Cells viability of plant growth promoting rhizobacteria in alginate-starch beads

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Introduction

Several inoculants carriers formulations in agriculture based on polymers have been evaluated in last decades, offering substantial advantage over other inoculants (Jung et al., 1982; Fages, 1990; 1992). These formulations immobilized cells, protect the microorganisms against environmental stresses and release them to the soil gradually when the polymers are degraded (Bashan, 1998).

Several studies have used alginate as the encapsulating material as it forms beads. Soil inoculants must be used in a dry form, but the drying of alginate beads reduces viable cells numbers to about 1% of the original cell count (Jung et al., 1982; Paul et al., 1993). For that, Bashan et al. (2002) proposed a secondary multiplication of the entrapped bacteria in the already formed beads. However, it would be expensive and difficult to implement at industrial level.

Starch increases the dry matter concentration of beads and thus reduces the time and cost of drying process. It is one of the most abundant and available biopolymers. Thus, it is a cheap material for capsules formation. It provides a carbon source for soil microorganisms and it permits a slow cell release into the soil (Ivanova, 2006).

The present study aims at improving cell survival of PGPR during encapsulation and storage using alginate supplemented with starch. It is part of an overall work of improvement of inoculants carriers for PGPR and represents a first promising step towards application at an industrial scale.

Material and Methods

Cell growth

Raoultella terrigena TFi08 and *Azospirillum brasilense* Sp245 were grown in 30 ml of sterile YEP medium adjusted to pH 6.5 containing peptone casein (Biokar Diagnostic, France) 10 g l^{-1}, yeast extract (Biokar Diagnostic, France) 5 g l^{-1}, sodium chloride (Fisher Scientific, UK) 5 g l^{-1}. In some cases, it was supplemented with trehalose (3mM). Cultures were performed on a rotary shaker (120 rev min^{-1}) at 30°C for 24 h. Cells were harvested in stationary growth phase.

Entrapment process

All the material used for the encapsulation process was previously sterilized at 120°C during 15 min and the process was carried out under aseptic conditions.

The matrix solution was prepared by mixing alginate and starch in distilled water in the following way. Three grams of sodium alginate (Satialgine S 60) were dissolved in 100 ml of distilled water and kept under stirring during 30 min to obtain a homogeneous solution. 44.6 g of standard corn starch (Sigma) and 2.4 g of modified starch (Cleargum®, Roquette) were mixed to the alginate solution.

Thirty ml of culture were centrifugated (8.720 *x g*, 10 min at 4°C). The pellet was resuspended in 3 ml of peptone solution 1%, transferred in 30 ml of matrix solution and mixed for 15 min to obtain

an homogeneous solution. The matrix mixture containing cells was transferred to the dropping device (50 ml syringe linked to a pump).The capsules were formed immediately with the contact of the droplets with sterile Ca^{2+} chloride or gluconate solution (0,1 M). Gelling of alginate-starch beads was completed after 30 min stirring.

Collected beads were placed on a filter paper in a Petri dish and dried in stove at 35°C, during 24 h. The final diameter of the dry beads was about 1-2 mm. The beads were stored in plastic flasks hermetically closed at 4°C.

Measurement of cell survival

Ten beads were dissolved in 10 ml sterile sodium citrate solution 6% for 30 min at room temperature in a rotary shaker, after that the beads were completely dissolved. One ml of sodium citrate solution were collected, and the number of released bacteria was determined by standard plate count method in YEP agar. Results are expressed in CFU Total/ batch.

Result and Discussion

Figure 1 presents the evolution of the total cell count at each step of the bioencapsulation process. The initial number of cells is $2.5x10^{11}$ for *R. terrigena* and $2.7x10^{10}$ for *A. brasilense*. For both bacteria, the cell number decreases when cells are transferred from the culture to the matrix solution. Indeed, 24% of cells are lost at this step for *R. terrigena* and 20% for *A. brasilense*. This could be due to the interaction of cells with starch granules. Actually, some authors report a strain-dependent cell adsorption to starch during bioencapsulation (Lahtinen et al., 2007). Such an adsorption could prevent cell multiplication. This hypothesis is at this time under investigation.

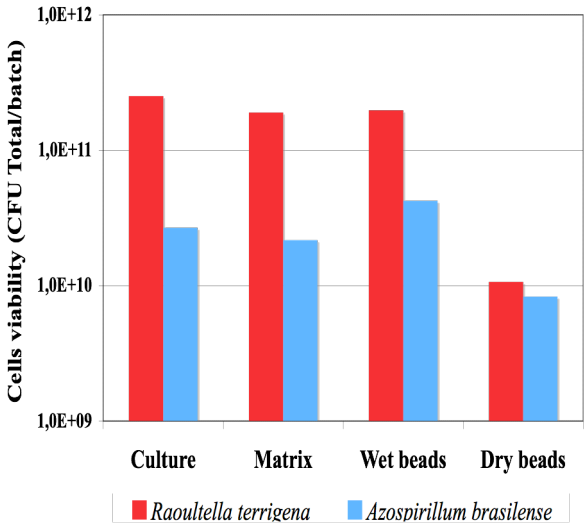

Figure 1. Number of living cells measured by CFU total/batch for *R. terrigena* and *A. brasilense* in each step of the bioencapsulation. Cells were grown without trehalose and beads were formed using Ca^{2+} chloride solution.

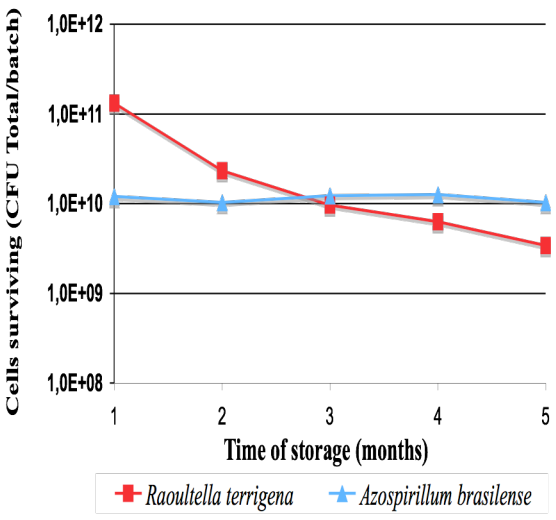

Figure 2. Number of living cells measured by CFU total/batch in dried alginate-starch beads during storage at 4°C.

In wet beads, the number of living cells increases in comparison with the matrix from $1.9x10^{11}$ to $2.0x10^{11}$ for *R. terrigena* and $2.2x10^{10}$ to $4.3x10^{10}$ for *A. brasilense*. The transfer of cells from matrix to wet beads lasts around 30 minutes and cell multiplication could occur in this step as the matrix contains 1% peptone as carbon source.

The drying process was deleterious for cell viability and particularly for *R. terrigena* as a decrease of 95% in cell count was measured in this step. It is well known that drying cells and especially bacteria leads to a high mortality, the rate of which is strongly strain dependent (Morgan, 2006).

During the capsule storage (Figure 2), the number of viable cells is constant for *A. brasilense* but decreases gradually for *R. terrigena*. This result demonstrates again that the behavior of bacteria cells to encapsulation is strongly specie dependant as already shown by Paul et al. (1993).

So as to improve the cell survival to the bioencapsulation process of *R. terrigena*, Ca^{2+} chloride and Ca^{2+} gluconate were tested for gelification and 3mM of trehalose was added to the cells growth medium.

The number of viable cells measured before and after the complete bioencapsulation process of *R. terrigena* is given in the table 1 for two Ca^{2+} salts: gluconate and chloride. Results show that the cell survival rate is significantly increased when Ca^{2+} gluconate is used as a gelification salt. The cell survival was measured at each step of the process and Ca^{2+} gluconate permits to improve cell survival in the drying step (results not shown). Gluconate is a glucose derivative that could protect cells during drying as glucose do (Hubalek, 2003).

Table 1. Cells viability of *R. terrigena* using two different source of Ca^{2+} in gelification process.

	Culture (CFU Total)	Dry capsules (CFU total)	Yield (%)
Ca^{2+} Chloride	**$2,5x\ 10^{11} \pm 3,4x10^{10}$**	**$1,1x10^{10} \pm 4,1\ x10^{9}$**	**4**
Ca^{2+} Gluconate	**$2,7\ x10^{11} \pm 1,0\ x10^{10}$**	**$8,4\ x10^{10} \pm 2,9\ x10^{10}$**	**30**

Additionally, *R. terrigena* was cultivated in a growth medium containing 3 mM of trehalose. Because, it has been reported to stabilize membrane structure under conditions of desiccation (Crowe et al, 1984). Table 2 shows an increase of 28% of cells viability, when *R. terrigena* was cultivated in a growth media containing trehalose. The use of disaccharides like as osmoprotectants in *Bradyrhizobium* obtained an increase of 294% of cells viability after 24 h of dehydratation (Streeter, 2003).

Table 2. Effect of the incorporation of substances osmoprotectants in YEP growth media

	Culture (CFU Total)	Dry capsules (CFU total)	Yield (%)
YEP	**$2,5x10^{11} \pm 3,4\ x10^{10}$**	**$1,1\ x10^{10} \pm 4,1\ x10^{9}$**	**4**
YEP + trehalose	**$2,4\ x10^{11} \pm 1,5\ x10^{10}$**	**$7,5\ x10^{10} \pm 5,7\ x10^{10}$**	**32**

Conclusions

In this study we observed during the bioencapsulation process, that the use of starch and alginate based formulation permits to preserve 4% of *R. terrigena* and 30% of *A. brasilense*. Only 1% of cell survival is reported for encapsulation with alginate alone.

This result shows the importance of using this type of biopolymer in beads formation, because protect the cells against the desiccation, principal part of the process of diminution of cells viability. Trehalose and Ca^{2+} gluconate were efficient to protect *R. terrigena* against desiccation under experimental conditions. The use of osmoprotectants in growth medium and bead formation increases cells viability in 26 to 28%.

After 5 months of storage at 4°C in alginate-starch beads, *R. terrigena* present 3% and *A. brasilense* 86% of cells survival, that shows that viability of cells depends directly on type of PGPR strain and beads formulations.

Optimization of the cells viability during long storage period and suitable formulations to ensure good protection of bacteria in soil and to provide a product easy to apply are crucial objectives. They can be stored dry for prolonged period, and can be manipulated easily according to the needs of specific bacteria or the crops. Counts over 10^{10} CFU Total, represent one log fold higher than classical soil inoculants carriers.

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