells. While trehalose and sucrose were able to stabilise cells from the first month, glucose and maltodextrin formulations did not further protect cells during storage as no significant difference to untreated cells could be revealed after six months (Figure 8e). Freeze dried cells without being protected by any compound suffered damage after the fourth month of storage, from this point in time no difference to non-treated fluidised bed dried *Ent. faecium* cells could be observed (Figure 8b,e). A constant decrease of viability was the trend for sucrose, trehalose and maltodextrin treated cells stored at 35°C, nevertheless sucrose turned out to be capable of stabilising *Ent. faecium* at this temperature significantly better than trehalose and maltodextrin, ending at a final viability of 48.86±4.30% after six months of storage (Figure 8f). As already observed in fluidised bed dried samples glucose was not effective in protecting *Ent. faecium* at this elevated temperature (1.46±0.55% after one month) being sevenfold worse than without the addition of a protective agent.

Effect of Protective Carbohydrates on St orage Stability of Fluidised Bed Dried *Lact. plantarum*

As less than 0.16±0.17% of the initial *Lact. plantarum* population survived the fluidised bed drying process without any protection by carbohydrates, this approach was not subjected to the storage trials. At 4°C storage temperature the best results in long-term stability were initially obtained when cells were formulated with the two disaccharides, nevertheless after the third month of storage no significant difference in stabilisation compared to glucose formulated cells could be observed. The three formulations secured

viabilities in the range between 8.23±2.63 (glucose), 10.22±0.93 (trehalose), and 13.75±2.01% (sucrose) after six months of storage (Figure 9a). Maltodextrin displayed significantly lower relative viabilities than the disaccharides over the whole period of investigation (except for trehalose after six months) ending with 2.03±1.28% residual viability. At 22°C storage temperature all formulations revealed a decline of more than a half of the relative viability, in the first place after one month and secondly from the first to the third month of storage (Figure 9b). The two disaccharides showed the same trend as at 4°C, not being significantly more efficient in the protection of *Lact. plantarum* compared to glucose and even maltodextrin formulations after the third month of storage. No compound among the carbohydrates tested turned out to be effective in the protection of *Lact. plantarum* cells at a storage temperature of 35°C dropping on average more than one log cycle after each month of storage (data not shown).
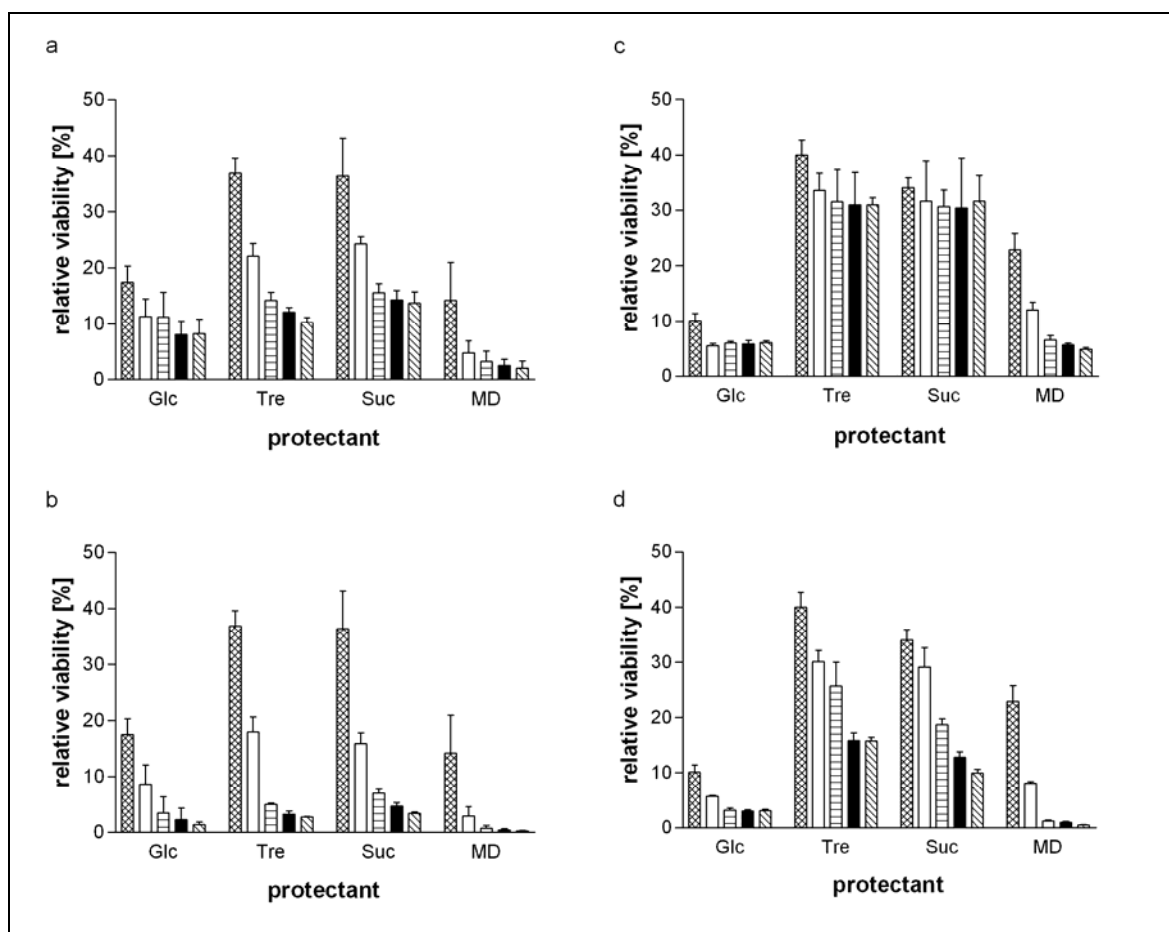
Figure 9 Relative viabilities of fluidised bed dried (a,b) and freeze dried (c,d) *Lact. plantarum* cells in the absence (w/o) and in the presence of the protective additives glucose (Glc), trehalose (Tre), sucrose (Suc), and maltodextrin (MD) after 0 (cross-hatched bars), 1 (white bars), 3 (lined bars), 4 (black bars), and 6 (hatched bars) months of storage at different temperatures. a,c Cells stored at 4°C. b,d Storage at 22°C.

Effect of Protective Carbohydrates on   Storage Stability of Freez   e Dried *Lact. plantarum*

At 4°C sucrose mediated best storage stability for *Lact. plantarum* cells without significant losses in viability over the entire period of investigation and a final viability of 31.60±4.72% (Figure 9c). In contrast, trehalose formulated cells suffered a significant decline in viability after the fourth month of storage, nevertheless single monthly viabilities did not differ from sucrose treated cells. Furthermore glucose and maltodextrin formulations were already reported not to promote process survival during freeze drying of *Lact. plantarum* (Figure 7b) nor did they exhibit a stabilising effect during storage compared to non-treated cells at the storage temperatures of 4 and 22°C. Cells without being protected retained viabilities of 2.06±0.20 and 0.64±0.04% at 4 and 22°C, respectively, after six months of storage (data not shown). After the third month at 22°C sucrose could not prevent an earlier decline of viability than trehalose, while no significant difference in stabilisation of freeze dried *Lact. plantarum* during storage between the two disaccharides could be observed from the fourth month (Figure 9d). Despite being reduced to more than a half and more than a third, respectively, the two disaccharides trehalose and sucrose maintained highest viabilities of 15.76±0.71 and 9.94±0.72%, respectively, among all compounds tested. With a focus on trehalose and sucrose formulations freeze dried cells were overall more stable than fluidised bed dried cells at both storage temperatures 4 and 22°C. Similarly to fluidised bed dried cells storage at 35°C led to extensive losses in viability and neither of the compounds used could prevent those (data not shown).

Dry Matter Contents of Bacterial Concentrates and Resulting Powders

The concentrating step yielded an average dry mass of 17.8 and 7.3% for the bacterial solutions of *Ent. faecium* and *Lact. plantarum*, respectively. In the case of *Ent. faecium* a ratio between protectant and cell mass of 1.8 was achieved, while the lower cell density of *Lact. plantarum* caused a ratio of 4.4.

Residual moisture contents of fluidised bed dried samples of both strains lay in the range between 3.76 and 6.05%, in contrast freeze dried samples varied to a higher extent from 1.90 to 7.98% (Table 2). Cells of both strains without any protectant added resulted in dried products of a narrow moisture range (3.32 to 4.05%). Maltodextrin formulated cells were mostly amongst the driest products while only glucose formulated freeze dried cells

showed smaller residual moisture contents of 2.35 and 2.81, respectively, than glucose formulated fluidised bed dried cells. The disaccharide formulations of *Lact. plantarum* did not differ in particular, while trehalose formulated *Ent. faecium* showed higher residual moisture contents than sucrose formulated *Ent. faecium* cells.

Table 2 Average residual moisture contents of the resulting formulations of both *Ent. faecium* and *Lact. plantarum* after freeze drying (FD) and fluidised bed drying (FBD).

Residual moisture content [%]	*Ent. faecium*		*Lact. plantarum*	
Additive	FBD	FD	FBD	FD
Without (w/o)	3.92	3.32	3.76	4.05
Glucose (Glu)	6.05	2.35	5.18	2.81
Trehalose (Tre)	5.72	7.98	4.87	4.25
Sucrose (Suc)	4.54	3.05	4.60	4.71
Maltodextrin (MD)	4.66	2.53	3.96	1.90

2.9 Discussion

This study provides a comparison of lyophilisation and fluidised bed drying in terms of survival of two lactic acid bacteria strains. Both industrial drying processes are employed for preserving sensitive bioactive compounds in order to achieve the preservation of high viability during the drying process and subsequent storage of bacterial starter cultures. Several previous surveys focused on the survival of lactic acid bacteria after lyophilisation and subsequent storage (Carvalho et al., 2004b), while previously published data related to fluidised bed drying of lactic acid bacteria is limited (Santivarangkna et al., 2007) and often refers to drying of cells granulated to pellets (Linders et al., 1997a; Mille et al., 2004), homogenised into powders (Mille et al., 2004) or entrapped in alginate beads (Selmer-Olsen et al., 1999b). In all the mentioned surveys bacteria are encapsulated in a certain matrix before being dried in contrast to spraying concentrated liquid cell suspensions onto fluidised carrier material as described in this study. Thus the technique applied is spray drying but in contrast to conventional spray drying lower temperatures can be applied in the fluidised bed preventing damage by heat.

Desiccation imposes severe stress on microorganisms as the removal of water induces conformational changes in proteins and cell membranes. It is apparent that induction of desiccation tolerance takes place under certain culture and pre-drying conditions. The exact mode of action of protective agents is indeed complex, and not fully understood to date being water replacement the essential mechanism underlying the protection mediated by certain additives (Potts, 2001). Fluidised bed dried *Lact. plantarum* cells turned out to be particularly susceptible to desiccation induced damage resulting in a residual viability of <0.2%, thus highlighting the need for formulations containing protective compounds that enhance process survival. Due to bad performance of the polyol sorbitol at a concentration of 32% (w/v) in the fluidised bed drying process and incomplete drying during lyophilisation (data not shown), no further investigations were carried out with this compound despite positive reports (Carvalho et al., 2002). In contrast to previous reports (Carcoba and Rodriguez, 2000; Linders et al., 1997c; Selmer-Olsen et al., 1999a) results obtained with the addition of skim milk at 10% (w/v) and skim milk in combination with trehalose and sorbitol were not significantly better than the above mentioned additives (data not shown) and therefore not considered in shelf life studies. The poor solubility of

lactose triggered clogging of the spray nozzle in the fluidised bed drying process and was excluded in this study.

Process survival rates after both drying methods revealed that without any protective compound added fluidised bed drying is more harmful to both bacterial strains than freeze drying. Similar, previous observations comparing freeze and spray drying showed that higher survival rates of lactic acid bacteria were achieved by the former drying method (Corcoran et al., 2006; To and Etzel, 1997; Wang et al., 2004). Another comparative study on this topic revealed contrary results, proving that susceptibility to certain drying methods is strongly dependent on the strain under evaluation (Zamora et al., 2006). Focusing on fluidised bed drying thermal inactivation encountered during spray drying (Corcoran et al., 2004) can be minimised in fluidised bed drying by applying gentle process conditions, thus dehydration inactivation remains the main problem in convective drying (Linders et al., 1997b). Furthermore the consequences of oxidation are very harmful especially to the membrane structure, thus oxygen-sensitive bacterial species suffer severe damage during convective air drying (Mille et al., 2004).

According to the literature mentioned above it can be summarised that in addition to dehydration inactivation microbial cells are mainly affected by ice crystal formation occurring during freeze drying or by oxidative stress encountered during fluidised bed drying. Our results correlate to a certain extent with those previous findings, nevertheless apart from the action of protectants there are several more factors being responsible for the poorer survival of fluidised bed dried cells that need to be considered in further studies. In the present experiments only one established processing condition per drying method was investigated, therefore variation of the processing parameters is likely to yield different results and should be used for further optimisation.

The results of the current study point out that the damage caused by either drying method may be reduced by incubating bacterial cells with certain protective carbohydrates as their presence during fluidised bed and freeze drying resulted at least in equal, in most cases in higher survival rates than those of non-treated cells. Especially trehalose was reported to be capable of stabilising biological material like cells and proteins (Schiraldi et al., 2002). Several physical principles underlying the mechanisms of stabilisation of cells and proteins in the dry state were mentioned: the prevention of fusion and phase transition of

phospholipid bilayers, direct interaction by hydrogen bonding of hydroxyl groups to polar residues and furthermore vitrification forming glassy amorphous solids (Crowe et al., 1992). Previous observations reported the ability of trehalose and sucrose to stabilise cell membranes by preventing the phospholipids' phase transition during drying and rehydration which in turn would be the cause of membrane leakage (Hoekstra et al., 1997; Leslie et al., 1995). Despite obvious variations in residual moisture contents each of the four carbohydrate formulations showed similar protective effects on *Ent. faecium* both during freeze drying and during fluidised bed drying. In contrast, the two disaccharides exhibited the best performance during fluidised bed drying of *Lact. plantarum* and furthermore they were the only compounds capable of enhancing survival during lyophilisation of *Lact. plantarum*. Appropriate water binding capacities may explain why the two disaccharides cause residual moisture contents that are particularly favourable for survival of *Lact. plantarum.* The low residual moisture contents of glucose and maltodextrin formulated freeze dried *Lact. plantarum* indicate the removal of structural water causing damage of important cell molecules. A certain amount of water must remain in the dehydrated state and the optimum moisture content needs to be determined for a satisfactory survival rate of the strain of investigation e.g. for freeze dried *Lact. salivarius* the optimum moisture content was reported to range from about 2.8 to 5.6% (Zayed and Roos, 2004).

Among the carbohydrates studied trehalose and sucrose furthermore proved to mediate equal if not the longest lasting protection during storage of both lactic acid bacterial strains and regardless of the drying method. While all compounds tested stabilised fluidised bed dried *Ent. faecium* at 4°C throughout the whole period of investigation, only the disaccharides secured initial viabilities from significant losses at an elevated storage temperature of 22°C. As observed previously the stability of dried samples decreased during storage showing higher losses in viability at elevated storage temperatures (Corcoran et al., 2004; Gardiner et al., 2000) which could correspond to a higher rate of fatty acid oxidation (Castro et al., 1995). In particular glucose must not be used in the drying medium at a storage temperature of 35°C as its glass transition temperature is exceeded (Bhandari and Howes, 1999) exerting negative effects on storage stability. Storage at 35°C led to significant losses of bacterial viability after one month, whereas the addition of the disaccharides resulted in a longer-lasting stabilisation in comparison to the

other two protectants tested. The residual moisture content was reported to be directly related to the type of drying medium (Zayed and Roos, 2004). In addition to the influence of certain protectants on storage stability the optimum moisture content of the individual strain needs to be determined in further studies. In general, dried *Lact. plantarum* showed poorer survival during storage after fluidised bed drying than after freeze drying, which is comparable to previous findings revealing higher survival of freeze dried than of spray dried *Lact. sakei* (Ferreira et al., 2005) and *Streptococcus thermophilus* (Wang et al., 2004). Interestingly, after three months of storage at 4 and 22°C both disaccharide formulations of fluidised bed dried *Lact. plantarum* lost in viability to such an extent that no difference to other formulations could be observed. With respect to a shelf life of half a year and depending on product requirements the more costly refrigerated storage could be avoided for optimally formulated *Ent. faecium* while it should be recommended for *Lact. plantarum* starter cultures. As sucrose is more cost-effective than trehalose, conventional sugar should be favoured if it exerts the same protective effects as trehalose.

Several facts explain why *Lact. plantarum* turned out to be more sensitive to both drying methods and subsequent storage than *Ent. faecium*. First, previous observations showed that *Enterococci* (i.e. small spherical cells) are apparently more resistant to freezing and freeze drying than rod-shaped *Lactobacilli* (Fonseca et al., 2000; Fonseca et al., 2004), which are more susceptible to membrane damage due to their higher surface area. Secondly, *Lact. plantarum* cells were harvested earlier than *Ent. faecium*, namely on the onset of stationary phase, which could impact negatively on resistance of *Lact. plantarum* to desiccation. Optimal survival of spray drying was reported of *Lactobacillus* cultures in the stationary phase of growth (Corcoran et al., 2004), which additionally proved to be more resistant to stress conditions (Kim et al., 2001). Finally, the 4.5-fold higher initial cell concentration could be made responsible for the better performance of *Ent. faecium* in both drying methods. The chosen experimental setup, however, does not allow a direct comparison between the two strains. Optimisation of all process steps is expected to result in improved survival rates for *Lact. plantarum*.

To sum up, further measures to enhance bacterial survival during drying processes need to be taken: Among those are optimising culture conditions (Palmfeldt and Hahn-Hagerdal, 2000; Saxelin et al., 1999) and cell density (Morgan et al., 2006), and testing of different protective compounds, concentrations and times of incubation thereof to strengthen

bacterial desiccation tolerance. By exhibiting stress conditions (pH, temperature, osmotic pressure etc) adaptive response can be provoked resulting in more desiccation-resistant bacterial cells (Abee and Wouters, 1999; Baati et al., 2000; Conrad et al., 2000; Kim et al., 2001; Morgan et al., 2006; Saarela et al., 2004). Response surface methodologies were reported to be particularly suitable to detect interactions and co-dependencies between the individual parameters mentioned (Schoug et al., 2006). Furthermore the findings underline the need of strain selection with regard to the performance during fermentation and downstream processing. In addition the fact that fluidised bed drying produces less than a fifth of the fixed and manufacturing costs estimated for freeze drying (Santivarangkna et al., 2007) needs to be considered when choosing a drying method on a large scale.

3   Sustained Release Formulations in Agriculture

3.1 Encapsulation of Agrochemicals

The intensive agricultural production of today is not feasible without the application of fertilisers and pesticides. This is contrary to the increasing and broad rejection of synthetic pesticides and especially their adverse impact on the environment and on consumer's health (Duke et al., 2003). Biological or ecological pest management is advisable for present-day high-tech agriculture to save crops from harm while at the same time enabling high level crop yields without polluting the important resources soil, groundwater and foods.

Depending on the method of application and climatic conditions, as much as 90% of conventional agrochemicals applied were reported never to reach their objective at the precise time and quantity required to exert the desired biological functions (Mogul et al., 1996). Sustained release formulations are one approach to reduce the amount of synthetic pesticides and fertilisers and to decrease the frequency of their administration by maintaining an effective concentration for a certain period of time thereby reducing the risk of contaminating the environment and foods with persistent residues of agrochemicals. The encapsulated granulate is applied to the field and the active ingredient is slowly released over time due to diffusion, microbial degradation and the impact of further environmental factors like rain, heat, etc. on the capsule material.

Apart from protecting the pesticide itself, controlled release formulations are able to eliminate repeated and over applications, decrease operator toxicity, pesticide leaching, runoff, and volatilisation while increasing persistence (Davis et al., 1996). Certain pests are active, when soil or air temperature reaches a critical level, therefore, temperature-controlled pesticide release is useful to protect the active substance from degradation and to release it at the right point of time to combat the pest (Greene et al., 1992). The difference between the terms controlled and sustained release is not pointed out clearly in literature. The former term was first introduced by the pharmaceutical industry referring to a precise time and site specific release of active ingredient. This characteristic is probably desired by the majority of industries for diverse applications, therefore mainly controlled

release formulations are cited. Despite being applied concurrently, the term sustained release may correspond to less defined release patterns.

Synthetic Polymers

Synthetic polymers utilisable for controlled release preparations of agrochemicals are polyvinyl pyrrolidone, polyvinyl alcohol, polyurethane, polyacryl amid, or polyhydroxy ethylmethacrylate (Alemzadeh and Vossoughi, 2002). The choice of the appropriate coating polymer may be facilitated by the determination of their water vapour permeability. Certain polymers revealed ascending permeability coefficients: polyhydroxybutyrate co-polyhydroxyvalerate (PHB/PHV), polylactic acid (PLA), polyhydroxyoctanoate (PHO), polyterephtalate (PET), polycaprolactone (PCL), and modified starch (Devassine et al., 2002). Temperature and coating layer thickness are the most important factors influencing diffusion of a polyurethane coated fertiliser (Du et al., 2006). A further approach to attain the desired diffusion character is to add release controlling agents, such as ethylene-vinyl acetate and surfactants to thermoplastic resins like polyvinylidene chloride that are highly impermeable to water (Jacobs, 2005).

Paraffin wax and aqueous paraffin emulsions used as carriers enable the application of pheromones for mating disruption of insect pests. The resulting formulations set pheromones free at a constant rate and over a certain period of time depending on the initial concentration, temperature and surface area (Atterholt et al., 1999).

Controlled release behaviour for the allelopathic compound β-napthol was achieved by co-polymerisation with acrylic acids derivatives. By the controlled, hydrolytic release of the plant growth regulator leaching to non-target environments may be prevented (Boudreaux et al., 1996).

Inorganic Salts

In addition to synthetics a further category of coating materials comprises non-polymeric delivery vehicles like inorganic salts or glasses (Mogul et al., 1996). Shell thickness of porous hollow silica nanoparticles fabricated by supercritical fluid technology turned out to be the main controlling factor for the controlled release rate and the UV-protection of the active agent (Li et al., 2006).

Biopolymers

Finally naturally occurring polymers and their application in sustained release formulations in the agricultural field need to be mentioned. Degradation kinetics of individual biopolymers need to be defined in order to be exploited in controlled release formulations making the products to fit in the picture of eco-compatibility.

Next to the encapsulation of microorganisms applied in the food industry (Hansen et al., 2002; Krasaekoopt et al., 2004), the water insoluble polysaccharide calcium alginate is employed in the agricultural field in sustained release formulations not only of microbial biocontrol agents (Manjula and Podile, 2005; Otsu et al., 2003; Zohar-Perez et al., 2005) but also of pesticides based on organic compounds (Connick et al., 1984; González-Pradas et al., 2008; Kulkarni et al., 2000; Pepperman and Kuan, 1995).

A coating containing microparticles with plant growth promoting rhizobacteria entrapped in a gelatine-polyphosphate polymer was evaluated for the bacterisation of seeds in order to yield crop improvement and subsequent reduction of pesticides of seeds (Amiet-Charpentier et al., 1998). Gelatine as well as alginate are maintaining a certain water content around the cells, thereby minimising the loss in viability due to dehydration (Manjula and Podile, 2005).

Comparably to controlled release formulations comprising active compounds in certain matrices, much research is done on the sector of seed coating with pesticides of any kind and further encapsulation in silicone gels, water insoluble polymers, and gellan gum coatings (Green and Beestman, 2007).

Despite its inhibitory activity on pathogens (Sebti et al., 2005) chitosan is a possible candidate for sustained release formulations in the agricultural field as it is insoluble at neutral and basic pH. Only an acidic pH causes complete solubilisation of the polymer, thereby releasing the encapsulated active agent all at once.

3.2 Allelopathic Compounds

There are several ways of organisms of different species to live together and form symbiotic relationships:

- Commensalism - one organism is profiting and the other one is not damaged.

- Mutualism - both organisms are benefiting.

- Parasitism - one organism is profiting, while the other one is harmed.

Pliny the Elder mentions in his "Naturalis Historia" (first century A.D.) the restraining influence of walnuts on the plants of its surroundings (Vyvyan, 2002). The active compound juglon is excreted by the leaves and washed into the ground by rain, where it affects on other plants. In 1937 the physiologist Hans Molisch found a narrow definition for the interaction among plants, he termed allelopathy: Plants influence the growth of other plants, while they deliver certain molecules to the environment.

Today every positive or negative effect on plants exerted by means of chemical messenger molecules derived from other plants as well as from organisms of other trophic levels like insects, fungi, and bacteria is placed under the concept of allelopathy. Therefore allelopathy may be found in all symbiotic relationships described above. According to the International Allelopathy Society (IAS) allelopathy "studies any process involving secondary metabolites produced by plants, algae, bacteria and fungi that influence the growth and development of agricultural and biological systems" (Macías et al., 2007).

The application of natural systems to prevent damage on agricultural crops and harvesting losses is to be recommended, in particular as diversity is maintained, soils are enriched and productivities stabilised. The major advantage of substituting synthetic with natural pesticides is the protection of the environment as conventional pesticides are characterised by persistence and environmental toxicity. A possibility could be the exploitation of natural, biochemical defence mechanisms - phytotoxins against insects, weeds and pathogens - employed by many plants.

Allelochemicals are released from crop plants through leachation, decomposition of crop residues, volatilisation, root exudates and from pollen (Khanh et al., 2005). Moreover, allelochemicals include compounds of several chemical classes, such as fatty acids,

benzoxazinoids, indoles, phenolic acids, phenylalkanoic acids and terpenoids. The entire path of allelochemicals - biosynthesis, accumulation in the plant, excretion to the environment, effect on target species - is dynamic and induced by various biotic and abiotic factors (Belz, 2007). The phytotoxic effects of the biologically active substances released contribute to the competitiveness and invasiveness of allelopathic plants in established ecosystems (Heisey and Kish Heisey, 2003).

The application of allelochemicals as biological pesticides is of particular interest. In terms of usability these substances should be safe and technologically sustainable, have new sites of action, must be active at low concentrations, and have a wide range of activities (Khanh et al., 2005). Isolation, identification and characterisation of active compounds are the main challenges of allelopathy research (Vyvyan, 2002). By means of bioassays the spectrum of activity of allelopathic compounds on different trophic levels like bacteria, fungi, algae, crustaceae and higher plants (crops and weeds) needs to be characterised. In addition, the localisation or distribution of the allelopathic active compound in the plant components root, stem, leaf, and flower is to be determined (Macías et al., 2007).

Several methods applying allelopathy in agricultural production systems are possible in order to reduce weeds and pathogens and to improve soil quality and crop yield:

- selection breeding of crops with allelopathic properties (wheat, rice, maize),

- allelopathic plants as undersown crops (crop rotation, companion cropping),

- composting/mulching of allelopathic plants,

- extracts or extraction of active substances of allelopathic plants, and

- chemical synthesis of allelopathic compounds.

As commercial plant activator application a seed extract of *Lychnis viscaria* L. is directly sprayed onto crop seeds, thereby promoting plant growth and resistance of the seedlings (Belz, 2007). Further applications of allelopathic crops, such as alfalfa, buckwheat, sunflower, or rye by means of plant residues, pellets, aqueous extracts or leachates were reported (Khanh et al., 2005). The only herbicide structurally based on a microbial product (bialaphos isolated from soil *streptomyces*) that has been successfully placed on the market is glufosinate (Macías et al., 2007). A further herbicide commercially exploited for the use

in maize is mesotrione, which is a synthetic analogue of the natural phytotoxin leptospermone excreted by *Callistemon citrinus* (Mitchell et al., 2001). Herbicidal effects of *Ailanthus altissima* bark extract, containing ailanthone as major active ingredient, were investigated under field conditions. As major drawbacks for a possible commercial application of ailanthone large scale production, lack of selectivity, and short persistence were cited (Heisey and Kish Heisey, 2003).

Due to their comparably good biological degradability allelochemicals on one hand do not reach the groundwater and are not recovered in harvest products, but on the other hand they show relatively short time of action (Macías et al., 2007). For an integrated crop protection it is therefore necessary to apply the active substance several times per cultivation period or to make use of an appropriate sustained release system. Encapsulation into biologically degradable materials is the method of choice including natural (not chemically modified) and semisynthetic materials (of natural origin). Synthetic polymers are to be excluded as they do not fit into the concept of sustained release formulations of allelopathic compounds to be used as biopesticides.

3.3 Sustained Release Behaviour of Coating Materials

In recent years the pesticide market has shown constant turnover being herbicides comprising the major part followed by fungicides and insecticides (Vyvyan, 2002). The use of pesticides and the residue-related impacts on environment, food and human health will not decrease significantly in the next years. Due to productive and environmental reasons a balance between active pest management and eco-compatible application needs to be established in systems like integrated plant protection.

Application of biopesticides is not only desirable for sustainable and ecological agriculture but also for conventional crop production in order to minimise residues in foods, soil, and waters. Over years several investigations comparing residues from pesticides derived from ecological and conventional agriculture came to the conclusion that products from the latter one are by far more contaminated than those produced biologically (Baker et al., 2002). Furthermore pesticide residues are carried off into the environment contaminating soil and waters. Market surveys revealed a clear tendency towards eco-friendlier products from conventional agricultural production as the world wide contamination of the environment by persistent pesticides has become a serious public concern (Mogul et al., 1996).

Allelopathic substances need to comply with the same important prerequisites like conventional synthetic pesticides. First they should be active for a certain period of time, secondly they must not harm the environment and they need to be biologically degradable at last. Allelochemicals are degraded rather quickly by microbes and are, therefore, eliminated from the environment in a relatively short period of time (Heisey and Kish Heisey, 2003) compared to synthetic compounds being persistent for several years or even decades in some cases (Boudreaux et al., 1996). This disadvantage is overcome by encapsulation of the active compound in rather slowly degrading materials of biological origin like polysaccharides, waxes, hydrogenated oils, and resins.

Allelochemicals are often stored and transported in plants as inactive precursor molecules that are activated after an external stimulus (e.g. injury) either by the plant's own enzymes or after being actively released in the rhizosphere by microbial degradation in the soil (Belz, 2007; Macías et al., 2007). Hence biologically degradable coatings exert two

functions: on one hand they act as encapsulating material and on the other hand they constitute an attractive substrate for microorganisms that activate allelochemicals by means of co-metabolisation.

The aim of this study was to develop sustained release systems for allelochemicals based on the degradation kinetics brought about by coating materials including substances of natural origin optionally chemically modified. Encapsulation was achieved by fluidised bed coating and sustained release behaviour was determined using an encapsulated inorganic model substance, which can be easily analytically detected. The compounds carnauba wax, hydrogenated soya oil, tall oil rosin, and shellac used in food, feed, pulp and paper were evaluated concerning the achievable product characteristics especially focusing on their ability to mediate sustained release. Apart from the aqueous shellac solution the coatings were applied as hot melts. This technique has been reported to be faster and cheaper than the conventional approaches, where evaporation and/or recovery of solvent can be expensive, tedious and time consuming (Barthelemy et al., 1999; Sinchaipanid et al., 2004). Top-spray fluidised bed coating is recommended for this purpose because it is possible to operate at the production temperature close to the congealing temperature of the molten mass, which is essential for producing a continuous coating on the particles. In addition, this coating technique enhances stability and provides sustained release characteristics to the pellets (Sinchaipanid et al., 2004).

As model substance aluminium sulphate was chosen as aluminium ions are easily detectable by inductively coupled plasma optical emission spectrometry (ICP-OES) and are not accumulated by microbial cells. Additionally aluminium sulphate is cheaper than other model substances such as gold particles. The detection of the amount of aluminium ions released enables the investigation of the quality and stability of the resulting encapsulation as well as the release kinetics, which were studied in degradation tests under simulated and optimised environmental conditions. Moreover, the use of an easily detectable model substance is necessary in order to investigate the sustained release systems periodically upon structural degradation of the encapsulating matrix, being independent from solubility or chemical stability of certain active ingredients in or isolated from plant extracts. The focus is not a complete biodegradation of the coating substance but the continuous structural decomposition of the encapsulating matrix. The mechanisms that control the release rate of coated pellets include dissolution of uncoated parts, leaching

through cracks and pores, furthermore diffusion and permeation through a hydrated, swollen film. A partial break-up facilitates pores and channels in the matrix, thereby enabling the release of encapsulated substances before the matrix is structurally disintegrated or even entirely degraded.

Independently, the biodegradation behaviour of the coating materials applied was determined using standardised aquatic laboratory tests (Modified Sturm Test according to OECD Guideline 301 B) in order to obtain a general idea of their degradation kinetics. During aerobic biodegradation in an aquatic environment, the organic carbon of the coating materials is microbially converted to carbon dioxide, microbial biomass, dissolved and volatile organic carbon. Biodegradation tests are commonly based on the measurement of carbon dioxide. In the aquatic environment almost optimal conditions in terms of distribution of nutritional sources, oxygen supply, and contact to the substance to be degraded can be achieved. In most cases the biodegradation kinetics of a substance in soil is derived from the kinetics obtained by aquatic tests.

3.4 Material and Methods

Fluidised Bed Aluminium Sulphate $Al_2(SO_4)_3$ Coating

3 kg $Al_2(SO_4)_3$ ground (17-18% Al_2O_3, Kemira Chemie GmbH, Krems, Austria) were solved in 7 L distilled water and were sprayed onto 25 kg of pharm-a-spheres Pellets neutral (min. 90% 0.5-0.6 mm, Hanns G. Werner Gmbh + Co.KG, Tornesch, Germany) previously loaded into a pilot scale fluidised bed drier (AMMAG). The solution was atomised by a two-fluid nozzle in top-spray position using a peristaltic pump (Watson Marlow) applying a spraying air pressure of 300 kPa. Bed temperature was set to 50°C which resulted in a maximal inlet temperature of 80°C, while the spraying rate was set to 220 g min^{-1} and no agglomeration of particles occurred. The fluidising air flow was held constant at 1300 m^3 h^{-1}. Finally the aluminium coated pellets were dried and cooled to room temperature. 1 g aluminium coated spheres were resolved in 400 mL distilled water, aluminium concentration was determined by inductively coupled plasma optical emission spectrometry (Ultima ICP-OES, HORIBA Jobin Yvon GmbH, Longjumeau Cedex, France) and ranged between 20 and 30 mg L^{-1} in the previously mentioned solution.

Fluidised Bed Shellac Coating

The coating solution consisted of 20 kg aqueous shellac solution (SSB Aquagold, Stroever GmbH + Co. KG, Bremen, Germany) and 0.5 kg glycerol (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The resulting mixture was further diluted to 15% solid content by the addition of 13.3 kg of distilled water. To achieve a 100% coating layer four volumes of the described coating solution were sprayed onto 20 kg of the previously prepared aluminium coated spheres loaded into a pilot scale fluidised bed drier (AMMAG). The solution was atomised by a two-fluid nozzle in top-spray position using a peristaltic pump (Watson Marlow) applying a spraying air pressure of 250 kPa. Bed temperature was set to 50°C which resulted in a maximal inlet temperature of 80°C, while the maximum spraying rate was 250 g min^{-1} in order to avoid agglomeration of particles. The fluidising air flow was held constant at 1300 m^3 h^{-1}. Finally the shellac coated aluminium pellets were dried and cooled to room temperature.

Fluidised Bed Hot Melt Coatings

Carnauba wax (Ph. Eur. 5.0, Gustav Heess GmbH, Stuttgart, Germany), hydrogenated soya oil (Ph. Eur. 5.0, Gustav Heess GmbH), and tall oil rosin (Sacotan 85, Kemira Chemie GmbH) were applied as hot melts. Prior to the coating process the spray lance was heated applying a spraying air temperature higher than the melting range of the individual coating in order to avoid clogging of lance and nozzle by solidified particles. 20 kg of hot melt were sprayed onto 20 kg of the previously prepared aluminium coated spheres loaded into a pilot scale fluidised bed drier (AMMAG) choosing appropriate process parameters (Table 3). The hot melts were atomised by a two-fluid nozzle in top-spray position using a peristaltic pump (Watson Marlow) and process parameters were set in order to avoid the formation of agglomerates. The fluidising air flow was initially set to 1000 m^3 h^{-1} and increased stepwise up to 1300 m^3 h^{-1} due to the weight gain of coated pellets. Finally the coated aluminium pellets were cooled to room temperature.

Table 3 Process parameters chosen for hot melt coatings.

Substance	Melting range [°C]	Spraying air temperature [°C]	Bed temperature [°C]	Spraying air pressure [kPa]	Maximum spraying rate [g min^{-1}]
Carnauba wax	80-88	100	65	250	200
Soya oil	66-72	85	55	250	200
Tall oil rosin	63-67	80	55	350	250

Electron Microscopy

A thin layer of dry coated pellets was mounted with conductive tabs on aluminium stubs, followed by the coating of pellets with gold (SCD 004 sputter-coater, OC Oerlikon Balzers AG, Balzers, Liechtenstein). Surface morphology of the coated pellets was examined and photographed with a digital scanning electron microscope (SEM, Zeiss DSM 940, Carl Zeiss AG, Jena, Germany) operating at 10 kV.

Determination of Average Particle Size

About half a kilo of coated pellets was poured into the top sieve of a nested column of sieves comprising mesh widths of 125, 500, 800 µm, and 1 mm. The column was placed in a mechanical shaker (VE1000, Retsch GmbH, Haan, Germany). Shaking amplitude was set 1 mm and each sample was shaken for ten minutes. The weight of the individual fractions

was expressed as percentage of total weight. The fraction containing the majority of particles was subjected to further analysis.

Preparation of Inoculum from Soil

Soil (Department of Agrobiotechnology, IFA-Tulln) used as inoculum was last treated with fertilisers (organic and mineral) and pesticides (of any kind) more than six months ago. 1-2 kg of soil were dispersed in about 5-7 L of tap water. The ratio is not critical. During one hour the suspension was stirred periodically (by hand) every 15 min so that all settled parts were whirled up. After a final settlement of 30 min, the supernatant was decanted through a fine sieve (preferably 63 or 100 µm). That suspension contained all fine particles and the majority of the microorganisms. It was centrifuged at 3.500 g for 20 min (Beckman J2-MI, rotor JP10, 4.500 rpm, Corona, USA) and the pellets were collected in a glass bottle (e.g. Schott AG, Mainz, Germany) and resuspended with a small amount of tap water.

A magnetic stirring rod was added and the inoculum was stirred overnight at room temperature in the open bottle to keep aerobic conditions, with active aeration on special demand. The homogenous suspension was of creamy consistence that could still be pipetted using a standard 1-mL-pipette (Eppendorf AG, Hamburg, Germany).

The inoculum was stored at 4°C for about three months. The stirring rod remained in the bottle so that the content could be easily homogenised on demand. Every withdrawal of parts of the inoculum was done under continuous stirring. The required amount of inoculum was approximately 5 mL per litre of test volume for the biodegradation test.

Determination of Biodegradability (Modified Sturm Test)

Figure 10 Experimental setup of the Modified Sturm Test according to OECD Guideline 301 B.

Table 4 Mineral medium, pH 7.2.

Substance	Amount / L
K_2HPO_4	1.0 g
KH_2PO_4	0.5 g
$MgSO_4$	0.1 g
NaCl	0.1 g
$(NH_4)_2SO_4$	0.3 g
$CaCl_2$	0.1 g
Solution of trace elements (Table 5)	1.0 mL

Table 5 Solution of trace elements.

Substance	Amount / L
H_3BO_3	0.50 g
$CuSO_4 \cdot 5H_2O$	0.04 g
KJ	0.10 g
$FeCl_3 \cdot 6H_2O$	0.33 g
$MnSO_4 \cdot H_2O$	0.40 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.20 g
$ZnSO_4 \cdot 7H_2O$	0.40 g
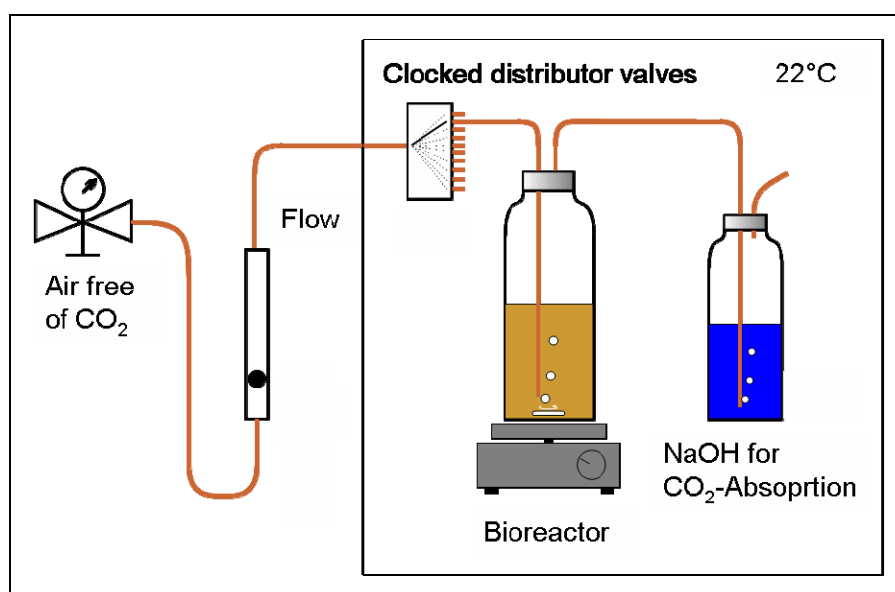
Figure 11 Schematic drawing illustrating the Modified Sturm Test according to OECD Guideline 301 B.

The aerobic biodegradability of the different coating materials was determined according to OECD Guideline 301 B by using an aqueous test system in 500 mL bottles (Schott) used as small bioreactors (Figure 10). The test mixture contained 400 mL of inorganic medium (Table 4), a magnetic stirring rod, 2 g of coated pellets (sample) as the sole source of carbon and energy and 2 g of the above described soil inoculum. Blank trials without the addition of any coated pellets were carried along. After inoculation the bottles were immediately closed, stirred, aerated and incubated in the dark.

Aeration was realised with decompressed air free of oil and almost free of carbon dioxide (CO_2), which was trapped by 2 M NaOH, at a flow rate of approximately 7.5 mL min^{-1} per bottle. For this purpose clocked distributor valves were applied, which enable the air flow consecutively to each bottle (Figure 11).

Experiments were carried out in duplicates and randomised at ambient temperature of 22°C. The exhaust air was piped through approximately 400 mL 0.5 M NaOH and the absorbed CO_2 was determined periodically by titration with 0.5 M HCl. After titration to the decolouration of phenolphthalein an aliquote of the solution was heated and shortly before boiling titrated against mixed indicator 5 (Merck, No.: 1.06130). Titrations were carried out in duplicates and means thereof were used to calculate the generated amounts of CO_2. All chemicals used were of analytical grade.

By applying the following equations the titrated volumes of HCl were converted into generated amounts of CO_2:

$$CO_2 \, [mg/g \, inoculum] = \frac{V_{BV} \times M_{HCl} \, [mol/L] \times 44 \times A}{weight \, of \, inoculum \, in \, blank \, trial \, [g]}$$

$$CO_2 \, [mg] = V_M \times M_{HCl} \, [mol/L] \times 44 \times A - CO_2 \, [mg/g \, inoculum] \times weight \, of \, inoculum \, [g]$$

V_{BV}....Volume HCl to colour change of mixed indicator of blank value [mL]

44......Conversion factor (1 mL 1 M HCl equals 44 mg CO_2)

A.........Aliquot of NaOH-absorption solution [50]

V_M.....Volume HCl to colour change of mixed indicator [mL]

The theoretical amount (Th-CO_2) per trial was calculated out of the TOC (total organic carbon) values of samples and inoculum determined by elementary analysis (CHNS elemental analyser, HEKAtech GmbH, Wegberg, Germany) according to the following equation:

$$Th - CO_2\,[mg] = TOC\,[\%] \text{ x amount } [g] \text{ x } 36.6 \text{ (conversion factor C in } CO_2, mg \text{ in g and } \%)$$

The level of biodegradation expressed in percent was determined by comparing the amount of evolved CO_2 with the theoretical amount (Th-CO_2) according to the following equation:

$$\text{degradation } [\%] = \frac{CO_2\,[mg] \text{ x } 100}{Th - CO_2\,[mg]}$$

To calculate the degree of degradation the individual determinations after certain time intervals were summed up consecutively. Mean degradation velocities were calculated by dividing the amount of generated CO_2 of a certain period of time by the individual sample weight and by the days of investigation.

Determination of Sustained Release of Aluminium Ions

Sustained release kinetics were determined using an aqueous test system in 500 mL Erlenmeyer flasks used as small bioreactors. 2 g coated spheres were suspended in 400 mL of inorganic medium (Table 4) set to pH 6.8 and inoculated with 2 g of the above described soil inoculum. After inoculation the bottles were immediately closed, aerated, shaken at 170 rpm and incubated in the dark. Aeration was realised with decompressed air free of oil and almost free of carbon dioxide (CO_2) at a flow rate of approximately 25 mL min^{-1} per flask in order to avoid anaerobic conditions during the experiments. Experiments were carried out in duplicates and randomised at ambient temperature of 22°C.

The sustained release of aluminium ions was determined periodically for eight weeks by acidifying a 6-mL-sample with 60 µL concentrated HNO_3. After filtration (45 µm membrane filter, BARTELT GmbH, Graz, Austria) the concentration of aluminium ions was detected by ICP-OES. To describe the degree of aluminium release, the individual determinations after certain time intervals were referred to the total amount of aluminium ions present in 2 g of the coated spheres.

3.5 Results and Discussion

Physical Characterisation of Coated Aluminium Pellets

The efficiency of coating aluminium sulphate onto the spherical carrier material was approaching 100% in all batches. Dusts resulting from abrasion during the process and insoluble Al_2O_3 were removed by sieving. SEM micrographs revealed the presence of surface adhered $Al_2(SO_4)_3$ crystals on the smooth surface of the pellets applied (Figure 12). Cracks and holes observed probably resulted from the generation of vacuum necessary to take SEM micrographs as inclusions of air in the porous pellet caused structural deformation.
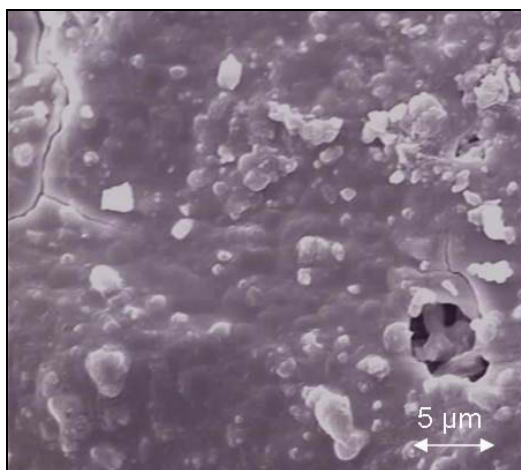
Figure 12 SEM micrograph (3000-fold magnification) of aluminium sulphate crystals adhering to the surface of the spherical carrier material used for subsequent encapsulation trials.

The deposited coating mass of the individual substances was related to the total weight of aluminium coated spheres used in one trial (20 kg), which was achieved by weighing the total amount of pellets before and after the process. in all cases. Depending on process efficiency the experiments resulted in the following coating amounts (Table 6).

Table 6 Amount of coating applied.

Coating substance	[%]
Carnauba wax	98.7
Shellac	90.1
Hydrogenated soya oil	80.0
Tall oil rosin	69.3

The aqueous shellac solution was most easily to handle, while the more delicate hot melt coatings needed much attention regarding process control. Nevertheless, the carnauba wax and hydrogenated soya oil coatings were finished successfully. Severe problems with agglomeration of particles and coverage of the spray nozzle and the reactor walls occurred only during coating with tall oil rosin. Despite several process interruptions at least a part of coated pellets could finally be recovered.

The resulting coated pellets were subjected to sieve analysis to determine their particle size distribution using four sieves of the mesh sizes 125, 500, 800 µm and 1 mm (Figure 13). The narrowest particle size distribution was achieved with the soya oil coating, more than 85% of pellets were found in the range between 800 µm and 1 mm, reflecting optimal process conditions. In comparison, the size distribution of the carnauba wax coated pellets showed slightly higher proportions in smaller sieve fractions (6.9% 125 - 500 µm and 19.7% 500 - 800 µm, respectively) which may be attributed to a higher susceptibility of carnauba wax to abrasion during the process. This finding may be explained by the friability of carnauba wax, which is reportedly the hardest, highest melting, commercial natural wax (Talens and Krochta, 2005). Nevertheless more than two thirds of pellets (70.4%) were recovered in the expected range between 800 µm and 1 mm.
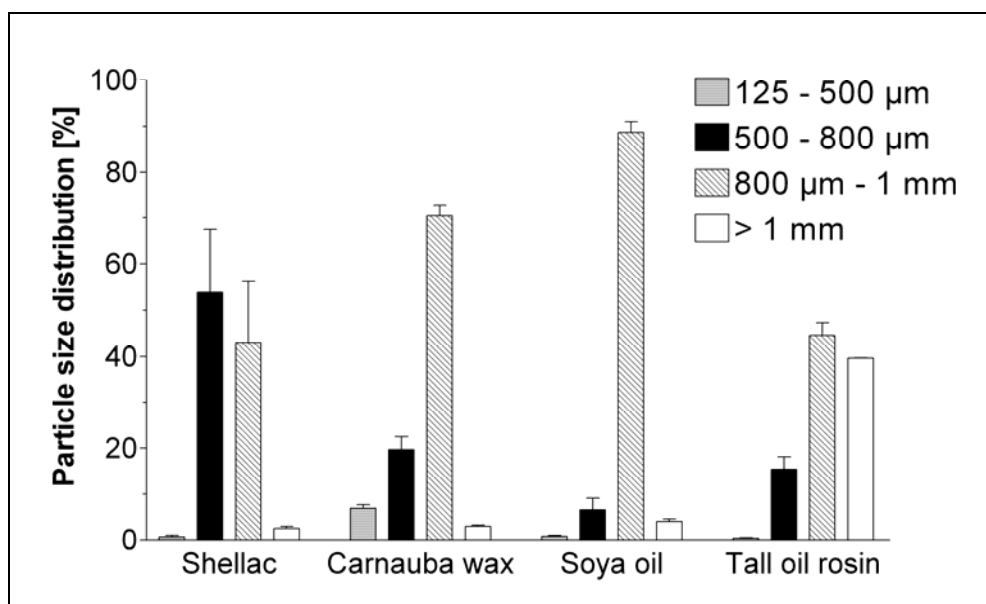
Figure 13 Particle size distribution of the resulting shellac, carnauba wax, soya oil, and tall oil rosin coated pellets. Values are means of triplicate analyses. Error bars indicate standard deviation.

Sieve analysis further revealed that pellets encapsulated with shellac resulted in a densely packed coating, which was expressed by the distribution of pellets to equal amounts in the

sieve fractions above and below 800 µm (53.8 and 43.0%, respectively). For further analyses the mentioned sieve fractions of shellac coated pellets were pooled.

Finally, the particle size distribution of tall oil rosin coated pellets shows that a rather small portion of pellets was found in the desired particle size. Due to the already mentioned difficulties in process control, on one hand a high amount of particles or agglomerates exceeded an average diameter of 1 mm (39.6%), while on the other hand friability residues and uncoated pellets were obtained below 800 µm (15.4%). For subsequent studies single coated pellets received from the sieve fraction ranging from 800 µm to 1 mm were used.

The uncoated carrier material possesses spherical shape and is optimally suited for the fluidised bed coating process because the coating can be applied most uniformly. Microscopic evaluation of the resulting pellets showed that a perfectly even distribution of the applied coating throughout the entire population of pellets is difficult to achieve as several parameters, such as spray rate, spraying air temperature, and product temperature influence the performance of the single process (Figure 14).
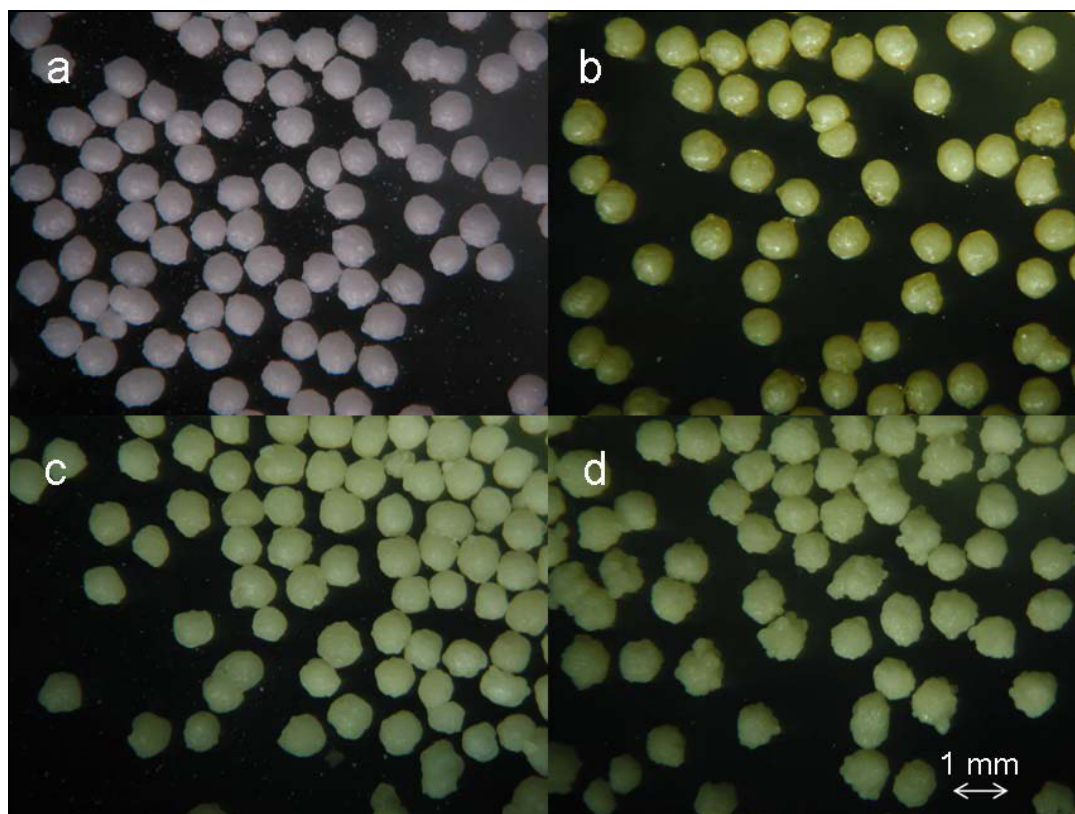
Figure 14 Microscopic pictures of resulting a. soya oil, b. shellac, c. tall oil rosin, and d. carnauba wax coated pellets. Hydrogenated soya oil, shellac, and tall oil rosin coatings show more uniform structures than carnauba wax coated pellets. Rare formation of "twins" was observed in all experiments.

Nevertheless, the individual coating procedures resulted in pellets that were more or less uniform in shape having a similarly structured surface. Solidifying liquid bridges or liquid binding are responsible for the formation of agglomerates (Hemati et al., 2003). The term twin growth agglomeration refers to the joining together of two particles of similar size to form a larger particle. The rare formation of such twins was observed in each of the four experiments and may be accounted as a common feature of coating processes, especially on pilot or larger scale.

Improved film formation was expected to occur during the aqueous shellac coating procedure due to optimal coalescence conditions which are achieved by the addition of an appropriate amount of plasticiser and by dilution to a lower dry matter content of the coating solution. SEM analysis of shellac coated pellets revealed a comparably uniform and smooth surface illustrating good film formation (Figure 15b). Furthermore, the resulting shellac coated pellets showed a yellow-brown gloss and smooth appearance, but sometimes the spherical shape of the original carrier material could not be maintained (Figure 14b). A disadvantage encountered during the top-spray coating process is premature evaporation of water from the atomised droplets by the heated air stream. Therefore the droplets are no longer able to spread on the surface of the core particles upon collision and consequently the droplets form small, solid beads on the surface of the core particles, which cause the uneven surface. Thus, the coating performance could be optimised by adjusting spraying air pressure and bed temperature. Another approach is limiting the travel distance of droplets by further submerging the spray nozzle in the bed of fluidised particles or by using a Wurster insert and the bottom-spray position of the spray nozzle (Desai and Park, 2005).

The quality of a hot melt coating depends strongly on the spraying and congealing rates. At lower product temperatures, the coat contains more pores and defects due to congealing prior to complete spreading. Therefore, the choice of fluidising air temperature is crucial. White, hydrogenated soya oil coated pellets appeared to have few imperfections and a quite uniform structure in both the microscopic (Figure 14a) and SEM analysis (Figure 15a), reflecting well selected process settings.

In contrast, particle-particle agglomeration occurred during tall oil rosin coating, which may be explained by choosing the bed temperature too close to the melting temperature of

the coating substance resulting in wet, sticky, and viscous beds (Dewettinck and Huyghebaert, 1999). Nevertheless, bright yellowish tall oil rosin coated pellets obtained after sieving, being separated from agglomerates and dusts, showed quite uniform appearance (Figure 14c).

Unlike the previously mentioned hot melt coatings the surface of yellow coloured carnauba wax coated particles was generally uneven and appeared bumpy (Figure 14d). Especially at the end of the process, the increase of the air flow to keep particles fluidised may have caused premature solidification of some molten wax droplets, which resulted in non-uniform coating patterns. An adaptation of atomisation pressure and bed temperature at the right point of time could prevent the trend that droplets are solidifying before they get in touch with the carrier material and can not further build up a coating layer.
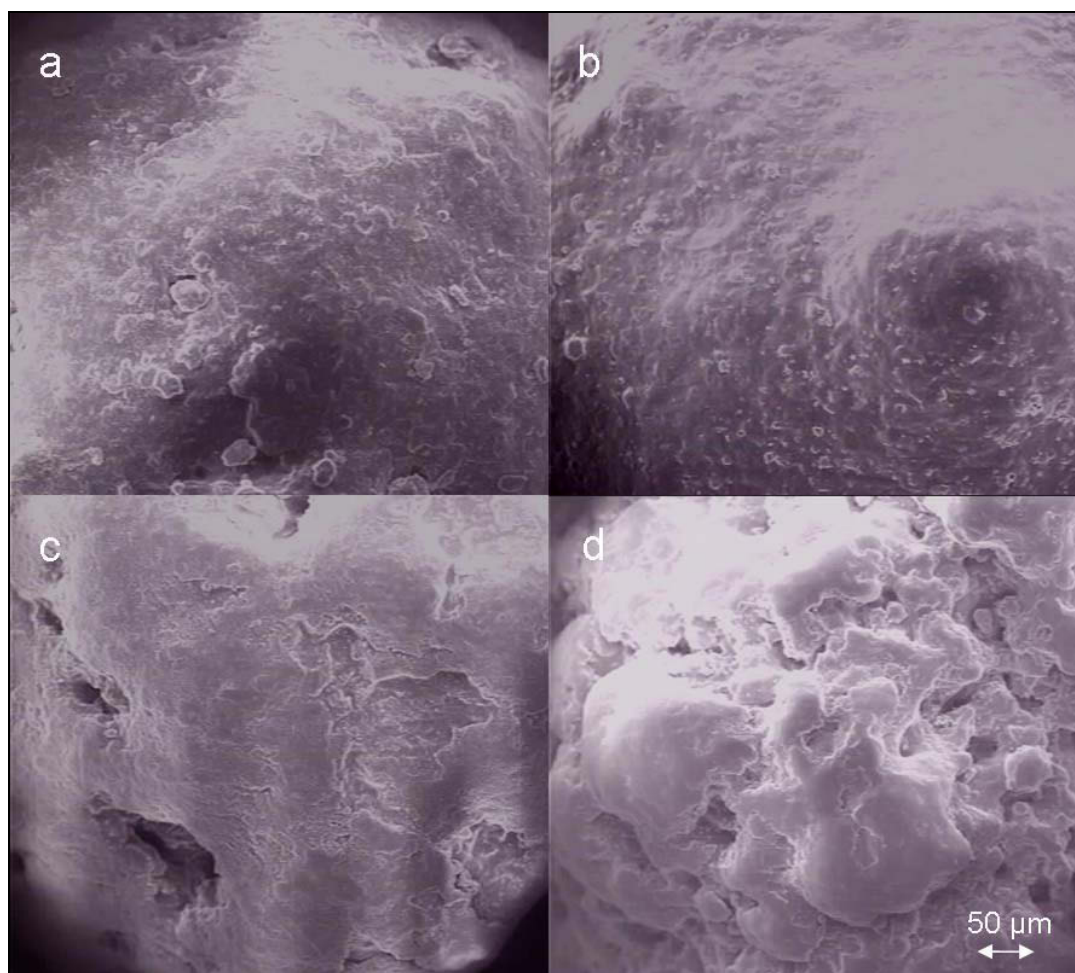
Figure 15 SEM micrographs (200-fold magnification) of resulting a. soya oil, b. shellac, c. tall oil rosin, and d. carnauba wax coated pellets. Hydrogenated soya oil and shellac coatings show uniform and smooth surface structures, while the surfaces of tall oil rosin and carnauba wax coated pellets are characterised by pores and cavities.

As far as SEM analysis of hot melt coated pellets is concerned, best coating results could be obtained by the application of hydrogenated soya oil as already mentioned (Figure 15a). In contrast, the surface of tall oil and carnauba wax coated pellets appeared less homogeneous, showing a rough morphology and including cavities (Figure 15c,d). The formation of a smooth surface structure of a certain coating depends on selected process parameters and the characteristics of the distinct hot melt applied. The less viscous coating substance applied in this study, hydrogenated soya oil, showed best film forming properties. In order to achieve more uniform hot melt coatings using tall oil rosin or carnauba wax, process conditions need to be optimised and the addition of plasticisers should be taken into consideration in order to improve film formation.

Biodegradability of Investigated Coating Materials

The Modified Sturm Test uses mineralisation in terms of carbon dioxide evolution as the primary end point in the assessment of the biodegradation potential of organic compounds in aqueous media. Those stirred batch release curve experiments were employed for practical reasons to rapidly evaluate the biodegradability of the four coating substances applied. Even though the produced degradation rates (Figure 17) are probably higher than anything that would be observed under natural, ambient conditions, the results give a good estimation well suited for comparative reasons.

By being submerged due to the experimental setup all the coated capsules softened due to film swelling, being extremely susceptible to abrasion. Therefore, capsules of all coating substances were already burst after the first day of incubation due to the mechanical forces exerted by the magnetic stirring rods used. This fact did not negatively influence the outcome of the degradation study, as optimised culture conditions are favourable and smaller particles, in particular lipophilics, are more bioavailable. The carrier material used for the coating experiments consists of starch and sucrose. The release of such easily metabolisable carbohydrates in the beginning of the test enhanced the adaptation of the microbial community present in the inoculum and could explain the relatively short lag phase observed (Figure 17). As the formulations are meant to be used in agricultural applications, a soil inoculum was used instead of the standardised Modified Sturm Test inoculum derived from activated sludge.
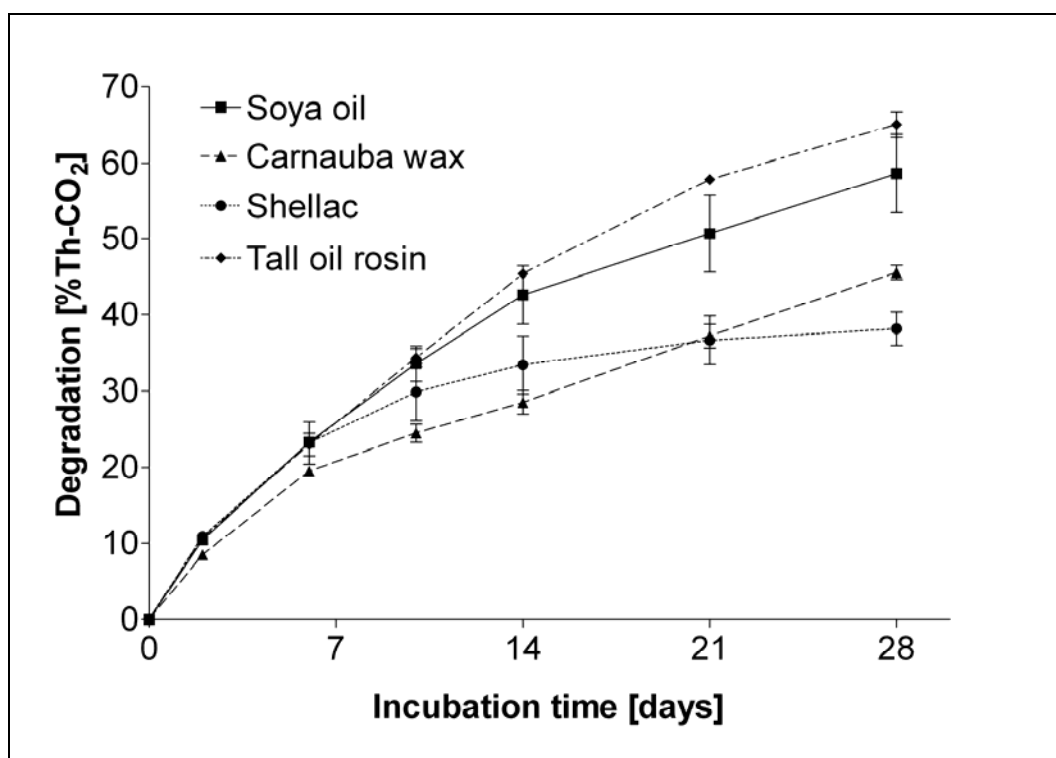
Figure 16 Biological degradation expressed in percent evolved CO_2 referred to the calculated theoretical CO_2-amount of the resulting soya oil, shellac, tall oil rosin, and carnauba wax coated pellets. Values are means of duplicate analyses and cumulative. Error bars indicate standard deviation.
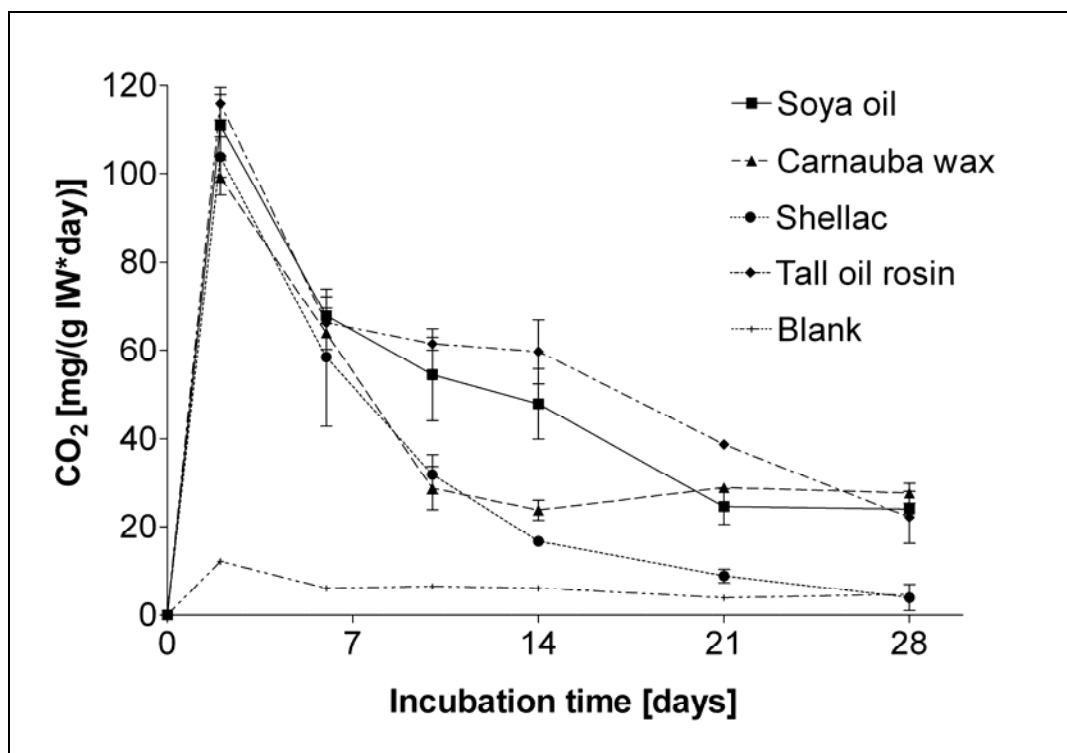
Figure 17 Biological degradation velocities expressed in mg generated CO_2 per g initial sample weight and day of the resulting soya oil, shellac, tall oil rosin, and carnauba wax coated pellets. Values are means of duplicate analyses. Error bars indicate standard deviation.

The Modified Sturm Test revealed that tall oil rosin is easily biologically degradable as more than 60% of the total theoretical amount of evolved carbon dioxide ($Th\text{-}CO_2$) is degraded within 28 days (Figure 16). This threshold value was failed marginally by hydrogenated soya oil ($58.7\pm5.2\%$), while carnauba wax and shellac turned out to be definitely more difficult to mineralise resulting in degradation degrees of $45.6\pm0.9\%$ and $38.2\pm2.2\%$, respectively.

As far as degradation rates are concerned, after 14 days degradation velocities of tall oil rosin, hydrogenated soya oil and carnauba wax were 3.6-, 2.9-, and 1.4-fold, respectively, of that of shellac (Figure 17). After 28 days degradation rates of the former three coating substances ranged between 22 and 28 mg carbon dioxide per g initial weight and day, while shellac caused about sixfold less degradation velocity of only 4 mg carbon dioxide per g initial weight and day at the end of the test.

Sustained Release Kinetics

The main objective of sustained release formulations is that the active ingredient is released at controlled rates over prolonged periods of time. To observe the sustained release of aluminium ions from the four resulting encapsulations, the experimental setup and process conditions were chosen similar to the Modified Sturm Test. In general the mechanisms of release for coated pellets are melting, diffusion, swelling, degradation, and particle fracture or erosion. In this case melting is negligible under normal ambient circumstances. In order to prevent or decline degradation of the capsules by abrasion, which is extremely forced by magnetic stirring rods, tests were carried out in Erlenmeyer flasks put on an orbital shaker instead of carrying out the experiments in stirred bottles. Thus, during examination of biodegradability over longer periods of time biological attack, erosion, and diffusion through the swollen matrix were the remaining incidents that affect the resulting formulations significantly. The objective of this study was to observe the disintegration in particular caused by biological degradation of the four natural coating films in simulated environmental conditions, which was monitored by the release of aluminium ions as a function of time.

Even though curing of shellac coatings was reported to enhance film formation (Pearnchob and Bodmeier, 2003b), such treatment did not alter the immediate release behaviour neither of the shellac nor of the tall oil rosin coating obtained in this study. On the

contrary, adequate thermal post treatment of hydrogenated soya oil and carnauba wax coatings reduced immediate release of aluminium ions. This procedure probably closed pores and holes resulting from improper film formation during the coating process and the resulting thermally treated pellets were subsequently used for the sustained release studies.

To describe the quality of the resulting coatings, they may be classified into the following types (Kleinbach and Riede, 1995):

- Type A or ideal coatings are intact on all particles and the mean and local coating thicknesses are the same. The relationship between the release of the active substance and time is linear.

- Type B coatings are also intact, however the mean and the local coating thicknesses vary. As a consequence of this, the quantity of active substance released with time is non-linear.

- Type C coatings differ from type B in that some of the particles have cracks and holes in their coating. These particles release their active ingredient almost immediately resulting in a discontinuity in the release curve at the beginning.

According to this classification scheme, the carnauba wax, hydrogenated soya oil, and tall oil rosin coatings are at least partially of type C, as an immediate release of aluminium ions of 12.8, 10.4, and 5.9%, respectively, could be observed on day zero. Uncoated or incompletely coated spherules remained in the final product due to suboptimal process conditions, such as premature congealing of molten droplets and lack of coalescence, hence failing proper film formation. The coating efficiency needs to be raised by optimising the coating temperatures triggering the atomised, molten particles to adhere to the heated pellet surface.

In contrast, the shellac coating exerted a good barrier effect, releasing less than 1% of aluminium ions immediately after submersion. The effectiveness may be explained first by the right choice and concentration of plasticiser in the shellac solution as the resin is brittle in nature and therefore does not have good film forming properties (Pearnchob et al., 2004). Secondly, the bed temperature during the coating process was near the T_g of shellac (50.4°C), and the addition of plasticisers decrease T_g resulting in optimal conditions to form a compact polymer layer around the pellets (Pearnchob and Bodmeier, 2003b).

After the quick, immediate release of active substance from the capsules obtained sustained release from the encapsulations follows that of a type B coatings as the shell thickness varies to a certain extent and release continues more slowly. The release course of polymer-coated controlled release fertiliser was described to consist of three distinct phases mainly caused by diffusion mechanisms (Du et al., 2006):

- a lag phase during which almost no release is observed,

- a constant release stage, and

- a stage of gradual decay of release rate.

Even though biological systems are often more difficult to interpret than physical processes, biodegradation as the main factor triggering sustained release desired in this study may be depicted in a similar manner.

The duration of the lag period of microbial growth is linked to the time bacteria need for adaptation to the conditions in the bioreactor. As already observed in the Modified Sturm Test, the lag phase turned out to be relatively short due to the presence or abundance of essential nutritional sources (Figure 17). This fact may explain the constant release of aluminium ions already observed during the first two weeks of investigation in particular from hydrogenated soya oil and shellac coatings and to a lesser extent from encapsulations of carnauba wax and tall oil rosin (Figure 18).

The stage of constant rate release did not last as long as initially expected. After the second week of investigation all formulations show decay in sustained release of aluminium ions, which may be due to nutrient depletion in particular in terms of easily metabolisable sources. Solubility in water is an important property for a substance to be referred to as bioavailable. As the coating films used in this study are insoluble in water in order to enhance product integrity, water resistance, and delayed release in terms of biodegradation, they are less bioavailable for the microbial community. Therefore, this stage may be termed a second lag phase, as bacteria need to open more sophisticated ways to be able to break down complex, hydrophobic compounds in order to subsist on them (Figure 18).
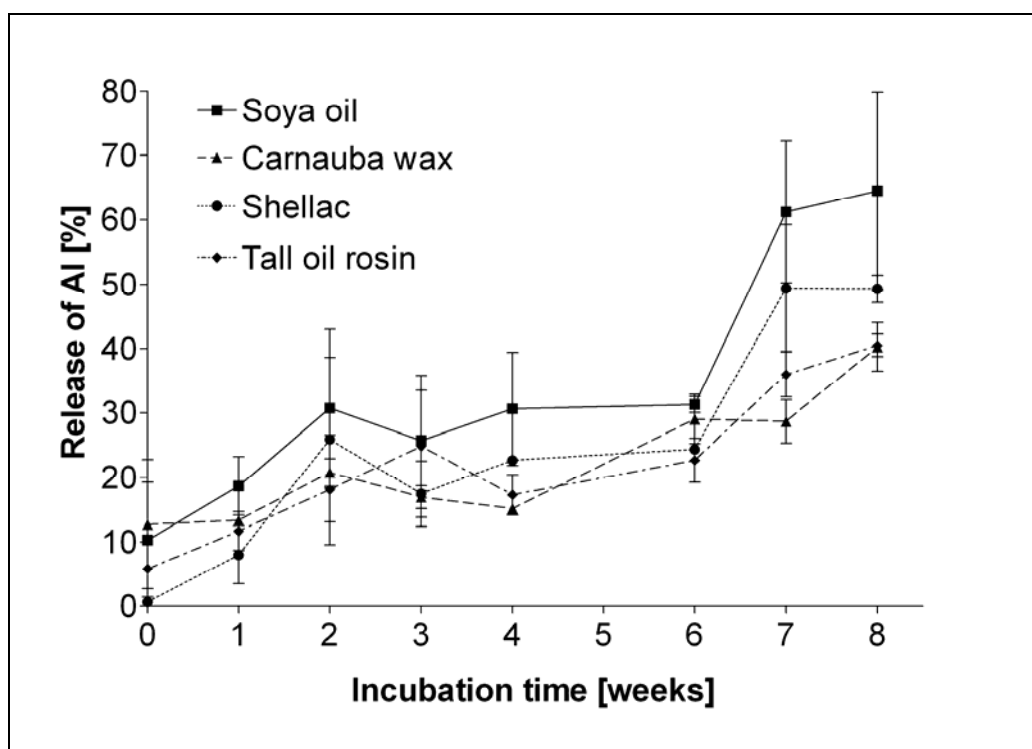
Figure 18 Sustained release behaviour of the resulting soya oil, shellac, tall oil rosin, and carnauba wax coated pellets expressed in percent released aluminium referred to total initial load. Values are means of duplicate analyses. Error bars indicate standard deviation.

An additional constant release stage expected to follow the second lag phase could be observed. During the final two weeks release from hydrogenated soya oil and shellac coatings increased, which for the former encapsulating matrix may be explained by the better biodegradability as observed in the Modified Sturm Test (Figure 16). Apart from biological degradation sustained release out of shellac coatings may be additionally attributed to slow dissolution in neutral pH media due to its pK_a of between 6.9 and 7.5 (Pearnchob et al., 2004), which may have contributed to enhanced release observed in the late phase of the experiment (Figure 18).

Comparatively slower release rates were obtained from the carnauba wax and tall oil rosin coating at this time of the experiment. In contrast to the findings of the Modified Sturm Test, where tall oil rosin dispersed by magnetic stirring rods turned out to be the best degradable of all substances analysed, it is not as easily degraded being present as compact, lipophilic capsules. In the case of carnauba wax a correlation between biodegradability revealed by the Modified Sturm Test and the sustained release behaviour of aluminium ions can be seen showing slow biodegradation as well as slow sustained release rates.

None of the formulations showed 100% release of encapsulated aluminium ions after eight weeks of investigation implying that remaining coating shells are still intact and total release of the active ingredient occurs only after complete erosion or degradation of the matrix. By varying the amount or thickness of certain coatings the sustained release kinetics may be altered with the view to establish desired product properties. It is assumed that particles with low coating thickness release their active ingredient quickly leaving the particles with higher coating thickness to release their active ingredient more slowly. Apart from coating thickness sustained release behaviour based on biodegradability and friability is intrinsic to the individual coating material used.

In general, the release rates achieved are higher than those that would be observed under field conditions. A factor must be determined in order to be able to compare the conditions in the aqueous test with ambient conditions present in agriculturally used areas. Therefore, further objectives are to measure the amount and rate of release of aluminium out of the sustained release formulations deposited on or in soil in simple packed columns. Under such conditions the water carries away the solute as it is released and thus may be detected.

It must be noted that formulation ingredients - in this case in particular the carrier material consisting of easily degradable carbon sources, such as sucrose and starch - often act as stimulants and may apparently increase the biological activity of the environment. This fact, desired or not, needs to be considered when designing a new formulation. It is expected, that the enhanced biological degradation of the coating material leads to increased release patterns of active ingredient due to the additional sources of nutrients (e.g. carbon, nitrogen).

When designing a new product formulation reduction of costs always plays a crucial role, therefore expenses for different materials and certain processes must be kept in mind. As far as material costs are concerned, the aqueous shellac solution particularly applied by the pharmaceutical and food industries is by far the most expensive coating substance employed in this study, being 19-fold and 9.5-fold more expensive than hydrogenated soya oil and carnauba wax, respectively, related to the applied amounts of coatings used in this study. In terms of process costs hot melt coating is known to be more cost-effective than processes including the evaporation of solvents (Ivanova et al., 2005).

Conclusion

From the commercial point of view particular attention needs to be paid to the rising consumer demand on less pesticide contaminated foods and biologically produced products. Thus, reducing the amount of synthetic pesticides is the driving force that makes modern agricultural crop management adopt biopesticides. Those products comprising bacteria, fungi, virus, pheromones, and plant extracts need to be biocompatible, biodegradable and non-polluting the environment. The exploitation of allelopathic plants is one approach in this context and an important contribution to a greener, meaning environmentally friendlier agriculture.

Depending on the period of activity, herbicides, insecticides, and fungicides are to be employed at different points of time next to or on crops. Production of small and uniformly sized pellets were reported to assure that conventional application equipment can be used for optimal distribution of the product in the field (Daigle et al., 1997). Therefore formulations need to differ and a series of appropriate sustained release systems needs to be designed in order to provide optimal product characteristics. As already outlined previously, the development of sustained release systems including biodegradable matrices and the detailed characterisation of mechanisms involved in their degradation will support the formulation goal (Mogul et al., 1996). A future outlook is to focus research on such systems that encapsulate biologically active ingredients in biodegradable substances that are effective and applicable in agriculture. The background is to substitute persistent synthetic pesticides for allelopathic compounds, to contribute to a more eco-compatible intensive agricultural production, to prevent negative environmental effects like contaminated soil and groundwater and to increase food safety in terms of preventing pesticide residues. Because of their natural origin, the coating materials tested are acceptable for the use in biopesticide applications, where synthetic polymers would not fit in the product image.

The development of such applications of allelopathy in agriculture and forest management was the basis of the current study. The investigated process conditions and coatings need to be adapted for further applications including sensitive biological compounds or plant extracts. The biological efficacy of the active components needs to be characterised due to

the influence of microbial degradation of the encapsulating matrix as well as of a possible microbial activation of precursor molecules.

Further optimisation includes choice and combination of coating materials and plasticisers, variation of process parameters, cost-effectiveness and additional encapsulating processes like extrusion in order to achieve a series of formulations comprising of distinct sustained release systems that show well defined release dynamics. Apart from those requirements ideal formulations of biopesticides based on allelochemicals need to have an extended shelf life, to be consistently effective against the target pest, feasible for large scale production, easy to handle and convenient for field application to be able to compete with their synthetic counterparts.

On one hand it is expected that the encapsulated active compounds show bactericidal or fungicidal activity and therefore have reducing effect on further biodegradation of the matrix. On the other hand the soil microflora could be strongly stimulated by the input of biodegradable coating substances so that sensitive allelopathic compounds are degraded by microbial co-metabolisation. This degradation may either deactivate the encapsulated compounds before they are even able to exert their designated properties, or as well activate as it is proposed for allelopathic compounds. The characterisation of certain allelopathic precursor molecules activated by the microflora involved in the biodegradation of the encapsulating matrix will be an important milestone.

4   Summary and Outlook

Fluidised bed technology is versatile: apart from minor modifications important processes for product formulation - drying, granulation and coating - may be exerted with the same equipment. Even though extensively used by the pharmaceutical industry new branches are adopting this technology and appreciating its broad range of applications.

According to recent consumers' behaviour there is an increasing interest in functional products, regardless of the industry - pharmaceutical, food, feed, agriculture, cosmetic, dye, chemicals, etc. - the product is derived from. Fluidised bed technology is an important processing tool in product development and formulation as this technology enables that compounds of interest are processed as dried substances, keeping the sensitive ingredient stable, viable, and active. Furthermore an improvement of the final product properties can be achieved by encapsulation and/or granulation. However, the activity, viability, and stability of active ingredients have been both a marketing and technological challenge for industrial producers always confronted with reduction of expenses.

When using fluidised bed technology for drying of microorganisms, several parameters influencing survival during and after preservation processes need to be considered in order to obtain optimal process performance. Among those crucial factors are the influence of the distinct bacterial species and even strains, growth conditions and cultivation phase, cell concentration, pH, use of protective agents, drying variables as well as storage and rehydration conditions. This list implies that each individual processing step during the production of starter cultures needs to be optimised with focus on the strain of interest. When isolating new strains selection criteria must include the technological suitability of the strain next to the desired product properties. Due to the comparably high cost-effectiveness in large scale production the fluidised bed process is a valuable alternative to the freeze drying approach and further investigations on this topic is therefore of scientific and industrial interest.

Preservation of LAB by drying processes brings about loss in cell viability. Previously published data shows the importance of the pre-drying stage on the viability of LAB, it is apparent that induction of desiccation tolerance takes place under certain culture and pre-drying conditions. The exact mode of action of protective agents is indeed complex, and

not fully understood to date, however there is no doubt that suitable selection of the protecting agent used as additive to the drying medium is essential for strong protection during storage of dried LAB. It is reasonable to suggest that the mechanism underlying this protection mediated by certain compounds is firstly to prevent aggregation and maintain solubility of cellular proteins and secondly to alter the physical properties of the cell membrane. Focusing on storage stability especially the influence of glass transition temperature on dry matrices needs to be taken into account, when optimising a certain formulation procedure.

Full comparison of the published data is difficult for a number of reasons; most reports have indeed focused on survival during the process of drying and not during the process of storage afterwards, and water is used as drying medium instead of a complex medium like reconstituted skimmed milk. Other sources of discrepancy arise from the different microorganisms or model systems employed and the chosen growth conditions, as well as the different drying methods or the distinct concentrations of protective agents used. Even though viability in terms of determination of CFU is predominant, further testing methods varied between different studies and are carried out additionally or alternatively. Among those are flow cytometry, enzymatic assays, lactic acid or bacteriocin production and fermentation activity, all of them enabling a better description of the complex processes associated with loss of bacterial survival during processing.

Fluidised bed coating is a prominent microencapsulation technique. Encapsulation of biological or generally sensitive compounds in protective coatings is documented and successfully exploited in many commercial applications. In the last decades microencapsulated formulations of agrochemicals have been extensively studied and applied. From an economic as well as an environmental standpoint, more basic research needs to be done in order to increase the effectiveness of the active agent without the harmful effects associated with exposing it to non-target areas. The development of more sophisticated formulations may optimise sustained delivery systems achieved by microencapsulation in terms of controlled release of the active ingredient, efficient utilisation of the active compound, greater safety to the operator, controlled biodegradation and protection of the environment.

Chemically diverse biologically degradable polymers are available on the market mainly as packaging materials. Degradation studies revealed a broad range of achievable degradation velocities in soil. Based on those findings sustained release systems with defined release characteristics may be developed. Furthermore waxes, resins and hydrogenated oils as well as some natural polymers that are generally insoluble in water may be employed to mediate sustained release of active ingredients. The mechanisms underlying controlled release are diffusion through the encapsulating matrix or enzymatic and microbial biodegradation, and abrasion of the matrix, whereby the active substance is discharged by the formation of pores or breaks.

Biologically degradable polymers and substances of natural origin used as sustained release coatings are perfectly suitable to be used in agricultural applications of allelochemicals or more general of biologically active substances. By the microbial degradation of the encapsulating matrix a further characteristic, the activation of allelochemicals by co-metabolisation may be additionally exploited. This property has yet not been investigated in much detail, but may further contribute to site and time directed release of the active compound. To sum up, the decomposition of the protective coating triggered by microorganisms does not only lead to sustained release of the active ingredient but also may activate precursor allelochemicals.

The developed formulations and their release characteristics can not only be used for allelochemicals but as well for sustained release systems of other conventional agrochemicals, such as fertilisers to prevent eutrophication of soil and waters. A further application could be the coating of seeds to enhance the growth of seedlings by applying nutritive elements directly, thereby reducing the quantity of pesticides and fertilisers. Moreover, coated seeds may be protected against moisture and temperature as well as harmful organisms or covered with symbiotic soil microorganisms applied in agriculture like *rhizobia* or *mycorrhiza.* Adequate coatings could encapsulate those living inocula capable of fixing atmospheric nitrogen or mineralising phosphorus in order to stabilise and/or release them over certain periods of time. Furthermore a useful bacterial formulation should have a long shelf life preferentially at elevated temperatures and remain maximum initial activity. In addition, microbial biocontrol agents may be protected by appropriate encapsulation from adverse environmental conditions like UV radiation, rain, etc.

When designing a distinct product formulation the physical-chemical properties of the active ingredient to be encapsulated as well as its compatibility with the carrier and the coating material need to be considered as important factors influencing the functionality of the desired added-value properties of the product. These interactions between formulation materials and active compounds are difficult to be estimated from theoretical models and therefore need to be determined for certain processing steps and in individual experiments. Furthermore losses in activity of certain active compounds encountered during processing need to be minimised especially when planning production on a larger scale. In spite of numerous applications already in use, there is still a potential of innovative technological solutions exploiting the fluidised bed technology, by either improving existing processes or by developing new approaches.

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6   List of Abbreviations

A	aliquot of NaOH-absorption solution
ADY	active dry yeast
a_w	water activity
CFU	colony forming unit
Ent.	*Enterococcus*
FBD	fluidised bed drying
FD	freeze drying
Glc	glucose
LAB	lactic acid bacteria
Lact.	*Lactobacillus*
MD	maltodextrin
MRS	growth medium recommended for cultivation of *Lactobacillus* sp.
MSG	monosodium glutamate
PLA	polylactic acid
SEM	scanning electron microscope
Suc	sucrose
T_g	glass transition temperature
TOC	total organic carbon
Tre	trehalose
V_{BV}	Volume HCl to colour change of mixed indicator of blank value [mL]
V_M	Volume HCl to colour change of mixed indicator [mL]
w/o	without
w/v	weight per volume

7   List of Figures

8 List of Tables

9 Acknowledgements

10 Raw data

Weight [g] of resulting freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium.*								
w/o FD	138.6	Glc FD	261.8	Tre FD	224.0	Suc FD	259.2	MD FD	248.7
w/o FBD	4982.6	Glc FBD	4931.6	Tre FBD	5433.0	Suc FBD	6167.6	MD FBD	4943.6
w/o FBD	4982.6	Glc FBD	5317.6	Tre FBD	5979.0	Suc FBD	6048.6	MD FBD	5795.6

CFU of resulting freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium.*									
w/o FD	3.80E+11	3.60E+11	3.40E+11	w/o FBD	4.80E+09	4.10E+09	4.20E+09	1.58E+10	1.51E+10	1.61E+10
Glc FD	3.90E+11	4.00E+11	4.10E+11	Glc FBD	4.60E+10	4.60E+10	5.00E+10	5.10E+10	5.40E+10	4.90E+10
Tre FD	4.00E+11	4.70E+11	5.00E+11	Tre FBD	4.30E+10	4.10E+10	5.20E+10	3.90E+10	3.00E+10	3.00E+10
Suc FD	3.80E+11	4.70E+11	4.40E+11	Suc FBD	4.80E+10	4.50E+10	5.20E+10	5.60E+10	5.90E+10	5.00E+10
MD FD	4.60E+11	4.40E+11	4.00E+11	MD FBD	5.60E+10	6.10E+10	5.60E+10	5.20E+10	5.00E+10	7.40E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 1 month at 4°C.									
w/o FD	4.20E+11	3.70E+11	3.60E+11	w/o FBD	3.70E+09	3.50E+09	2.80E+09	1.31E+10	1.20E+10	1.34E+10
Glc FD	3.20E+11	3.30E+11	3.00E+11	Glc FBD	4.90E+10	4.80E+10	4.60E+10	5.40E+10	5.40E+10	5.00E+10
Tre FD	3.70E+11	3.50E+11	2.60E+11	Tre FBD	4.60E+10	4.80E+10	3.90E+10	3.40E+10	3.70E+10	3.40E+10
Suc FD	4.20E+11	3.50E+11	2.70E+11	Suc FBD	5.30E+10	4.80E+10	4.80E+10	5.50E+10	5.10E+10	5.60E+10
MD FD	2.80E+11	3.30E+11	3.00E+11	MD FBD	4.10E+10	6.10E+10	6.80E+10	5.70E+10	5.20E+10	5.00E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 3 months at 4°C.									
w/o FD	3.90E+11	2.70E+11	3.30E+11	w/o FBD	3.00E+09	2.40E+09	3.20E+09	1.01E+10	1.03E+10	9.50E+09
Glc FD	2.90E+11	3.60E+11	2.80E+11	Glc FBD	4.40E+10	4.90E+10	5.50E+10	5.60E+10	4.90E+10	4.80E+10
Tre FD	4.10E+11	3.90E+11	3.60E+11	Tre FBD	3.50E+10	3.90E+10	4.50E+10	3.60E+10	3.60E+10	3.20E+10
Suc FD	4.10E+11	3.10E+11	3.60E+11	Suc FBD	5.20E+10	5.30E+10	4.60E+10	5.10E+10	4.20E+10	5.60E+10
MD FD	3.50E+11	3.10E+11	3.10E+11	MD FBD	6.10E+10	6.20E+10	5.40E+10	5.50E+10	5.00E+10	5.10E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 4 months at 4°C.										
w/o FD	2.40E+11	2.20E+11	3.30E+11	w/o FBD	2.80E+09	2.60E+09	2.40E+09	8.80E+09	9.00E+09	7.40E+09
Glc FD	2.90E+11	3.10E+11	3.30E+11	Glc FBD	4.70E+10	4.70E+10	4.30E+10	4.60E+10	5.10E+10	5.30E+10
Tre FD	4.10E+11	3.80E+11	3.90E+11	Tre FBD	3.70E+10	4.20E+10	3.40E+10	3.60E+10	3.80E+10	3.80E+10
Suc FD	3.00E+11	3.00E+11	3.20E+11	Suc FBD	4.70E+10	5.40E+10	5.00E+10	4.90E+10	5.00E+10	4.20E+10
MD FD	3.50E+11	3.10E+11	3.20E+11	MD FBD	5.70E+10	5.40E+10	4.50E+10	5.40E+10	5.20E+10	4.80E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 6 months at 4°C.										
w/o FD	2.02E+11	1.93E+11	2.24E+11	w/o FBD	2.00E+09	1.80E+09	2.40E+09	7.00E+09	7.60E+09	5.90E+09
Glc FD	2.06E+11	1.97E+11	1.88E+11	Glc FBD	3.50E+10	4.10E+10	3.70E+10	4.20E+10	4.20E+10	3.50E+10
Tre FD	3.20E+11	3.40E+11	4.40E+11	Tre FBD	3.80E+10	3.80E+10	4.10E+10	2.90E+10	3.20E+10	4.10E+10
Suc FD	2.90E+11	3.10E+11	2.90E+11	Suc FBD	4.70E+10	4.20E+10	4.60E+10	4.90E+10	3.80E+10	4.40E+10
MD FD	3.10E+11	2.80E+11	2.70E+11	MD FBD	3.90E+10	3.70E+10	4.50E+10	4.90E+10	4.90E+10	4.60E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 1 month at 22°C.										
w/o FD	4.00E+11	3.50E+11	3.90E+11	w/o FBD	2.20E+09	2.40E+09	2.60E+09	9.20E+09	8.90E+09	7.40E+09
Glc FD	2.90E+11	2.90E+11	3.20E+11	Glc FBD	4.60E+10	4.40E+10	4.30E+10	5.20E+10	5.30E+10	4.50E+10
Tre FD	3.40E+11	2.90E+11	3.70E+11	Tre FBD	4.30E+10	4.20E+10	4.50E+10	3.80E+10	3.10E+10	2.90E+10
Suc FD	3.60E+11	3.20E+11	3.30E+11	Suc FBD	5.20E+10	5.00E+10	4.20E+10	5.10E+10	5.70E+10	4.60E+10
MD FD	3.10E+11	3.30E+11	2.50E+11	MD FBD	4.30E+10	5.80E+10	6.80E+10	6.20E+10	4.80E+10	5.80E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 3 months at 22°C.										
w/o FD	2.30E+11	2.80E+11	2.90E+11	w/o FBD	1.50E+09	1.90E+09	2.10E+09	6.40E+09	7.10E+09	6.60E+09
Glc FD	2.10E+11	2.50E+11	2.70E+11	Glc FBD	4.30E+10	4.50E+10	4.60E+10	5.20E+10	5.10E+10	4.50E+10
Tre FD	3.90E+11	3.50E+11	3.00E+11	Tre FBD	3.70E+10	3.50E+10	3.80E+10	3.40E+10	3.40E+10	3.20E+10
Suc FD	3.80E+11	3.30E+11	2.90E+11	Suc FBD	4.30E+10	4.40E+10	5.00E+10	5.20E+10	4.60E+10	5.60E+10
MD FD	2.60E+11	2.80E+11	2.90E+11	MD FBD	5.90E+10	5.70E+10	5.50E+10	5.30E+10	5.80E+10	4.90E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 4 months at 22°C.										
w/o FD	1.91E+11	1.76E+11	1.83E+11	w/o FBD	9.40E+08	9.70E+08	1.03E+09	4.80E+09	5.10E+09	4.90E+09
Glc FD	2.10E+11	2.80E+11	2.00E+11	Glc FBD	3.30E+10	3.70E+10	3.20E+10	3.30E+10	4.20E+10	4.10E+10
Tre FD	3.10E+11	3.60E+11	3.30E+11	Tre FBD	3.60E+10	3.30E+10	3.40E+10	3.10E+10	3.80E+10	3.20E+10
Suc FD	3.40E+11	3.40E+11	2.50E+11	Suc FBD	4.10E+10	5.10E+10	4.50E+10	4.20E+10	4.70E+10	4.80E+10
MD FD	1.63E+11	1.97E+11	1.88E+11	MD FBD	4.60E+10	4.90E+10	4.40E+10	4.70E+10	3.80E+10	3.80E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 6 months at 22°C.										
w/o FD	1.61E+11	1.36E+11	1.45E+11	w/o FBD	6.60E+08	6.70E+08	7.50E+08	3.90E+09	4.90E+09	4.20E+09
Glc FD	1.74E+11	1.63E+11	1.59E+11	Glc FBD	3.00E+10	2.30E+10	3.20E+10	3.80E+10	3.90E+10	2.90E+10
Tre FD	3.10E+11	3.20E+11	4.00E+11	Tre FBD	2.90E+10	3.40E+10	3.00E+10	2.90E+10	3.30E+10	3.70E+10
Suc FD	2.30E+11	2.40E+11	3.10E+11	Suc FBD	4.60E+10	3.90E+10	3.80E+10	4.00E+10	4.20E+10	3.70E+10
MD FD	1.53E+11	1.54E+11	1.72E+11	MD FBD	3.10E+10	4.10E+10	3.10E+10	3.90E+10	3.50E+10	3.40E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 1 month at 35°C.										
w/o FD	9.20E+10	9.40E+10	1.04E+11	w/o FBD	1.50E+09	1.92E+09	1.67E+09	5.50E+09	5.20E+09	6.30E+09
Glc FD	5.00E+09	1.00E+10	6.00E+09	Glc FBD	2.00E+08	4.00E+08	4.00E+08	3.00E+08	0.00E+00	6.00E+08
Tre FD	2.40E+11	3.50E+11	4.10E+11	Tre FBD	3.50E+10	4.10E+10	4.70E+10	3.00E+10	3.30E+10	2.00E+10
Suc FD	3.20E+11	3.90E+11	2.70E+11	Suc FBD	5.10E+10	3.90E+10	4.60E+10	4.70E+10	4.40E+10	4.90E+10
MD FD	3.00E+11	2.90E+11	3.10E+11	MD FBD	4.50E+10	4.50E+10	3.40E+10	4.30E+10	4.30E+10	4.00E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 3 months at 35°C.										
w/o FD	1.54E+10	1.32E+10	1.38E+10	w/o FBD	3.40E+08	4.70E+08	5.30E+08	2.60E+09	2.40E+09	2.90E+09
Glc FD	4.10E+06	5.80E+06	6.10E+06	Glc FBD	1.39E+08	1.24E+08	1.45E+08	8.30E+07	7.60E+07	6.90E+07
Tre FD	3.00E+11	2.70E+11	2.30E+11	Tre FBD	3.30E+10	2.80E+10	2.90E+10	2.80E+10	2.50E+10	2.50E+10
Suc FD	3.60E+11	3.00E+11	3.20E+11	Suc FBD	3.50E+10	2.70E+10	3.60E+10	4.10E+10	3.10E+10	3.50E+10
MD FD	1.90E+11	1.80E+11	2.50E+11	MD FBD	3.70E+10	3.90E+10	4.20E+10	3.40E+10	3.90E+10	3.90E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 4 months at 35°C.										
w/o FD	9.60E+09	1.09E+10	1.24E+10	w/o FBD	6.20E+08	4.50E+08	4.20E+08	1.88E+09	2.17E+09	2.03E+09
Glc FD	2.50E+06	2.30E+06	2.00E+06	Glc FBD	1.21E+08	1.02E+08	1.01E+08	5.20E+07	4.50E+07	4.40E+07
Tre FD	1.66E+11	1.67E+11	1.61E+11	Tre FBD	2.80E+10	2.90E+10	2.00E+10	2.00E+10	2.50E+10	2.20E+10
Suc FD	3.10E+11	3.10E+11	2.30E+11	Suc FBD	4.10E+10	3.80E+10	3.40E+10	3.20E+10	3.50E+10	3.70E+10
MD FD	1.27E+11	1.16E+11	1.04E+11	MD FBD	4.20E+10	4.10E+10	3.80E+10	2.70E+10	3.00E+10	2.70E+10

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Ent. faecium* after 6 months at 35°C.										
w/o FD	5.90E+09	4.80E+09	5.00E+09	w/o FBD	9.40E+07	1.05E+08	9.80E+07	1.17E+08	1.06E+08	1.08E+08
Glc FD	1.26E+06	1.38E+06	1.45E+06	Glc FBD	6.30E+07	5.90E+07	6.90E+07	3.40E+07	3.80E+07	4.10E+07
Tre FD	1.12E+11	1.05E+11	1.18E+11	Tre FBD	2.16E+10	2.21E+10	2.08E+10	1.94E+10	1.81E+10	1.72E+10
Suc FD	2.60E+11	2.30E+11	2.20E+11	Suc FBD	2.00E+10	2.80E+10	3.00E+10	2.80E+10	3.30E+10	3.20E+10
MD FD	6.80E+10	6.50E+10	7.00E+10	MD FBD	2.40E+10	2.10E+10	3.00E+10	3.00E+10	2.70E+10	2.10E+10

Weight [g] of resulting freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum*.								
w/o FD	88.7	Glc FD	183.6	Tre FD	219.8	Suc FD	230.7	MD FD	224.1
w/o FBD	4039.6	Glc FBD	5248.6	Tre FBD	4949.6	Suc FBD	4845.6	MD FBD	5725.6
w/o FBD	4059.6	Glc FBD	4937.6	Tre FBD	5705.6	Suc FBD	5342.6	MD FBD	5301.6

CFU of resulting freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum*.										
w/o FD	4.90E+10	4.30E+10	4.60E+10	w/o FBD	3.00E+06	3.00E+06	2.00E+06	6.90E+07	6.30E+07	6.60E+07
Glc FD	1.41E+10	1.54E+10	1.18E+10	Glc FBD	2.80E+09	3.00E+09	3.40E+09	2.30E+09	2.60E+09	3.20E+09
Tre FD	4.30E+10	4.90E+10	4.50E+10	Tre FBD	6.70E+09	6.40E+09	6.20E+09	5.70E+09	4.70E+09	5.50E+09
Suc FD	3.90E+10	3.70E+10	3.50E+10	Suc FBD	6.30E+09	5.90E+09	5.80E+09	4.90E+09	5.30E+09	7.90E+09
MD FD	2.80E+10	2.70E+10	2.20E+10	MD FBD	2.70E+09	2.40E+09	3.60E+09	1.40E+09	1.40E+09	1.30E+09

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 1 month at 4°C.									
Glc FD	7.60E+09	7.10E+09	8.10E+09	Glc FBD	2.60E+09	2.10E+09	1.90E+09	1.46E+09	1.63E+09	1.47E+09
Tre FD	4.20E+10	3.80E+10	3.50E+10	Tre FBD	3.50E+09	3.20E+09	3.80E+09	3.40E+09	3.30E+09	3.80E+09
Suc FD	4.20E+10	3.50E+10	2.60E+10	Suc FBD	4.00E+09	3.90E+09	4.50E+09	4.00E+09	3.80E+09	3.90E+09
MD FD	1.52E+10	1.30E+10	1.23E+10	MD FBD	1.06E+09	9.30E+08	9.70E+08	4.20E+08	4.00E+08	5.30E+08

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 3 months at 4°C.									
Glc FD	8.10E+09	8.80E+09	7.90E+09	Glc FBD	2.70E+09	2.50E+09	2.00E+09	1.25E+09	1.31E+09	1.31E+09
Tre FD	3.70E+10	2.90E+10	4.20E+10	Tre FBD	2.40E+09	2.30E+09	2.20E+09	1.90E+09	2.20E+09	2.50E+09
Suc FD	3.10E+10	3.70E+10	3.20E+10	Suc FBD	2.40E+09	2.60E+09	2.80E+09	2.80E+09	2.70E+09	2.20E+09
MD FD	6.60E+09	7.20E+09	8.40E+09	MD FBD	6.80E+08	7.40E+08	7.20E+08	2.50E+08	2.50E+08	3.00E+08

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 4 months at 4°C.									
Glc FD	8.90E+09	8.10E+09	7.20E+09	Glc FBD	1.79E+09	1.51E+09	1.58E+09	9.10E+08	1.05E+09	1.12E+09
Tre FD	3.10E+10	4.30E+10	3.20E+10	Tre FBD	1.93E+09	2.05E+09	1.87E+09	1.82E+09	1.99E+09	1.85E+09
Suc FD	4.40E+10	3.00E+10	2.50E+10	Suc FBD	2.00E+09	2.40E+09	2.70E+09	2.20E+09	2.60E+09	2.30E+09
MD FD	6.70E+09	6.50E+09	6.00E+09	MD FBD	4.90E+08	5.20E+08	5.40E+08	2.20E+08	2.60E+08	2.50E+08

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 6 months at 4°C.									
Glc FD	7.90E+09	8.40E+09	8.80E+09	Glc FBD	1.88E+09	1.60E+09	1.54E+09	1.14E+09	9.80E+08	9.50E+08
Tre FD	3.50E+10	3.40E+10	3.70E+10	Tre FBD	1.74E+09	1.57E+09	1.51E+09	1.69E+09	1.63E+09	1.55E+09
Suc FD	3.30E+10	4.00E+10	3.00E+10	Suc FBD	2.20E+09	2.20E+09	1.90E+09	2.60E+09	2.20E+09	2.50E+09
MD FD	5.60E+09	5.80E+09	5.10E+09	MD FBD	4.20E+08	4.40E+08	5.30E+08	1.45E+08	1.32E+08	1.47E+08

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 1 month at 22°C.									
Glc FD	7.50E+09	8.00E+09	7.80E+09	Glc FBD	2.00E+09	1.80E+09	1.80E+09	8.90E+08	1.04E+09	9.60E+08
Tre FD	3.70E+10	3.30E+10	3.30E+10	Tre FBD	2.80E+09	3.50E+09	2.50E+09	3.20E+09	2.40E+09	2.70E+09
Suc FD	2.90E+10	3.00E+10	3.60E+10	Suc FBD	2.90E+09	2.80E+09	2.40E+09	2.70E+09	2.10E+09	2.90E+09
MD FD	9.20E+09	9.30E+09	8.60E+09	MD FBD	6.80E+08	6.60E+08	6.00E+08	2.60E+08	2.20E+08	2.30E+08

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 3 months at 22°C.										
Glc FD	4.90E+09	3.80E+09	4.30E+09	Glc FBD	8.60E+08	1.07E+09	9.80E+08	1.43E+08	1.35E+08	1.27E+08
Tre FD	2.70E+10	3.50E+10	2.60E+10	Tre FBD	9.00E+08	8.20E+08	8.60E+08	7.10E+08	7.50E+08	7.60E+08
Suc FD	1.93E+10	2.15E+10	2.03E+10	Suc FBD	1.24E+09	1.38E+09	1.25E+09	1.08E+09	1.12E+09	9.30E+08
MD FD	1.34E+09	1.51E+09	1.34E+09	MD FBD	1.78E+08	1.57E+08	1.73E+08	6.70E+07	5.90E+07	5.30E+07

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 4 months at 22°C.										
Glc FD	3.60E+09	4.20E+09	4.40E+09	Glc FBD	6.70E+08	5.80E+08	7.20E+08	6.60E+07	7.60E+07	8.60E+07
Tre FD	1.64E+10	1.83E+10	1.96E+10	Tre FBD	4.10E+08	6.40E+08	5.50E+08	4.10E+08	5.10E+08	5.50E+08
Suc FD	1.28E+10	1.50E+10	1.41E+10	Suc FBD	6.40E+08	7.60E+08	9.70E+08	6.90E+08	8.00E+08	7.90E+08
MD FD	8.70E+08	1.17E+09	1.12E+09	MD FBD	9.80E+07	1.02E+08	8.90E+07	4.00E+07	3.70E+07	3.60E+07

CFU of freeze dried (FD) and fluidised bed dried (FBD) powders of *Lact. plantarum* after 6 months at 22°C.										
Glc FD	4.00E+09	3.90E+09	4.60E+09	Glc FBD	3.70E+08	3.50E+08	4.40E+08	4.70E+07	4.00E+07	5.50E+07
Tre FD	1.81E+10	1.71E+10	1.87E+10	Tre FBD	3.90E+08	4.80E+08	4.10E+08	4.10E+08	4.50E+08	4.40E+08
Suc FD	9.90E+09	1.13E+10	1.12E+10	Suc FBD	6.20E+08	6.80E+08	6.50E+08	5.10E+08	5.20E+08	4.10E+08
MD FD	4.80E+08	5.80E+08	5.70E+08	MD FBD	6.30E+07	7.20E+07	6.30E+07	2.40E+07	2.40E+07	2.70E+07

Weight [g] of sieve fractions of the resutling coatings.												
Sieve fraction	125 - 500 μm			500 - 800 μm			800 μm - 1 mm			> 1 mm		
Shellac	4.75	7.03	3.05	421.09	268.09	565.87	263.45	389.71	311.22	20.60	20.40	16.15
Carnauba wax	37.59	30.93	35.70	87.50	116.16	93.30	349.39	346.13	366.05	14.32	16.73	13.57
Soya oil	5.22	4.10	2.70	50.01	29.46	22.73	457.23	448.54	463.77	20.90	23.02	17.84
Tall oil rosin	1.84	2.21	3.01	82.61	91.69	102.33	228.73	302.82	280.31	239.56	260.93	218.54

Data used for the calculation of carbon dioxide generation.									
	Blank value		Soya oil		Carnauba wax		Shellac		Tall oil rosin	
TOC [%C]	-		57.83		63.51		51.90		58.99	
Inoculum [g]	2.00	2.04	2.02	1.98	2.03	2.07	2.05	2.07	2.01	2.05
Sample [g]	-		2.01	2.01	2.00	2.00	2.04	2.06	2.05	2.05

Volumes HCl needed for titration of evolved carbon dioxide [mL].						
Day	2	6	10	14	21	28
Blank value	0.045	0.046	0.047	0.043	0.054	0.063
	0.044	0.044	0.049	0.047	0.048	0.057
Soya oil	0.468	0.532	0.500	0.436	0.403	0.367
	0.431	0.549	0.391	0.353	0.328	0.367
Carnauba wax	0.395	0.511	0.232	0.207	0.420	0.433
	0.416	0.511	0.284	0.231	0.420	0.394
Shellac	0.442	0.397	0.262	0.168	0.182	0.140
	0.422	0.566	0.312	0.173	0.154	0.087
Tall oil rosin	0.467	0.506	0.514	0.528	0.558	0.295
	0.487	0.571	0.499	0.452	0.555	0.405

Concentration of aluminium ions [mg/L].									
Week	0	1	2	3	4	6	7	8	total
Soya oil	0.83	4.48	8.07	6.39	4.99	6.58	10.94	10.98	20.39
	3.41	3.17	4.50	4.14	7.53	6.22	14.12	15.42	20.55
Carnauba wax	3.87	2.54	4.35	3.06	3.04	5.14	5.13	8.38	19.63
	1.11	2.69	3.75	3.55	2.88	6.19	6.06	7.33	19.40
Shellac	0.13	1.98	6.16	3.72	4.01	4.32	7.49	8.44	17.60
	0.10	0.86	2.98	2.45	3.99	4.28	9.95	8.98	17.71
Tall oil rosin	1.14	1.98	2.49	3.52	3.11	4.18	6.90	8.09	20.41
	1.29	2.84	4.97	6.72	4.02	5.17	7.93	8.61	20.82