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An optimized process for manufacturing an *Azospirillum* inoculant for crops

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Summary. An optimized process for manufacturing a crop inoculant was developed with an *Azospirillum lipoferum* strain. This process involves the entrapment of living cells in alginate beads and dehydration. The influence of several parameters, alginate concentration, additions of adjuvants at different stages, dilution of culture broth, water activity and dehydration method on bacterial survival is presented. The highest survival was obtained by addition of skim milk and controlled air-dehydration of the alginate beads. Finally, a powdered inoculant was obtained, containing more than 10 billion cells/g, easy to store and to handle, which can be used in the field as a microgranule or as a seed coating. Furthermore, the biodegradability insures that there is no environmental pollution.

Introduction

Several micro-organisms can exert beneficial effects on plant growth. However, the release of such micro-organisms in soil does not always lead to the expected effects (Smith et al. 1984; Okon and Hadar 1987). The inoculum application method used in the field could be a major parameter explaining inconsistency of the results.

In the last few years, several new inocula formulations for agriculture have been proposed including polyacrylamide-based inoculants (Dommergues et al. 1979) and several biopolymers have been shown to be good carriers for agricultural inoculants (Jung et al. 1982). These carriers permit entrapment of living cells, protecting the organisms against stresses. In addition, microorganisms are released into the soil when the polymer is degraded, assuring protection until degradation occurs.

One of the main problems in inoculant technology is the survival of micro-organisms during storage. Several parameters have an influence on this survival: the culture medium, the physiological state of the micro-organisms when harvested (Chen and Alexander 1973), the process of microencapsulation with biopolymers, the process of dehydration, the rate of drying (Mary et al. 1985), the temperature of storage and the water activity of the inoculum (Hahn-Hägerdal 1986).

It has been reported that the lower the water activity, the higher the survival rate during storage with *Rhizobium, Agrobacterium, Arthrobacter* and some fungi (Mugnier and Jung 1985). However with lactobacilli, it has been found that a minimal amount of water must be left to obtain a satisfactory survival rate (de Valdez et al. 1985).

The purpose of this study is the development of a dehydrated inoculum with bacteria of the genus *Azospirillum* and the elucidation of the effects of several physical parameters on their final survival. Azospirilla can fix dinitrogen and are found living in close association with the roots of several grasses and cereals (Döbereiner and Pedrosa 1987). Their inoculation on grain grasses affects root development (Fallik et al. 1988; Jain and Patriquin 1984; Okon and Kapulnik 1986) and causes increases in dry matter of the vegetative parts of maize (O'Hara et al. 1981), wheat (Mertens and Hess 1984) and others crops and in grain yield (Okon and Hadar 1987).

Formulation and application methodology optimization might be crucial for obtaining crop yield benefits and are necessary for appropriate and economical manufacture of beneficial bacteria (Bashan 1986; Graham-Weiss et al. 1987).

Materials and methods

Strain. Azospirillum lipoferum crtl was used as a model organism in this study. It was isolated in our laboratory from maize roots as previously described (Fages and Mulard 1988).

Media. The media used in this study, AFM 10, 20 and 21, were derived from Albrecht and Okon (1980). Their minimal basal composition is (per litre): $CaCl_2$, 20 mg; NaCl, 100 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $Na_2MoO_4 \cdot 2H_2O$, 2 mg; K_2HPO_4, 600 mg; KH_2PO_4, 400 mg; yeast extract, 3 g. Glucose was used as carbon source at 5 g/l for AFM 10 medium, 30 g/l for AFM 20 medium, and 50 g/l for AFM 21 medium. Ammonium chloride was used as nitrogen source in AFM 20 and 21 media at 2.5 and 3.0 g/l respectively. Media were sterilized by autoclaving for 30 min at 115° C. Glucose was sterilized separately under the same conditions. All chemicals obtained from various commercial sources were of analytical grade.

Culture conditions. Cultures were carried out in 2-l and 20-l fermentors (SGI, Toulouse, France) with AFM 20 and 21 media respectively. Inocula for 2-l fermentors were cultured for 24 h in 250-ml erlenmeyer flasks containing 150 ml AFM 10 medium. A 24-h culture in a 2-l fermentor was used as inoculum for the 20-l fermentor. In fermentors, the pH was maintained at 7.0 by automatic addition of 1 *N* NaOH and the temperature at 35° ± 0.1° C. Dissolved oxygen tension was followed by an amperometric probe (Ingold France, Paris, France). Biomass was followed using direct counts (Thoma cell, OSI, Paris, France), dry weight and plate dilution counts.

Microencapsulation process (Fages and Mulard 1986). Sodium alginate (10 g/l; Satialgine SG 500, S.B.I., Baupte, France) was added to the cell suspension in the fermentor after adding (or not) an adjuvant (skim milk, glucose, sucrose, or glycerol). The mixture was then vigorously stirred in order to allow a homogeneous dissolution of the polysaccharide. Then a pressure of 500 hPa was applied to the liquid surface and the mixture was extruded through plastic nozzles with a diameter of 1 mm. The resulting drops were then projected into a 6 g/l $CaCl_2$ solution where gelation of sodium alginate into a calcium alginate matrix occurred. Small (1 mm < diameter < 2 mm), well-separated beads, containing entrapped bacteria were obtained. Beads were then quickly washed with an aqueous rinsing solution in order to obtain only a surface reticulation of the beads and to eliminate residual $CaCl_2$. Depending on the experiments, the adjuvant used was added either to the culture broth, $CaCl_2$ solution and/or the rinsing water. The beads thus obtained, containing entrapped bacteria, where then dehydrated as described below.

Air drying. Beads were placed on trays in a layer approximately 5 mm thick. These trays were disposed in a convection oven with hygrometric regulation. Drying was achieved at 35° C and 15% < relative humidity (RH) < 20% and lasted between 18 and 96 h.

Freeze drying. Beads were placed on a special bulk tray in a layer 1 cm thick. This tray was disposed in the vacuum chamber of a freeze-dryer (CIRP RP2V, Argenteuil, France). Samples were frozen and cooled to −45° C and then freeze-dried under vacuum.

Secondary dehydration. Samples were put into an oven at ambient temperature for 48 h with a continuous flow of dehydrated air.

Measuring water content and water activity. Water content was measured by setting samples in an oven at 105° C overnight. Water activity is considered to be equal to the RH of the atmosphere in equilibrium with the sample (air is considered as a perfect gas). Samples were put in a small air-tight plastic chamber, the only opening of which was closed by a rubber stopper through which the probe of a hygrometer was fixed. All the measurements were done after equilibrium was reached and at 21° C. With this apparatus, due to the high sample volume/air volume ratio, we can avoid the problem of not reaching equilibrium, which often occurs with the classical method, where water activity is, by hypothesis, equal to the theoretical RH of the atmosphere above saturated salt solutions.

Storage of inoculum samples. All samples were stored at room temperature (21° C) in the presence of silica gel.

Enumeration of viable cells in the dried inoculum. A sample (about 50 mg dehydrated inoculum) was weighed precisely and put in 50 ml phosphate buffer for 1 h on an orbital shaker at 100 rpm. Then the mixture was vigorously homogenized with a turbine (Ultra-turrax N10G, Ika, Staufen, FRG) in order to allow a good dereticulation of the polysaccharide matrix. Decimal dilutions were then plated on petri dishes, and the counting of colony forming units (cfu) was done after 48 h incubation at 35° C.

Results

Unless otherwise specified, the adjuvants were added to the culture, the $CaCl_2$ solution and the rinsing water, and dehydration was performed by air-drying at 35° C during 24 h.

Culture conditions

The microencapsulations were done after 24 h, in the logarithmic growth phase, with a cell density of 10^{10}/ml, corresponding to a biomass of 12 g/l. Experiments with older cultures led to a reduced survival rate.

Alginate concentration

Table 1 shows the influence of three concentrations of sodium alginate on survival rate just after dehydration and after 4 weeks' storage. No major differences could be noticed. Very few bacteria were lost in the reticulation solution (0.3%–0.5%) and in the rinsing solution (less than 0.1%).

Influence of four different adjuvants

Several adjuvants were tested: glucose and glycerol, which can be used by *A. lipoferum* as carbon

Table 1. Influence of alginate concentration on survival of *Azospirillum lipoferum* in entrapped beads

Alginate Conc. (g/l)	dry wt. Sample (g)	Survival rate[a] at			
		$t=0$ day		$t=28$ days	
		10^9 cfu/g (SD)[b]	10^8 cfu/ml (SD)	10^9 cfu/g (SD)	10^8 cfu/ml (SD)
10	3.64	16.6 (4.9)	6.0 (1.7)	2.6 (0.7)	1.0 (0.3)
20	4.78	10.5 (2.5)	5.0 (1.2)	3.1 (0.4)	1.5 (0.2)
30	5.27	5.7 (1.2)	3.0 (0.6)	2.7 (0.1)	1.4 (0.5)

[a] Data shown are means of five replicates; survival is expressed per gram of sample dry weight and per millilitre of culture broth to avoid including alginate weight
[b] cfu: colony-forming units; SD: standard deviation

sources; sucrose, which is not metabolized (Döbereiner and Pedrosa 1987); and skim milk (SM). Their effects on survival just after dehydration is shown in Table 2. All the adjuvants were used at a concentration of 10 g/l. The best adjuvant in terms of bacterial numbers recovered was SM, which allowed the recovery of at least twice the number of bacteria recovered as compared to the other adjuvants tested.

Table 2. Influence of adjuvants on survival of *A. lipoferum* in alginate beads

Adjuvant (log/l)	Sample dry weight (g)	Survival[a] at $t=0$ day	
		10^8 cfu/g (SD)	10^7 cfu/ml (SD)
Skim milk	7.12	18.5 (1.8)	4.5 (0.4)
Glucose	5.90	9.4 (1.3)	1.8 (0.2)
Sucrose	6.53	4.0 (0.1)	0.9 (0.03)
Glycerol	6.32	4.6 (0.5)	1.0 (0.1)

[a] Data shown are means of three replicates; survival is expressed per gram sample dry weight and per millilitre culture broth

Table 3. Influence of skim milk concentration on survival of *A. lipoferum* in alginate beads

Smik milk conc. (g/l)	Culture no.	Survival[a] 10^7 cfu/ml (SD)	
		$t=0$ day	$t=120$ days
0	1	4.1 (0.7)	NC[b]
	2	4.1 (0.9)	NC[b]
10	1	31.7 (11.1)	2.3 (1.3)
	2	49.0 (17.1)	2.3 (0.9)
20	1	21.0 (6.7)	9.3 (5.4)
	2	37.8 (9.4)	5.5 (6.0)

[a] Data shown are means of three replicates; survival is expressed per millilitre culture broth to avoid including skim milk weight
[b] NC, not counted ($<10^5$ cfu/ml)

Influence of SM concentration

Two concentrations of SM, 10 and 20 g/l, were tested in two different cultures and compared with a control without SM. The concentration of 20 g/l led to the highest values of viable cells. However, the difference between 0 and 10 g/l was much larger than between 10 and 20 g/l (Table 3). Further experiments with concentrations higher than 20 g/l showed that the survival rate was not improved.

Where should SM be added?

Using 20 g/l as the model concentration, all combinations of SM addition within the three solutions were tested. The data shown in Table 4

Table 4. Timing of skim milk addition for best survival of *A. lipoferum* during storage

Sample	Skim milk[a] in			Survival[b] at	
	Broth	$CaCl_2$	H_2O	$t=0$ day 10^7 cfu/ml (SD)	$t=110$ days 10^6 cfu/ml (SD)
A	+	+	+	28.7 (3.5)	110.4 (28.6)
B	+	+	−	2.7 (0.7)	0.2 (0.3)
C	+	−	+	4.5 (1.6)	60.3 (19.7)
D	+	−	−	1.3 (0.8)	0.5 (0.2)
E	−	+	+	4.4 (1.9)	20.1 (5.2)
F	−	+	−	1.2 (0.5)	1.2 (0.7)
G	−	−	+	7.9 (1.8)	13.3 (2.5)
H	−	−	−	1.6 (0.8)	0.4 (0.2)

[a] Addition of 20 g/l skim milk (+); no addition of skim milk (−)
[b] Data shown are means of five replicates; survival is expressed per millilitre culture broth

Table 5. Effect of dilution of culture broth on survival of *A. lipoferum* in alginate beads

	Survival[a] at $t=0$ day	
	10^9 cfu/g (SD)	10^8 cfu/ml (SD)
Without dilution	16.6 (4.9)	6.0 (1.7)
In H_2O (1/1; v/v)	6.1 (1.7)	2.8 (0.8)
In AFM 10 (1/1; v/v)	21.6 (3.1)	13.8 (2.0)

[a] Data shown are means of five replicates; survival is expressed per gram sample dry weight and per millilitre culture broth to avoid weight variations due to dilutions

Table 7. Drying process

Drying method	Water activity $t=0$ day	Survival[a] 10^8 cfu/g (SD)	
		$t=0$ day	$t=80$ days
Air-drying	0.21	9.5 (5.2)	1.4 (0.3)
Freeze-drying	<0.05	2.6 (1.4)	NC[b]

[a] Data shown are means of five replicates; survival is expressed per gram sample dry weight
[b] NC, not counted ($<10^6$ cfu/g)

clearly indicate that survival was best when SM was added to the rinsing water. This can be seen by comparing sample G with samples D, F and H, and sample B with samples A, C and E. The addition of SM to the other two solutions also had a positive effect on survival rate: compare sample G with samples A, E and C.

Dilution of broth culture

Using a standard process of encapsulation (20 g/l SM in all solutions) we tried dilution of the culture broth in demineralized sterile water and in AFM 10 medium. Table 5 shows that dilution in water had a negative effect on survival, whereas dilution in fresh medium increased the number of bacteria recovered per millilitre of initial culture. This effect was repeatedly observed in several more experiments.

Dehydration process and water activity

Comparing samples from the same culture encapsulated at the same time under the same conditions but dehydrated during 24 and 89 h, leading to water activity values of 0.32 and 0.23 respectively, showed that the lowest water activity resulted in the best survival during storage (Table 6). One part of each sample was dehydrated twice. This process led to water activity of 0.10; no significant improvement was observed in survival following this process.

Under the same water activity conditions, faster dehydration (obtained by diminishing the thickness of the beads layer) led to a much lower number of viable cells compared to those dehydrated slowly. However, a compromise in shortening the duration of the process has to be found, since the process is performed under non-sterile conditions and too slow a dehydration rate could result in development of contaminant micro-organisms. When drying was slower, beads gradually turned to a pink-red colour, indicating pigment synthesis. This coloration did not occur in the fast dehydration experiments.

Comparison between freeze-drying and air-drying

Table 7 shows that air-drying gave the best results both immediately after dehydration and during storage.

Table 6. Effect of dehydration and water activity on survival of *A. lipoferum* in alginate beads

Duration of dehydration	Water activity		Survival[a] 10^8 cfu/ml (SD)	
	At $t=0$ day	After secondary dehydration	At $t=0$ day	At $t=79$ days
Short	0.32		20.0 (4.5)	3.6 (0.8)
	0.32	0.10		5.5 (1.6)
Long	0.23		13.1 (3.5)	7.0 (1.9)
	0.23	0.10		5.2 (1.9)

[a] Data shown are means of five replicates; survival is expressed per millilitre initial culture broth to avoid weight variations due to different dehydration processes

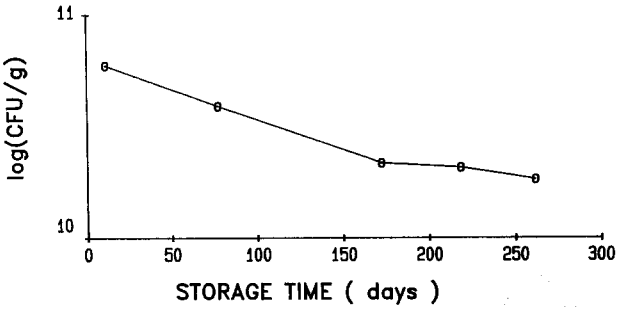

Fig. 1. Time course of survival of dehydrated *Azospirillum lipoferum* crtl during storage

Optimized inoculum

The optimized encapsulation process produced a high-density inoculum — more than 10^{10} cfu/g dry weight of beads — with minimal losses after several months storage. A time course of survival is shown in Fig. 1.

Discussion

Within the range tested, alginate concentration had no effect on the survival rate of *A. lipoferum*. This was probably due to the fact that 10 g/l sodium alginate was sufficient for proper entrapment of an *Azospirillum* culture with a cell concentration of between 10^9 and 10^{10} cells/ml. This is in good agreement with what is known about the ultrastructure of the polysaccharide matrix of calcium alginate (Musgrave et al. 1983).

The positive effect of the dilution of culture broth with fresh medium was probably due to the lower concentration of inhibitory substances produced by metabolic activity of the strain. The benefit cannot be credited to glucose, since the concentration of this carbohydrate in the culture broth ranged from 15 to 20 g/l. Reticulation was performed at that stage in order to lower water activity through water solvation and to allow a better revival at rehydration.

SM is a well-known cryoprotector and commonly used in freeze-drying. Its protective effect in our dehydration process was therefore not surprising. The protective power of SM is probably correlated with its hydration properties: non-freezable water content and water activity were modified by the solutes. However, some mechanical protection of the cell wall cannot be excluded, since dehydration and rehydration are known for their effects on membranes (Beker et al.1984); SM improved survival both after dehydration and during storage.

The negative results obtained with freeze-drying were not clear. However, it is well-known that (i) some bacteria cannot be freeze-dried without significant damage, and (ii) the matrix of alginate can be destroyed by freezing. Besides, the water activity levels reached (less than 0.05) were probably too low to allow good survival of this strain.

Air-drying is a simple and efficient method when properly used. It can be carried out under non-sterile conditions, does not require sophisticated equipment, and is not costly in terms of energy. However, it requires fine-tuning so as to (i) allow the maturation phase where physiological modifications, including pigment synthesis (Nur et al. 1981), of the cells occur, leading to improved resistance to dehydration stress, (ii) avoid the development of contaminant organisms, and (iii) adequately lower water activity for good survival during storage.

Optimizing the process of microencapsulation and dehydration of *A. lipoferum* led to a powdered product, containing more than 10^{10} viable cells/g dry weight after several months storage at ambient temperature, easy to store and to handle, that can be spread in furrows with the same microgranulator used for classical insecticide treatment. This inoculum is also biodegradable and can be tested for seed coating, which is probably the most efficient way to use an inoculum in the field.

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