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Development of Formulations for Plant Beneficial Bacterial Inoculants

Dissertation

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Wie komm ich am besten den Berg hinan? Steig nur hinauf und denk nicht dran!

Friedrich Wilhelm Nietzsche

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CONTENT

1 ABSTRACT

Plants are known to live in close association with a vast variety of microorganisms, some of which may promote plant growth, increase nutrient uptake or enhance plant resilience to biotic and abiotic stresses. Therefore, specific microorganisms may be applied in agriculture as a sustainable alternative to synthetic chemical fertilizers and pesticides. In many cases, however, their susceptibility to environmental factors and short shelf life are limiting the inoculation efficiency in the field and thus their practical use. To address this issue, the development of protective formulations is crucial. Encapsulation in a biopolymer matrix (alginate) or granule formation based on inorganic carriers (zeolites) were investigated as formulation techniques with the aim of obtaining stable inoculants carrying a high number of viable cells of the sensitive, Gram negative model organism *Parabukholderia phytofirmans* PsJN.

Alginate beads for bacterial immobilization were produced by a range of lab-scale methods. The throughput and compatibility of the respective methods with different matrix compositions as well as the morphology of resulting beads, the controlled release of bacteria and suitability for seed coating were described. Alternatively, granules and powders carrying the inoculant were developed based on a matrix of zeolite, a film forming agent and protectants. Their morphological characteristics were investigated by microscopy and laser diffraction.

Additionally, strategies for attenuation of desiccation stress occurring during the production of dry inoculants were investigated. For this, 20 chemically diverse protectants were checked regarding their ability to maintain a high viability of PsJN after lyophilization or air drying. Furthermore, secretion of exopolysaccharides was triggered in PsJN and tested as a protectant during desiccation. The bacterial viability was monitored over a period of up to 7 months of storage at different temperatures.

We demonstrated that the selection of appropriate protectants and their combination with suitable drying methods is highly important and dramatically increases the survival rate of PsJN by up to 100,000-fold. A high shelf life of more than three months at room temperature was achieved applying skimmed milk powder or PsJN's exopolysaccharide as protectants. Small sized alginate beads (< 100 µm) proved suitable for seed coating, whereas zeolite granules may be a feasible means to deliver the inoculant in-furrow.

These observations contribute to identifying suitable formulations, not only for PsJN but also for other promising, plant beneficial bacteria. This is a major prerequisite for successful application of inoculants in the field and thus utilizing the vast potential of these microorganisms.

2 ZUSAMMENFASSUNG

Pflanzen bilden eine enge Gemeinschaft mit einer unglaublichen Vielfalt von Mikroorganismen. Von einigen dieser Mikroorganismen weiß man, dass sie das Pflanzenwachstum fördern, die Nährstoffaufnahme verbessern oder die Pflanze widerstandsfähiger gegenüber biotischem und abiotischem Stress machen. Daher könnten spezifische Mikroorganismen als nachhaltige Alternative zu herkömmlichen Agrarchemikalien in der Landwirtschaft eingesetzt werden. In vielen Fällen wird eine erfolgreiche Beimpfung und damit die praktische Anwendung jedoch dadurch verhindert, dass die Mikroorganismen sehr empfindlich gegenüber Umwelteinflüssen sind und eine geringe Lagerstabilität aufweisen. Um dem entgegenzuwirken, ist die Entwicklung schützender Formulierungen unerlässlich. Verkapselung in einer Matrix aus Biopolymeren (Alginat) oder die Herstellung von Granulaten basierend auf anorganischen Trägermaterialien (Zeolith) wurden als Formulierungstechniken untersucht. Oberstes Ziel war, stabile Impfmittel mit einer hohen Anzahl lebensfähiger Zellen zu erhalten. Als Modellorganismus diente das empfindliche, Gram negative Bakterium *Paraburkholderia phytofirmans* PsJN.

Zur Immobilisierung der Bakterien wurden Alginatkapseln mittels verschiedener Methoden im Labormaßstab hergestellt. Die Durchflussrate und die Kompatibilität der Methoden mit unterschiedlichen Matrixzusammensetzungen sowie die Morphologie der Kapseln, kontrollierte Freisetzung der Bakterien und die Anwendbarkeit als Saatgutbeschichtung wurden beschrieben. Alternativ wurden Granulate und Pulver basierend auf Zeolith, Filmbildnern und Schutzstoffen als Träger für die Impfmittel entwickelt und mikroskopisch und sowie mittels Laserbeugung charakterisiert.

Zusätzlich wurden Ansätze zur Reduktion des während der Herstellung der Impfmittel auftretenden Trockenstresses untersucht. Dafür wurden 20 Schutzstoffe unterschiedlicher chemischer Struktur hinsichtlich ihres Effekts auf die Überlebensrate von PsJN nach Lyophilisierung oder Lufttrocknung getestet. Darüber hinaus wurde die Sekretion von Exopolysacchariden in PsJN getriggert und diese als Schutzstoffe während der Trocknung untersucht. Die Lebensfähigkeit der Bakterien wurde über einen Zeitraum von bis zu sieben Monaten und bei unterschiedlichen Lagertemperaturen beobachtet.

Es wurde gezeigt, dass der Auswahl geeigneter Schutzstoffe und deren Kombination mit adäquaten Trocknungsmethoden höchste Wichtigkeit zukommt und die Überlebensrate von PsJN um das 100.000-fache verbessern kann. Bei der Verwendung von Magermilchpulver oder bakteriellen Exopolysacchariden wurde eine gute Lagerstabilität bei Raumtemperatur von mehr als drei Monaten

erreicht. Alginatkapseln kleiner Größe (< 100 µm) erwiesen sich als geeignet für Saatgutbeschichtung, wohingegen Zeolithgranulate bei der Aussaat in die Ackerfurche eingebracht werden könnten.

Diese Beobachtungen tragen dazu bei, adäquate Produktzusammensetzungen für Beimpfungsmittel zu identifizieren – nicht nur für PsJN, sondern auch für andere, vielversprechende Bakterien mit einem positiven Einfluss auf die Pflanze. Dies ist eine bedeutende Voraussetzung für die erfolgreiche Anwendung im Feld und somit die Nutzbarmachung des großen Potentials, das in diesen Bakterien steckt.

3 INTRODUCTION

3.1 IMPROVING PLANT PERFORMANCE IN AGRICULTURE

Agricultural plant production is the basis for food, feed and fiber industry and thus plays a central role in supplying goods for a large range of demands in our daily lives. The capability of agricultural production as we know it today originates in the Green Revolution in the 1960s, which marked the onset of the application of modern technologies and high-performance crops. Despite having led to record yields, agriculture today is facing new challenges, which make further improvements and the development of innovative strategies necessary. These challenges include a growing global population, which is proposed to reach 9 billion by 2050. Combined with the trend towards a diet based on animal products, this will lead to a further increase in the demand of crops for food and feed. Simultaneously, extensive yield losses occur due to climate change, which brings about a higher frequency of extreme weather events, such as droughts, floods or severe storms. Furthermore, a decline in soil quality can often be observed as a consequence of inappropriate soil management, resulting in erosion, salinization and leaching of nutrients. In addition, the occurrence of crop pests and diseases account for up to 25 % of annual yield losses worldwide (Lugtenberg, 2015) and are increasingly difficult to combat as resistances emerge. It is obvious that taking counteractions is necessary in order to ensure the future supply of plant-based products in high quality and sufficient quantity.

3.1.1 APPLICATION OF AGROCHEMICALS

Applying agrochemicals has been a key strategy to improve crop performance and agricultural productivity. Comprising pesticides, insecticides, herbicides, fungicides, nematicides and others, agrochemicals generally consist of an active ingredient in conjunction with an inert carrier as well as adjuvants, which, in combination, represent the formulation that is commercialized and distributed. The main aim of formulating the active ingredient is to obtain a stable product, which is safe and simple in handling and displays a high efficacy (Knowles, 1970).

The active ingredient describes the component responsible for biological effectiveness. A large number of active ingredients from diverse sources are available – they may be derived from plant extracts such as pyrethrum or may be of mineral origin as in the case of copper or sulphur treatments. The majority, however, is chemically synthesized. In any case, the active ingredients differ regarding their chemical properties. In this regard, their solubility is of special interest, as it determines compatible carriers and

potential ways of delivering the active ingredient to the target site. On the one hand side, liquid formulations may be considered, including emulsions, solutions or so called flowables (particles suspended in liquid). Alternatively, dry or solid formulations may be applied, including dusts, granules, wettable powders, soluble powders or water dispersible granules. The decision not only depends on the chemical characteristics of the active ingredient, but also on the mode of application. Dusts, for example, are suitable for seed treatments, whereas granular formulations are frequently applied as soil amendments.

In addition to the carrier material, adjuvants may be applied. These include adhesives, plant penetrants, buffers, anti-foaming agents, surfactants, and other substances, which enhance the performance of the active ingredient and improve handling during application (Green and Beestman, 2007; Zabkiewicz, 2000).

Despite an evident contribution to pest management and resulting improvement of yield in terms of quantity and quality, synthetic pesticides come with some serious drawbacks (Aktar et al., 2009). Since they are substances designed to kill living organisms, they pose an inherent risk to human health as well as ecosystems. Agricultural farm workers are most concerned due to their direct exposure to agrochemicals during handling, but also consumers may be affected of residues in food and drinking water. Unintended drift or run-off may transport pesticides from the application site to adjacent ecosystems, where they pose a threat to non-target organisms and thus endanger biodiversity and beneficial organisms (Damalas and Eleftherohorinos, 2011; Werf, 1996). In this process, the potential human and environmental toxicity does not necessarily arise from the active ingredient alone, but may be amplified by the presence of certain adjuvants in the formulation (Surgan et al., 2010). In order to keep residues of hazardous substances in food low, farmers are bound to adhering to a certain period of time (so called “pre-harvest interval”) between the last pesticide application and harvesting.

Disadvantages associated with the use of synthetic fertilizers - commonly referred to as NPK-fertilizers – include exploitation of limited phosphorus resources, nitrate pollution of groundwater, a high energy input in form of fossil fuels (Kliopova et al., 2016) as well leaching or runoff and subsequent eutrophication of aquatic ecosystems (Conley et al., 2009).

In view of such serious shortcomings of synthetic pesticides and fertilizers regarding health and environmental impacts, it is obvious that the development of sustainable alternatives is imperative. This

agenda is supported by regulatory authorities and the public calling for non-polluting, eco-friendly agricultural practices.

3.1.2 PLANT ASSOCIATED, BENEFICIAL BACTERIA AS SUSTAINABLE ALTERNATIVES

Utilizing naturally occurring, biotic interactions for the benefit of plants instead of chemically synthesized compounds has caught increasing attention in the last decades. These interactions may occur for instance between plants and microorganisms. The most prominent example in this regard is the association of legumes with rhizobia, which fix nitrogen and provide this essential nutrient to their host. Rhizobia on peat carriers have been commercially produced and knowingly delivered to the field to enhance soil fertility since the late 19th century (Brockwell and Bottomley, 1995). Since then, many other beneficial mechanisms in the relationship between plants and microorganisms have been described. Basically, they may be assigned to three major categories: biofertilizers, biopesticides and plant growth-promoters. Biofertilizing microorganisms increase the availability of nutrients and include not only the aforementioned nitrogen fixers, but also microbes which help sequestering iron or solubilize phosphate from the soil (Barea and Richardson, 2015).

Biopesticides encompass microbes which are enhancing plants' resilience in the face of disease attack. They may do so indirectly by triggering the plants' defense mechanism, known as induced systemic resistance (Pieterse and Wees, 2015), and by outcompeting pathogens in the fight for nutrients and space. Alternatively, they may compromise pathogens directly by producing antibiotics or cell wall degrading enzymes, a strategy called antibiosis (Thomashow and Bakker, 2015).

Plant growth-promotion is mainly conferred by microbes' production of phytohormones influencing the plants' physiology. These include for example auxins and cytokinins, which are involved in cell division and root development (Spaepen, 2015). Furthermore, microbes may modulate the level of ethylene, a plant stress hormone, and thereby prevent growth retardation as a negative response of plants in face of biotic and abiotic stress (Glick, 2015).

Looking at this broad range of mechanisms, it is obvious that plants' reactions to environmental factors are greatly influenced by the microbial community they are associated with. This suggests the modification of the plant microbiome as a promising tool to improve plant performance and ultimately agricultural production. Plant-associated microorganisms are thus a non-toxic, sustainable alternative to synthetic agrochemicals. Furthermore, microbe-based products may be convenient for the farmer in

terms of application timing, since they do not – in contrast to pesticides – impose an obligatory pre-harvest interval.

3.2 FORMULATION OF MICROBIAL INOCULANTS

Similarly to the formulation of synthetic pesticides, microbial products include not only the inoculant itself, but need to be combined with specific compounds to enhance their performance at the target site and facilitate their practical use by farmers. This implies that formulation development is an integral part of the commercialization of microbial inoculants as an alternative to standard agrochemicals.

3.2.1 NECESSITY AND REQUIREMENTS OF PROTECTIVE FORMULATIONS

The lack of adequate formulations of microbial products and concomitant low inoculant quality is regarded as one of the major constraints to their successful, widespread use (Stephens and Rask, 2000). The delivery of a high number of viable cells to the plant is required to reach a satisfactory colonization rate, which in turn influences the extent of the inoculated microbes' effect on the host crop. The viability of inoculants may suffer at different stages both prior and during application. A good maintenance of viability prior to application is commonly described as a high shelf life or storage stability. This term comprises the production process itself, packaging, storage and transport conditions (Arora et al., 2011). Considering the distribution of inoculants to remote farms or in less developed countries, it is obvious that inoculants cannot always be stored under ideal conditions but may be exposed to high temperatures, humidity or light (Herrmann and Lesueur, 2013). A good formulation process should ideally address these pre-application issues and contribute to an increased shelf life of the inoculant, thereby allowing for meeting the seasonal demand of farmers.

During application on the field, the inoculant is confronted with further factors that are detrimental to its viability. These include UV radiation from sunlight (Zohar-Perez et al., 2003) - particularly when applied on above-ground plant parts - fluctuating soil properties such as texture, temperature and pH (Arora et al., 2011) and repeated drying-rewetting cycles depending on the frequency of precipitation. For inoculants applied directly to seeds, the inherent seed coat toxicity may be harmful (Deaker et al., 2012). Furthermore, biotic interactions with the native microflora and -fauna present a major challenge to the artificially introduced strains. Frequently, their cell numbers decline quickly after application to non-sterile soil since they are out-competed by indigenous microbes or diminished by predators such as protozoa (Arora et al., 2011; Bashan, 1998). Formulations aim to provide a protective microenvironment, in which

the inoculant is physically shielded and optionally supplied with protective substances, such as osmoprotectants or xeroprotectants, and nutrients. Simultaneously, the formulation must guarantee the release of viable cells in due time to colonize the plant at a preferably early developmental stage (Bashan, 1998).

Obviously, pre-application stress factors are exacerbating the problem of insufficient target site colonization – the lower the cell survival rate upon arrival at the field the less likely is a successful establishment in the soil and/or plant. A formulation, which takes into consideration all stress factors occurring over the course of production and application is therefore of vital importance.

Apart from cell viability, a formulation should be optimized considering requirements from the manufacturer's or farmer's point of view. This concerns for example physical, chemical and biological consistency of the carrier material to establish routine processing (Stephens and Rask, 2000) as well as non-toxicity to humans, plants and ecosystems (Catroux et al., 2001). The aspect of environmental-friendliness further comprises biodegradability as well as the sustainable nature of raw materials and production process (Bashan et al., 2014). Furthermore, the carrier should be sterile or easily sterilized to avoid contamination and with this a potential suppression of the inoculant strains by the presence of competing microbes (Bashan et al., 2014). To increase acceptance by farmers, the formulation should be compatible with the practices they are used to, for example applicable with standard machinery, not associated with additional work steps and combinable with traditional techniques such as seed treatments (Catroux et al., 2001). Finally, the cost of microbial products should be minimized to allow for competition with standard agrochemicals.

Obviously, it is hardly possible to bring a formulation to perfection regarding all the aforementioned aspects. However, it is necessary to be aware of them and to come as close to meeting all requirements as possible in order to circumvent potential criteria for exclusion.

3.2.2 POSSIBLE MODES OF APPLICATION – STATE OF THE ART

A variety of technologies for the delivery of inoculants to the field are in practice, as shown in Fig. 1. The feasibility of a given technique is directly dependent on the physical state the formulation is available in. On the other hand, the physical appearance of the formulation may be developed specifically for a desired application technology.

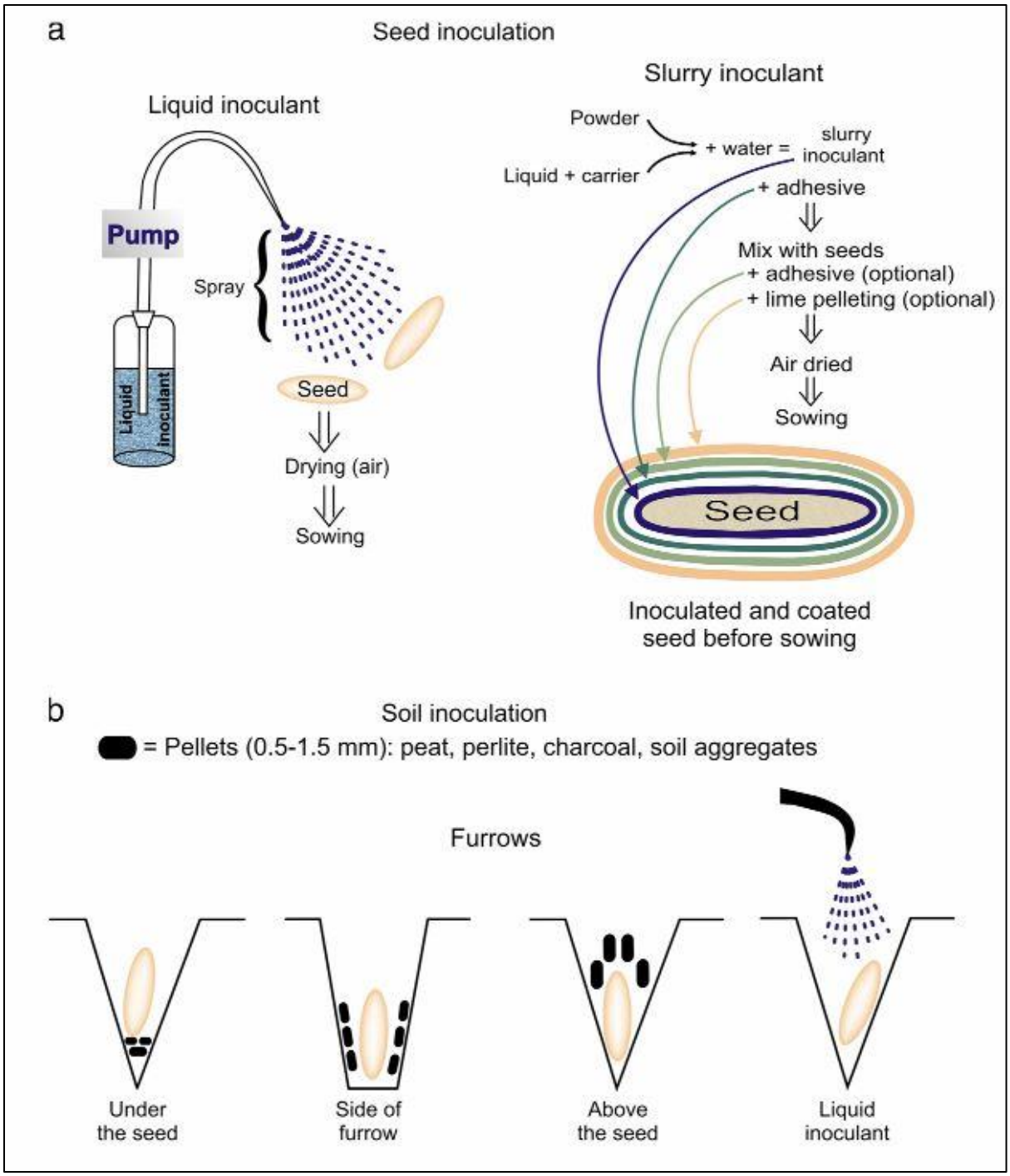

Figure 1: Application techniques of bacterial inoculants in the field. Seed treatment (a) or soil inoculation (b) is possible with either liquid or solid formulations of different particle sizes (*source: Bashan et al. 2014*).

As in the case of standard agrochemicals, microbial products may come in solid or liquid form. Solid formulations may be subdivided into powders and granules depending on their particle sizes. Dry formulations based on peat have a long history in application of rhizobia (Brockwell and Bottomley, 1995) and continue to be the most widely used type (Herrmann and Lesueur, 2013). They may be

sterilized prior to impregnation with the inoculant broth or suspension (Stephens and Rask, 2000). As is true for other dry powder formulations, peat flours are commonly coated onto seeds, mostly with the support of an adhesive to prevent detachment during handling (Bashan et al., 2014). Regarding seed treatment, two different approaches can be distinguished: pre-inoculation, which is commonly performed by the seed manufacturer prior to sale or customer inoculation, which is done by the farmer on demand (Brockwell and Bottomley, 1995; Deaker et al., 2004). While pre-inoculation is labor saving for farmers, it may lead to unwanted germination if the seed coating contains too much moisture (John et al., 2011). Furthermore, the survival rate of live inoculants on the seed may be lower than in customer inoculation, due to an enhanced storage period prior to sowing. Seed coating may be cost-efficient due to a minimal application rate necessary; however, this simultaneously constitutes a limit, since the maximum load of inoculant depends on the seed surface area. Especially in small sized seeds, the subsequent limitations in cell numbers per seed may restrict the feasibility of this practice (Stephens and Rask, 2000). Also, this technique may be incompatible with other seed treatments such as fungicide coatings or may be prohibited in crops with a delicate seed coat (Bashan et al., 2014).

To circumvent these issues, dry formulations have alternatively been applied as soil amendments. For this purpose, they are frequently produced as granules of approximately 0.5 – 1.5 mm size. These are less dusty than powders, plus, the applied quantity can easily be controlled and they may be purposefully placed in-furrow or lateral of the seed bed during sowing (Bashan et al., 2014). Apart from peat as carrier materials for dry formulations, several other options have been investigated (Bashan et al., 2014; Malusá et al., 2012). These include soil derived carriers (e.g. charcoal, clays, turf), organic carriers (e.g. sawdust, wheat/soy/oat bran, grape bagasse, vermicompost, animal manure, sewage sludge, cork compost, vermicompost) and inert materials (e.g. perlite, vermiculite, bentonite, kaolin, silicates, talc, polymers). Pure lyophilized cultures, where desired in presence of a lyoprotectant, may also be an option and can be used directly or in combination with a solid carrier (Malusá et al., 2012).

A major issue in any dry formulation is the desiccation live cells have to endure, which is often critical for organisms sensitive to drying. This is one of the reasons liquid formulations have been developed as an alternative. They comprise oil- or water-based suspensions of cell concentrates, emulsions or slurries containing solid particles (Malusá et al., 2012). A range of additives may be incorporated, such as nutritive substances, protectants, stabilizers or adhesives (Bashan et al., 2014). It has been argued that liquid formulations allow for straightforward handling and application and are thus increasing in

popularity (Stephens and Rask, 2000). In some cases, they confer a better shelf life than dry formulations (Bashan et al., 2014), however, they frequently require cooling (Herrmann and Lesueur, 2013; Stephens and Rask, 2000). Furthermore, the lack of a protective carrier may render them more susceptible to environmental factors once applied in the field. Thus, liquid formulations have been found to be occasionally inferior to solid carrier based formulations (Albareda et al., 2008).

Nevertheless, liquids are suitable for a wide range of application technologies. Like dry formulations, they may be applied directly (together with an adhesive) to the seed immediately prior to sowing (Bashan et al., 2014). Also, liquids may be delivered to the soil in-furrow during sowing or at a later stage via fertigation systems (Malusá et al., 2012). The latter technique is particularly relevant for the inoculation of perennial crops, where beneficial microorganisms need to be introduced into an already established orchard or plantation (Malusá et al., 2012). Furthermore, liquids permit the treatment of above ground plant parts, for example in form of foliar spray (Jambhulkar et al., 2016). This may be desirable if the inoculant organism features a plant colonization pathway using aerial plant parts (e.g. stomata, flowers) as entry ports, as has been observed for endophytic bacteria such as *Paraburkholderia phytofirmans* PsJN (formerly *Burkholderia phytofirmans* PsJN) (unpublished data). Similarly, an epiphytic lifestyle or the site of action of a biocontrol agent may require its above-ground application. This is the case for example when combating fire blight caused by *Erwinia amylovora* in some Rosaceae or *Botrytis cinerea* in viticulture with antagonistic microorganisms.

As an innovative formulation technology providing extra protection to microorganisms and controlled release in the soil, encapsulation of inoculant cells has been proposed (Bashan, 1986; Dommergues et al., 1979). Essentially, all delivery techniques mentioned above may be considered for application of these capsules, but are dependent on capsule size. Small microcapsules may be coated onto seeds (Bashan et al., 2002) or sprayed onto aerial plant parts (Wiwattanapatapee et al., 2013), whereas macrobeads are more suitable for soil amendments. The formation of capsules to provide a protective microenvironment constitutes one of the approaches that was pursued in the present study, thus the theoretical background will be discussed in detail in chapter 4.3.1.

Figure 2 is summing up the facts about current formulation and delivery techniques for microbial inoculants: there are solid (powders, granules) and liquid (suspension, emulsions, slurries) formulations, which may be applied as seed treatment (powders, liquids), delivered to soil (powders, granules, liquids) or to above ground plant parts (liquids, powders).

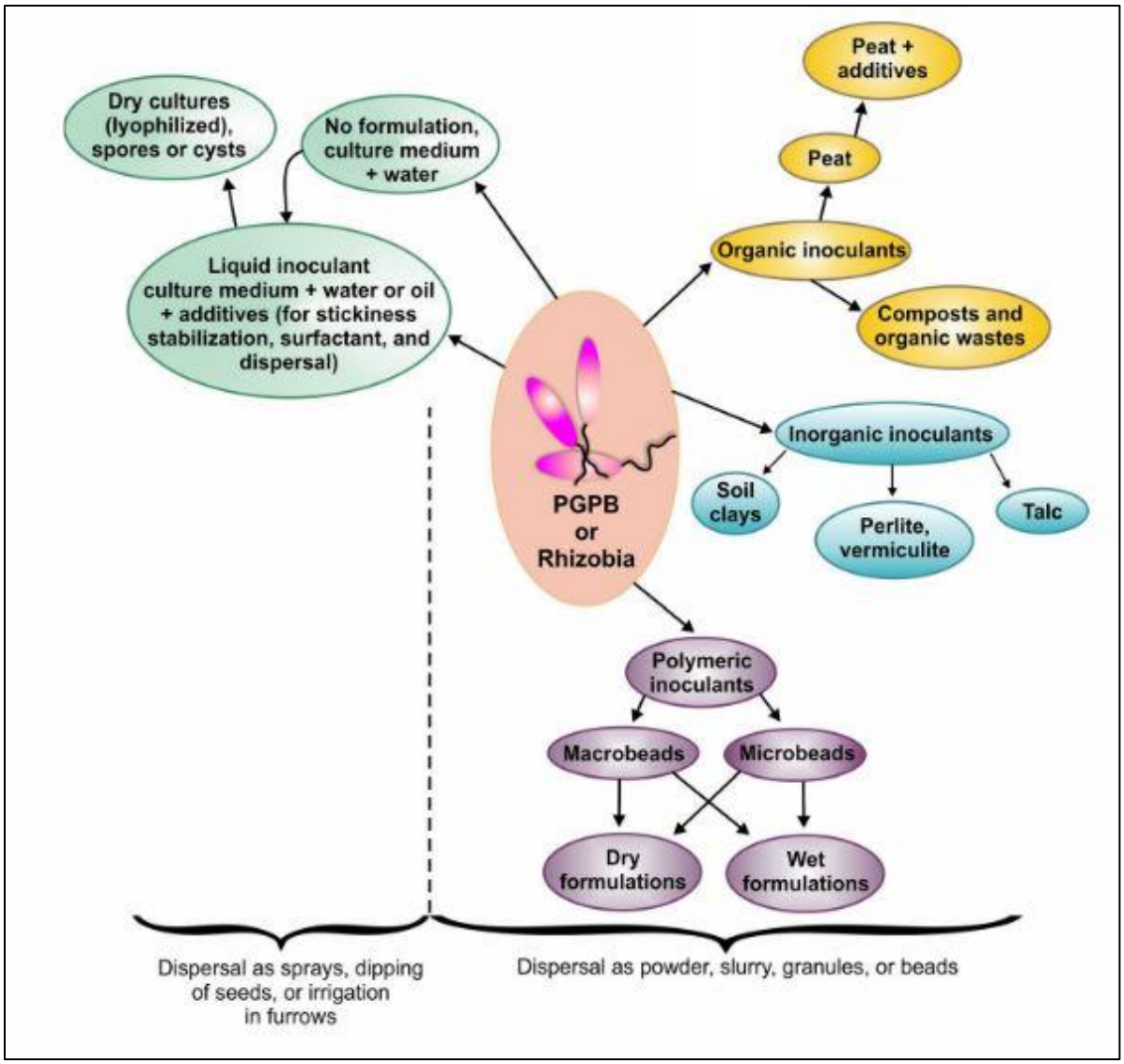

Figure 2: Overview of formulation techniques commonly used for bacterial inoculants in agriculture (*source: Bashan et al. 2014).*

The choice of formulation and application technology depends on the equipment available, farmer's convenience, presence or absence of additional treatments, plant inherent characteristics (seed size, perennial/annual crop production, seed coating sensitivity), plant developmental stage, cost, site of action and infection of the inoculant (Bashan et al., 2014; Deaker et al., 2004; Malusá et al., 2012).

3.2.3 SUCCESSFUL BACTERIAL PRODUCTS ON THE MARKET

Commercialization of bacterial products, precisely rhizobia, for improvement of crop performance in agriculture started as early as 1895 in the US and UK (Brockwell and Bottomley, 1995). Since then, many more beneficial, plant-associated microorganisms have been commercialized as "biopesticides" and "biostimulants". Products released as biostimulants comprise agents, whose mode of action is

based on enhancing plant vigor and abiotic stress tolerance, for example by increasing nutrient availability or hormone production. As opposed to biopesticides, they do not display any direct effect against plant pests or diseases. Only microbial products immediately targeting biotic plant stressors are considered biopesticides and thus need to undergo similar registration processes as standard chemical plant protectants. The first biopesticide was marketed in France under the name “Sporeine” in 1938 and contained live cells of *Bacillus thuringiensis* (Ravensberg, 2015).

According to the EU pesticides database on active substances (regulation EC No 1107/2009) the number of bacteria approved in the EU is limited to 12 strains (as per 31/03/2016). The majority can be ascribed to the genus *Bacillus* and comprises 5 species (*Bacillus firmus*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus subtilis and Bacillus thuringiensis* with 4 subspecies). Furthermore, two *Pseudomonas* species (*Pseudomonas chlororaphis* and *Pseudomonas* sp.) have been authorized, as well as two *Streptomyces* species (*Streptomyces lydicus* and *Streptomyces* K61). Globally, the amount of registered bacterial strains as agents for plant protection is 77 (Ravensberg, 2015).

The spectrum of available bacteria commercialized as biostimulants on the market is much less clear. This is because they do not need to be approved by legal authorities, which makes it impossible to provide a comprehensive list of products and inoculant strains used. Mostly, they involve bacteria from the genus *Bacillus*, *Pseudomonas* and *Rhizobium* (Ravensberg, 2015). In some cases, microbial consortia are contained in the product, which are not further specified. However, looking at formulation types and recommended application technologies of such products may allow for drawing conclusions regarding farmer’s preferences and thereby marketing abilities. Table 1 provides some examples of commercially available products, the strains and amount of CFU they contain, as well as the application technology they are suitable for. Additionally, the expected shelf life is given. Comparing this non-exhaustive collection of products available in different countries all over the world to information from scientific literature helps in identifying the most important aspects when it comes to commercialization of a product.

Table 1: Example descriptions of commercially available biofertilizers and biopesticides.

Product / company name	Strain / minimum cell count	Form / carrier	Application	Storage conditions / Shelf life
Biofertilizers				
FZB24® flüssig (ABiTEP GmbH, Germany)	*Bacillus amyloliquefaciens ssp. plantarum,* 3 x 10^9 CFU/g	Liquid concentrate (water, residues of 1,2-Propandiol, spores)	Dilution in water • Dipping • Seed treatment • Soil drench	• 0 °C to • + 30 °C • 2 years
FZB24® TB (ABiTEP GmbH, Germany)	*Bacillus amyloliquefaciens ssp. plantarum,* 1 x 10^9 CFU/g	Dry powder (talcum, corn starch, skimmed milk powder, glycerol, lyophilized spores)	• Dry treatment of seeds or tubers • Soil amendment	• 0 °C to • + 30 °C • 3 years
FZB24® WG (ABiTEP GmbH, Germany)	*Bacillus amyloliquefaciens ssp. plantarum,* 5 x 10^{10} CFU/g	Wettable powder (corn starch, skimmed milk powder, glycerol, lyophilized spores)	Slurry in water • Tuber treatment • Seed treatment • Soil drench	• 0 °C to • + 30 °C • 2 years
Bactofil® B10 (Kwizda Agro GmbH, Austria)	*Azospirillum lipoferum, Azotobacter vinelandii, Bacillus megaterium, Bacillus circulans, Bacillus subtilis, Pseudomonas fluorescens, Micrococcus roseus,* 5 x 10^9 CFU/mL	Liquid concentrate (water, nutrients)	Dilution in water • Soil drench	• +5 °C to +10 °C • 6 months
Rizoliq® Top S plus Premax® (Rizobacter, Argentina)	*Bradyrhizobium japonicum* strains SEMIA 5079 and SEMIA 5080, 2 x 10^9 CFU/mL	Liquid concentrate + separate liquid protectant (cellulose, disaccharides, nutrients)	Mix inoculant and protectant prior to use • Seed treatment	• < +20 °C • 2 years
BIODOZ® Soja plus microgranulate BIODOZ® Soja M (Novozymes, Denmark)	*Bradyrhizobium japonicum* strain G49, 1 x 10^9 CFU/g	Wettable powder (peat based) or dry granules	Slurry in water (BIODOZ® Soja only) • Seed treatment Microgranules (BIODOZ® Soja M • Soil amendment (in-furrow)	• 0 °C to +25 °C • Shelf life not declared
NITROFIX™ - AC (Agri Life, India)	*Azotobacter chroococcum* strain MTCC 3853, 5 x 10^7 CFU/g	Wettable powder (kaolin, dextrose, lignite, spores)	Slurry in water, add sugar: • Seed coating Slurry in water, add manure: • Seedling root dip Mix with compost • Soil amendment Add to irrigation stream • Soil drench	• 1 year • No storage specifications

K Sol B ® (Agri Life, India)	*Frateuria aurantia,* 5 x 10^7 CFU/g	Wettable powder (talc, dextrose, lignite)	Slurry in water, add sugar: • Seed coating Slurry in water, add manure: • Seedling root dip Mix with compost • Soil amendment Drench (irrigation stream)	• 1 year • No storage specifications
Biopesticides				
Lipel ™ (Agri Life, India)	*Bacillus thuringiensis* var. kurstaki, 18,000 IU/mg	Wettable powder (nutrient medium residues, sodium chloride, dextrose, spores, endotoxin)	Slurry in water: • Foliar spray Dry powder: • Foliar dusting	• 1 year • No storage specifications
Bionemagon ™ (Agri Life, India)	*Bacillus firmus* strain NCIM 2673, 1 x 10^8 CFU/g	Wettable powder (kaolin, spores)	Dry: • Soil amendment Mix in water, filter, decant: • Drip irrigation	• 1 year • No storage specifications
Sheathguard™ (Agri Life, India)	*Pseudomonas fluorescens* strain IIHR-PF2, 1 x 10^8/g	Wettable powder (Carboxymethylcellulose, talc, cells)	Slurry in water, add sugar: • Seed coating Dry: • Nursery bed treatment • Soil application • Compost enrichment	• 1 year • No storage specifications
Cedomon® (Lantmännen BioAgri AB, Sweden)	*Pseudomonas chlororaphis* MA342, 10^9 – 10^{10} CFU/mL	Emulsion (rape seed oil)	Seed coating	• + 4 °C to + 8 °C for 8 weeks • Room temperature for 3 weeks • Storage of treated seeds 1 year
Cerall® (Lantmänne BioAgri AB, Sweden)	*Pseudomonas chlororaphis* MA342, 10^9 – 10^{10} CFU/mL	Flowable suspension (water based)	Seed coating	• + 4 °C to + 8 °C for 8 weeks • Room temperature for 1 week • Storage of treated seeds 1 year
Serenade® Opti (Bayer CropScience LP, USA)	*Bacillus subtilis* QST 713, 1 x 10^{10} CFU/g	Wettable powder	Dilute in water: • Foliar spray • Soil drench	• Cool, dry place, not exposed to sunlight • Shelf life not specified
Galltrol®-A (AgBioChem, Inc., USA)	*Agrobacterium radiobacter* strain K84, 1 x 10^{11} CFU/plate	Bacterial culture on agar plates	Cell suspension in water • Spray • Root dip • Root drench	• +2 °C to +4°C • 120 days

The table shows that many manufacturers offer the same active ingredient in different application forms, e.g. solid and liquid. Offering the inoculant as wettable powders, the farmer may choose between processing it into slurry and delivering it via liquid delivery systems or applying it in a dry state. This product diversification emphasizes the importance of catering to farmers' convenience and allow for maximum flexibility regarding the application technique they may choose.

Another interesting strategy taken on by some manufacturers is providing inert additives enhancing the performance of the active ingredient as additional products. For example the inoculant Rizoliq® may be applied in combination with Premax®, which does not contain viable cells itself, but functions as a protectant and adhesive. Keeping both viable cells and protectants separated until immediately prior to application is an interesting strategy to prevent bacterial metabolization and thus exhaustion of nutrients and possible accumulation of toxic metabolites during storage.

The table also shows that there are alternative forms of products available, which are commonly not considered actual "formulations". This is the case in Galltrol®-A, which is sold as bacterial cultures on agar plates. Despite a very limited shelf life in the range of 3 months and the necessity of refrigeration, it is obvious that such a product may be successful if it can be produced on demand and shows a good efficiency.

However, most products guarantee a shelf life of at least 6 months and up to 2 – 3 years and frequently do not require specific storage conditions such as refrigeration. Such long storage stability is most readily achieved when using spore-forming bacteria. Indeed, this is the case in most inoculant strains listed in the table, many of which belong to the genus *Bacilli*. Clearly, the commercialization of non-spore forming bacterial inoculants is more challenging due to their susceptibility during the production process, storage and handling. This implies that sensitive bacterial strains, albeit displaying strong plant-beneficial effects, hardly make their way into commercially available products. Herein lies a challenge for formulation development and the prospect of utilizing highly potent bacterial strains when overcoming this obstacle.

3.2.4 *PARABURKHOLDERIA PHYTOFIRMANS* PSJN AS AN ENDOPHYTIC MODEL ORGANISM

Formulation aspects such as process survival rate, shelf life and delivery were tailored to *Paraburkholderia phytofirmans* PsJN. The reason for choosing this bacterium as a model organism lies in its way of interacting with plants and its physiological characteristics.

The bacterial isolate PsJN was initially obtained from the endorhizosphere of onions, by Dr Jerzy Nowak (Frommel et al., 1991). At first classifying it as a non-fluorescent *Pseudomonas* sp., it was described as Gram-negative, aerobic, rod-shaped with a width of 0.5 – 0.8 µm and a length of 0.8 – 1.8 µm and featuring a flagellum for motility. Its plant growth-promoting effect was first shown on potato plantlets, which displayed higher stress resistance during transplanting and a better developed root structure after inoculation with PsJN. Follow-up investigations based on chemotaxonomic and phenotypic characteristics as well as biomolecular tools eventually clarified the classification of this strain to the genus *Burkholderia.* Its ability to promote plant growth was the decisive factor for its final nomenclature *Burkholderia phytofirmans* PsJN (Sessitsch et al., 2005).

Subsequently, this strain has become one of the most studied endophytic bacterial strains. In addition to potato (Frommel et al., 1991; Kurepin et al., 2015), PsJN has been demonstrated to colonize tomato (Pillay and Nowak, 1997), grapevine (Barka et al., 2000; Barka et al., 2006; Fernandez et al., 2012), switchgrass (Kim et al., 2012; Lowman et al., 2015), wheat (Naveed et al., 2014a), maize (Naveed et al., 2014b), thale cress (Su et al., 2015), ryegrass (Afzal et al., 2013), cucumber and sweet pepper (Nowak et al., 2002). This large host range, comprising both dicotyledons and monocotyledons, illustrates the potential of PsJN to serve for improvement of agriculturally highly important field crops (maize, wheat) as well as for vegetable cultivation and production of energy crops in low-input systems. The possibility of a broad application range instead of a mere treatment of niche cultivars is an important aspect to consider when it comes to commercialization.

Regarding its mode of action, PsJN has mostly been classified as a plant growth-promoter, with a beneficial effect on plant vigor particularly in the face of abiotic stresses, for example arising from adverse properties of the growth substrate. In this context, PsJN was shown to increase the salt tolerance of *Arabidopsis thaliana* (Pinedo et al., 2015), enhance biomass production of plant species used for phytoremediation when irrigated with industrial effluent (Afzal et al., 2014) and promote root and shoot development in switchgrass on low fertility soils (Lowman et al., 2015). Abiotic stresses may also be caused by climatic variations in temperature or precipitation. The feasibility of PsJN for increasing plant tolerance towards cold temperatures has been demonstrated in grapevine (Barka et al., 2006; Fernandez et al., 2012) and thale cress (Su et al., 2015). Plants' performance was also improved under drought conditions, for example for wheat (Naveed et al., 2014a) and maize (Naveed et al., 2014b).

It is the ability of PsJN to modulate the levels of hormones linked to plants' vigor, such as indolacetic acid and ethylene that is for a large part responsible for its growth-promoting effect. Peaks in ethylene levels commonly occur as a plant's response to stress and trigger phenomena such as growth retardation, chlorosis, leaf abscission, plant senescence and finally yield losses. Enhanced ethylene levels may be prevented by PsJN, since it synthesizes the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase cleaving the precursor of ethylene. This means that plants colonized by PsJN show a less pronounced negative stress response (Glick, 2015).

This mechanism renders PsJN a potential tool in helping prevent yield losses as a consequence of extreme weather events and thereby may be a way of mitigating the impact of climate change on farmers' livelihoods. It may also help achieving higher yields under suboptimal conditions, such as degraded lands. Considering global issues such as climate change and lack of arable land, application of PsJN as an active agent meets a demand in agriculture, which will further increase in the future.

As the effect of PsJN on plants is predominantly a growth-promoting one, it may be cost-efficiently registered as a "biostimulant" and does not need to undergo a costly authorization process.

Another important feature of PsJN with consequences for practical application is its endophytic lifestyle. This implies that it does not only colonize the rhizosphere and rhizoplane of plants, but is also found in the inner tissues of plants, such as root interior, stem, leaf (Compant et al., 2005) as well as inflorescence tissue (Compant et al., 2008) and seeds (unpublished data). Not only is PsJN flexible regarding its exact location in the plant, but also in terms of colonization pathways. Mostly, penetration into the plant tissue occurs via the root (cracks, sites of lateral root emergence), however, PsJN has also been shown to colonize the interior of above ground plant parts – including flowers and the developing fruit – when applied as foliar spray (unpublished data).

These observations are of relevance regarding practical application for two reasons: Firstly, an endophytic organism holds the promise of successful long-term colonization in the plant, since it is protected from the fierce microbial competition of the rhizosphere, which has been assumed a major reason for the ineffectiveness of introduced strains. Secondly, the flexibility in colonization pathways (via the soil or above ground plant parts) allows for different application techniques. The bacteria may be delivered by standard seed or soil treatments but potentially also by spraying of plants at advanced stages of development.

Obviously, choosing a non-sporulating model bacterium for formulation development is linked to challenges when it comes to maintaining viability during processing. However, investigating strategies to stabilize PsJN may shed light on scientifically relevant questions regarding its behaviour in natural environments when exposed to stress. In addition, it may allow for defining feasible ways of formulating other non-sporulant plant beneficial bacteria.

3.3 THE FORMULATION PROCESS

Formulating bacteria as biofertilizers or biopesticides means dealing with a multi-step process, in which numerous parameters are involved and multiply to a literally infinite amount of possible combinations. This requires an elaborate and high throughput screening to narrow down the possible approaches and to end up with an optimized overall process. Ideally, this approach commences with cheap, straightforward screening of a large range of variables and concludes with more sophisticated and labor-intensive steps including only the parameters selected in previous steps (Köhl et al., 2011).

Early considerations are centered on strain selection, which may depend on proposed target crop, marketing ability, the safety and efficacy of a bacterial agent and its ability to be mass-produced (Köhl et al., 2011; Slininger and Schisler, 2013). While these aspects are not "formulation" steps in a narrower sense, they do constitute basic considerations that may decide over failure or success of the inoculant in its later stages of development. In case of the present study, the strain *Paraburkholderia phytofirmans* PsJN had already been thoroughly characterized and was selected as a model organism due to the aspects mentioned in chapter 4.2.4.

The first actual formulation steps concern the culture conditions and harvesting of the cultivated strain. Since many biopesticides or biofertilizers are commercialized as dry products, a drying step is what frequently follows. This requires choosing a suitable drying method out of a pool of available techniques. Finally, storage conditions need to be evaluated. In some cases, rehydration conditions are also taken into account since they have been shown to influence bacterial viability after reconstitution. However, results may be difficult to implement in practice, especially when aiming at developing dry soil applications, where rehydration conditions vary. At each single step, a high bacterial viability is the major criterion for selection of the respective parameters. The present work focuses on different techniques for maintenance of the bacterial viability using encapsulation, inorganic carriers and protective substances.

3.3.1 ENCAPSULATION

Encapsulation commonly describes the immobilization of key active agents of (bio-)technological processes to improve their performance. The classification of encapsulation approaches may be based on different parameters. Focusing on the bead size, microspheres (10-100 µm) are distinguished from macrospheres (>100 µm) (John et al., 2011; Rathore et al., 2013). Microspheres were shown to display enhanced mechanical stability and to better allow for diffusion of nutrients and oxygen (Rathore et al., 2013) than macrospheres. Considering they are also more suitable for seed coating or foliar spraying in agricultural applications (John et al., 2011), the focus was on production of small sized capsules in the present work.

The encapsulation process may also be characterized regarding bead shape (spherical, elliptical, irregular) (John et al., 2011) or morphology. Solid spheres are commonly referred to as beads, whereas hollow spheres, possibly with a liquid core, are termed capsules. Further variations include coated beads or multicompartment beads as well as lens-like shaped capsules (Vemmer and Patel, 2013).

Encapsulation has been applied for stabilization and controlled release of biomolecules, such as enzymes (Santagapita et al., 2011), but also in case of whole cells. Depending on the industrial process these cells are involved in, the main reasons for encapsulation are keeping them isolated to allow for easy cell recovery and prolonged, high activity, protecting them and/or releasing them in a controlled manner (Rathore et al., 2013). Thus, encapsulation has proved suitable for a broad scope, including food technology, environmental protection, pharmaceutical and environmental applications. One of the most investigated medical applications has been the encapsulation of isolated pancreatic islet cells to protect them from the body's immune system during transplantation to patients suffering from Diabetes mellitus (Robles et al., 2014). Whole cell encapsulation in food technology is often aimed at delivering probiotics to positively influence human gut microflora. In this context, encapsulation has been shown to protect probiotic bacteria during exposure to deleterious gastric and intestinal juices (Chávarri et al., 2010; Ding and Shah, 2009) as well as to maintain viability during storage (Brinques and Ayub, 2011) and high food-processing temperatures (Abbaszadeh et al., 2014; Mandal et al., 2006). Also during fermentation processes in food industry, which aim at obtaining certain metabolites produced by living cells, encapsulation has proved beneficial. This was shown for example in case of the conversion of sugar to ethanol by *Saccharomyces cerevisiae*, which may be improved by immobilizing the yeast cells (Najafpour et al., 2004). Encapsulation has also been used in environmental applications, for example

to provide a physical barrier for microorganisms used in waste water treatment against native ones (Covarrubias et al., 2012). In animal husbandry, encapsulation of swine semen has been shown to support preservation, delivery and controlled release for successful sow artificial insemination (Torre et al., 2000). Finally, aiming at agricultural application, entrapment of bacterial inoculants in polymeric gels for delivery to the field was pioneered around the 1980s (Bashan, 1986; Jung et al., 1982). As main advantages of encapsulated inoculants over standard peat based formulations, Bashan (1998) proposed a better protection against environmental stresses and a controlled release in the soil precisely at the target site and, ideally, upon germination of adjacent seeds. This may be achieved by a slow degradation of the encapsulation matrix by native soil microbiota, thereby setting free the immobilized cells. Furthermore, encapsulation provides a well-defined microenvironment to viable cells, which may be amended with nutrients according to specific needs of the strains. Thus, in the last decades, several studies have aimed at investigating the delivery of bacterial inoculants to plants by help of encapsulation. It was found that encapsulation protected *Bacillus megaterium* used for control of rice sheath blight against harmful UV radiation (Wiwattanapatapee et al., 2013). A controlled release of encapsulated *Pseudomonas putida* and *Bacillus subtilis* in sand substrate followed by efficient colonization and plant growth promotion of lettuce was demonstrated by Rekha and co-authors (2007). *Pseudomonas fluorescens* and *Burkholderia cepacia* encapsulated in alginate beads displayed higher phosphate solubilisation activity than the control and improved the growth of wheat plants under semi-arid conditions (Minaxi and Saxena, 2011). Encapsulation also proved suitable for supporting a long shelf life in case of *Azospirillum brasilense* (Schoebitz et al., 2012).

Thus, the feasibility of encapsulated formulations for plant beneficial bacteria has been demonstrated in a range of individual cases. However, despite these promising results, encapsulation has not been widely adopted for agricultural purposes, presumably due to high production costs and technical handling (Herrmann and Lesueur, 2013). Since the production costs largely depend on matrix material, production method and desired capsule size, which in turn need to be tailored towards the desired field application technique, it is necessary to consider these intertwined factors. This frequently means having to balance a trade-off between those.

The main difference between agricultural applications and the ones in industrial fermentation is the controlled release in the first case and the prevention of release in the latter case. Both goals may be

achieved with the right matrix composition and inherent capsule physical characteristics. For shaping these, several methods and a variety of materials are available.

3.3.1.1 ENCAPSULATION TECHNIQUES

One of the most straightforward and mild encapsulation techniques is extrusion (John et al., 2011; Rathore et al., 2013). In this approach, an encapsulation matrix is forced through an orifice installed over a hardening bath, in which the spheres are collected. The size and morphology of the resulting capsules is dependent on the orifice diameter, the viscosity of the matrix, the distance from the hardening bath as well as the concentration of the hardening agent and its temperature (Rathore et al., 2013). The formation of spheres by extrusion may be further improved regarding throughput and size control (i.e. miniaturization) by a controlled break-up of the laminar jet flowing through the orifice. As outlined in Fig. 3, this can be achieved by applying electrostatic force, vibration frequency, rotating blades ("jet-cutter") or coaxial air flow (Prüsse et al., 2008). The different extrusion methods may further be subdivided depending on the gelation mechanism, which in turn is linked to the material applied. Hardening of the encapsulation matrix may take place by thermal gelation, ionic gelation or complex coacervation (Vemmer and Patel, 2013). Since the mechanisms are dependent on the materials used, they will be described in section 4.3.1.2 (matrix composition).

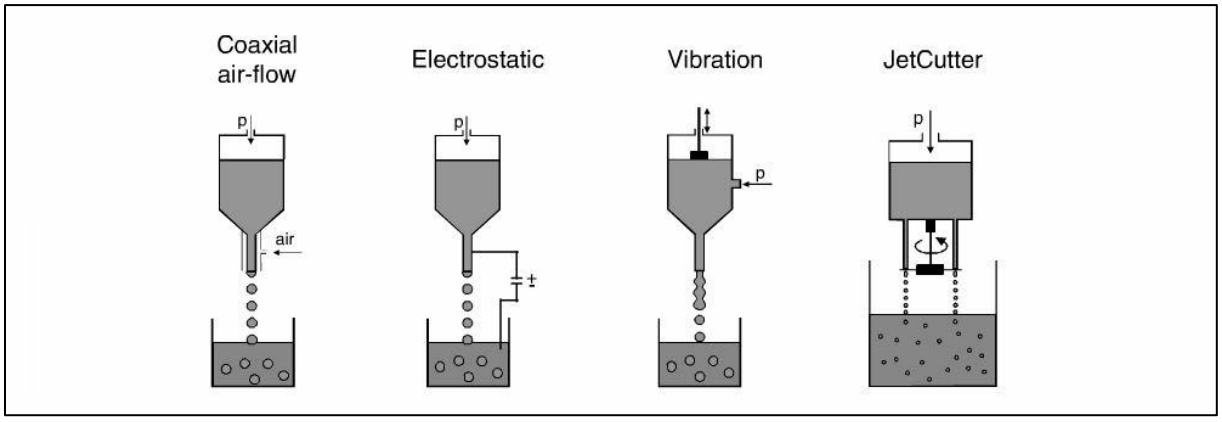

Figure 3: Possible modes of laminar jet break up in encapsulation by extrusion. *Source: Prüsse et al. 2008*

Although extrusion per se is a low-cost, mild process suitable for sensitive, live cells, it has some drawbacks which need to be considered. These include limitations in throughput (Krasaekoopt et al., 2003) and a lack of compatibility with matrices of high viscosity. Both aspects are particularly relevant if aiming at the production of small sized microspheres.

An alternative method is the encapsulation of cells by formation of microspheres in an emulsion (Vemmer and Patel, 2013). Commonly, a matrix of cells in water-soluble polymers is dispersed in an oil-based or organic continuous phase. The dispersed phase forms microdroplets when homogenized by stirring, which are conserved upon initiation of the gelation mechanism. The resulting spheres are then separated from the continuous phase by filtration or centrifugation. Although emulsification has been applied as a mild method for cell encapsulation, its weak points are the requirement of removal of the oil-phase, for which an additional purification step is needed and the difficulty in controlling the size of the microspheres, which results in a wide size distribution (Ding and Shah, 2009; Rathore et al., 2013). Both extrusion and emulsification yield wet beads.

Spray drying, despite not being considered a classical encapsulation method, has been applied for cell immobilization obtaining a dry final product (Vemmer and Patel, 2013). In this case, a liquid encapsulation matrix including the cells is atomized into a chamber of hot air, resulting in a fast evaporation of moisture from the mist of droplets. Due to the quick removal of moisture and high temperatures involved in this process, a loss of cell viability is often a major issue (John et al., 2011b). Thus, spray drying may not be suitable for encapsulation of sensitive bacterial agents.

Variations of these encapsulation methods are known from pharmaceutical applications. For example, drug carriers for controlled release have been produced by coacervation, in which liquid core droplets are separated from the polymeric solution by initiating the formation of pre-membrane component. The solidification of the membrane is induced for example by changing the temperature or pH (Park and Chang, 2000). Solvent extraction/evaporation relies on the emulsification of a matrix carrying the cells in a continuous phase, followed by extracting or evaporating the solvent (John et al., 2011b). However, both approaches are considered less suitable for encapsulation of bacterial inoculants in agriculture, since cell damage may result from the compounds used in solvent extraction/evaporation (John et al., 2011b) and the costs associated with coacervation may be rather high (Park and Chang, 2000; Rathore et al., 2013).

Among all methods, extrusion has been the one firstly used for encapsulation of bacteria for plant inoculation (Bashan, 1986) and has since remained the most popular approach in agriculture-related applications (Gurley and Zdor, 2005; Liu et al., 2007; Minaxi and Saxena, 2011; Schoebitz et al., 2012; Wu et al., 2011).

3.3.1.2 MATRIX COMPOSITION

Numerous materials have been tested for encapsulation of bacterial cells in alimentary industry, agriculture and biotechnology. Among the most popular ones is alginate, a biodegradable, readily available and inexpensive biopolymer derived from different species of algae (John et al., 2011b; Kailasapathy, 2002; Prüsse et al., 2008). Alginates are essentially linear polysaccharides composed of D-mannuronic and L-guluronic acid residues, whose ratio and sequence differs depending on the source of extraction. Alginate sodium salts are water soluble and form a solid network upon contact with divalent cations such as Ca^{2+}, which displace the sodium ions from the guluronic acid (Gombotz and Wee, 1998; Lee and Mooney, 2012). This mechanism, known as ionic gelation or crosslinking, leads to the formation of a characteristic "egg-box" structure (Grant et al., 1973), in which the guluronic acid groups are stacked and the resulting alginate chains dimerized, forming a three-dimensional gel matrix (Fig. 4). This provides a mild process for biological entities such as bacterial cells to be entrapped in a protective microenvironment. The alginate network is disintegrated to release cells upon contact in presence of chelating agents such as phosphate or sodium citrate.

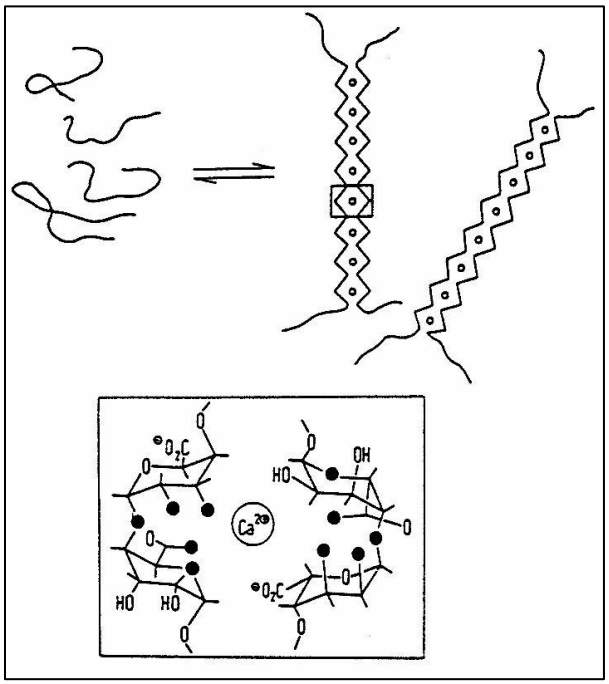

Figure 4: Formation of alginate gel network (egg box structure) by cross-linking with Ca^{2+} ions (*source: Gombotz and Wee, 1998*).

Apart from alginate, many other natural or synthetic polymers have been applied for encapsulation of bacterial cells. Rathore et al. (2013) provide a review of the most common polymers, including agar, K-

carrageenan, chitosan, gellan gum, gelatin, xanthan gum, polyacrylamide and polyvinyl alcohol. As in case of alginate, the entrapment of cells is achieved by ionic gelation when using K-carrageenan (potassium or calcium ions), xanthan gum (divalent ions such as calcium) or chitosan (anions). Capsules may be formed based on gelatin, agar and agarose by thermal gelation without requiring ions for stabilization. This is commonly done by dripping the warm matrix into a collecting solution of low temperature or by emulsifying the water soluble matrix in warm oil, followed by temperature decrease to initiate gelation (Vemmer and Patel, 2013).

Capsule characteristics may not only be designed by choosing a specific bulk encapsulation material and method, but also by incorporating synergistic additives into the matrix (Vemmer and Patel, 2013). Starches from different botanical sources such as corn and rice have frequently been applied as fillers and were found to enhance flowability, sphericity and mechanical strength of dried alginate beads and rendered them less hygroscopic (Chan et al., 2011). The starch content was also shown to influence cell release, swelling ratio, biodegradability (Wu et al., 2011) as well as cell viability (Schoebitz et al., 2012). Alginate has also been blended with glycerol and/or chitin, bentonite or kaolin, by which a number of textural features such as bead size, surface roughness and porosity were manipulated (Zohar-Perez et al., 2004). The same mixtures were also evaluated regarding their UV-transmission properties, showing that beads made from alginate-glycerol-kaolin matrix provide the highest degree of UV protection for encapsulated cells (Zohar-Perez et al., 2003). The addition of chitin, cellulose, olive oil and gelatin was investigated regarding its effect on pore size and mechanical properties of alginate beads and the resulting diffusion characteristics and controlled release of immobilized bacteria (Liu et al., 2007). Finally, the encapsulation matrix may be supplemented with protectants aiming to prevent cell damage from the drying procedure, such as glycerol, adonitol, skimmed milk (Kearney et al., 1990; Selmer-Olsen et al., 1999) or humic acid (Young et al., 2006). This maintenance of a high titer of viable cells is a key aspect in formulation development.

Apart from blending into the matrix, additional processing steps may be included to alter bead characteristics such as release or mechanical stability. A typical example is capsule coating by exploiting the interaction of polyanionic and polycationic polymers. This process, known as ionic polymer coating, may be performed on alginate beads by coating with chitosan or poly-L-lysin (Vemmer and Patel, 2013). For example, alginate beads carrying probiotic bacteria were found to provide better protection from gastric juice when coated with chitosan (Chávarri et al., 2010).

These examples illustrate the vast amount of substances available for designing capsules with desired properties. However, it is often difficult to predict the outcome of the interplay between supplements, many of which exhibit different functions simultaneously. Due to the difficulties in establishing a systematic categorization of the multitude of additives and combinations thereof, formulation development currently is performed in a rather empiric fashion (Vemmer and Patel, 2013).

3.3.1.3 CRITICAL FACTORS IN ENCAPSULATION OF BACTERIA FOR AGRICULTURE

Although many approaches of encapsulation are similar across a range of application fields, immobilization of plant associated, beneficial bacteria for agricultural purposes has to meet specific requirements. Firstly, a controlled release of bacterial agents at the target site is required. This is in contrast to many biotechnological applications aiming at retrieving metabolic products, meaning that a leakage of cells from capsules is not desirable. When applied in the soil, the gradual release of bacteria is facilitated by degradation of the immobilization network. In case of alginate, this is achieved upon contact with organic acids or phosphates acting as chelating agents. Furthermore, degradation by soil microorganisms leads to disintegration of the capsules, thus ensuring a constant supply of introduced bacteria over an extended period of time (Liu et al., 2007). The degradation rate may be influenced by the presence of additives. For example, it was approximately two weeks for alginate microbeads amended with skimmed milk (Bashan et al., 2002).

While controlled release – ideally at the time of germination of the inoculated plant – is desirable, an overly fast release into soil matrix is counterproductive. Once released, the inoculant is exposed to competition by native soil microorganisms, predation and abiotic stress, which may lead to a lower inoculation efficiency especially when the inoculant has to overcome a certain distance to arrive at the plant (Bashan et al., 2002). This trade-off requires fine-tuning of capsule properties to achieve an adequate release time.

Alternatively, microcapsules with powder-like properties have been developed for seed coating to place the inoculant in closer proximity to the target site. Herein it is necessary to ensure a high throughput in capsule production to keep manufacturer's costs low. In contrast to pharmaceutical applications, where sophisticated capsule compositions and production methods may be feasible, the cost factor is a criterion for exclusion in agriculture if not kept low enough to compete with standard agrochemicals.

3.3.2 IMMOBILIZATION ON INORGANIC CARRIER MATERIAL – EXAMPLE ZEOLITE

As an alternative to encapsulation in a biopolymer matrix, bacteria may be immobilized in inorganic carriers (Bashan, 1998). For this, cheap, readily available, non-toxic and inert materials are commonly chosen, for example clay (Anandham et al., 2007), talcum powder, vermiculite (Tripathi et al., 2014) or kaolin (Mejri et al., 2012). Offering the ability to adsorb bacterial cells to its surface (Kubota et al., 2008), zeolite is a prospective carrier and has been applied in many industrial and environmental fields, such as waste water treatment and soil remediation. Zeolites are crystalline aluminosilicates building a three-dimensional, negatively charged, microporous network. The negative charges are compensated with exchangeable cations such as calcium, magnesium, potassium and sodium. Zeolites are available in different particle sizes averaging around several micrometers (De Smedt et al., 2015). Considering agricultural application, they offer the additional advantage of increasing the water binding capacity of the substrate and acting as a cation exchanger, thus providing nutrients to plants (Mumpton, 1999). Bacteria may be immobilized onto this carrier by adsorptive growth (Hrenović et al., 2007) and/or by coating them onto zeolite with the support of additives such as vegetable oil or xanthan gum (Stelting et al., 2012; Swaminathan and Jackson, 2008). Improving handling properties, granules may be formed based on zeolite-“dough” for incorporation of bacteria and subsequent soil application. Another option may be the use of zeolite powders as dusts for treatment of above-ground plant biomass, where the formation of a particle film allows for release of active agents. This may be feasible for microorganisms who unfold their effect at this site or can colonize the plant *via* above ground structural features. As is true for encapsulation in biopolymers, a range of potential additives may be incorporated.

3.3.3 THE DRYING PROCESS

Any products based on live bacterial cells are required to display a certain shelf life to be successfully commercialized. It is therefore one task of formulation development to achieve microbial preservation for as long as possible while preventing a change in genetic or physiological characteristics of the active agent (Prakash et al., 2013). This has been an issue for example in food industry aiming at stabilizing probiotic bacteria for dietary intake, in conserving reference strains in pharmacy and research and is similarly relevant in case of biocontrol agents (García, 2011; Morgan et al., 2006). Drying of microorganisms has been recognized as an efficient way of long-term preservation. In the state of desiccation of an organism – also known as anhydrobiosis – its vital functions come to a complete or

partial halt and a state of dormancy is acquired. Upon rehydration, the organism is resuscitated and resumes its vital functions (García, 2011). Apart from achieving a high shelf life, dry products also reduce the costs associated with storage and distribution under refrigeration and are less prone to contamination (Meng et al., 2008). In establishing efficient, cheap and straightforward drying protocols, choosing an appropriate drying method, set-up and matrix has to be considered (Prakash et al., 2013).

3.3.3.1 DRYING METHODS

A range of drying methods have been explored in food industry when formulating probiotic bacteria and the insights may well serve as a reference for microbial biocontrol agents. The most commonly used methods are freeze drying, fluidized bed drying, spray drying and vacuum drying (Fig. 5), all of which differ in their properties and consequently the product characteristics they result in (García, 2011).

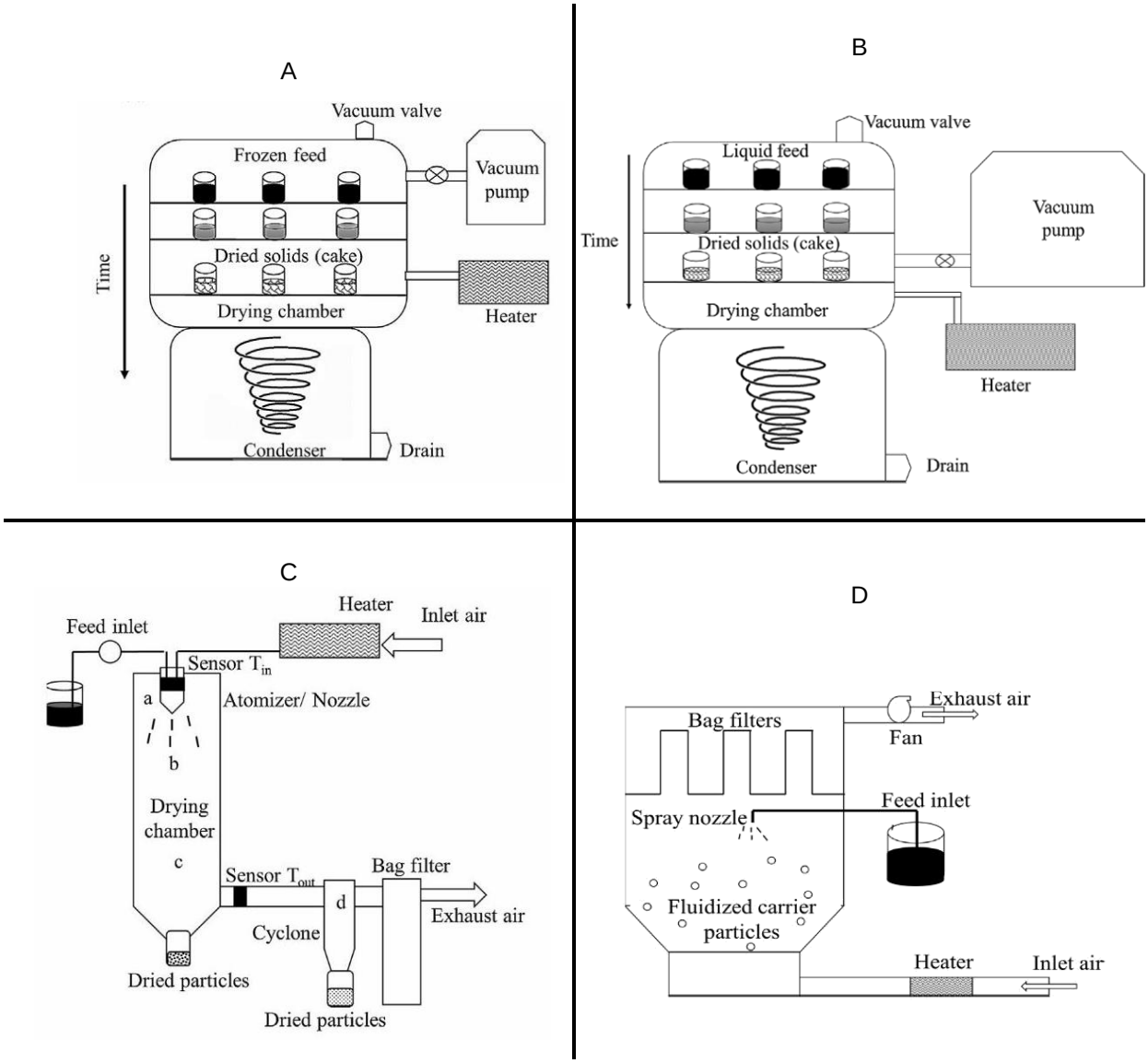

Figure 5: Set-up of different drying methods commonly used for drying of formulations: Freeze drying (A), vacuum drying (B), spray drying (C) and fluidized bed drying (D) (*Source: Broeckx et al. 2016 – modified).*

Freeze drying or lyophilization essentially consists of two processing steps: pre-freezing and sublimation of water by exposing the sample to high vacuum conditions. Sublimation describes the phase transition of the sample from solid to vaporous and is dependent on its temperature and the surrounding vacuum. Below a certain value, which differs depending on the sample composition, a phase transition occurs immediately from solid (ice) to vaporous without going through a liquid state. The fact that melting of the sample is omitted renders the process a rather mild one and helps maintaining product characteristics throughout the drying process. The final outcome of the sublimation process is influenced by the pre-freezing temperature, pressure in the drying chamber, input temperature, amount of sample, endpoint of drying and instrument properties, which implies that there are countless combinations of process parameters to be evaluated when optimizing a freeze drying protocol for a given sample (Morgan et al., 2006). Drawbacks of lyophilization are the high costs it is associated with (Santivarangkna et al., 2007) and the limited volume of this batch-type operation (Morgan et al., 2006).

Similarly to freeze drying, vacuum drying relies on driving evaporation of water out of the sample through the application of low pressure. However, the sample is not pre-frozen but goes through a phase transition from liquid to solid. The low pressure conditions decrease the boiling point of the sample and thus facilitate evaporation at low temperatures (Broeckx et al., 2016). Despite this potentially mild method, studies about its application in drying live bacteria are limited – possibly due to the fact that lyophilization mostly confers higher survival rates and has been established as a standard drying method (Broeckx et al., 2016) or due to relatively long drying times of vacuum drying of up to 100 hours (Santivarangkna et al., 2007).

Spray drying involves the atomization of a liquid matrix into a drying chamber with hot air of up to 200 °C, leading to quick evaporation of water, which in turn is cooling the sample until dry powders are formed (Morgan et al., 2006). This process is the one predominantly used in industry and has already been described in the context of encapsulation. Manufacturing costs are estimated to be 20 % those of freeze drying, which makes this process a more economically feasible one.

Another popular drying method is fluidized bed drying, which operates at temperatures of around 40 °C, thus being potentially milder than spray drying (García, 2011). It is mostly applied as a second drying method to lower the water activity in solid particles or granules (Broeckx et al., 2016). For this, the particles are suspended in an upward blowing stream of warm or hot air, conferring a fluid-like behaviour to the bulk of the granules. Bacterial agents may either be sprayed onto this moving mass of carrier

granules, may be incorporated into carriers prior to drying, e.g. as done during encapsulation in alginate or may be provided as pure dry mass and then coated with a protective shell in a fluidized bed (Broeckx et al., 2016). It is thus also considered as a method for encapsulation of bacterial cells or granule formation. Although it is a rather low cost drying method, studies on fluidized bed drying are limited, possibly due to the infeasibility when having slurry-like original matrices.

Despite the necessity to dry live bacterial cells to preserve them in the long-term and bring them in a form that is most easily to handle, the drying process itself often decreases the bacterial viability. Therefore, the bacterial survival rate is one of the main quality parameters when evaluating suitable drying methods. There are several approaches to preventing cell damage during desiccation, however, studies dealing with this issue are mostly strain specific and based on empiric measurements resulting in a lack of generic theories (Morgan et al., 2006).

3.3.3.2 DAMAGE BY DRYING AND STRATEGIES TO PREVENT IT

The removal of water from live bacterial cells is a physiologically challenging process and frequently coincides with a loss of viability. This is particularly true for non-spore forming bacteria, which do not display the ability to adopt a highly resistant, dormant form to outlast adverse environmental conditions (Potts, 1994). The damages resulting from desiccation depend in part on the drying method used, but are generally based on three main deleterious processes: oxidative damage, phase transition and browning reactions (García, 2011). In a water deficient system, the formation of reactive oxygen species (ROS) is a major cause for lesions of cell components. The reduced functionality of dehydrated proteins responsible for trapping such ROS and an enhanced rate of chemical processes producing ROS result in the accumulation of these free radicals (García, 2011). Subsequently, they lead to lipid peroxidation, protein denaturation and DNA mutation (Billi and Potts, 2002).

When phospholipids in the cell membrane are dehydrated, their packing density and consequently van der Waal's interactions increase. The resulting rise in the phase transition temperature (T_m) causes the lipids to pass from a liquid crystalline phase to a gel-phase, thus losing membrane fluidity. This renders the membrane leaky – a fact that becomes lethal particularly upon rehydration (Potts, 1994). In addition, browning reactions (Maillard reactions) cause damage derived from condensation between reducing sugars and lysin and methionine residues of proteins (Potts, 1994).

The response to these deleterious mechanisms and the resulting tolerance of desiccation depends not only on the bacterial strain, but also its momentary growth phase, cell concentration, rehydration and

storage conditions as well as presence of protective additives during the drying process (Morgan et al., 2006). These observations provide different approaches to influencing the desiccation tolerance of microorganisms. The most straightforward one is the external application of protectants. The list of potential protectants is extensive and comprises chemically diverse substances such as sugars, polymers and amino acids. Examples are skimmed milk, trehalose, liquid growth medium, horse serum (Peiren et al., 2015), sucrose, Ficoll, hydroxyethylcellulose, hydroxypropylmethylcellulose, polyvinylalcohol (Wessman et al., 2011), glucose, sucrose, maltodextrin (Strasser et al., 2009), fructose, lactose, sodium glutamate, cysteine, dextran, polyethylenglycol and glycerol (Costa et al., 2000).

The non-reducing disaccharide trehalose is certainly one of the most frequently studied desiccation protectants. The mechanism underlying its protective effect is known as the “water replacement hypothesis” and is related to the ability of trehalose to lower the phase transition temperature of phospholipids in the membrane by replacing the water molecules around the lipid head groups (Leslie et al., 1995). This helps maintaining the membrane fluidity and thus integrity. Sugar molecules may also replace the water in hydrogen bonds of other biomolecules, thus preventing for example protein denaturation after desiccation (García, 2011). Furthermore, vitrification, that is the glass formation of trehalose and other sugars, is assumed to aid in protection of cells from drying by stabilizing the cytoplasm (Potts, 2001). In most cases, the protectants are added externally to the bacterial cells prior to drying, however, it has also proven feasible to add trehalose to the culture medium and achieve a protective effect through the uptake and accumulation of this disaccharide into the cytoplasm (Streeter, 2003).

As an alternative to the external application of protectants, cellular protective mechanisms may be triggered by applying sub-lethal stress prior to desiccation. This provokes the modulation of cell physiology to adapt to the perceived environmental stress and thereby indirectly enhances desiccation tolerance. For example, fermentation under sub-optimal pH or temperature conditions was found to influence the composition of membrane lipids in so far as it increased the ratio of unsaturated to saturated fatty acids. The lower phase transition temperature exhibited by unsaturated fatty acids thus renders the cell membrane less prone to damage when drying (Liu et al., 2014; Schoug et al., 2008). Growing the bacterial culture to stationary phase may also induce certain stress responses due to the depletion of nutrients and accumulation of toxic metabolites (Morgan et al., 2006). These adverse conditions may, similarly to sub-optimal temperature or pH, aid in preparing bacteria for the following

desiccation stress, for example by synthesizing different stress proteins. However, it has been observed that the correlation between growth phase and desiccation tolerance is also strain dependent (Schoebitz et al., 2012).

Another bacterial defence mechanism includes the secretion of exopolysaccharides to form a shield against environmental stressors such as drought, predation, competition and toxic compounds (Patel and Prajapati, 2013). For example, soil inhabiting *Pseudomonas* sp. were found to produce EPS as a response to drying stress and thereby create a microenvironment with increased water retention capacity and reduced drying rate (Roberson and Firestone, 1992). The same is true for several *Paraburkholderia* strains, whose EPS secretion is triggered in presence of certain carbon sources during cultivation, for example sugar alcohols such as mannitol and glucitol (Bartholdson et al., 2008). The most common EPS type among *Burkholderia* and *Paraburkholderia* strains consists of a branched acetylated heptasaccharide repeat-unit with D-glucose, D-rhamnose, D-mannose, D-galactose and D-glucuronic acid and has been termed cepacian (Cescutti et al., 2000; Cérantola and Lemassu-Jacquier, 1999). It was shown to provide protection against desiccation and metal ion stress in several environmental strains (Ferreira et al., 2010), which makes it a prospective protectant in formulation development. Indeed, the approach of including EPS as a matrix component has been explored in formulation of *Pseudomonas fluorescens* (Slininger et al., 2010) and *Pseudomonas aeruginosa* (Tewari and Arora, 2014) and may thus be a viable option.

Obviously, drying methods and associated parameters also have a profound effect on bacterial survival rates. While thermal inactivation is mostly an issue in spray drying or fluidized bed drying, ice crystal formation is the major detrimental event in lyophilization. These stresses may be controlled by evaluating the strain specific response to different drying methods to determine the most suitable approach and fine tuning the drying set-up. Such multi-parameter experiments have shown significant interdependencies for example between concentration of lyoprotectant, cell density and freezing rate in lyophilization of lactobacilli (Schoug et al., 2006) or between lactic acid bacteria strains, drying technology and protectants (Strasser et al., 2009).

These examples illustrate the extensive number of variables that must be considered when setting the parameters for one of the most crucial steps in formulation development – the drying process. Enhancing the survival rate of bacteria by external addition of protectants and/or by inducing the cells' own

physiological stress adaptation mechanisms are two of the most promising ways of achieving a high titer of viable bacteria in dry products.

3.3.4 CHARACTERIZATION OF FORMULATIONS

Defining suitable methods for characterization of formulations are highly relevant to determine the outcome of different production technologies and parameters and to ensure the feasibility of serving the final purpose: practical application in the field followed by a high colonization rate of inoculated plants. It is obvious that the analysis of a given formulation comprises the cell viability of the bacterial agents, but other factors are equally relevant for successful commercialization of a biocontrol or biofertilizer product. These include the storage stability or shelf life as well as the morphology, which has a significant effect on important parameters such as handling properties and timely release of bacteria at the target site.

3.3.4.1 CELL VIABILITY

The multitude of variables involved in formulation development makes necessary a reliable, high-throughput and cost efficient method for the determination of bacterial viability to facilitate the screening of a large range of processing parameters and adjust them accordingly. Next to fulfilling this requirement, it is important to consider the detection limit and dynamic range of a given method as well as its suitability to investigate different types of matrices, including solid and/or opaque ones (Braissant et al., 2014).

Cultivation of bacteria on solid media followed by counting the colony forming units (CFU) has been the gold standard for enumeration of live bacteria. It comes with the highest dynamic range, theoretically allowing for detection of a single cell. However, it is laborious and time-consuming, delivering results only after 24 to 48 hours of incubation – in some cases of slow growing bacteria even longer (Braissant et al., 2014). Furthermore, it considers only those cells, which can grow at the instant of incubation and neglects the fraction of cells, which may have acquired a so called “viable but not culturable” (VBNC) state due to stress during technological processing. These are structurally intact and functional and may thus be resuscitated under appropriate circumstances. Furthermore, they are known to contribute to fermentation processes for example in food industry despite their incapability to form colonies on solid growth media (Bensch et al., 2014; Diaz et al., 2010). Assessment of survival rates is therefore also a question of defining bacterial viability, which may be either based on culturability or on structural integrity and functionality.

Focusing on the latter aspects and aiming at achieving a high throughput, automatable process, several alternative methods discriminating viable from dead cells have been developed. For the most part, they are based on optical detection by spectrophotometry (e.g. measuring optical density at a specific wavelength) or microscopy. Viability assays may also be based on biochemical reactions with certain cell components, e.g. sugars or amino acids, on metabolic activity or on the turnover of chromogenic or fluorogenic substrates by cellular enzymes (Braissant et al., 2014). For evaluation of viability of lactic acid bacteria starter cultures in food industry, flow cytometry has been suggested as an efficient and time saving detection method (Diaz et al., 2010; Kramer et al., 2009). Flow cytometry measures viability at the single cell level by creating optical signals from scattering or fluorescence when the cell passes through a laser beam. The resulting signal may then be correlated to structural or functional parameters, but also allows for investigation of membrane integrity and metabolic activity by using specific fluorescent dyes.

A multiparameter assessment of viability based on plate counting as well as cultivation independent methods including membrane integrity, membrane potential, esterase and respiratory activity has been performed for different bacterial strains subjected to air drying (Nocker et al., 2012). Although the authors could gain valuable insight into stress levels at a given point of measurement, the cultivation independent methods could not fully replace standard plate counting. This was because of the limited dynamic range of signals detectable by the plate reader. The signal frequently fell below the detection threshold especially when dealing with severe stresses and sensitive bacteria.

For estimation of viability in encapsulated formulations, biochemical assays can be advantageous in so far as they allow in situ measurements in contrast to standard plate counting, which requires dissolution of the encapsulation matrix to release cells. This approach has been performed for bacteria encapsulated in alginate and polyvinylalcohol, targeting bioreducible tetrazolium salt and adenosine triphosphate and proved suitable in case of the alginate matrix (Wadhawan et al., 2010).

However, an issue in all assays based on biochemical reactions is the lack of specificity. Since these methods detect all bacteria in a given sample, it is necessary to maintain sterile conditions throughout the formulation, drying and re-suspending process. Depending on the systems used for encapsulation and drying – for example air drying under laminar flow – they may be rather prone to contaminations at some point of processing.

As an alternative, DNA amplification methods may be applied to specifically detect the strain of interest. However, DNA may persist even if the bacterial cell is already compromised and is thus does not allow for discrimination between live and dead cells. To achieve this, samples may be incubated with ethidium monoazide (EMA) or propidium monoazide (PMA), which are DNA intercalating dyes only able to penetrate the cells through permeabilized membranes (Nocker and Camper, 2009). Subsequent to incubation with this dye, the samples are exposed to bright light to induce the covalent binding of the dye to DNA. As a result, amplification of these DNA strands is inhibited during PCR, so only DNA from cells with intact membranes that prevented the penetration of EMA or PMA is amplified. Combining this approach with quantitative real-time PCR allows for an estimation of the actual number of bacterial cells with an intact membrane. This promising approach, however, still needs to be evaluated for different bacterial strains and stress regimes, since these are relevant parameters for the conditions of cell membranes and the ability of the dyes to enter (Løvdal et al., 2011; Nocker and Camper, 2009). The exclusion of false positive requires thorough optimization of the protocol especially when dealing with a high background microflora (Gensberger et al., 2014). Frequently, there seem to be issues with false positive results, which may arise from an inefficient light activation especially in turbid samples or in high initial cell concentrations (Løvdal et al., 2011).

Despite the multitude of viability detection methods available, it still seems unfeasible to replace standard plate counting with an alternative, stand-alone technique. Due to its broad dynamic range covering the full logarithmic scale and its compatibility with a variety of formulation matrices, cultivation on solid medium is still the method of choice in most studies investigating bacterial viability and was also employed in the one at hand. The method can be sped up however by preparing samples in multi-well plates, using multichannel pipets and incubating small drop volumes on the plates instead of larger volumes for streaking (Nocker et al., 2012).

3.3.4.2 STORAGE STABILITY

Ensuring high viability immediately after drying is not sufficient for successful commercialization of a biocontrol or biofertilizer product. Much rather, shelf life or storage stability should be considered. Information on the minimum storage period desirable varies, ranging from two to three months at room temperature (Malusá et al., 2012) to a minimum of one to two years (Bashan et al., 2014). Although a fast rate of deterioration may to a certain degree be balanced by a high initial cell number (Bashan et al., 2014), economic considerations call for focusing on the stabilization of bacterial agents in the long

term. Factors detrimental to storage stability include exposure to oxygen, high temperature, moisture, microbial contamination and light (Morgan et al., 2006). Investigating the effect of these parameters in detail, it was found that the mortality rate during storage could be decreased by lowering the storage temperature. For example, the half-life of a lactic acid bacterial strain was increased when lowering the temperature from 25 °C to 4 °C (Achour et al., 2001; Jean-Noel et al., 2012). However, cooling of the product is not always an option. Investigating the effect of packaging, it was found that storing samples under vacuum or nitrogen proved a feasible way to enhance stability of *Campylobacter jejuni* compared to air as a head space gas (Portner et al., 2007). High barrier plastic bags or glass vials were more suitable in maintaining cell viability of *Pantoea agglomerans* than low barrier plastic bags, presumably due to the exclusion of oxygen and moisture (Costa et al., 2002).

Apart from temperature, water activity, which describes the availability of water in a sample, has been recognized as a critical parameter influencing storage stability (Weinbreck et al., 2010). Highest survival rates of rhizobia coated onto seeds were achieved at water activities between 0.47 and 0.38, however, survival was also dependent on the seed species, inoculum preparation, coating ingredients and coating technique (Deaker et al., 2012). The composition of the storage matrix, or, specifically, the type of protectant present, appears to be another significant variable to influence bacterial shelf life (Peiren et al., 2015).

Thus, while there are some generally valid strategies to enhance shelf life, the fine tuning of storage conditions regarding the aforementioned parameters seems to be strain specific as is the case in the desiccation process. This requires again a high throughput screening of different combinations of parameters.

Since the evaluation of shelf life over prolonged storage periods slows down this process of formulation optimization, accelerated shelf life tests are frequently performed. Modelling the loss of viability over time, the most common approach is based on the Arrhenius model, assuming a temperature dependent deterioration rate. Viability data acquired from short term storage at elevated temperatures thus allows for predicting the survival rate at lower temperatures for any period of time. This model was in good agreement with actual cell counts in freeze dried lactic acid bacteria (Achour et al., 2001; King et al., 1998), *Pseudomonas fluorescens* (Jean-Noel et al., 2012) and *Campylobacter jejuni* (Jean-Noel et al., 2012; Portner et al., 2007). An exact prediction by help of such models may not always be possible, especially if factors other than temperature are involved and influencing the degradation rate. However,

a good estimation may help excluding unsuitable conditions in a large scale screening process to only continue evaluating the most promising ones.

3.3.4.3 OTHER CHARACTERISTICS

In addition to physiological conditions of immobilized bacterial agents, physical characteristics of the formulation play an important role in application. Depending on the mode of application, dry particles need to be compatible with seeding machinery, free flowing, homogenous in size and spherical rather than angular. Miniaturization of capsules or granules carrying the active agent is of concern when applied as seed coating or dusting of above ground plant parts, where a homogenous particle layer is desirable. Physical properties are also of special interest when the active agent is encapsulated and relies on being released in a timely manner and at adequate rates.

The most frequently used instrument to determine capsule or bead characteristics is scanning electron microscopy. Herein, a beam of electrons is focused on the sample, generating signals from its interaction with the sample surface. This high-resolution technique followed by image analysis by specific software such as ImageJ has been applied for determining capsule characteristics such as pore size, relative porosity (Chan et al., 2011), surface roughness (Zohar-Perez et al., 2004) and location and distribution of encapsulated bacteria (Liu et al., 2007). Enabling the investigation of a larger and thus representative fraction of powder-type samples, laser diffraction analysis has been used to determine bead size and size distribution (Wu et al., 2011). Stereomicroscopy has been applied to determine shrinkage after drying (Rassis et al., 2002) and swelling upon rehydration, considering diameter expansion in addition to gravimetric measurements (Wu et al., 2011). Mechanical stability of beads is commonly investigated by compressing them between flat plates and measuring the force necessary to achieve a certain deformation of the particles (Chan et al., 2011; Rassis et al., 2002). Bead shape characteristics may be described by calculating the sphericity factor, measuring bulk and tapped density and calculating the Hausner ratio as an indicator for flowability by dividing the tapped density by the bulk density (Chan et al., 2011). Hygroscopicity, which may be relevant for storage stability, has been determined gravimetrically (Chan et al., 2011).

Although a thorough physical characterisation allows estimating the release of encapsulated agents, controlled release is usually checked by submerging beads in physiological saline, drawing samples at specific intervals and checking them for the presence of released cells by plate counting (Liu et al., 2007; Wu et al., 2011). Since the release is also governed by decomposition in soil, the degradation rate of

alginate beads was investigated by burying them in fine-meshed nylon bags and checking bead integrity by microscopy at specific time intervals (Bashan et al., 2002).

Using formulation techniques other than encapsulation, a thorough physical characterisation is not commonly found. This may be because particles are already described regarding size and size distribution by the manufacturer, e.g. in case of zeolite powders. Furthermore, release kinetics may be less complex if bacteria are simply immobilized onto particles rather than inside a polymer matrix.

3.4 FUTURE PROSPECTS OF BACTERIAL INOCULANTS FOR AGRICULTURE

Numerous potent biocontrol and biofertilizer strains are continuously being described and the market of bacteria-based products for application in agriculture is growing steadily. Currently, the worldwide global turnover of biopesticides amounts to 1.8 billion US$ - this is not considering products categorized as biostimulants (Ravensberg, 2015). In any case, there is a growing interest and an increasing number of products available, whose use as alternatives to standard agrochemicals is getting more and more popular. The key to making use of diverse, potential plant beneficial strains and to increase the market share is the development of suitable, strain-specific formulations. This is a labor intensive process due to the vast number of parameters, which need to be considered and tested. Despite this fact, formulation development currently is still a niche in the research in agricultural microbiology. In their review, Bashan et al. (2014) give several recommendations regarding future formulation development. Apart from the obvious necessity of fine tuning parameters such as the ratio of components in a formulation and putting a focus on improving survival rate, the authors highlight the importance of understanding the interaction between bacteria and formulation material such as the protectants. This requires an interdisciplinary, close collaboration between microbiologists, material scientists and agricultural scientists.

To bring bacteria based products to the market more easily, it may be feasible to target nursery grown plants rather than field crops, since conditions in the green house can be more easily controlled.

It also needs to be considered that the interaction between plants and microbes is taking place in a highly complex network, which is not yet fully understood. This ability of a strain to compete in a given environment and to efficiently colonize a given plant individuum are additional factors deciding over success or failure of the introduced inoculant strains. In some cases, it has proven more efficient to inoculate with a synergistic strain-mix rather than single strains. The sophisticated interactions among

microbes and of microbes and plants are slowly being elucidated, especially by help of novel molecular tools such as high throughput sequencing and genome annotation. These may also help in tracing the communication between plant and microbe and determine the microbial traits necessary for successful host colonization. This implies also that the prediction of a plant's response to inoculants may be possible in the near future and may even allow breeding for a desired response to microbes (Mitter et al., 2016).

An increase in research efforts to provide highly efficient biofertilizers and biopesticides for agricultural production is expected to pay off in the long term – supporting plants in their defense against pathogens, enhancing their stress tolerance and nutrition status and thus making agriculture more sustainable and productive.

4 TABLE

5 FIGURES

6 REFERENCES

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7 MAIN OBJECTIVES OF THE WORK

The central objective of this work was the formulation of beneficial, plant associated microorganisms – specifically growth-promoting bacterial endophytes – to obtain stable inoculants for practical application in agriculture. For this, several approaches were pursued, namely encapsulation in alginate beads and immobilization in zeolite granules. In doing so, the following aspects were in the focus:

- Establishing and evaluating various lab-scale extrusion methods regarding their feasibility to produce alginate beads carrying bacterial inoculants. This included the definition of the matrix composition suitable for extrusion, as well as the thorough characterization of the methods regarding throughput, resulting bead size, controlled release and applicability of the resulting beads in seed coating.
- Improving the survival rate and shelf life of desiccation sensitive bacterial inoculants during the manufacturing process and storage. This involved the testing of different drying methods, storage conditions and times as well as the evaluation of suitable protectants. In this context, artificial additives as well as inherent bacterial protection strategies such as the production of exopolysaccharides were investigated.
- Producing granular or powder formulations based on combinations of zeolite and previously evaluated additives, carrying immobilized bacterial inoculants. For this, different zeolites and matrix compositions were evaluated and characterized as in case of alginate beads.

8 PUBLICATIONS

8.1 MANUSCRIPT 1: THE SMALLER, THE BETTER? THE SIZE EFFECT OF ALGINATE BEADS CARRYING PLANT GROWTH-PROMOTING BACTERIA FOR SEED COATING

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RESEARCH ARTICLE

The smaller, the better? The size effect of alginate beads carrying plant growth-promoting bacteria for seed coating

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ABSTRACT

A range of lab-scale methods for encapsulation of plant growth-promoting bacteria in alginate beads intended for seed coating was evaluated: contact-spotting, extrusion through syringe with/without vibration, ejection by robotic liquid handler, extrusion by centrifugal force and commercial devices (nanodispenser, aerodynamically assisted jetting, encapsulator). Two methods were selected based on throughput (encapsulator: 1.5–5 mL/min; syringe with subsequent pulverisation: 5 mL/min). Four bead sizes ($55 \pm 39\,\mu m$, $104 \pm 23\,\mu m$, $188 \pm 16\,\mu m$ and $336 \pm 20\,\mu m$ after lyophilisation) were produced. Bacterial viability, release, bead morphology, seed surface coverage and attrition were investigated. Release from the smallest bead size was approximately 10 times higher than from the largest. Seed surface coverage was highest ($69 \pm 3\%$) when alginate beads produced with nozzle size $80\,\mu m$ were applied. Pulverised macrobeads are an alternative option, if high throughput is top priority.

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Introduction

Encapsulation is a widely employed strategy for protection and delivery of chemical substances, enzymes or whole cells in diverse biomedical or -technological fields, such as drug delivery, tissue engineering (Hoffman, 2002), probiotic food amendment (Dong et al., 2013), fermentation (Najafpour et al., 2004) or bioremediation (Covarrubias et al., 2012). In the 1980s, encapsulation was introduced to agricultural research (Bashan, 1986) for maintenance of viability of beneficial microbial inoculants. Their controlled release in the soil results in the establishment of high populations in association with the host plant, which is a prerequisite for successful plant growth promotion (John et al., 2011; Schoebitz et al., 2013).

Generally, the bulk encapsulation matrix consists of a water-soluble polymer (Rathore et al., 2013), with alginate being the most popular material utilised. This is due to the simplicity and mildness of its capsule forming process, which relies on chemical cross-linking of sodium–alginate with some divalent ions, such as Ca^{2+}, thus forming a stable polymer network (Gombotz and Wee, 1998; Lee and Mooney, 2012). The most straightforward approach for obtaining alginate beads is extrusion of the matrix through a needle orifice into a hardening bath of $CaCl_2$. This method has been applied in the majority of publications describing encapsulation of agriculturally relevant microorganisms (Zohar-Perez et al., 2003; Young et al., 2006; Liu et al., 2008; Wu et al., 2011; Guo et al., 2012; Schoebitz et al., 2012). The resulting alginate beads are of diameters of several mm and are thus exclusively suitable as soil amendments. In contrast to seed coating, this may entail an unfavourable distance between the location of inoculant organisms and the seed, which is mostly insurmountable by active movement of bacteria (Bashan et al., 2002). There are only few agriculture-related reports about the production of small-sized alginate beads between 20 and 200 μm diameter, which were applied in form of a leaf spray (Wiwattanapatapee et al., 2013) or seed coating (Bashan et al., 2002). It has been estimated that the latter application strategy requires 10 times less inoculant compared to soil amendment, as it delivers the active agent on the spot (Bashan, 1998). Therefore, reducing alginate bead size to obtain a powder-like formulation is expected to be advantageous for application efficiency. However, beads in the low μm range are increasingly difficult to produce, especially when the matrix viscosity is high and/or additives are present. This implies a trade-off between bead size and throughput. Therefore, the central objectives of our study were (1) to test a range of lab scale alginate bead production methods and to highlight their respective strengths and peculiarities, (2) to examine a range of bead sizes between 50 and 400 μm regarding their influence on controlled release and seed coating efficiency and (3) to identify the alginate bead sizes offering a reasonable compromise in terms of throughput and suitability for the target application.

Methods

Evaluation of different lab-scale extrusion methods for alginate bead production

Alginate with a viscosity of 350–550 cP (1% w/v in dH_2O) was obtained from Carl Roth GmbH (Karlsruhe, Germany). Alginate with a viscosity of 4–12 cP (1% w/v in dH_2O) was obtained from Sigma Aldrich (St. Louis, MO). Alginate solutions of 0.5%, 1% and 2% (w/v) were prepared in dH_2O. A 0.2 M $CaCl_2$ solution served as the hardening bath.

Contact-spotting (Figure 1A): Alginate particles were spotted with an automated microarray system (Gene Machines OmniGrid, Digilab, Marlborough, MA) onto the bottom of multi-channel reservoir troughs, employing a solid pin (SSP015, Arrayit Corporation, Sunnyvale, CA) and a split pin (SMP5, Arrayit Corporation, Sunnyvale, CA). The alginate dots were gelled by sliding a drop of

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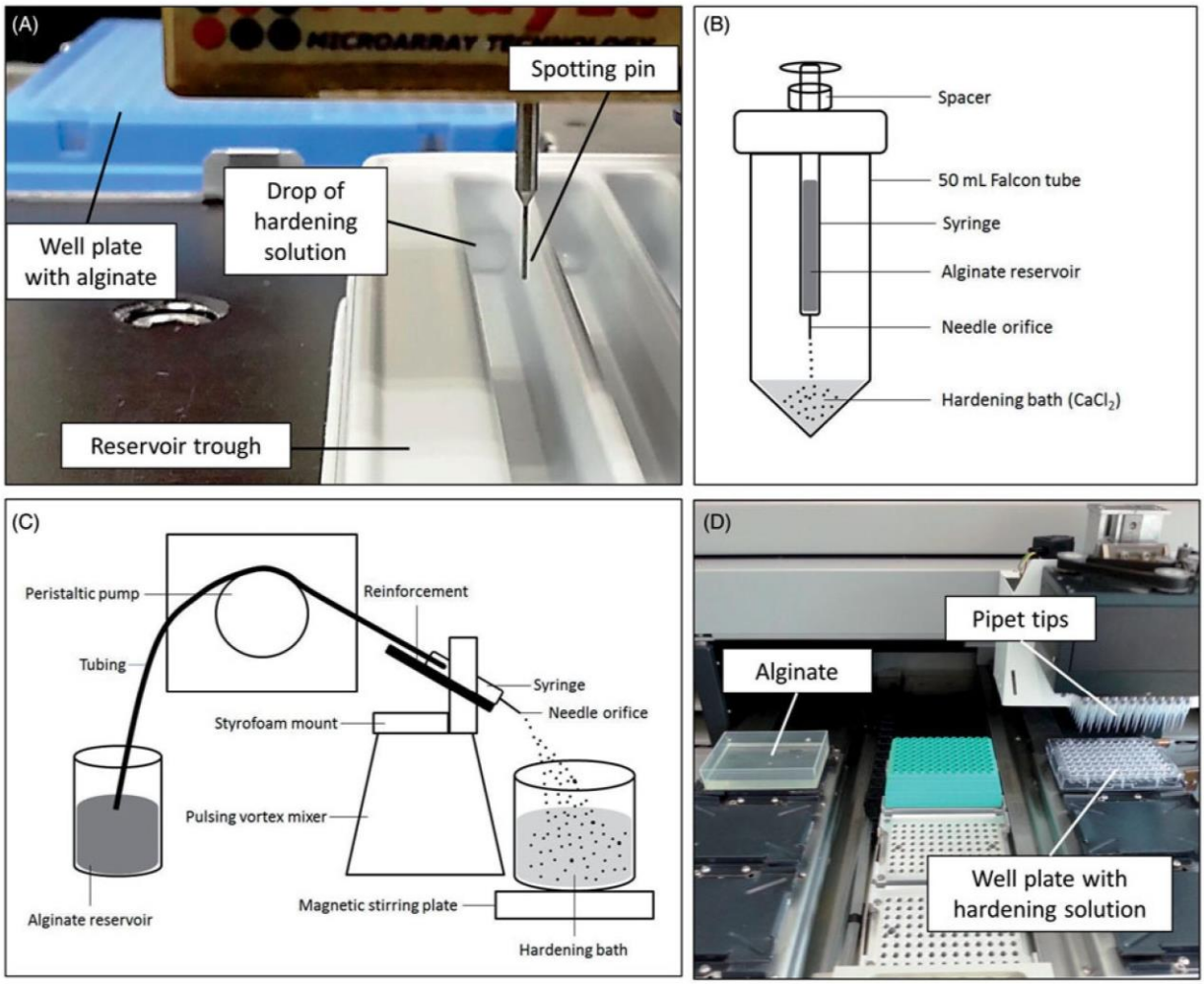

Figure 1. Set-up of the tested lab-scale bead production methods. Contact-spotting of alginate into multi-channel reservoir troughs by help of spotting pin (A), syringe inserted into falcon tube and subjected to centrifugal force (B), syringe coupled to a peristaltic pump and a vibration unit (C), ejection of alginate by automated liquid handler into wells filled with hardening solution (D).

0.2 M $CaCl_2$ immediately after spotting of each single dot over the bottom of the reservoir trough.

Syringe under centrifugal force (Figure 1B): An insulin syringe (Omnican 50 U-100, B. Braun Melsungen AG, Melsungen, Germany) was loaded with alginate and inserted into a bored lid of a 50 mL falcon tube carrying the hardening solution. The tube was centrifuged (Eppendorf centrifuge 5702 with swing-out rotors, Eppendorf, Hamburg, Germany), thus ejecting the alginate horizontally into the hardening bath (Haeberle et al., 2008). The minimum and the maximum rpm for extruding each concentration were identified by centrifuging in intervals of 100 rpm.

Syringe coupled to peristaltic pump and vibration unit (Figure 1C): The same type of insulin syringe was connected to a tubing (Ismatec Idex Health & Science GmbH, Wertheim, Germany) and a peristaltic pump (Reglo Digital, Ismatec Idex Health & Science GmbH, Wertheim, Germany). The part ending in the syringe was reinforced by a small plastic stick and led through a styrofoam construction fixed to a pulsing vortex mixer (VWR International GmbH, Darmstadt, Germany). The alginate solutions were pumped through the tubing at a rate of 3 mL/min and a vibration of the vortex mixer of 2500 rpm was applied simultaneously.

Handheld syringe: The alginate solution was extruded manually through a syringe (Sterican 26 gauge needle, B. Braun Melsungen AG, Melsungen, Germany), aiming at a high flow rate while maintaining a dripping regime. Alginate drops fell into a beaker carrying 0.2 M $CaCl_2$, which was stirred at 500 rpm by help of a magnetic stirrer.

Robotic liquid handler (Figure 1D): With an automated pipetting system (Genesis RSP 200, Tecan Group Ltd, Männedorf, Switzerland), alginate was ejected in a 96-well plate filled with $CaCl_2$. The ejection speed was adjusted to 600 µL/sec and volumes were 1, 0.5 and 0.1 µL.

Pipejet P9: Sample alginate beads from 2% low-viscosity alginate were kindly produced in test runs by BioFluidix GmbH (Freiburg, Germany) with their Pipejet P9 nanodispenser. The distance to the hardening agent was adjusted to 25 mm and the frequency amounted to 5 Hz.

Aerodynamically assisted jetting VAR J30: Sample alginate beads from 2% low-viscosity and 1% high-viscosity alginate were kindly produced by Nisco Engineering AG (Zurich, Switzerland), employing their encapsulation unit VAR J30. Both alginate solutions were handled with a pressure of 80 mbar and a flow rate of 0.5 mL/min.

Encapsulator B-390: By help of the encapsulator B-390 (Büchi Labortechnik GmbH, Flawil, Switzerland), beads were extruded into a beaker filled with 0.2 M $CaCl_2$ under fast agitation. The smallest nozzle size available (80 µm diameter) and an alternative one allowing for a higher throughput (200 µm diameter) were tested.

The maximally achievable throughput of all methods was either metered from the container of the bulk matrix (in case of handheld syringe, encapsulator and syringe subjected to centrifugation or vibration) or it was derived from the known pipetting speed and volume or frequency of contact spotting in case of robotic liquid handler and microarray system, respectively.

Beads obtained from all production methods were hardened for 30 min prior to evaluation by microscopy and photographic documentation. The bead projection area was measured by ImageJ 1.47v (Schneider et al., 2012) and the diameter of a circle of equal projection area (EQPA) was calculated by the formula: $d = 2 \times \sqrt{A/\pi}$, where d is the diameter and A is the projection area. The roundness was calculated with the formula: $roundness = 4 \times \frac{A}{\pi \times [major\ axis]^2}$.

Bacterial cultivation and encapsulation

Bacillus amyloliquefaciens FZB42 was obtained from the Bacillus Genetic Stock Centre (Columbus, OH) and cultured in three replicate runs in liquid Luria–Bertani (LB) at 28 °C on a shaker at 200 rpm to late stationary phase (48 h). Bacteria were harvested by centrifugation at 4500 rpm and 4 °C for 10 min and re-suspended in sterile 0.9% NaCl. The suspension was adjusted to 10^8 CFU/mL based on OD_{600} measurement (NanoDrop 1000 3.7.1., Wilmington, DE). The exact initial amount confirmed by plate counting on LB agar was 4×10^7 CFU/mL. For bacterial encapsulation, a 2% (w/v) low-viscosity alginate solution was prepared in dH_2O and mixed thoroughly with the bacterial suspension in a ratio of 1:10. Thus, the encapsulation matrix contained 4×10^6 CFU/mL.

For follow-up tests, sub-mm beads were produced with the encapsulator B-390 (Büchi GmbH, Flawil, Switzerland) with nozzle sizes 80, 200 and 450 µm. Macro-beads (>2 mm) for size modification after drying were produced manually by extrusion through a syringe. Beads were hardened in $CaCl_2$ for 30 min, filtered off with a vacuum pump, rinsed with dH_2O, filtered again and dried by lyophilisation. Size, shape and structure were observed by scanning electron microscopy (Hitachi TM3030, Hitachi High-Technologies Corporation, Tokyo, Japan).

To examine encapsulation efficiency, a matrix volume of 1 mL for each bead size was extruded into 50 mL of 0.2 M $CaCl_2$ and hardened for 30 min. Subsequently, a serial dilution was prepared from the hardening solution and the CFU were determined by plating counting.

Determination of cell viability

About 50 mg of dried beads were dissolved in 5 mL sodium citrate (50 g/L). A serial dilution was prepared and the amount of CFU was determined on LB agar plates.

Bacterial release from dried beads

Dried beads were submersed in sterile 0.9% NaCl (10 mg/1 mL) in a tube and gently shaken at 4 °C. After 30 min, 24 h, 48 h and 72 h, the shaking was stopped and beads were allowed to settle. The supernatant was serially diluted and released FZB42 cells were determined by plate counting. Release from beads coated onto seeds was performed accordingly (10 seeds/5 mL NaCl). The amount of beads corresponding to a given weight, encapsulated and released CFU/bead, depth of release and total surface area were calculated. The morphological changes during rehydration were observed under a stereomicroscope (SZX16 Olympus; lens: SDF Plapo 1 XPF Olympus, Tokyo, Japan).

Seed coating

Applying a commercially available seed coater (Satec Concept ML 200, Satec Handelsges.m.b.H, Elmshorn, Germany), 50 g of maize seeds were coated with 2 mL of 40% gum arabic (Elzein et al., 2006) and 3 g of alginate beads. In order to make superfluous bead material stick to maize seeds, again 1 mL of adhesive was introduced, followed by 1 g of talc to take up residual moisture and to prevent seeds from sticking to each other. Ten seeds were randomly picked and photographed with an SLR camera (Nikon AF-S Micro Nikkor 40 mm, NIKON CORPORATION, Tokyo, Japan). ImageJ 1.47v (Schneider et al., 2012) was used to determine coating quality.

Attrition test

Thirty seeds were transferred by forceps into 50 mL falcon tubes and weighed. The tubes were placed horizontally on a roller mixer (RS-TR05, Phoenix Instruments, Garbsen, Germany) and mixed for 20 min at a speed of 35 rpm. The seeds were then picked out from the tube by forceps making sure that detached coating material remained in the tube. The seeds were weighed again and the amount of abraded material was calculated.

Results and discussion

Microarray system

Generally, the formation of alginate beads is based on one of two principles: either droplets are formed by allowing an air gap to break up the jet of alginate during extrusion (direct method) or by formation of droplets in an emulsion with a hydrophobic phase (indirect method) (Haeberle et al., 2008). We investigated contact-spotting as a third principle (Figures 1A and 2, row H). Hereby, bead formation relies on the transfer of alginate matrix adhering to a pin to a receiving base by direct contact. Gelation is achieved by exposing the receiving base to hardening solution. This way, spherical beads with a diameter (EQPA) of <100 µm were produced from high-viscosity alginate (Figure 2, H2/H3/H4 and Figure 3A). More precisely, the diameter (EQPA) for 0.5%, 1% and 2% high-viscosity alginate beads spotted with a solid pin were 82 ± 18 µm (Figure 2, H2), 89 ± 9 µm (Figure 2, H3) and 99 ± 15 µm (not shown), respectively. Spotting 0.5 and 1% low-viscosity alginate was not possible, probably due to the low surface tension and/or lower amount of material adhering to the pin. Two percent low-viscosity alginate resulted in beads with a wide size distribution of 342 ± 210 µm (Figure 2, H1).

By applying a split pin (lower contact area), the size of beads produced from 2% high-viscosity alginate could further be decreased to 52 ± 8 µm (Figure 2, H4).

The production of small sized beads from highly viscous solutions comes at the cost of an extremely low throughput of approximately 10^{-7} mL/min. Even though this amount may be enhanced by the parallel installation of several pins, contact-spotting does not qualify for large-scale production. However, when aiming to encapsulate expensive or limitedly available compounds for example in early stage research, a method which can handle small quantities is advantageous. The minimum amount of encapsulation matrix required to run the system is less than 50 µL. This plus factor has also been mentioned in context of alginate bead production by inkjet printing (Dohnal and Štěpánek, 2011). The latter method produces beads in the same size range as contact-spotting (Dohnal and Štěpánek, 2010). However, inkjet printing is incompatible with high contents of mechanical stabilisers or other additives, such as starch filler, which may be applied in amounts of

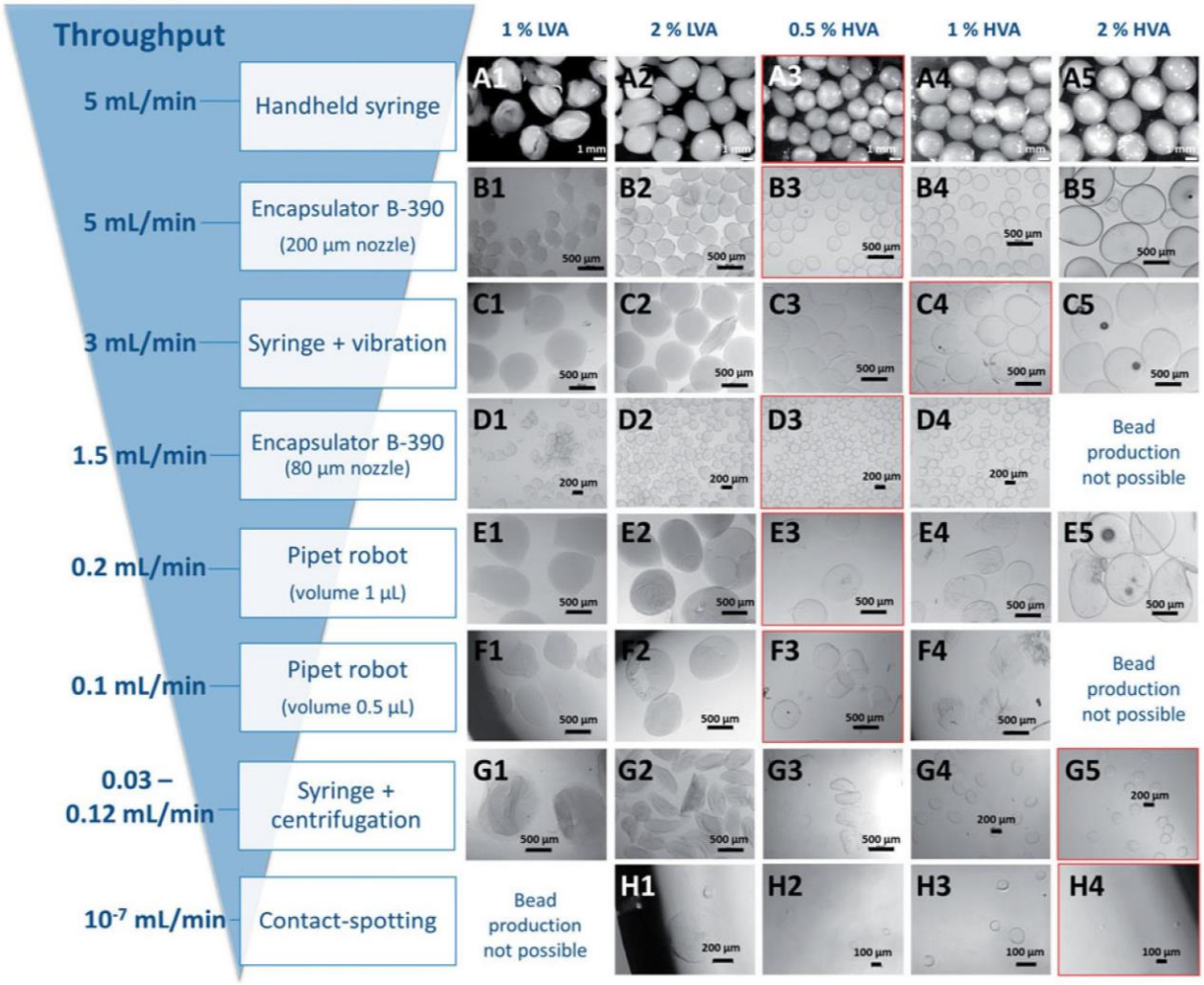

Figure 2. Overview of alginate beads produced by different methods. The maximally achievable throughput is given on the left (decreasing from top to bottom). Columns show the performance of methods applying low-viscosity alginate (LVA) and high-viscosity alginate (HVA).

up to 600 g/L (Chan et al., 2011). In this regard, contact-spotting is superior and is, therefore, an option for miniaturisation of beads from highly viscous matrices, although it requires a compromise regarding throughput.

Robotic liquid handler

Another low-throughput method is bead formation by a robotic liquid handler (Figure 2, rows E and F and 3B), which allows for production of beads with an exactly pre-defined volume.

Intake volumes of 0.5 µL of 1% and 2% low-viscosity alginate formed beads with diameters (EQPA) of 738 ± 76 µm (Figure 2, F1) and 860 ± 74 µm (Figure 2, F2), respectively. High-viscosity alginate of 0.5% was the concentration processed best and resulted in beads of 533 ± 30 µm diameter (Figure 2, F3). Using 0.5 µL of 1% high-viscosity alginate, the diameter increased again and was 879 ± 337 µm (Figure 2, F4). The large size inhomogeneity derives from uneven intake or ejection of alginate solution due to its high-viscosity.

Increasing the intake volume to 1 µL enhanced the range of processable viscosities up to 2% high-viscosity alginate. Applying this volume, bead sizes were 1 mm ± 181 µm (Figure 2, E1), 1 mm ± 100 µm (Figure 2, E2), 680 ± 126 µm (Figure 2, E3), 695 ± 106 µm (Figure 2, E4) and 992 ± 52 µm (Figure 2, E5) for 1% and 2% low-viscosity alginate as well as 0.5%, 1% and 2% high-viscosity alginate, respectively. As in the case of 0.5 µL intake volume, the lowest bead size was achieved with 0.5% high-viscosity alginate (Figure 2, E3).

The throughput of this system was 0.1 mL/min (intake: 0.5 µL) or 0.2 mL/min (intake: 1 µL). Automated pipetting allows the simultaneous testing of different hardening agents and concentrations, as the beads are collected in separate compartments (wells of the receiving well plate). A range of hardening agents is known, such as $CaCl_2$, $BaCl_2$, $AlCl_3$ (Bajpai and Sharma, 2004) and calcium gluconate (Schoebitz et al., 2012). Screening for the appropriate type and concentration is facilitated with the robotic liquid handler system.

Syringe under centrifugal force

Beads formed by subjecting a syringe to centrifugal force were characterised by their small size and superior roundness and homogeneity (Figure 3C). The set-up described by Haeberle et al. (2008) was improved regarding reservoir volume by constructing a conjunction between a syringe and a 50 mL falcon tube (Figure 1B). There was a clear trend of decreasing bead size with increasing alginate viscosity, spanning 1316 ± 70 µm (Figure 2, G1) and 478 ± 42 µm (Figure 2, G2) when applying 1% and 2% low-viscosity alginate and 394 ± 49 µm (Figure 2, G3), 255 ± 26 µm (Figure 2, G4)

and 226 ± 4 μm (Figure 2, G5) with 0.5, 1 and 2% high-viscosity alginate, respectively. Simultaneously, roundness and homogeneity increased with increasing alginate concentration (Figure 3C). Based on the observations, 2% high-viscosity alginate was regarded as the most suitable concentration for this method (Figure 2, G5).

The ideal rotation speed of the centrifuge was 300 rpm and 900 rpm for 1% and 2% low-viscosity alginate as well as 800 rpm, 1400 rpm and 2000 rpm for 0.5%, 1% and 2% high-viscosity alginate, respectively (Figure 3C). Additionally, extrusion of 3% high-viscosity alginate was investigated and proved feasible for bead production at 2900 rpm (not shown). Flow rates of 0.1 mL/min were achieved and are in good agreement with the ones determined by Haeberle et al. (2008). The flow rate was true for all alginate concentrations and types except 1% low-viscosity alginate with a maximum throughput of 0.03 mL/min. The total flow rate may be enhanced by parallel insertion of syringe-tube-constructions in the centrifuge. This way, it may reach values comparable with the ones achieved by coaxial air-flow or electrostatically aided extrusion (Prüsse et al., 2008), while resulting in even smaller beads from more viscous solutions.

Extrusion through syringe

Manual extrusion through a syringe (Figure 2, row A) is the most commonly applied and readily available technique. The flow rate varies slightly depending on the operator, but was estimated in the range of 5 mL/min for all alginate concentrations tested except 2% high-viscosity alginate, which was extruded at 3 mL/min. The resulting bead sizes lay in the mm-range, precisely 2.86 ± 0.21/0.23 mm (Figure 2, A1/A2) for 1 and 2% low-viscosity alginate as well as 2.19 ± 0.12 mm (Figure 2, A3), 2.57 ± 0.20 mm (Figure 2, A4) and 3.06 ± 0.07 mm (Figure 2, A5) for 0.5%, 1% and 2% high-viscosity alginate, respectively. The bead size was minimal when using 0.5% high-viscosity alginate (Figure 2, A3) and increased with increasing viscosity.

The bead size was reduced when applying vibrations at high frequencies (Figure 3D). Low-viscosity alginate of 1% and 2% yielded beads of a diameter (EQPA) of 803 ± 43 μm (Figure 2, C1) and 723 ± 60 μm (Figure 2, C2). 0.5, 1 and 2% high-viscosity alginate resulted in beads of 598 ± 20 μm (Figure 2, C3), 612 ± 47 μm (Figure 2, C4) and 833 ± 44 μm (Figure 2, C5). The values are in the range of those obtained with commercially available devices relying on the extrusion–vibration technique (Prüsse et al., 2008). High-viscosity alginate of 1% is standing out as the best workable concentration with a high degree of roundness and homogeneity (Figure 3D).

Commercially available devices

The Pipejet P9 (BioFluidix GmbH, Freiburg, Germany), a highly precise dispenser, produced homogeneous droplet-shaped particles of 169 ± 2 μm diameter (EQPA) with a volume of 2–3 nL when 2% low-viscosity alginate and a frequency of 5 Hz were applied (Figure 3E). These settings led to a flow rate of roughly 300 beads/min or 0.6–0.9 μL/min. The VAR J30 (Nisco Engineering AG, Zurich, Switzerland), based on the aerodynamically assisted jetting, reached a flow rate of 0.5 mL/min with 2% low-viscosity alginate and produced beads as small as 20 ± 10 μm from 1% low-viscosity alginate (Figure 3E). The encapsulator B-390 (Büchi Labortechnik GmbH, Flawil, Switzerland) (Figure 2, rows B and D) reached a flow rate of approximately 1.5 mL/min and formed beads of 163 ± 45 μm (Figure 2, D1) and 180 ± 44 μm (Figure 2, D2) when solutions of 1 and 2% low-viscosity alginate and the smallest nozzle size available (80 μm) were applied. Using 0.5% and 1% high-viscosity alginate yielded beads of 149 ± 33 μm (Figure 2, D3) and 191 ± 41 μm (Figure 2, D4), respectively. Bead production with the 80 μm nozzle was not possible when applying 2% high-viscosity alginate. For a nozzle diameter of 200 μm, an increased flow rate of up to 5 mL/min was reached (Figure 2, row B). Beads of a diameter (EQPA) of 377 ± 36 μm (Figure 2, B1) and 384 ± 63 μm (Figure 2, B2) were produced from 1% and 2% low-viscosity alginate. Applying 0.5 and 1% high-viscosity alginate resulted in beads of 299 ± 3 μm (Figure 2, B3) and 311 ± 9 μm (Figure 2, B4). Furthermore, these viscosities led to improvement of roundness and homogeneity (Figure 3E). With the 200 μm nozzle, it was also possible to process 2% high-viscosity alginate, albeit with a higher bead diameter (EQPA) of 610 ± 240 μm (Figure 2, B5) and a less favourable homogeneity and roundness (Figure 3E).

Considering that a high throughput is top priority in agricultural applications, the encapsulator B-390 was applied for further tests. Moreover, exchangeable nozzle sizes enable the production of a range of different bead sizes to study their effect on bacterial viability, release and seed coating quality. As the extrusion by syringe offers an equally high flow rate, it was employed in combination with size modification after drying.

Lyophilisation of alginate beads

The encapsulated formulation was dried to enhance suitability for seed coating and to avoid unwanted germination of seeds (Bashan, 1986; John et al., 2011). Moreover, desiccation is commonly applied to increase shelf life (Morgan et al., 2006; García, 2011). Alginate beads after lyophilisation were largely free of agglomerates and exhibited free flowing properties, but experienced a certain degree of collapse during drying (Figure 4). The mean diameters (EQPA) of dried beads were 104 ± 23 μm (Figure 4A), 188 ± 16 μm (Figure 4B) and 336 ± 20 μm (Figure 4C) when produced with nozzle sizes 80 μm, 200 μm and 450 μm, respectively. Crushing macro-beads resulted in the smallest mean particle size, but showed the largest size distribution (55 ± 39 μm, Figure 4D). Thus, crushing macro-beads may be a feasible way to combine large throughput in manufacturing with powder-like properties of the final product.

Viability of bacterial cells after lyophilisation

As a stable, spore forming model organism, *Bacillus amyloliquefaciens* FZB42 (Priest et al., 1987) was applied in encapsulation. Nevertheless, during the process of bead production and desiccation, its viability decreased from an average of 3.5×10^7 CFU/100 mg beads to 1.3×10^6 CFU/100 mg beads, corresponding to a survival rate of 4%. The expected amount of CFU/100 mg was calculated knowing that 1 mL matrix corresponds to 25 mg dried beads. A significant loss of cells during the encapsulation process was excluded by examining the hardening solution, which was found to be free of bacterial cells (detection limit: 0.5% of initial amount of CFU in alginate matrix, corresponding to an encapsulation efficiency of >99.5%). The decrease in CFU may thus be due to uncompleted spore formation and/or the partial incapability to form colonies upon reactivation. However, provided an adequate initial cell concentration, a survival of 4% of encapsulated bacteria represents an acceptable value for inoculation, as it is assumed that the survivors establish a sufficiently high population (Morgan et al., 2006). It is important to note that the value of 4% is calculated based on the actual arithmetic counts, whereas a calculation based on geometric values (logarithmic cell counts) would display a survival rate of 80% (initial CFU/100 mg dropped from log 7.5 to log 6). This inconsistency in presenting bacterial survival rates has to be

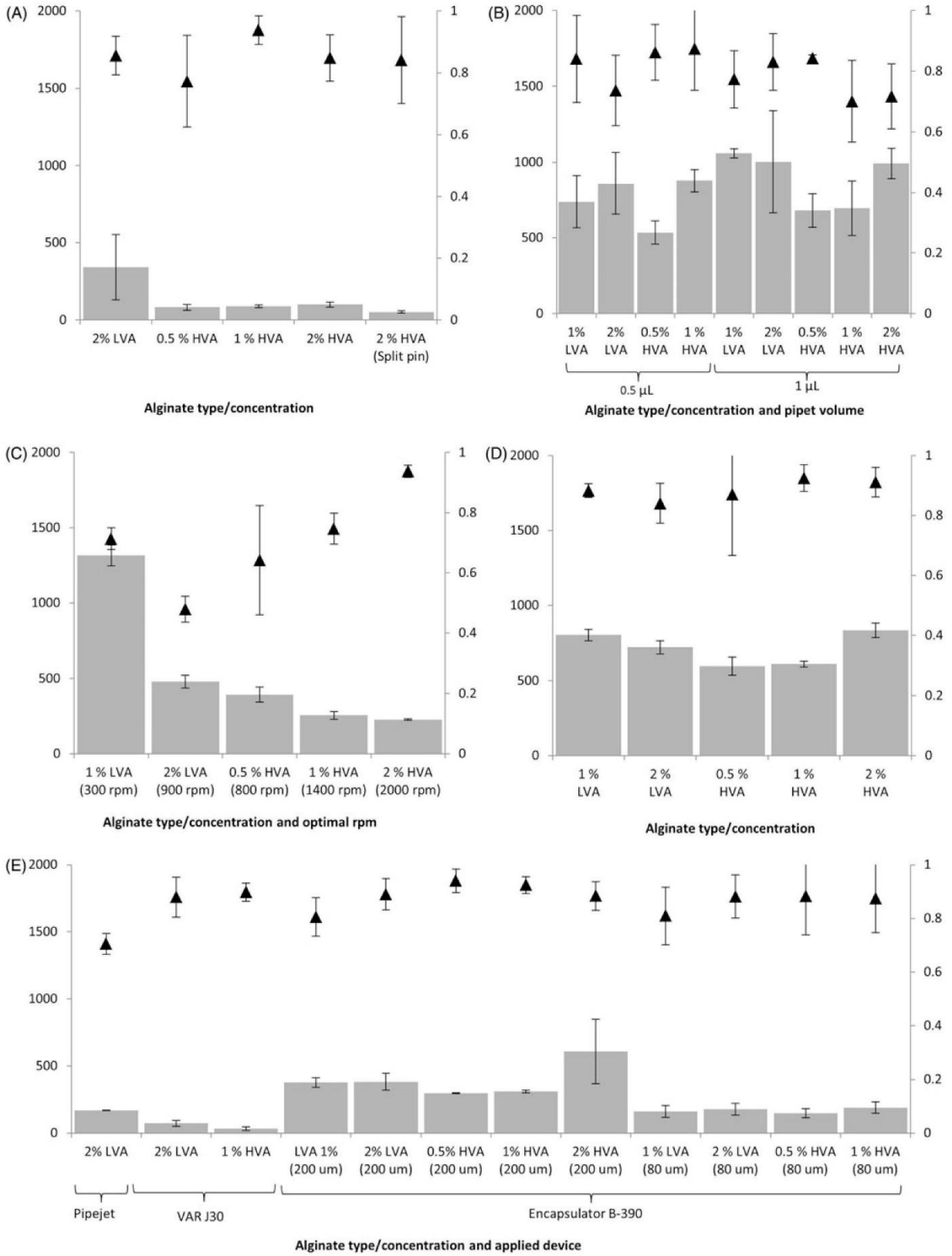

Figure 3. Characteristic bead sizes and roundness. Beads were obtained by contact spotting (A), ejection by robotic liquid handler (B), syringe subjected to centrifugation (C), syringe subjected to vibration (D) and commercially available devices (E). Left *y*-axis and bars show bead size (µm), right *y*-axis and triangles represent roundness (dimensionless).

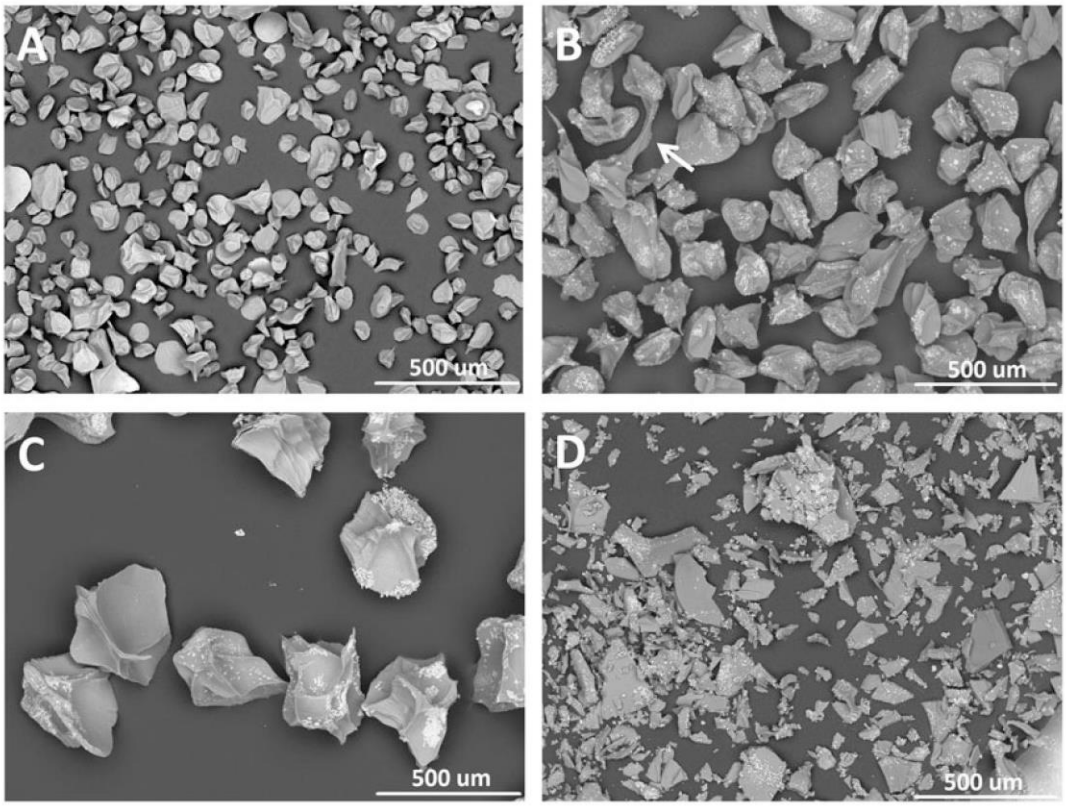

Figure 4. Images of alginate beads after lyophilisation. Alginate beads produced with the encapsulator and applying nozzle sizes 80 μm (A), 200 μm (B) and 450 μm (C) showed a collapsed structure. Macro-beads produced by extrusion through syringe were crushed after drying and yielded small sized, free-flowing slivers (D). All procedures resulted in powder-like formulations with free flowing properties. Lack of bead separation during production yielded dumbbell-shaped particles (B: arrow).

considered when comparing data from literature (Morgan et al., 2006).

All bead sizes protected bacterial cells of FZB42 equally well. Crushing of macro-beads did not have a detrimental effect on the bacteria. These observations suggest that for stable organisms such as *Bacillus amyloliquefaciens* FZB42, the parameter bead size can be optimised to primarily suit throughput, coating efficiency and release efficiency without taking into account cell viability.

Rehydration and bacterial release

The cumulative release from freshly dried beads in a model solution of 0.9% NaCl was compared with the release from beads subjected to seed coating and stored for 14 weeks. This was done to verify the integrity of beads during storage at ambient conditions and to investigate the effect of the adhesive gum arabic applied in seed coating. All release measurements were terminated after 72 h, due to the fact that this time marks the onset of degradation in soil and thus, after few days release mechanisms governed by factors other than moisture become dominant (Bashan, 1986; Bashan et al., 2002).

Freshly produced, lyophilised alginate beads released bacterial cells in a sharp burst, essentially reaching a plateau 30 min after submersion in NaCl (Figure 5A). The amount of maximally released bacteria was highest in crushed macro-beads (log 4.7 ± 0.1 CFU/100 mg), medium in 80 μm and 200 μm beads (log 4.1 ± 0.2 CFU/100 mg and log 3.9 ± 0.2 CFU/100 mg) and lowest in large beads produced with the 450 μm nozzle (log 3.7 ± 0.3 CFU/100 mg). Thus, decreasing bead sizes yielded higher release efficiency.

Release kinetics of dried beads after coating onto seeds and 14 weeks of storage (Figure 5B) differed from freshly produced dry beads in two essential aspects. First, the maximum of cell release was slightly decelerated, so the plateau was reached only after 24 h. We assume that initially, the gum arabic coating impeded a sharp burst release. This effect quickly became obsolete due to the high water solubility of gum arabic. After 72 h, released cell numbers amounted to log 3.4 ± 0.3 CFU/seed when coated with 80 μm beads, log 3.2 ± 0.4 CFU/seed when coated with 200 μm beads, log 3.0 ± 0.2 CFU/seed with crushed macro-beads and log 2.4 ± 0.5 CFU/seed when coated with 450 μm beads. In this enumeration, the second vital difference becomes obvious. Crushed macro-beads no longer displayed the highest release. Furthermore, the maximum release of all bead sizes/types per seed reached merely about 10% compared with the one from 100 mg of freshly produced beads. These observations reflect the difference in the reference amount (CFU/100 mg versus CFU/seed), which was lower after coating and differed between bead sizes.

Microscopic swelling analysis (Figure 6) showed that upon contact with moisture, the dry seed coating cluster (Figure 6A) fell apart within seconds (Figure 6B) and rehydration led to unfolding of the collapsed structure (Figure 6C–F). The unfolding of the alginate network is regarded as a major cause for release of entrapped bacteria. All beads were fully rehydrated within 30 min with the exception of beads produced with the 450 μm nozzle, which required 24 h.

Overall, alginate beads were able to deliver encapsulated cells upon contact with moisture, albeit a relatively small fraction of initially encapsulated cells. In the best case (crushed macro-beads), a cumulative release of 1% was achieved (arithmetic values). Similar low release percentages are known from the literature (Bashan et al., 2002; Liu et al., 2008; Wu et al., 2011). As the alginate matrix is characterised by a pore size in the low nm range

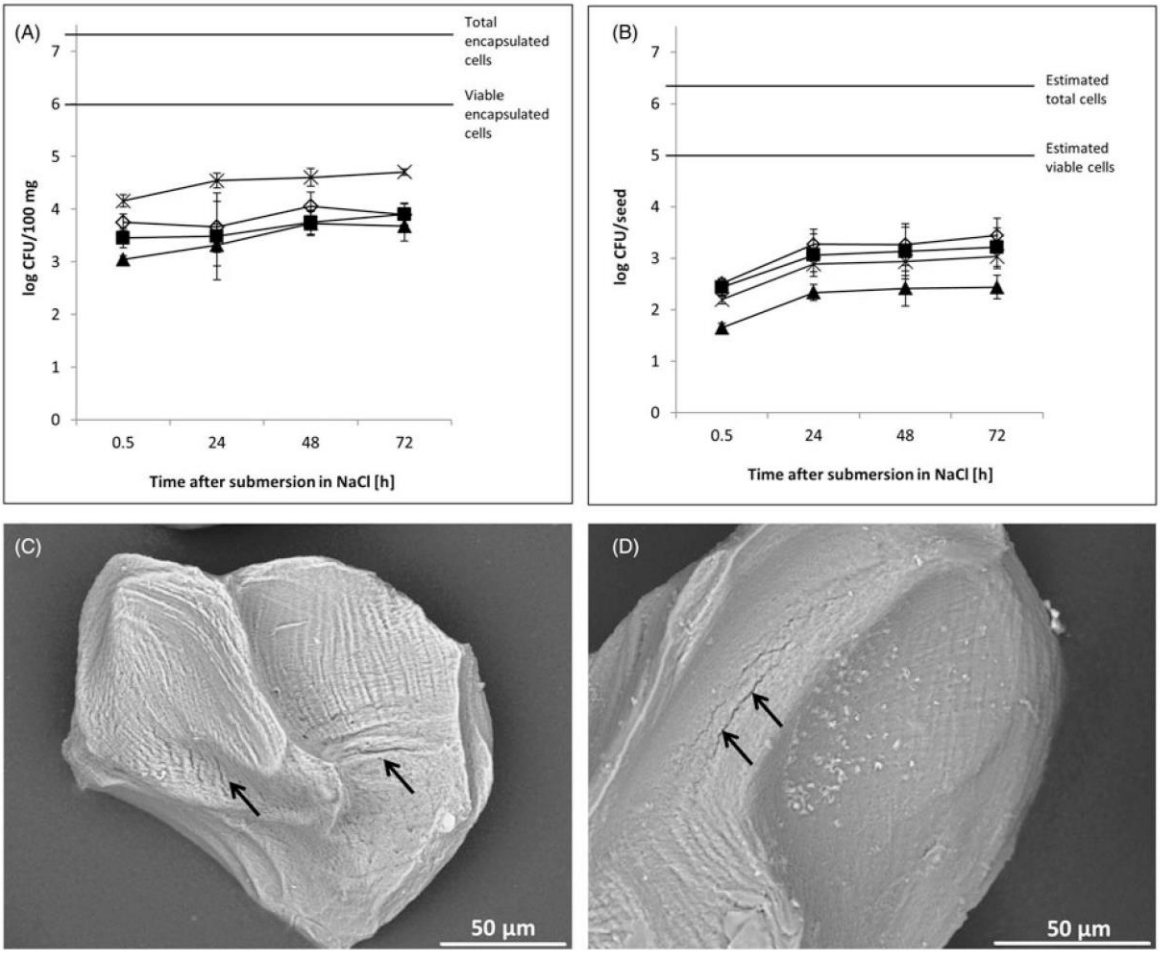

Figure 5. Release of bacteria from lyophilised alginate beads of 80 µm (open diamonds), 200 µm (solid squares), 450 µm (solid triangles) and crushed macro-beads (crosses). Bacterial release reached a plateau after 30 min in experiments immediately after production of beads (A). Crushed macro-beads released the highest amount of CFU/100 mg, 450 µm beads the lowest. In beads from seed coating and after storage, the release is slowed down (B). Total released amounts are lower due to lower reference value. Release is facilitated by crack formation during drying (C and D). Values show means of $n = 3$, error bars show standard deviation.

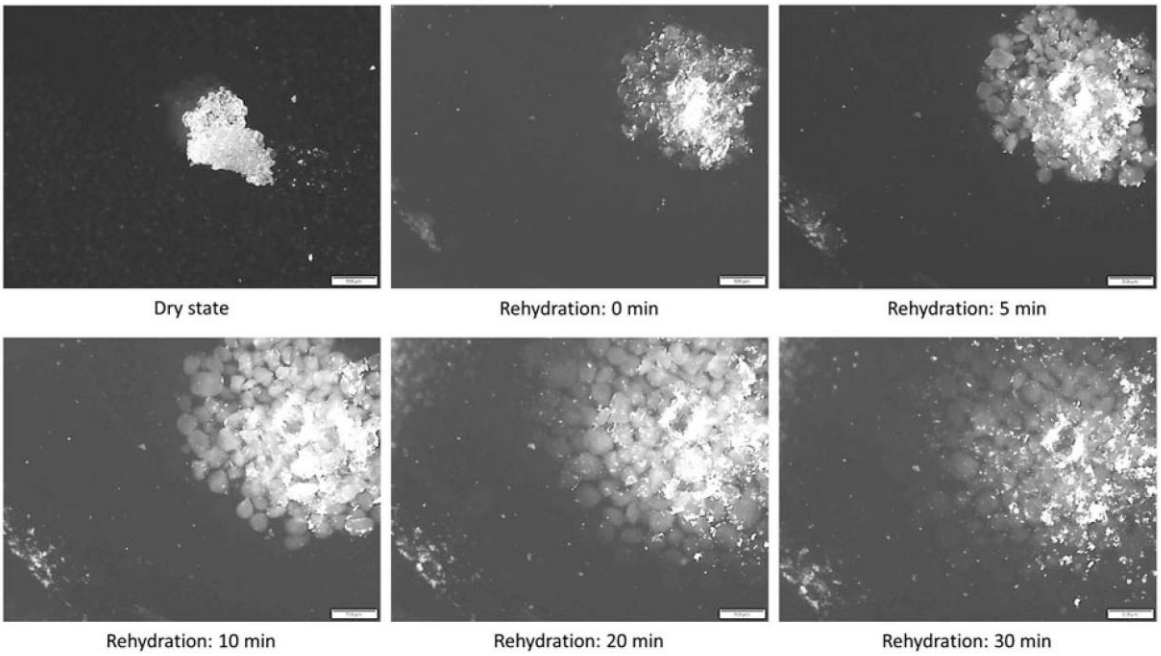

Figure 6. Swellability of alginate beads (in this example: beads produced with 200 µm nozzle). Seed-coating clusters consisting of alginate beads, talc particles and gum arabic are rehydrated within 30 min.

Table 1. Calculations of release depending on bead characteristics.

	Amount of beads in 100 mg	Cells encapsulated per bead	Cells released per bead	Radius of release (μm)	Total surface area of 100 mg beads (mm^2)
rB * = 0.080 mm*NE* † = log 6.0 ± 0.5 CFU/mL*NR* ‡ = log 4.1 ± 0.5 CFU/100 mg*SR* ¥ = 0.04	1 865 096	19	0.2	6.5	150 000
rB * = 0.200 mm*NE* † = log 5.9 ± 0.5 CFU/mL*NR* ‡ = log 3.9 ± 0.2 CFU/100 mg*SR* ¥ = 0.03	119 366	293	2.1	14.7	60 000
rB * = 0.450 mm*NE* † = log 6.0 ± 0.4 CFU/mL*NR* ‡ = log 3.7 ± 0.2 CFU/100 mg*SR* ¥ = 0.04	10 479	3334	12.4	13.0	26 666

Notes: A standardised radius of wet, spherical beads (*rB* = nozzle size) was used for calculations due to the regaining of sphericity of beads after rehydration. The smaller is the bead radius, the higher is the amount of beads in 100 mg and the higher is the total surface area in 100 mg. The depth of release in all sizes is in the low μm range, indicating the limitation of diffusion of bacteria.

**rB* is the radius of bead estimated from the nozzle size applied for extrusion.

†*NE* is the viable amount of CFU after drying.

‡*NR* is the viable amount of CFU released.

¥*SR* is the survival rate after drying.

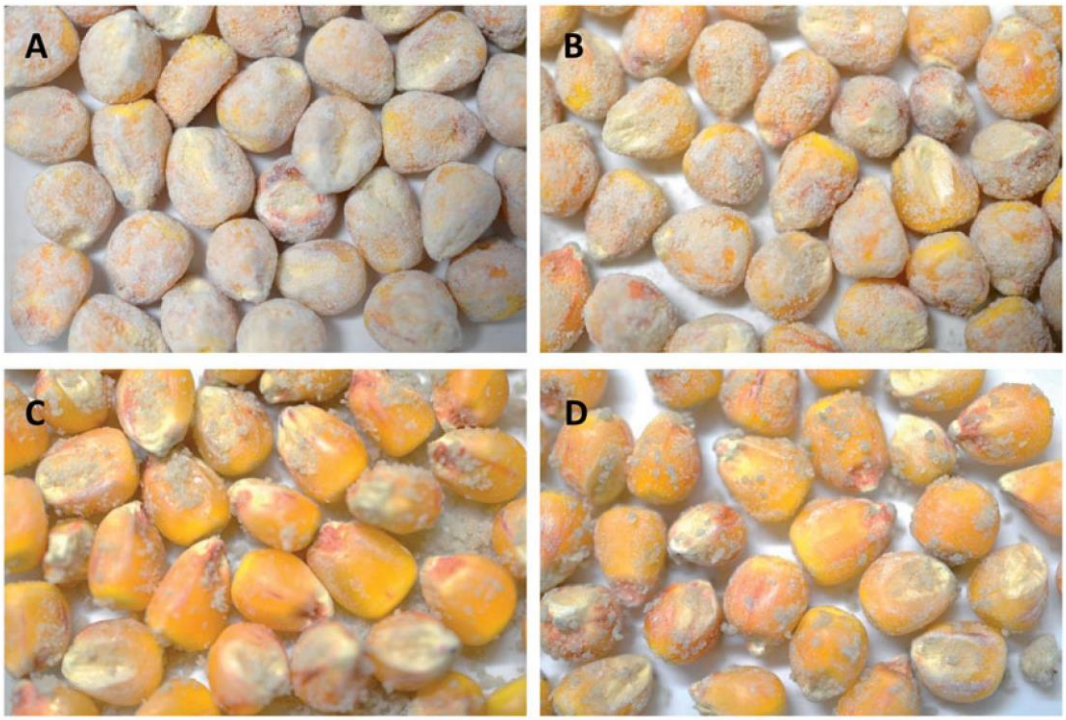

Figure 7. Maize seeds coated with four different alginate bead/particle sizes. Seed lots coated with alginate beads produced with nozzle size 80 μm (A), 200 μm (B) and 450 μm (C) as well as crushed macro-beads (D) are shown. The smaller is the bead size, the more homogeneous is the coating.

(Gombotz and Wee, 1998; Lee and Mooney, 2012), it does not allow for easy passage of bacteria lying typically in the range of 0.5–1.5 μm. It appears that delivery of bacteria from intact alginate particles takes place in close proximity to the bead surface as shown by the calculation of release depth (Table 1). Size reduction as achieved by crushing of macro-beads increased total surface area and thus enhanced release rate (Table 1). Furthermore, crack formation during dehydration (Figure 5C and D) increased the surface and rendered it prone to erosion (Gombotz and Wee, 1998), thus facilitating release of near-surface bacteria. Erosion of the calcium alginate network may thus be considered as a second important reason for bacterial release in addition to bead swelling.

Seed surface coverage and attrition rate

Maize seeds were coated with a powder-like formulation of alginate particles (bacterial carrier), gum arabic (adhesive) and talc (moisture absorbent). The best seed surface coverage as measured by ImageJ was 69 ± 3% in case of alginate beads produced with the nozzle size 80 μm (Figure 7A). With increasing nozzle/bead size, the percentage of surface coverage declined to 58 ± 3 and 44 ± 4% for beads produced with 200 μm (Figure 7B) and 450 μm nozzle (Figure 7C), respectively. Coating with crushed macro-beads (Figure 7D) showed a low coverage of 44 ± 7% similar to the largest bead size, but revealed its advantages when testing for susceptibility to attrition (lost material: 0.8 ± 0.3 mg/seed). Despite their better surface coverage, alginate beads produced with nozzle sizes 80 μm and 200 μm detached increasingly easily during the abrasion test in amounts of 1.5 ± 0.9 mg and 2.2 ± 1.3 mg/seed. Alginate beads produced with the 450 μm nozzle lost up to 2.7 ± 1.0 mg/seed due to the fact that they present higher asperities on the surface, which are susceptible to rounding off. Large slivers in the treatment with crushed macro-beads were concerned of the same effect, whereas the bulk of the inoculant consisting of small sliver sizes was tightly bound. Thus, a finer grinding may render a powder well suitable for seed coating.

A three-dimensional inspection of the seed coating revealed that alginate beads are present not as a single-layer coating but rather as agglomerates as defined by Bika et al. (2001). This way, satisfactory loads can be achieved despite limited seed surface area, especially if particle packing density is high. Generally, packing density increases with increasing particle size distribution, as smaller particles fill the voids between larger ones. This perception supports the application of crushed macro-beads.

Conclusions

Encapsulation of plant growth-promoting bacteria for agricultural applications requires a high-throughput, low-cost approach resulting in a free flowing powder suitable for seed coating, from which bacteria are readily released. Focussing on seed coating efficiency, 80 μm beads proved highly suitable. However, based on the inherent large throughput and observed bacterial release, finely ground macro-beads were an alternative option. Providing pulverisation to particles of < 100 μm and a suitable seed-coating protocol, encapsulation of plant growth-promoting bacteria in macro-beads is a prospective approach.

Declaration of interest

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8.2 MANUSCRIPT 2: ZEOLITE-BASED, DRY FORMULATIONS FOR CONSERVATION AND PRACTICAL APPLICATION OF *PARABURKHOLDERIA PHYTOFIRMANS* PSJN

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Zeolite-based, dry formulations for conservation and practical application of *Paraburkholderia phytofirmans* PsJN

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Abstract

Aim

Producing dry, zeolite-based granular and powder inoculants of the Gram negative, plant growth-promoting bacterium *Paraburkholderia phytofirmans* PsJN. Key aspects were maintenance of cell viability during desiccation and throughout storage at ambient conditions.

Methods and Results

20 additives and exopolysaccharide (EPS) produced by PsJN were screened for conserving cell viability of PsJN in air drying and lyophilization. Suitable combinations (e.g. skimmed milk + air drying) increased survival of PsJN up to 100,000-fold and maintained it for > seven months. EPS performed as good as skimmed milk during air drying, but was second-rank regarding shelf life. Combinations of zeolite, skimmed milk and gelatin as a film forming agent were extruded and processed into granules and powders, both displaying relatively stable viability for over four weeks at ambient conditions. Gelatin promoted brittleness of zeolite-based inoculants.

Conclusions

Viability of highly sensitive PsJN was successfully conserved in dry formulations, taking into account the interplay between carrier, protectants, drying method and coating agent.

Significance and Impact of Study

This is the first study to provide ways of maintaining viability of PsJN during desiccation stress and to investigate the applicability of its EPS as a protectant, thus ultimately facilitating successful plant inoculation especially under field conditions.

Keywords

Paraburkholderia phytofirmans PsJN; zeolite; formulation; desiccation; exopolysaccharide

Introduction

Beneficial, plant-associated bacteria have been recognized as a sustainable alternative to agrochemicals such as synthetic fertilizers or pesticides. One promising bacterial agent with a wide host range is *Paraburkholderia,* formerly *Burkholderia phytofirmans* PsJN (Sawana *et al*. 2014), which was shown to ameliorate abiotic stress (Barka *et al.* 2006; Fernandez *et al.* 2012; Naveed *et al.* 2014a; Naveed *et al.* 2014b) and promote plant growth (Kim *et al.* 2012; Lowman *et al.* 2015). However, effects of this and other potential strains observed under controlled conditions often lack reproducibility in field trials, so only a small fraction is eventually commercialized (Ravensberg 2015). Non-spore forming bacteria such as PsJN are particularly concerned, since their viability rapidly declines due to stress factors during technological processing and/or at the site of application (Schisler *et al.* 2016).

This issue may be overcome by help of suitable formulations, which maintain high bacterial viability over a long period of time without requiring sophisticated storage conditions (Herrmann and Lesueur 2013). Formulation in a dry product has been a popular strategy for conserving bacterial viability (Morgan *et al.* 2006). Common application forms comprise granules, which have been preferred for soil amendment, since they allow for precise dosage, are compatible with chemical seed treatments and suitable when seed sensitivity prohibits coating (Malusá *et al.* 2012; Bashan *et al.* 2014). Alternatively, powder formulations may be used for seed coating or foliage dusting. If wettable, powders may be processed into slurry for root dipping, seed coating or soil amendment (Malusá *et al.* 2012).

In both granules and powders, the organic or inorganic carrier material makes up the bulk of the product and thus constitutes a crucial parameter in formulation. Granular formulations have frequently been based on alginate (Müller-Stöver *et al.* 2004; Chung *et al.* 2005; Schoebitz *et al.* 2012), clay (Anandham *et al.* 2007) or flour, also known as “Pesta” (Daigle *et al.* 2002; Müller-Stöver *et al.* 2004). Powders have been produced, for instance, from talcum powder and vermiculite (Sarma *et al.* 2011), clay or sawdust (Tripathi *et al.* 2015). Important selection criteria for carriers include price, availability, environmental sustainability, chemical stability, non-toxicity and farmer’s convenience regarding handling and flexibility (Malusá *et al.* 2012; Bashan *et al.* 2014).

Zeolite as a carrier material meets all of these requirements. It is described as a crystalline aluminosilicate with a microporous structure (Mumpton 1999; Ramesh *et al.* 2010) and high chemical and thermal stability (De Smedt *et al.* 2015), which allows for easy sterilization. Zeolite has been applied in agriculture for the controlled release of standard pesticides and in biotechnology for immobilization of

microorganisms (De Smedt *et al.* 2015). It may furthermore function as a cation-exchanger in soil, providing plants with nutrients such as calcium and potassium and increasing water binding capacity (Mumpton 1999). When applied together with plant growth-promoting bacteria in soil, this may provide an additional benefit. Similar to other clay minerals, zeolite powders may form a particle film on the plant surface when applied as dust (De Smedt *et al.* 2015), thus delivering endophytic beneficial bacteria to the plant *via* structural features such as stomata on above-ground biomass.

Frequently, the carrier materials are amended with adjuvants such as wheat bran (Müller-Stöver *et al.* 2004), glycerol, carboxymethylcellulose (Sarma *et al.* 2011), glucose, rice bran (Anandham *et al.* 2007), maltose, lactose (Daigle *et al.* 2002), sucrose and oil (Mejri *et al.* 2012). These fulfil a variety of tasks such as nutrient provision, improvement of physical characteristics and protection of bacterial cells. The latter is highly relevant, since in dry formulations, bacteria undergo a physiologically demanding transition to an inactive state.

The application of (osmo)protectants has been recognized as a feasible means to enhance survival rate of bacteria during desiccation and storage (Costa *et al.* 2000; Schoebitz *et al.* 2012; Yánez-Mendizábal *et al.* 2012; Schisler *et al.* 2016). For example, the disaccharides trehalose and sucrose are known to stabilize cell membranes and preserve structure and function of proteins (Leslie *et al.* 1995). Skimmed milk is another popular protectant, whose effect has been ascribed mostly to the protein fractions and their function as membrane stabilizers (Morgan *et al.* 2006; Meng *et al.* 2008). However, the efficiency of a given protectant is largely species dependent and thus requires individual optimization for each biocontrol candidate (Morgan *et al.* 2006).

As a largely unexplored way of protection, the capability of bacteria to produce exopolysaccharides (EPS) as a shield against environmental stressors (Patel and Prajapati 2013) may be exploited in the context of formulation. Several *Paraburkholderia* strains are able to produce EPS, precisely, cepacian, which has been shown to provide protection against desiccation and metal ion stress (Ferreira *et al.* 2010). EPS has been successfully tested as an essential component in formulation of *Pseudomonas fluorescens* (Slininger *et al.* 2010) and *Pseudomonas aeruginosa* (Tewari and Arora 2014) and as a desiccation protectant for *Escherichia coli* (Nocker *et al.* 2012), but still knowledge about the compatibility of cepacian with different drying methods and its behaviour during storage is scarce.

The present work investigates the production of granular and powder formulations based on zeolite, protectants and coating agents regarding their morphology and efficiency in conserving cell viability of PsJN to obtain inoculant formulations with suitable properties for application in the field.

Material and methods

Cultivation of bacteria in liquid broth

Paraburkholderia phytofirmans PsJN::*gfp*2x was previously obtained by tagging the strain with a fluorescent marker gene and antibiotic resistance to kanamycin to ensure selective detection (Compant *et al.* 2005). This is particularly relevant since the formulations were prepared in aseptic, but not sterile conditions. PsJN::*gfp*2x was cultured in LB (Luria Bertani) broth (Bertani 1951) consisting of 10 g^{-l} tryptone, 5 g^{-l} yeast extract, 5 g^{-l} NaCl amended with 25 μg^{-ml} of the selective antibiotic kanamycin at 28 °C on a shaker for 24 h. Bacterial cells were harvested by centrifugation at 4400 x g for 15 min, washed with sterile 0.9 % NaCl, pelleted again and resuspended in the original growth medium or in sterile 0.9 % NaCl. The cell density was adjusted to approximately 5 x 10^9 CFU^{-ml} and confirmed by viable plate count.

Cultivation of bacteria on agar plates and preparation of exopolysaccharides

For desiccation tests involving EPS, PsJN::*gfp*2x was cultured on YEM agar plates (2 g^{-l} yeast extract, 20 g^{-l} mannitol, 15 g^{-l} agar) amended with 25 μg^{-ml} kanamycin. For this, an aliquot of overnight culture grown in liquid LB was plated on YEM plates and incubated at 28 °C for four days. The suspension of bacterial cells in EPS was harvested by adding sterile 0.9 % NaCl onto each plate, scraping off and vortexing thoroughly. An aliquot of the matrix was kept separately. In order to adjust the cell density, the bacterial suspension in EPS was first diluted 1:2 in sterile dH_2O to decrease the viscosity and thus allow for better separation of cells by centrifugation. The diluted matrix was centrifuged in a super speed centrifuge (Sorvall RC 6 Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA) at 48 000 x g, 15 °C, for 30 min, the supernatant was retrieved and again subjected to centrifugation at the same settings. In order to again concentrate the diluted EPS, the supernatant was collected in a beaker and the surplus dH_2O was evaporated under vacuum (Rotavapor R-210, Büchi Labortechnik AG, Flawil, Switzerland) at a water bath temperature of 45 - 50 °C and a vacuum of 40 – 45 mbar. To check for residual bacterial

cells, a serial dilution of the extracted EPS was prepared and plated on LB agar plates. Its protective effect was investigated on cells, which had produced the EPS ("self-produced") and on cells, which were originally grown in LB ("added"). In the first case, the aliquot of original EPS with bacterial cells was diluted in the one with reduced cell content, the cell concentration was adjusted to 5 x 10^9 CFU^{-ml} by measuring the OD_{600} and confirmed by viable plate count. In the second case, the EPS was treated according to the artificial additives, using it as a protectant for PsJN grown in LB.

Screening of additives combined with lyophilization or air drying

A range of chemically dissimilar, potential desiccation protectants were evaluated in four independent replicate tests. D-(+)-trehalose-dihydrate, sucrose, D-galactose, lactose, D-sorbitol, D-mannitol, glycerol, carboxymethylcellulose sodium salt (CMC), gelatin from cold water fish skin, gum arabic from acacia tree, Ficoll PM 400 and humic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Maize starch Meritena 100 and maltodextrin Maldex G190 were obtained from Tereos Starch & Sweeteners (Marckolsheim, France). Skimmed milk powder and dimethyl sulfoxide (DMSO) were obtained from Carl Roth GmbH & Co. KG (Karlsruhe, Germany), yeast extract from Merck KGaA (Darmstadt, Germany). All additives were applied in a concentration of 7.5 % (w/v), except Ficoll 400, CMC, alginate and DMSO, which were applied in 2.0 % (w/v and v/v respectively). This was done to maintain an osmotic pressure in the range of physiological saline (0.9 % NaCl: 0.3 Osm^{-L}). Due to their different molecular weights, the sugars require concentrations between 5 % (e.g. D-galactose) and 10 % (w/v) (e.g. sucrose) to reach an osmolarity of 0.3 Osm^{-L}. To avoid a large deviation from the physiological osmotic pressure and thus reduce osmotic stress, we chose the intermediate concentration of 7.5 % (w/v) for sugars. This value was adopted for high-molecular weight substances in order to allow for direct comparison. In case of Ficoll 400, CMC and alginate, the solubility was limited and the concentration therefore adjusted to 2.0 % (w/v). DMSO is known to be toxic for cells in high concentrations, which was the reason for choosing a reduced concentration of this additive. In case of small, osmotically active molecules (trehalose, sucrose, galactose, lactose, sorbitol, mannitol, glycerol, DMSO), dH_2O was used as a solvent. High-molecular weight compounds (all others) were prepared in 0.9 % NaCl to maintain iso-osmotic conditions for bacteria (Wessman *et al.* 2011).

Bacterial suspensions were mixed with the additives in a ratio of 1:10 in 2 mL Eppendorf tubes to obtain a final volume of 200 µL, thus diluting the bacterial suspension 10-fold. All tubes were prepared in duplicate to be tested in two different drying methods. One set of tubes was pre-frozen at -80 °C,

transferred to a freeze drying chamber (Alpha 2-4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany) and lyophilized at 0.120 mbar for 45 h. The other set of tubes was air dried in an oven at 25 °C under ventilation for 65 h.

Immediately after drying, the dried matter was rehydrated in sterile dH_2O (200 µL). The suspensions were transferred to a sterile 96-well plate and 10-fold serially diluted in sterile 0.9 % NaCl using a multichannel pipet (Nocker *et al.* 2012). 5 µL of each well was transferred by help of a multichannel pipet onto quadratic petri dishes (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with LB agar amended with 25 μg^{-ml} kanamycin and incubated at 28 °C for 48 - 72 h. False positive colony counts were prevented by using the selective medium containing kanamycin as well as by considering the characteristic morphology of PsJN and observing the fluorescence of the *gfp*-labelled variant under UV light.

In the viable cell counting, those samples which did not show any colony growth were assumed to lie just below the theoretical detection limit of 30 CFU^{-ml}, which was calculated from the total drop plated volume per sample. Therefore, a value of log 1.5 instead of 0 was inserted in order to avoid a distortion of standard errors.

Shelf life test with selected combinations of additive-drying method

The best performing “artificial” protectant from the previous screening test and the “natural” protectant (EPS) with their respectively suitable drying methods were investigated regarding viability maintenance during storage. Apart from bacterial survival rate after drying, another reason for the selection was a completely dry appearance under the given drying conditions, therefore dismissing for example trehalose.

The samples were prepared in parallel to the samples for desiccation test and comprised duplicate tubes for each storage time interval and temperature. The tubes were vacuum packed in R-Vac vacuum bags (Landig + Lava GmbH, Saulgau, Germany) including 5 g of silica beads each. Samples were stored at suboptimal conditions of 22 °C for 7, 28, 56 and 82 d and at conservative conditions of 4 °C for 28, 54, 82, 140 and 196 days in parallel. At each point of measurement, duplicate samples were destructively analysed as done immediately after drying.

Combination of the best additive with carrier and film forming agent

The additive and drying method leading to the best immediate and long-term survival (skimmed milk powder, air drying) was chosen for combination with zeolite as a carrier and gelatin as a film forming agent. The suitability of skimmed milk was also confirmed in a pre-test subjecting PsJN to air drying in zeolite with skimmed milk or EPS, demonstrating the latter was significantly less efficient (see supplementary information).

The possibility to form extrudates depending on the zeolite content was tested by mixing 40, 50, 60 or 70 % (w/v) of zeolite LithoFill BM (particle size 0-25 µm, kindly provided by LITHOS Industrial Minerals GmbH, Ennsdorf, Austria) with 0.9 % NaCl and extruding it manually through a syringe.

To identify suitable ratios of protectant, carrier and film former, skimmed milk powder (0 %, 7.5 %, 15 % or 30 % w/v) and gelatin (0 %, 1 % or 10 % w/v) were mixed with suspensions of PsJN::*gfp*2x in 0.9 % NaCl, prepared as during protectant screening. All skimmed milk concentrations were paired with all gelatin concentrations, giving a total of 12 combinations. 200 µL of each combination were blended with the amount of zeolite previously determined as suitable for extrusion, air dried as during additive screening and re-suspended when appearing dry (after 45 h). Cell viability was determined as described previously.

Formation and characterization of granules and powders

Based on the observed handling properties, 70 % (w/v) zeolite was applied for production of granules and powders with PsJN. 15 % (w/v) skimmed milk powder and/or 10 % (w/v) gelatin were chosen based on considerations regarding bacterial viability for further processing to obtain granular and powder formulations. For this, the components were mixed thoroughly, extruded and allowed to dry for 48 hours under ambient conditions. Then, they were processed to granules of approximately 1 – 5 mm size by manual crushing or to fine powders of sub-mm size by grinding in a mortar. Morphological characteristics of the granules were observed by scanning electron microscopy (Hitachi TM3030, Hitachi High-Technologies Corporation, Tokyo, Japan). Powder particle size was additionally characterized by laser diffraction analysis (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Bacterial viability in differently composed granules and powders was checked immediately after drying and was monitored periodically during storage in Eppendorf tubes at ambient conditions after 3, 7, 14 and 28 days by viable plate counting as done in the additive screening test.

Statistical methods

Statistical significance of the protective effect of additives was evaluated by performing a one-way ANOVA followed by Dunnett's Multiple Comparison test, comparing every additive to the control (null hypothesis: a given additive does not enhance cell viability of PsJN after drying compared to the control NaCl) . The effect of EPS in air drying and lyophilization was evaluated by a two-way ANOVA followed by a Bonferroni post-test (null hypothesis: EPS "added" or "self-produced" does not improve cell viability of PsJN after drying compared to the control NaCl in neither air drying nor lyophilization). All analyses were done with GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Cell viability after air drying or lyophilization in presence of additives

Survival rate of PsJN was investigated in different protectants dried by standard lyophilization compared to a more economically feasible method (air drying) to identify the most suitable approach for later preparation of zeolite-based inoculants. The primary criterion was maximization of survival rate while taking into account the trade-off between economic feasibility and high cell numbers.

In lyophilization, 15 out of 20 tested additives significantly improved survival compared to the control NaCl (Fig. 1A). The highest cell viabilities of > log 8.0 CFU^{-ml} (suspension before drying: log 8.8 ± 0.4 CFU^{-ml}) were achieved when adding skimmed milk or trehalose. Good viability maintenance within the same order of magnitude was also observed in sucrose and lactose as well as galactose. Some of the high molecular weight polymers (corn starch, carboxymethylcellulose, maltodextrin and gum arabic) as well as complex compounds consisting of proteins and sugars (yeast extract, gelatin and growth medium) allowed for similar survival rates. A very low degree of protection was found when adding sorbitol, Ficoll, or humic acid. Mannitol, glycerol, alginate, PEG and DMSO did not provide protection during drying compared to the control (NaCl).

In air drying, only eight out of 20 tested additives provided significant protection to PsJN. However, three of those did not appear completely dry (trehalose, sucrose, sorbitol). More stringent drying conditions, e.g. at elevated temperatures, may have solved this issue but were avoided to keep the process as mild

as possible. This leaves skimmed milk powder standing out as the best protectant, maintaining a viability of log 8.4 ± 0.2 CFU^{-ml}. A significant degree of protection – albeit with a loss of viability of up log 3 CFU^{-ml} – was provided by lactose, mannitol, maltodextrin and gelatin.

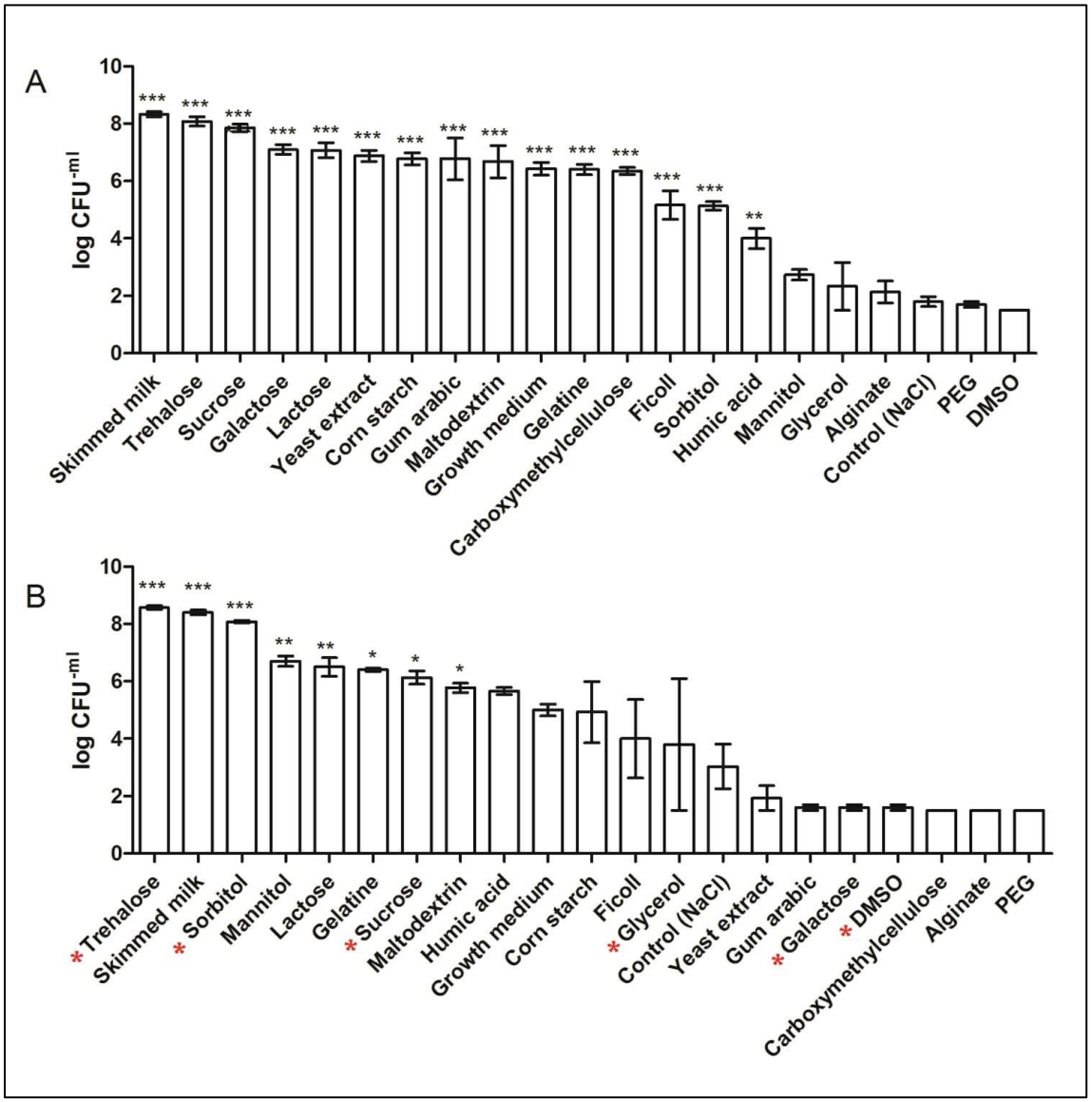

Figure 1: Cell viability of PsJN after lyophilisation (A) or air drying (B) depending on protectants. Bars show mean viabilities (n=4) with SE. Survival was significantly improved compared to control (NaCl) where bars are marked with *** at p<0.001, ** at p<0.01 or * at p<0.05 (Oneway ANOVA, Dunnett's Multiple Comparison test). Initial concentration was log 8.8 ± 0.4 CFU^{-mL}. Additives marked with a red asterix did not dry thoroughly at ambient conditions.

Clearly, there is a strong interaction between the drying method and the additives, which becomes particularly obvious in samples containing mannitol (low protection in lyophilization, high protection in air drying) as well as in those containing CMC, gum arabic or yeast extract (high protection in

lyophilization, low protection in air drying). This may reflect the differences in stress mechanisms conferred by the respective drying methods, for example cell damage by ice crystal formation.

Cell viability after air drying or lyophilization in presence of EPS

PsJN produced EPS when grown on YEM agar plates. For testing of the capabilities of EPS to prevent cell death during desiccation, it was either extracted and applied on PsJN harvested from LB broth ("added") or it was used along with the inherent PsJN cells responsible for its production ("self-produced"). This was done to check whether the protective effect is related to the physiological state of the bacteria, in which they produce EPS or if the mere external presence of EPS is sufficient.

Figure 2 shows that the survival rate of PsJN was significantly improved after desiccation in presence of EPS compared to the control (NaCl) during both lyophilization and air drying. This was true for both EPS "self-produced" and "added". In both cases, the protective effect was much more pronounced in air drying than in lyophilization. This means only a minor loss of cell viability of less than one order of magnitude in presence of EPS during air drying, which is a value comparable to the best performing artificial additives tested in this study.

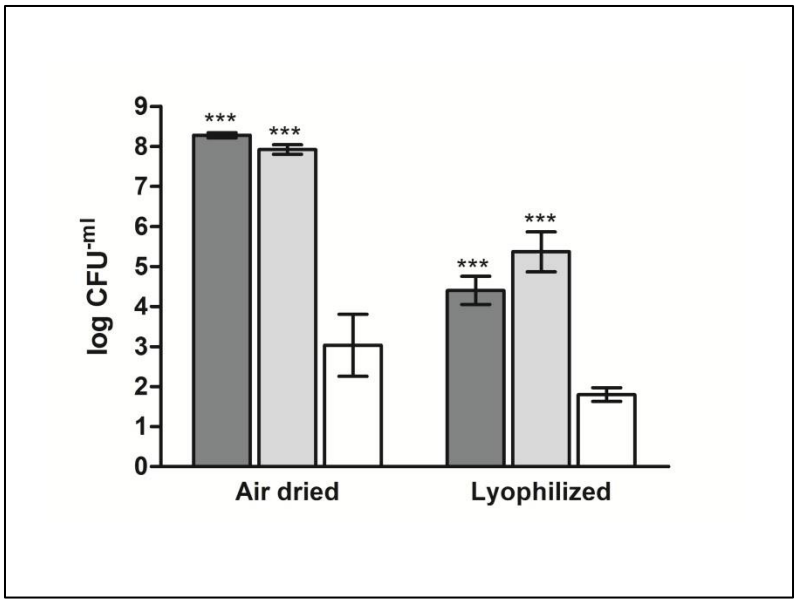

Figure 2: Cell viability of PsJN after lyophilization or air drying in presence of EPS (dark grey bars: EPS "added", light grey bars: EPS "self-produced", white bars: control). Bars show mean viabilities (n=4) with SE. Survival was significantly improved in presence of EPS compared to control ($p<0.001$, Bonferroni post-hoc test), regardless of whether the EPS was self-produced or added to cells grown in LB. The protective effect was more pronounced in air drying. Initial concentration was log 9.1 ± 0.8 CFU^{-mL}.

Shelf life of PsJN in selected additive-drying combinations

Skimmed milk (the best performing “artificial” additive) and EPS (as a “natural” protectant) were chosen for a storage test, since storage stability is also a critical factor in subsequently produced zeolite-based inoculants. Both drying methods were applied with skimmed milk, but EPS was only air dried since it had already been shown to result in very low bacterial survival rates during lyophilisation. Thus, samples of PsJN in skimmed milk (lyophilized or air dried) and EPS (air dried) were stored at 22 °C for a shelf life test under suboptimal conditions and at 4 °C for conservative conditions. At higher storage temperature, the cell viability declined more rapidly (Fig. 3).

In both temperature regimes, viability was best maintained in skimmed milk (air dried), which showed stable values over more than three months at 22 °C and more than seven months at 4 °C. EPS (air dried) was slightly inferior in its ability to conserve viability, which dropped by about one order of magnitude after three months when samples were stored at 22 °C. In contrast, viability was relatively stable at 4 °C throughout the test period of seven months.

Interestingly, samples lyophilized in skimmed milk proved much less suitable, since the cells decreased in viability much faster even at conservative conditions of 4 °C.

Based on the fact that samples air dried in skimmed milk performed best during the shelf life test, this combination of additive and drying method was chosen for further formulation of PsJN in zeolite matrix.

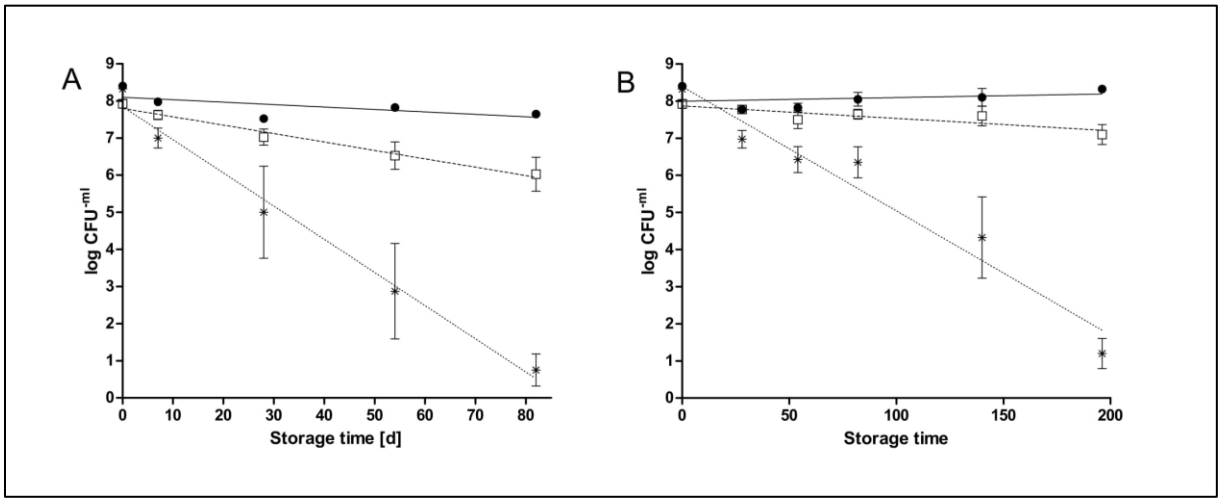

Figure 3: Decrease of cell viability during storage at 22 °C (A) or 4 °C (B) in presence of skimmed milk air dried ( ), skimmed milk lyophilized ( ) and EPS air dried ( ). Means of n=4 with SE and best-fit linear regression lines are depicted. Note that the scaling of the x-axis differs at each temperature depending on the maximum duration of storage.

Suitable ratios of matrix components for production of granules and powders

A pasty matrix consistency is desirable for extrusion. The minimum concentration of zeolite to achieve this was shown to be 70 % (w/v) (Fig. 4 A), which explains why granules and powders produced subsequently were based on this value.

Bacterial viability after air drying in this zeolite matrix combined with 0, 7.5, 15 and 30 % (w/v) skimmed milk and 0, 1 or 10 % gelatin (w/v) was tested (Fig. 4 B) to identify suitable ratios of both additives. Gelatin was chosen as a film forming agent since it is a biodegradable, readily available and inexpensive additive (Gomez-Guillen *et al.* 2011) and performed well during protectant screening.

In absence of zeolite, viability was maintained – as expected – at a high level when skimmed milk was present, regardless of the concentrations tested. There was no synergistic effect between gelatin and skimmed milk. The presence of gelatin alone also improved the survival rate of PsJN, but not as efficiently as skimmed milk. Adding 70 % zeolite lowered PsJN survival rate by approximately 2.5 orders of magnitude in almost all compositions. The drop in viability was even more pronounced in absence of skimmed milk. The exception were samples with 10 % gelatin only, in which protection of PsJN was better with 70 % zeolite than without.

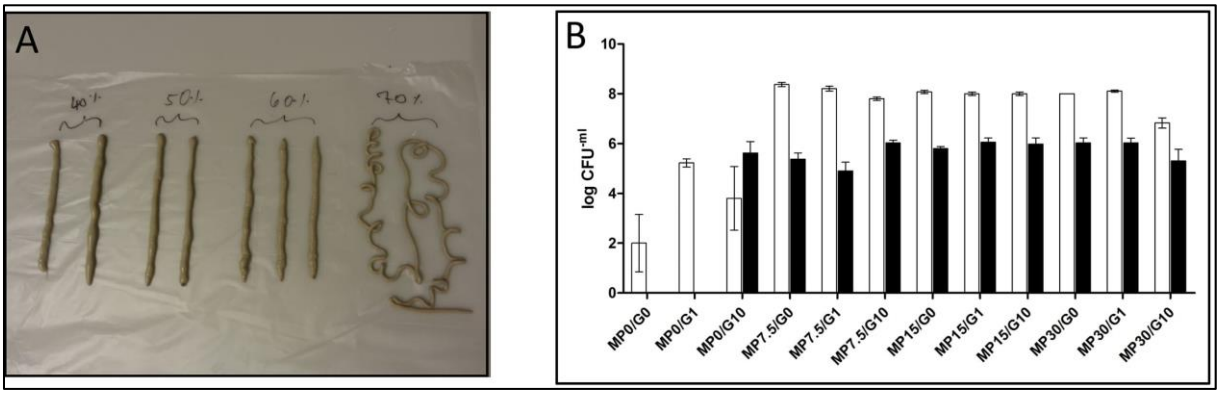

Figure 4: Matrix consistency and extrudability are dependent on zeolite concentration in NaCl (A). Cell viability (B) of PsJN after air drying with or without 70 % (w/v) zeolite (white bars: no zeolite; black bars: 70 %) and different ratios of skimmed milk powder (MP) and gelatine (G). Numbers in combination with MP or G denote the concentration in % (w/v). Bars show mean viabilites (n=4) with SE. Original cell concentration before drying was log 8.6 ± 0.3 CFU^{-mL}.

Production and morphological characterization of zeolite-based granules and powders

The combinations of 70 % (w/v) zeolite with 15 % (w/v) skimmed milk and/or 10 % (w/v) gelatin carrying bacteria were extruded and processed into granules and powders after drying. The presence of gelatin had a profound effect on physical properties of the zeolite matrix both before drying and after drying (Fig. 5). It rendered the matrix more fluid and the diffluent behavior made the formation of clearly defined extrudates impossible. The zeolite-skimmed milk matrix was the only composition with a pasty appearance, resulting in a cylindrical form of extrudates, which was maintained throughout the drying process. As a consequence, dry granules composed of zeolite-gelatin or zeolite-gelatin-skimmed milk resembled flattened disks, whereas granules made from zeolite-skimmed milk were cylindrical. The size of all dried granules was in the range of approximately 1-5 mm. There was no perceptible difference in physical characteristics during handling between samples containing bacteria and the no-bacteria control of correspondent composition.

The particle size characteristics of powders examined by laser diffraction analysis also differed depending on composition. Powders made from zeolite amended with skimmed milk and gelatin and including bacteria showed a surface area mean diameter (Sauter mean) of 62 µm, with 10 % of the volume of particles (D(0.1)) lying below 32 µm and 90 % (D(0.9)) below 1294 µm. Values of the same composition but without bacteria were only slightly different, with a surface area mean diameter of 54 µm, D(0.1) of 32 µm and D(0.9) of 958 µm. Powders produced from zeolites with only one of the two additives were distinguished by a smaller surface area mean diameter of 27 µm for zeolite-milk powder and 13 µm for zeolite-gelatin compositions. The D(0.1) of zeolite-milk powder lay at 9 µm and the D(0.9) at 1193 µm. Zeolite-gelatin showed an even lower value for D(0.1) of 5 µm and a D(0.9) of 1300 µm. All frequency curves obtained from laser diffraction analysis show a bimodal distribution, with the low size range peak being most pronounced in zeolite-gelatin powders. The latter quickly disintegrated upon the force of the mortar during grinding, whereas zeolite-gelatin-skimmed milk and zeolite-skimmed milk extrudates required more effort.

Analysis of zeolite granules by SEM supported these observations. Combination of zeolite with both skimmed milk and gelatin resulted in mechanically strong agglomerates, which displayed hardly any abrasion. Air bubbles from foaming created during mixing were enclosed in the extrudates and were maintained to form porous structures after drying. In contrast, granules made of zeolite-skimmed milk lacked larger cavities and displayed even, solid surfaces at the sites of breakage. Also zeolite-gelatin

granules were free of large voids, but places of fracture were more irregularly shaped than in presence of skimmed milk alone and a high amount of abraded material was visible.

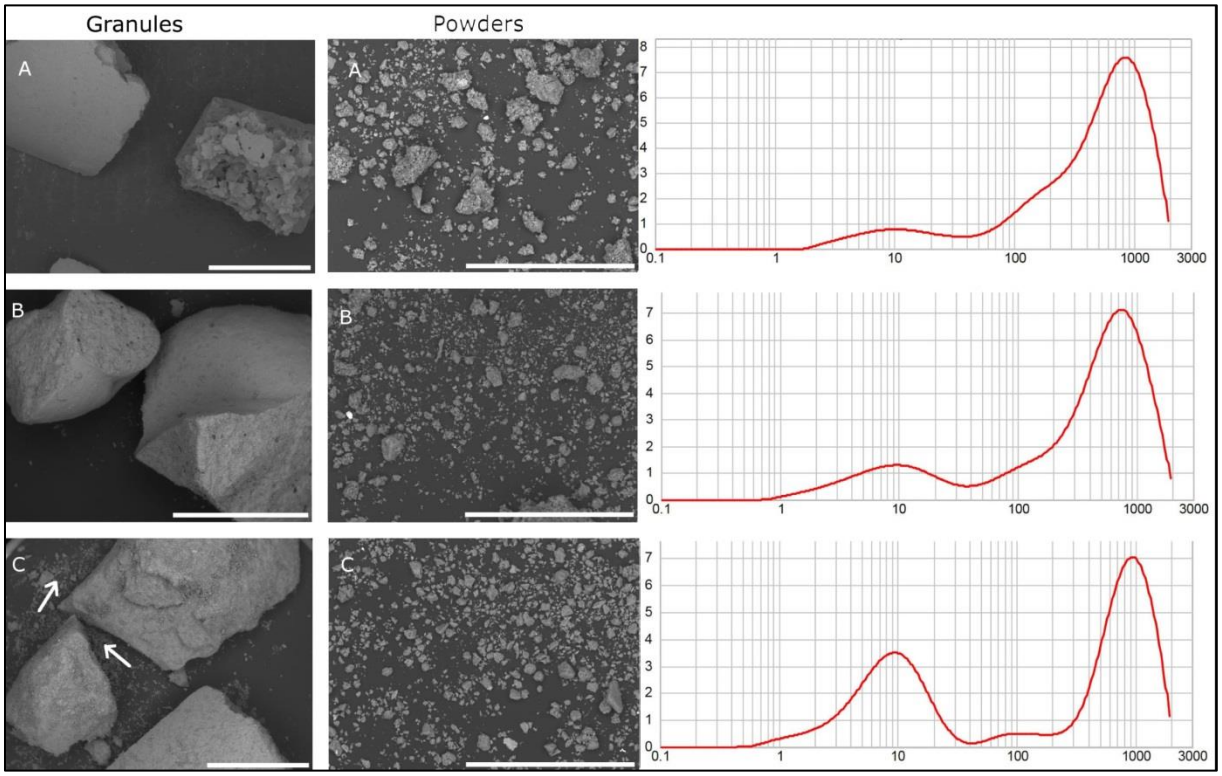

Figure 5: Morphological characterization of zeolite particles carrying PsJN. SEM images show differently composed zeolite granules and powders, including milk powder and gelatin (A), milk powder only (B) and gelatin only (B). Presence of gelatin renders the particles more brittle (see abraded fraction in granules C, indicated by arrow). Scale bars denote 2 mm in granule images, 200 µm in powder images. x-axis in particle size distribution denotes particle size [µm], y-axis volume [%].

Viability of PsJN in zeolite granules and powders during storage

Granules and powders made from zeolite and additives and carrying immobilized PsJN were stored at ambient conditions and cell viability was measured periodically over the course of one month (Fig. 6). The most pronounced drop in viability of 2.5 to 3.5 orders of magnitude was observed as an immediate result of desiccation. In the storage time following, the viability was comparably stable particularly in zeolite-gelatin mixtures. Despite suboptimal storage conditions, by the end of one month cell viability laid only one order of magnitude below the one measured immediately after drying. These general trends were observed in parallel in both granules and powders.

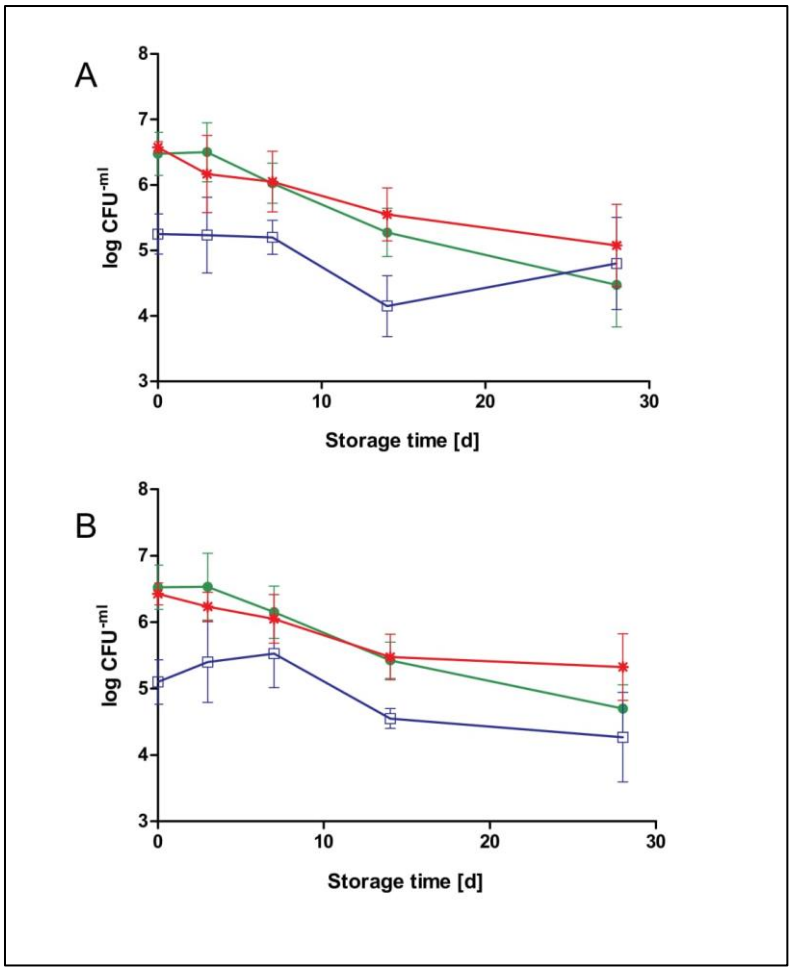

Figure 6: Cell viability of PsJN during storage at ambient conditions in zeolite granules (A) or powders (B) including 15 % skimmed milk and 10 % gelatin ( ), 15 % skimmed milk only ( ) or 10 % gelatin only ( ). Values show means of n=4, error bars show SE. Original cell concentration before drying was 9.2 ± 0.4 CFU^{-mL}.

Discussion

This study firstly investigates the development of granular and powder formulations for the plant growth-promoting bacterium *P. phytofirmans* PsJN with a focus on long-term conservation of cell viability. Our approach yielded a high titer of viable cells of PsJN in dry matter, which is an important prerequisite for successful application of a biofertilizer product. Protective formulations proved particularly relevant for PsJN due to its high sensitivity during drying, where viable cell numbers decreased by log 5 – log 6 CFU^{-mL} in the absence of protectants. Other Gram negative bacteria such as *Pantoea agglomerans* or *Campylobacter jejuni* (Costa *et al.* 2000; Portner *et al.* 2007) dropped by merely log 2 – log 3 CFU^{-mL}

during desiccation, whereas Gram positive, industrially relevant bacteria such as *Lactobacilli* are even less sensitive and decreased by approximately log 1 (Siaterlis *et al.* 2009).

Zeolite has previously been suggested as a carrier for bacteria due to its microporous structure and large surface area (Hrenović *et al.* 2007) and offers the possibility to form either powder or granular inoculants. However, zeolite alone did not sufficiently protect bacteria from desiccation, thus requiring the screening of potential additives to maintain cell membrane integrity during drying and storage.

Most studies investigating protectants were performed with lyophilization as a drying method (Costa *et al.* 2000; Abadias *et al.* 2001; Carvalho *et al.* 2004; Siaterlis *et al.* 2009), since it is regarded as a preferred drying process in industry (Morgan *et al.* 2006). However, it is also an energy-consuming procedure (Santivarangkna *et al.* 2007) and lacks comparability to the mode of drying as it occurs under natural conditions, for example in the field. Nevertheless, we performed lyophilization to include a standard technique for comparison.

During lyophilization, the disaccharides trehalose and sucrose as well as skimmed milk proved highly protective. These protectants are amongst the ones most frequently used for drying of prokaryotes (Hubálek 2003; Morgan *et al.* 2006; García 2011). They may also serve as prebiotics to facilitate quick recovery and enable cell growth upon rehydration.

Reports about the improvement of survival of Gram negative bacteria in air drying are limited, again mostly taking into account sugars as osmoprotectants (Nocker *et al.* 2012; Schisler *et al.* 2016). The sugars trehalose, lactose and sucrose proved efficient in protecting PsJN during air drying. However, trehalose, sucrose and others did not appear dry after a given time due to their hygroscopicity. This fact may have implications for physical characteristics of a formulation, such as reduced flowability.

Several high molecular weight additives were efficient in stabilizing bacteria during drying, such as skimmed milk and gelatin. Alginate, however, displayed no significant protective effect during either drying method. This is a relevant finding, since alginate has frequently been promoted as a suitable material for protection of microorganisms by encapsulation (Bashan and Gonzalez 1999). Although encapsulation with alginate comprises a cross-linking step, which was omitted here, the results suggest that pure alginate is not sufficient for conservation of viability in case of PsJN. This assumption is supported by our observations of rapid cell death of PsJN when formulated as dry alginate beads (Berninger *et al.* 2014).

In addition to “artificial” protectants, bacterial EPS was investigated regarding its protective effect. Our results showed that the addition of EPS as a protectant during the process of drying of PsJN is very efficient. The protective effect appears to be conferred mostly externally to the cell, since there was only a minor difference in “self-produced” or “added” EPS. Interestingly, the protective effect of EPS was much higher in air drying than in lyophilization, possibly due to an inability of EPS to prevent cell damage from ice crystal formation during pre-freezing. These effects seem to be specific for PsJN’s EPS and cannot be generalized as they were not observed in alginate, which is essentially also an EPS.

The significance of the drying method was also obvious in galactose, yeast extract, gum arabic (all performing better in lyophilization) as well as mannitol and EPS (better in air drying). This finding highlights the necessity to investigate combinations of drying methods, protectants and strains rather than single parameters in a sequential mode.

During long-term storage, survival of PsJN was dependent on temperature, protectant and drying method. Elevated temperatures led to a faster degradation than lower ones, which is in line with reports about the sensitive, Gram negative bacterium *Campylobacter jejuni* (Portner *et al.* 2007).

The influence of the drying method on long term stability was apparent in skimmed milk, in which viability of PsJN decreased much faster when lyophilized than when air dried. This may be a result of differences in residual moisture content in samples, which is expected to be lower in lyophilization, since the vacuum provides a stronger force driving the removal of water than is the case in ambient conditions. Air dried samples in skimmed milk were successfully stabilized over the whole testing period of three months at room temperature and seven months at 4 °C, thus highlighting the possibility to provide stable inoculants of PsJN with a certain flexibility in storage temperature. EPS was the second-best protectant during storage and preserved cell viability almost as efficiently as skimmed milk air dried when stored at 4 °C.

Zeolite-based granules and powders were produced incorporating skimmed milk as a protectant. Using porous substrates like zeolite as carriers, the addition of coating agents such as xanthan gum has been promoted for shaping morphological properties and thereby release of immobilized agents (Swaminathan and Jackson 2008). Moreover, the physical characteristics determine the compatibility of inoculants with seeding machinery. Ideally, the coating agent also positively influences the long-term viability of cells, as was shown for the combination of xanthan gum and olive oil on *Pseudomonas sp*. (Stelting *et al.* 2012). We chose including gelatin as a coating agent due to its beneficial effects on PsJN

observed during air drying. Gelatin is also known for its film forming properties, thereby providing a shield from exposure to light and oxygen (Gomez-Guillen *et al.* 2011).

In determining suitable ratios of zeolite, gelatin and skimmed milk powder, it turned out that the addition of carrier has a detrimental effect on bacterial viability after drying. An immediate drop in bacterial viability of around two orders of magnitude (or more) when immobilized onto a high ratio of zeolite was also observed in case of *Pseudomonas sp.* (Stelting *et al.* 2012). The authors propose that moisture adsorption properties in zeolite play an important role for bacterial survival. The adsorption of moisture into the porous structure of zeolites may confer a change in drying kinetics. This may in turn influence the physiological response of entrapped bacteria. Nevertheless, when considering extrusion for the production of granular formulations, a pasty consistency of bacterial entrapment matrix is required and may only be reached with elevated levels of zeolite.

Gelatin was observed to influence the matrix properties both in the wet and dry state. It is known to act as a steric barrier in suspensions (Likos *et al.* 2000) and may thereby prevent agglomeration of zeolite particles in bacterial suspensions. Therefore, only mixtures of zeolite and skimmed milk displayed the desired pasty consistency for extrusion. This may not be very relevant for production of powders, but is more so if aiming at producing cylindrical granules, which was not possible to achieve in the presence of gelatin. On the other hand, a faster disintegration of granules may facilitate the release of entrapped bacteria once applied in the soil.

The assumption that PsJN is better protected inside the microenvironment of large granules compared to powders did not prove true. Viability immediately after drying as well as during storage was similar in both particle sizes. Possibly, the survival of the bacteria tested in our study is more dependent on the protectant and film forming agent being present in close proximity to the cell than being surrounded by a bulk zeolite matrix. This can be achieved with powders and granules alike and thus allows for flexibility in agricultural delivery technique – for example, powders may be applied as slurries or dusts for seed treatment, whereas granules may serve as soil amendments.

In contrast to particle size, the composition of zeolite-based formulations did have an influence on bacterial survival during storage, albeit not a very pronounced one. Compositions of zeolite with gelatin and gelatin-skimmed milk were slightly more stable during the first weeks of storage than granules made of zeolite-skimmed milk. This may be an indication for the protective role of the film-forming agent gelatin. Interestingly, this finding is in contrast to samples dried in absence of zeolite, in which the

protection provided by skimmed milk alone was sufficient. This may indicate that in presence of zeolite, the hygroscopic nature of gelatin unfolds a higher impact on the drying kinetics in favor of cell viability than it does when applied purely.

Our findings highlight the necessity of taking into account the interplay between carrier, protectants, drying method and coating agent in formulation development. For *P. phytofirmans* PsJN, our observations suggest a process based on air drying and including skimmed milk powder as a protectant to obtain highly viable inoculants. Incorporation of gelatin into the matrix proved beneficial especially when aiming at producing powders or granules, which disintegrate readily. Future investigations may focus on improving the immediate survival rate of bacteria when combined with additives and carrier, aiming at reaching similarly high survival rates and shelf lives as those obtained in pure protectants.

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Conflict of interest

No conflict of interest declared

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Supplementary information

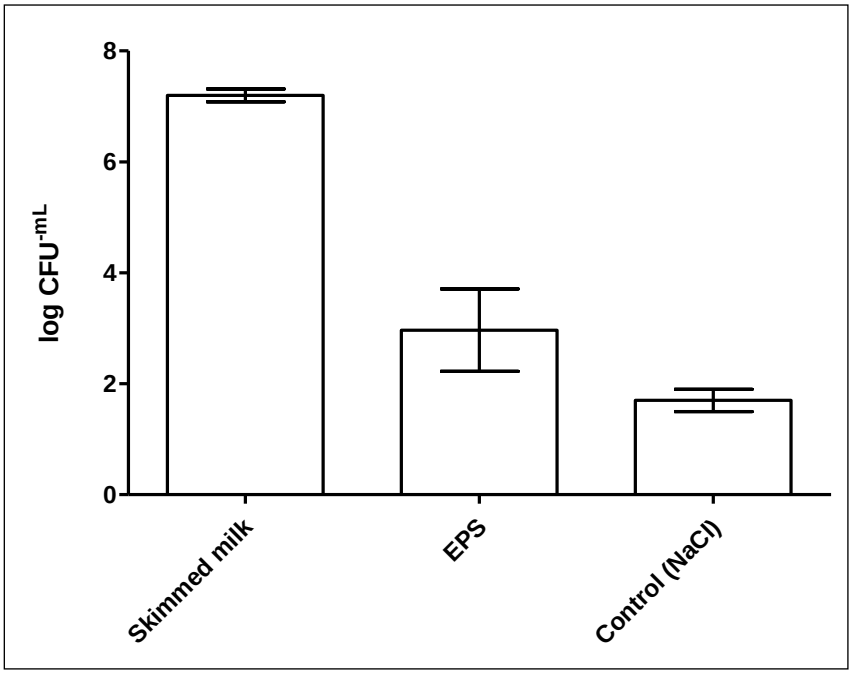

Figure A: Survival rate of PsJN in skimmed milk or EPS and 20 % zeolite when air dried. Original cell concentration before drying was log 8.7 ± 0.1 CFU^{-mL} in skimmed milk and control, and log 8.8 ± 0.2 CFU^{-mL} in EPS. With zeolite, skimmed milk was the most effective protectant during air drying and was therefore chosen over EPS for follow-up formulation steps.

9 CONCLUSION AND OUTLOOK

In this work, two different approaches for formulation of plant beneficial bacteria were pursued - encapsulation in alginate beads and immobilization in zeolite particles.

Both methods yielded inoculants with morphological characteristics suitable for seed coating and in-furrow placement respectively.

During alginate bead production, a critical factor was the trade-off between bead size and throughput. This could be balanced to a certain extent by optimizing the matrix composition and choosing an appropriate extrusion method. However, in comparison, production of zeolite granules proved to be a more straightforward approach. The physical characteristics of zeolite-based inoculants, such as brittleness and extrudability, could be shaped by the ratio of zeolite and incorporated additives.

The maintenance of bacterial viability during the drying of the inoculants was shown to be a major challenge in both carrier materials. This was true especially when formulating the Gram negative bacterium *Paraburkholderia phytofirmans* PsJN, whose viability decreased much more dramatically during desiccation than the one of the tested spore-former, *Bacillus subtilis*.

Putting the focus on the bacterial viability during technological processing and later storage, suitable protectants were identified out of a range of externally added substances tested. Furthermore, the use of bacterial exopolysaccharides proved to be a feasible and innovative approach to protect PsJN from desiccation stress. Interestingly, the efficiency of a given protectant proved largely dependent on the drying method applied. This emphasizes the necessity of evaluating combinations of process parameters rather than merely looking at isolated aspects.

Regarding future investigations, the observation of the influence of the drying process on a molecular level might provide valuable insights. This may comprise on the one hand side looking at the integrity of the cell membrane and on the other hand the physiological response of the microorganisms such as the expression of certain genes in response to drying stress. It may also be interesting to elucidate the mode of protection conferred by exopolysaccharide and investigate in how far this is species specific or general. However, despite using sophisticated scientific methods, the practical applicability – including material cost, production procedure and handling - should continuously be kept in the focus to allow for bacterial inoculants to take the step from the laboratory to the field.

10 ADDITIONAL SCIENTIFIC OUTPUT

10.1 ORAL PRESENTATIONS

- T. Berninger, B. Mitter, C. Preininger: Developing a formulation for the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN to be applied as a “biofertilizer”. Workshop on Application Techniques of Endophytes, COST action FA1103, 14. – 15.07.2014, Bielefeld, Germany

- T. Berninger, B. Mitter, C. Preininger: Optimization of alginate bead production and drying methods for the application of plant growth-promoting bacteria in the form of seed coating. XXII International Conference on Bioencapsulation, 17.-19.09.2014, Bratislava, Slovakia

- T. Berninger: Breaking the wall of sustainable agriculture. Falling Walls Lab Vienna, 04.06.2014, Vienna, Austria

- T. Berninger: Breaking the wall of sustainable agriculture. Falling Walls Lab Finale, 07.11.2014, Berlin, Germany

- T. Berninger (invited): Formulierung von Agrarprodukten: Bakterienverkapselung. BÜCHI GmbH Anwenderseminar, 09.06.2015, Vienna, Austria

- T. Berninger (invited): Nutzung mikrobieller Ressourcen zur Verbesserung der Pflanzenproduktion und Nachhaltigkeit in der Landwirtschaft. European Forum Alpbach, Technology Symposium, Plenary Session on Bioeconomy, 28.08.2015, Alpbach, Austria

- T. Berninger, B. Mitter, C. Preininger: Suitable protectants in formulation of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN. DocDay 2015, 13.10.2015, Tulln, Austria

- T. Berninger (invited): Bioökonomie für eine nachhaltige Landwirtschaft. Jugendtag der Landwirtschaftskammer Steiermark, 01.02.1016, Graz, Austria

- T. Berninger (invited): Practical application of beneficial bacteria in agriculture – opportunities and challenges. Forschungszentrum Jülich, Institute of Plant Sciences, 10.02.2016, Jülich, Germany

- T. Berninger, B. Mitter, C. Preininger: Formulation development: Maintaining viability of bacterial inoculants in face of various stresses. Tagung DPG-Arbeitskreis Biologische Kontrolle von Pflanzenkrankheiten, 17.03.2016, Tulln, Austria

10.2 POSTER PRESENTATION

- T. Berninger, B. Mitter, C. Preininger: Improving desiccation tolerance and shelf life of *Burkholderia phytofirmans* PsJN as a prerequisite for application in agriculture. Microp Symposium, 23.-25.11.2015, Vienna, Austria

10.3 OTHER

- Radio radio feature on *Ö1*: Dimensionen: Bioökonomie. Broadcast on 09.07.2015

- Press release in *Die Presse*: Bakterien sicher zur Pflanze bringen. Released on 21.08.2015. URL: http://diepresse.com/home/science/4804244/Bakterien-sicher-zur-Pflanze-bringen?from=gl.home_wissenschaft

- Press release in *Kurier*: Mit weniger Fleischkonsum die Welt nachhaltig ernähren. Released on 28.08.2015.
  URL: http://futurezone.at/science/mit-weniger-fleischkonsum-die-welt-nachhaltig-ernaehren/145.170.092

- Press release APA Science: Zukunftshoffnung Bioökonomie. Released on 25.06.2015.
  URL:
  https://science.apa.at/site/natur_und_technik/detail?key=SCI_20150625_SCI63213285023950786

10.4 AWARDS

FALLING WALLS LAB 2014 VIENNA

Teresa Berninger

has been awarded

1st PLACE

for **BREAKING THE WALL OF**

Sustainable Agriculture

at the Falling Walls Lab Vienna, organised by the Max F. Perutz Laboratories, the Campus Vienna Biocenter, the Austrian Institute of Technology, the Falling Walls Foundation and supported by A.T. Kearney and Festo on June 4, 2014.

Prof. Dr. Jürgen Mlynek
Chairman of the Board of Trustees
Falling Walls Foundation

Prof. Dr. René Schroeder
Chairman of the Jury Vienna
MFPL

DI Anton Plimon
Managing Director
AIT

DI Dr. Michaela Fritz
Head of Health & Environment
AIT

Prof. Dr. Graham Warren
Scientific Director
MFPL

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AIT AUSTRIAN INSTITUTE OF TECHNOLOGY

Wissenschaftspreis Rotary Club Tulln

Der Rotary Club Tulln verleiht laut Beschluss der Jury

Prof. Dr.med.vet. Martin Wagner
Dr. Birgit Tauber
Norbert Payr
Mag. Irma Priedl
DI Andreas Scheed
DI Heinrich Feketitsch

an Frau *Teresa Berninger*

den **3. Preis**

für ihre hervorragende wissenschaftliche Arbeit
„Small scale production of bacteria encapsulated beads by extrusion and spraying"

Präsident Rotary Club Tulln, Mag. Ismail Sadek

Vorsitzender Jury, DI. Heinrich Feketitsch

Tulln, 16. Juni 2014

INTERNATIONAL SYMPOSIUM

MICROBE-ASSISTED CROP PRODUCTION – OPPORTUNITIES, CHALLENGES & NEEDS

NOV. 23 – 25, 2015

SCHLOSS SCHÖNBRUNN TAGUNGSZENTRUM
APOTHEKERTRAKT | VIENNA, AUSTRIA

miCROPe Best Poster Award 2015

in the amount of € 200,-

for

Teresa Berninger

in recognition for her/his excellent performance at the international miCROPe 2015 Symposium

for presenting the work with the title:

Improving desiccation tolerance and shelf like of Burkholderia phytofirmans PsJN as a prerequisite for application in agriculture

Vienna, November 25th 2015

Doz. DI. Dr. Angela Sessitsch

Organized by: ÖGMBT - Austrian Association of Molecular Life Sciences and Biotechnology, AIT - Austrian Institute of Technology

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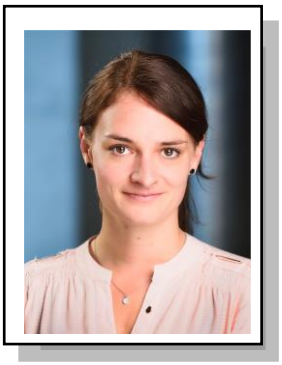

August 2013 – present

Dissertation in Microbiology/Agricultural Sciences:

- BOKU University of Natural Resources and Applied Life Sciences, Vienna
- Doctoral research at AIT Austrian Institute of Technology GmbH, Health & Environment Department, Unit Bioresources, Tulln

March 2011 – August 2013

DI Agricultural Biology

- BOKU University of Natural Resources and Applied Life Sciences, Vienna
- Master's thesis at AIT Austrian Institute of Technology GmbH Health & Environment Department, Unit Bioresources, Tulln
  Title: "Colonization of reproductive organs of pepper and tomato plants by the beneficial endophyte *Burkholderia phytofirmans* PsJN"

September 2007 – February 2011

B.Sc. Nutritional Science and Home Economics

- Justus Liebig University, Gießen (Germany)
- Erasmus semester at Corvinus University of Budapest (Hungary)
- Bachelor's thesis at Institute of Food Chemistry and Food Biotechnology
  Title: „The novel food regulation"