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Starch filler and osmoprotectants improve the survival of rhizobacteria in dried alginate beads

Mauricio Schoebitz[1,2], Hélène Simonin[1] and Denis Poncelet[1]

[1]*Département de Génie des Procédés Alimentaires, ONIRIS, Rue de la Géraudière, BP82225, 44322 Nantes Cedex 03, France and* [2]*Facultad de Ciencias Agrarias, Instituto de Producción y Sanidad Vegetal, Universidad Austral de Chile, Valdivia, Chile*

Abstract
This work deals with optimising the cell survival of rhizobacteria encapsulated in alginate beads filled with starch. Immobilisation of rhizobacteria was done by dripping alginate-starch solution mixed with rhizobacteria into a calcium solution. Beads were analysed based on matrix formulation, bacteria growth phase, osmoprotectants and nature of calcium solution. Maximum cell recovery was obtained on *Raoultella terrigena* grown in medium supplemented with trehalose and calcium gluconate as gelling agent. Furthermore, dried beads containing *Azospirillum brasilense* presented 76% of viable cells after one year of storage. The survival of rhizobacteria during the bioencapsulation process can be improved by incorporating starch on beads composition, varying the growth phase of cells and using trehalose in growth culture medium. This work provides a selection of appropriate methods to improve the surviving rate of encapsulated cells during their production and long-term storage (~1 year at 4°C).

Keywords: bioencapsulation, Azospirillum, Raoultella, cell survival, inoculants, alginate beads

Introduction

Synthetic chemical carriers are being efficiently replaced by agroindustrial-based carriers for inoculant production (Singh et al., 2011). Microbial inoculants are based on peat formulations that cannot ensure a high cell density. For example, Fallik and Okon (1996) showed reduction of cell concentration after 6 months of storage of peat inoculated with *A. brasilense* (decreased from 10^{10} to 10^5 CFU g^{-1}).

Cell immobilisation in biopolymer gels has been shown to be more valuable than peat inoculation (Cassidy et al., 1996), protecting the microorganisms against environmental stressors and competitors (Bashan, 1998). Bioencapsulation of active compounds on calcium alginate beads achieves certain desirable effects, such as stabilisation, protection and controlled release (Chan et al., 2009). Furthermore, dried beads can be stored for months and are easier to handle for agricultural applications. In 1983 dry alginate beads were produced containing *A. brasilense* and recovered in 1996 with significant cell survival, around 10^5–10^6 CFU g^{-1} of beads, and good maintenance of the original physiological features of the cells (Bashan and Gonzalez, 1999). However, loss of cell activity or viability during the drying step continues to be the main difficulty of the living cell bioencapsulation process. According to several authors (Fages, 1990, 1992; Paul et al., 1993), the number of viable cells decreases (0.1% of viability) during the whole bioencapsulation process, especially during the drying step, as it is essential to retain biological activity of the beads during long storage periods (Bashan and Gonzalez, 1999) and to enable commercialisation of the inoculants carriers.

On the other hand, alginate beads are structured as a loose network filled with a large quantity of water. Thus, using alginate alone for cell bioencapsulation would not

Address for correspondence: Mauricio Schoebitz, Campus Isla Teja s/n Valdivia, Chile. Tel: +56-6329-3950. Fax: +56 6322 1233. E-mail: mschoebitz@gmail.com

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adequately protect cells during the drying process and results in slightly distorted beads. Filler materials, such as starch or clay, may be added to the formulation to increase the dry matter in the beads, increasing mechanical resistance and allowing for a progressive release of cells into the soil (Bashan et al., 2002).

Bacteria are not able to physiologically respond to stress when they are desiccated. The use of osmoprotectants may increase cells survival during desiccation (Vriezen et al., 2007). Osmoprotectants, like sugars, provide protection against desiccation by maintaining membrane integrity during drying and rewetting (Leslie et al., 1995).

In this study, two rhizobacteria, *Azospirillum brasilense* and *Raoultella terrigena*, were encapsulated in alginate-starch and alginate-clay beads. *A. brasilense* was isolated from tropical regions where its effect on the growth and yield of numerous plant species had been documented in the last decades (Okon and Labandera-Gonzalez, 1994). However, *R. terrigena* is a recently re-classified bacterium (Drancourt et al., 2001) and a promising strain to promote plant growth in temperate zones.

The aim of this study was to improve the survival of these two rhizobacteria species by encapsulating it in alginate beads supplemented with starch, which allows the stable production of dried beads containing a high cellular concentration.

Materials and methods

Micro-organisms and culture conditions

Azospirillum brasilense Sp245 and *Raoultella terrigena* TFi08 were provided by the Catholic University of Leuven, Belgium, and the Austrian Research Center, Vienna, Austria, respectively. Both strains were grown in 30 ml of sterile YEP medium adjusted to pH 6.5 and containing 10 g l^{-1}peptone casein (Biokar, France), 5 g l^{-1}yeast extract (Biokar Diagnostic, France), and 5 g l^{-1}sodium chloride (Fisher Scientific, UK). In some cases, YEP medium was supplemented with 3 mmol l^{-1} of trehalose (Sigma-Aldrich, France) or 3 mmol l^{-1} of fructose (Merck, Germany). Cultures were incubated on a rotary shaker (120 rev min^{-1}) at 30°C to harvest cells in their exponential (12 h) or stationary (24 h) growth phase.

Bioencapsulation process

All the material used for the bioencapsulation process was previously sterilised and the process was carried out under aseptic conditions. The matrix solution was prepared by mixing alginate and starch (or clay) in distilled water in the following way. Three grams of sodium alginate Satialgine S60 (Cargill, France) were dissolved in 100 ml of distilled water and stirred for 30 min to obtain a homogeneous solution (viscosity of alginate at 1% solution: 34 centipois). The composition of alginate-starch matrix was given as follows: 45 g of standard corn-starch (Sigma-Aldrich) and 2 g of modified starch (Cleargum, Roquette, France) were added to the alginate solution. For the alginate-clay matrix, 7 g of bentonite clay (Sigma-Aldrich) were added to the alginate solution. The matrices were then stirred for 30 min for homogenisation. Next, 30 ml of the culture was centrifuged (8720 × *g* for 10 min at 4°C). The pellet was re-suspended in 3 ml of 1% peptone and the suspension was mixed with 30 ml of matrix solution. The matrix mixture containing cells was transferred to the syringe (50 ml).

The matrix solution was placed in a pump-syringe and dropped into sterile calcium chloride or calcium gluconate (0.1 $moll^{-1}$). Gelling of alginate-starch beads was completed after 30 min in contact with the calcium solution. The collected beads were placed on a filter paper in a Petri dish and dried in an oven at 35°C for 24 h (35% final dry matter and 0.3–0.5 final water activity). They were then stored in hermetically sealed plastic flasks at 4°C.

Bead bacterial counts

Ten beads were dissolved in 10 ml sterilised sodium citrate solution (pH 8.5; 60 g l^{-1}) for 30 min at 20°C in a rotary shaker until complete dissolution. Then the number of released bacteria was determined by the standard plate count method on YEP agar. Results were expressed in total CFU (obtained from 30 ml of cell growth culture or matrix solution).

Statistical analysis

In this study, trials were replicated three times and then the results presented are the averages of all experiments. Statistical analysis was carried out by Student's test with $p \leq 0.05$ and data were analysed by Statgraphics statistics software (STATGRAPHICS Centurion XV.II).

Scanning electron microscopy

Dried alginate-starch beads containing *R. terrigena* were prepared for observation under a scanning electron microscope (JEOL JSM-6400 F, Japan). Cell fixation was achieved by adding 2% (v/v) glutaraldehyde solution, following the protocol of Guillaumin (1980).

Results

Change in the number of viable cells during the whole bioencapsulation process

The number of viable cells was counted at different steps of the bioencapsulation process: initial culture, matrix, wet beads and dry beads. The stationary growth phases of rhizobacteria were used to form dried alginate-starch beads. The results are presented in Figure 1. For *A. brasilense*,

alginate and alginate-clay beads were also produced to study the influence of the matrix formulation on cell survival. All these results are reported in Table 1A.

The total cell number in the standard bioencapsulation process was not modified significantly during bead gelling. However, cell viability dropped drastically after the drying process and the cell number decreased by one log during this step for both bacterial species, meaning that it was the critical step of the bioencapsulation process. In alginate beads, the initial cell number in the growth medium decreased after the drying process and cell recovery was lower than 1% of the original cell count (Table 1A). The addition of bentonite clay to the matrix formulation did not lead to a significant improvement in *A. brasilense* survival after drying. The alginate-starch beads gave the best yield for *A. brasilense* survival cell during the bioencapsulation process reaching 10% of the initial cell number. Alginate-starch beads were also tested for their ability to protect *R. terrigena* cells against drying. After bead drying, the free living cell density in beads was around 10^9 CFU g^{-1}. The matrix formulation containing alginate and starch provided the best result regarding cell survival during the bioencapsulation process. Nevertheless, the loss of viable cells remained significant during the drying step, regardless of the bead formulation. Thus, various experiments were carried out to improve cell survival in the drying process using alginate-starch beads.

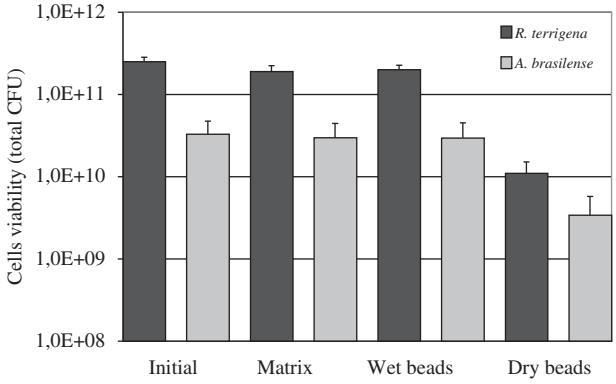

Figure 1. *A. brasilense* and *R. terrigena* survival at different steps of the bioencapsulation process in alginate-starch beads. Cells were cultivated in YEP standard medium and gelling was carried out with $CaCl_2$. YEP medium (10 g l^{-1}, peptone casein 5 g l^{-1}, yeast extract and 5 g l^{-1} NaCl).

Table 1A. Number of viable *A. brasilense* cells (total CFU) in dried alginate, alginate-clay and alginate-starch beads.*

Encapsulated gels type	Initial culture total CFU	Dry beads total CFU	Yield %
Alginate	$(5.6 \pm 2.0) \times 10^{10}$	$(3.7 \pm 5.1) \times 10^{8}$ (a)	0.63
Alginate-clay	$(7.4 \pm 0.7) \times 10^{10}$	$(4.5 \pm 0.5) \times 10^{8}$ (a)	0.60
Alginate-starch	$(3.3 \pm 1.4) \times 10^{10}$	$(3.4 \pm 2.3) \times 10^{9}$ (a)	10.4

Notes: *Cells were cultivated in YEP standard medium and gelling was carried out with $CaCl_2$. Mean ± standard errors. Significant differences according to Student's test with $p \leq 0.05$. YEP medium (10 g l^{-1}, peptone casein 5 g l^{-1}, yeast extract and 5 g l^{-1} NaCl).

Microscopic observation of alginate-starch beads

Dry alginate-starch beads were spherical in shape with a diameter ranging from 2.3 ± 0.16 mm (the decrease in diameter during drying was about 50% of the wet beads), whereas the alginate-clay matrix provided smaller spherical beads, around 1.33 ± 0.03 mm in diameter, and alginate presented very small wrinkled beads, 1.05 ± 0.11 mm in diameter (Figure 2). The distribution of alginate and starch in dried beads was homogeneous and rhizobacteria were spread at the starch granule surface and homogeneously distributed throughout the whole bead volume (Figure 3).

Influence of the physiological phase of cells on their survival during the drying step

Alginate-starch beads were prepared using cells collected during the exponential or stationary growth phase of cultures. The results of cell survival for *A. brasilense* and *R. terrigena* after bead drying are presented in Table 1B. *A. brasilense* in the log phase presented only 0.3% cell survival after the bioencapsulation process. In contrast, when cells were immobilised in the stationary phase, the survival yield reached 10%. For *R. terrigena,* survival rates were around 6% and 4% from the exponential and stationary growth phase, respectively, showing no significant difference ($p < 0.05$).

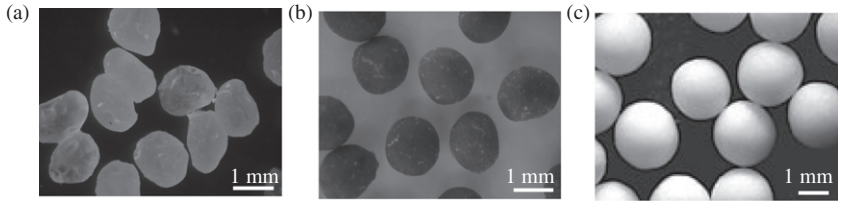

Figure 2. Macroscopic appearance of beads for different matrix formulations: (a) alginate; (b) alginate-clay; (c) alginate-starch.

Influence of osmoprotectants on cell survival during the drying step

As drying is the critical point of the bioencapsulation process, the addition of osmoprotectants could improve cell survival for that different osmoprotectants were tested as follows. YEP medium was supplemented with 3 mmoll^{-1} trehalose or fructose and the cell survival rate was compared with cells grown in standard YEP medium without osmoprotectants. The results are presented in Table 2.

The initial cell count was not influenced by addition of sugar to the medium. All the cultures reached about 10^{10}–10^{11} total CFU at the end of the stationary growth phase. For *A. brasilense*, the survival rate for standard medium and standard medium supplemented with trehalose or fructose was 10, 4 and 12%, respectively. The statistical test ($p < 0.05$) showed that these results were not significantly different. The initial cell count in cultures of *R. terrigena* reached about 10^{11} total CFU at the end of the stationary growth phase. The survival rate was 4, 7 and 19% in dried beads for cells cultivated in standard medium, and medium supplemented with fructose or trehalose, respectively. In fact, the use of trehalose in the culture medium reached 7.5×10^{10} total CFU at the end of the process. Trehalose had no effect on cell survival rate when it was added to the bioencapsulation matrix (result not shown). Thus, trehalose only improved *R. terrigena* survival during the drying process when it was added to the culture medium.

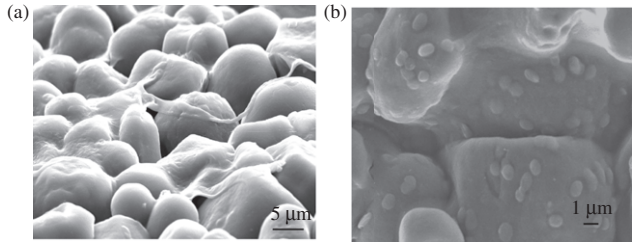

Figure 3. Scanning electron microscope pictures of alginate-starch beads: (a) distribution of alginate and starch granules on the surface of dried beads; (b) *R. terrigena* immobilised on and adhered to the surface of starch granules.

Influence of the calcium source on cell survival

A. brasilense was encapsulated in beads formed with calcium gluconate and the initial cell number was 2.8×10^{10} total CFU, which decreased to 6.5×10^{6} total CFU after drying. This represents a yield of only 0.02% in cell survival, which is significantly lower than that obtained by using the traditional calcium chloride for alginate gelling (10%) ($p < 0.05$). Furthermore, a significant decrease in the living cell number was observed during the transfer into the matrix as only 27% of the initial cell count could be recovered after this step. In contrast, *R. terrigena* encapsulated on beads formed with calcium gluconate presented a survival yield of 31% after drying. This is significantly higher

Table 1B. Number of viable *A. brasilense* and *R. terrigena* cells (total CFU) encapsulated in alginate-starch beads.*

Encapsulated gels type	Growth phase	Initial culture total CFU	Dry beads total CFU	Yield %
A. brasilense	Logarithmic	$(1.3 \pm 0.3) \times 10^{10}$	$(3.7 \pm 0.5) \times 10^{7}$ (b)	0.3
	Stationary	$(3.3 \pm 1.4) \times 10^{10}$	$(3.4 \pm 2.3) \times 10^{9}$ (a)	10.4
R. terrigena	Logarithmic	$(4.3 \pm 1.3) \times 10^{11}$	$(2.6 \pm 0.4) \times 10^{10}$ (a)	6.1
	Stationary	$(2.5 \pm 0.3) \times 10^{11}$	$(1.1 \pm 0.4) \times 10^{10}$ (a)	4.2

Notes: *Cells were cultivated and immobilised in the logarithmic or stationary growth phase. Mean ± standard errors. Significant differences according to Student's test with $p \leq 0.05$.

Table 2. Influence of addition of trehalose and fructose to cell growth medium on *A. brasilense* and *R. terrigena* viability after bioencapsulation.

Encapsulated gels type	Growth medium	Initial culture total CFU	Dry beads total CFU	Yield %
A. brasilense	YEP	$(3.3 \pm 1.4) \times 10^{10}$	$(3.4 \pm 2.3) \times 10^{9}$ (a)	10.4
	+fructose	$(2.7 \pm 0.6) \times 10^{10}$	$(1.1 \pm 5.1) \times 10^{9}$ (b)	4.2
	+ trehalose	$(5.2 \pm 0.4) \times 10^{10}$	$(6.5 \pm 1.6) \times 10^{9}$ (a)	12.4
R. terrigena	YEP	$(2.5 \pm 0.3) \times 10^{11}$	$(1.1 \pm 0.4) \times 10^{10}$ (a)	4.2
	+ fructose	$(1.3 \pm 0.2) \times 10^{11}$	$(8.9 \pm 3.9) \times 10^{9}$ (a)	6.6
	+ trehalose	$(8.4 \pm 0.2) \times 10^{11}$	$(4.2 \pm 5.7) \times 10^{10}$ (a)	19.0

Notes: Mean ± standard errors. Significant differences according to Student's test with $p \leq 0.05$. YEP medium (10 gl^{-1}, peptone casein 5 gl^{-1}, yeast extract and 5 gl^{-1} NaCl).

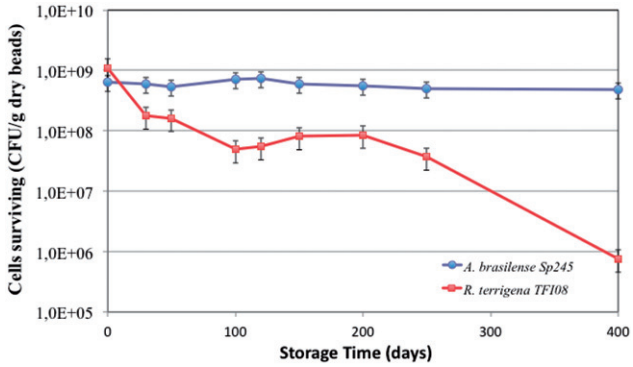

Figure 4. *A. brasilense* and *R. terrigena* survival during storage at 4°C in dried alginate-starch beads. Cells were cultivated in YEP standard medium and gelling was carried out with $CaCl_2$. YEP medium (10 g l^{-1}, peptone casein 5 g l^{-1}, yeast extract and 5 g l^{-1} NaCl).

than the yield obtained by using the traditional $CaCl_2$ for alginate gelling (4%) ($p < 0.05$).

Cell survival in dried beads during storage at 4°C

Cell survival during storage at 4°C was monitored over one year. The results obtained for the two bacterial species are shown in Figure 4. Both bacteria were cultivated in standard YEP medium and harvested in the stationary growth phase. Beads were formed using $CaCl_2$. The viability was expressed as CFU g^{-1} of dried beads. The number of viable cells in alginate-starch beads containing *A. brasilense* remained constant at around 10^9 CFU $g^{-1}$of dried beads during one year of storage at 4°C. In contrast, *R. terrigena* showed a gradual decline in cell survival during storage. The initial cell number was 10^9 CFU $g^{-1}$of dried beads but this decreased to about 10^8 CFU g^{-1} after 100 days of storage. It remained constant during the following 100 days and then decreased drastically to reach 10^6 CFU g^{-1} after one year of storage.

Discussion

This work shows drying step as the critical step of the bioencapsulation process for cell survival. This is in agreement with the results obtained by Paul et al. (1993) who demonstrated that a large proportion of cells are destroyed during bead dehydration. In fact, a decrease in the number of cells was observed (Figure 1). Cell mortality during the drying of encapsulated cells has been recognised as the critical point of the bioencapsulation process by various authors (Paul et al., 1993; Cassidy et al., 1995; Bashan et al., 2002). In these latest studies, the survival yield after drying did not exceed 1% of the initial cell count for various Azospirillum and Pseudomonas species. Drying involves great physical stress for viable cells, including an increase in osmotic pressure that causes a water outflow leading to plasmolysis, and cell death is mainly attributed to the disruption of the plasma membrane (Mille et al., 2002). Nevertheless, the survival of dehydrated cells depends on various factors such as: drying conditions, micro-organism species, adjuvant used, bacterial strain and culture conditions (Morgan et al., 2006). The drying kinetics has been shown to be of particular importance for cell survival with a detrimental effect of fast drying (Paul et al., 1993; Poirier et al., 1997). Alginate beads contain about 97% water and such a matrix failed to protect cells during drying as a two log decrease in *A. brasilense* cell number was observed. Using starch in the bioencapsulation matrix enabled the water content to be reduced to 65% and significantly improved cell survival. This could be due to a decrease in bead drying speed resulting from the lower water content. There is a wide choice of materials for bioencapsulation ingredients such as clay (Marshall, 1968), skim milk powder (Bashan et al., 2002), humic acid (Young et al., 2006) and starch (Wang et al., 1999; Crittenden et al., 2001; O'Riordan et al., 2001), which have been used on living cell carrier formulations. In addition, it is necessary to take into account that starch is an inexpensive material for bead formation as it is one of the most abundant natural biopolymers (Hickman, 1999) and, as such, could be used as a nutrient by several soil bacteria, which could help with the release of cells into the soil and their development within the soil. Previous studies on probiotic carriers have revealed a protective effect of starch due to cell adhesion to granules (Wang et al., 1999; Crittenden et al., 2001). Besides slowing the drying rate, the ability of granular starch to protect the rhizobacteria from drying stress may be due to cell adhesion to this matrix. This hypothesis is supported by microscopic observations of bead sections showing bacteria at the surface of the starch granules. Cell adhesion to starch may depend on the strain encapsulated since there is a link between adhesion to starch and its use as a carbon substrate by the cells (Crittenden et al., 2001). After testing the best survival rates with the alginate-starch matrix, various strategies were analysed to improve cell survival during the drying process using this formulation.

It is generally admitted that stationary phase cells are more resistant to physical stresses, such as dehydration, than cells harvested in the exponential growth phase (Vriezen et al., 2006). Our results showed that the survival of *A. brasilense* in dried beads was dependent on the growth phase, whereas no influence of the growth phase was observed for *R. terrigena*. This difference in the behavior of the two bacterial species is not surprising because the optimal growth phase for desiccation survival has been found to be largely dependent on the micro-organism (Boumahdi et al., 1999). Nevertheless, stationary phase cells are generally more resistant to physical stresses such as drying (Mary et al., 1986; Vriezen et al., 2006). This is due to the stress response triggered by carbon starvation and exhaustion of available food sources (Morgan et al., 2006). *A. brasilense* is able to produce high levels of polyhydroxyalkanoates (PHA) in the stationary phase since during nutritional starvation, the bacteria can accumulate up to 75% of the cell dried matter as PHA (Kadouri et al., 2003).

Table 3. Influence of calcium salt type (chloride or gluconate) used as a gelling agent on the survival of *A. brasilense* and *R. terrigena* at different steps of the bioencapsulation process.

Encapsulated gels type	Gelling solution	Initial culture total CFU	Bead formation total CFU	Dry beads total CFU	Total yield %
A. brasilense	Calcium chloride	$(3.3 \pm 1.3) \times 10^{10}$	$(2.3 \pm 0.2) \times 10^{10}$	$(3.4 \pm 2.3) \times 10^{9}$ (a)	10.4
	Calcium gluconate	$(2.8 \pm 0.1) \times 10^{10}$	$(7.7 \pm 0.4) \times 10^{9}$	$(6.5 \pm 0.8) \times 10^{6}$ (b)	0.02
R. terrigena	Calcium chloride	$(2.5 \pm 0.3) \times 10^{11}$	$(2.0 \pm 0.3) \times 10^{11}$	$(1.1 \pm 0.4) \times 10^{10}$ (a)	4.2
	Calcium gluconate	$(2.7 \pm 0.1) \times 10^{11}$	$(2.0 \pm 0.4) \times 10^{11}$	$(8.4 \pm 2.9) \times 10^{10}$ (a)	31.0

Notes: Mean ± standard errors. Significant differences according to Student's test with $p \leq 0.05$.

This energy and carbon storage compounds may increase bacterial survival under stress conditions.

In contrast, no survival increase was found in *R. terrigena* in stationary phase. The metabolism of this recently isolated bacterial species is not yet well known. This may mean that *R. terrigena* does not produce any intracellular storage substances during desiccation or nutrient starvation.

Protective agents can be added either during the growth of microorganisms or previous drying process improving many living cell species. These are mainly sugars (fructose, glucose) and non-reducing sugars (e.g. trehalose) (Morgan et al., 2006). For this reason the use of osmoprotectant molecules, such as trehalose and fructose, were also tested. The addition of trehalose to the growth medium increased the survival of *R. terrigena* during the drying process. Moreover, adding trehalose to the growth medium was much more effective in protecting against desiccation than adding it to the matrix solution just prior to drying. This could mean that trehalose must be accumulated by cells to implement its protective effect. This result is consistent with the work by Streeter (2003) on the survival of *Bradyrhizobium japonicum* during desiccation. In this study, the accumulation of trehalose within the cell cytoplasm contributed to membrane stabilisation during desiccation. However, the accumulation of intracellular trehalose is only possible if the microorganisms cannot use it as a carbon source (Streeter, 2003).

Supplementation with fructose did not protect the bacteria studied against the drying phase either to the growth medium or to the matrix prior to bead formation. This could be explained by the fact that *A. brasilense* is able to degrade fructose by using it as a carbon source.

Gluconate could also act as an osmoprotectant given that it provided strong protection against dehydration for *R. terrigena*. Gluconate arises from the oxidation of glucose and is a possible gelling agent not yet tested in cell bioencapsulation for environmental applications. Actually, $CaCl_2$ is normally used with this aim (Krasaekoopt et al., 2004). On the other hand, the use of calcium gluconate was detrimental to *A. brasilense* survival, during both cell transfer to the matrix and bead drying (Table 3). This result confirms that the effect of protective agents depends on the microorganism and should be evaluated in each individual case.

Finally, the number of viable cells was assessed during long-term storage over one year at 4°C (Figure 4). Making sure good protection of bacteria during a long storage period is a crucial objective of living cell bioencapsulation (Fages, 1992; Bashan et al., 2002). The alginate-starch beads kept a constant number of living *A. brasilense* (10^8 CFU g^{-1}). In the literature, *Azospirillum lipoferum*, *Bacillus subtilis* and *Pseudomonas corrugata* have been shown to survive long-term storage in the dried state (Fages, 1992; Trivedi and Pandey, 2008). However, our results show that *R. terrigena* did not exhibit good resistance to storage because a decrease on living cells number was observed after one year (10^6 CFU g^{-1}). This could be attributed to the inability of this strain to produce carbon storage compounds under stress conditions. Besides, it would be interesting to study the influence of adding protective sugars to the matrix on the survival of this strain during storage.

As we can see, improving cell survival during bioencapsulation is not a simple procedure since it depends on diverse factors: growth media composition, strain on the cells, physiological state of cells and bioencapsulation process parameters. Each factor should be optimised to ensure the best activity of inoculants after drying and during storage. By combining these factors, we could obtain biodegradable and dried inoculants with a high living cell density for the two bacterial strains tested.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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