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SCIENTIFIC PAPER

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ALGINATE BASED MACROCAPSULES AS INOCULANT CARRIERS FOR THE PRODUCTION OF NITROGEN FIXING BIO FERTILIZERS

This paper presents the first steps of the development of dry capsules of biofertilizer to replace chemical fertilizers that cause environmental problems. This new product consists of macrocapsules (large size beads, about 3 to 4 mm diameter) containing nitrogen fixing bacteria: Azospirillum. They are produced by quick encapsulation and drying processes, are made of alginate (3%), standard starch (44.6%) and modified starch (2.4%) and can contain up to 10^6 CFU/capsule. This large size inoculum carrier was formulated to reduce production costs, favour its storage and application in the field and the results show that this new form of inoculum carrier is far better than the liquid and powdered forms.

Key words: Encapsulation, Nitrogen, Bacteria, Biofertilizers.

Cereals such as wheat have high nitrogen fertilization requirements for maximum yield. This essential element is required for the synthesis of wheat protein, nucleic acid, chlorophyll and other cellular constituents. Today, to insure sufficient nitrogen, agriculture applies chemical fertilizers. The use of chemical nitrogen fertilizers has increased 10–fold over the last 40 years, representing a worldwide cost of $20 billion (Stuchinsky, 1982). Nitrogen is accumulated in plants and vegetables, but is also drained into ground water streams and rivers, risking human and animal health (Shelby, 1996). The industrial production of nitrogen fertilizers consumes a large amount of natural gas and releases carbon dioxide, which could lead to global warming.

The European Union is aware about these problems and in the past few years, they have encouraged farmers to reduce the use of chemical fertilisers by optimising their application and, especially, by replacing them, partly or completely, with natural bio–fertilizers.

Bio–fertilizers are living, microbial inoculants that are added to the soil to improve plant growth (Pereira, 2004). Their beneficial effects are: – the absorption and fixation of atmospheric nitrogen in the root–zone, providing 30–50% of nitrogen requirements – the production of plant growth hormones (auxins and cytokinins) – enhancing the germination efficiency, plant immunity and the yield (25%) – assisting in the uptake of mineral nutrients from the soil – the excretion of antibiotics, which protect against minor root pathogens (KKM; Kloepper, 1989; Barea, 1997).

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Particular attention has been given to the genus *Azospirillum*. These bacteria can fix atmospheric nitrogen and are found living in close association with roots of several grasses and cereals (Fages, 1990, Pedrosa 1987). *Azospirillum* inoculation on grain grasses can affect root development (Jain, 1984) and cause an increase in grain yield (Okon, 1987) and of dry matter of the vegetative parts of maize (O'Hara et al., 1981), wheat (Mertens, 1984) and other crops. This bacteria was used as a model for this study and it must be underlined that the new carrier could be applied to various nitrogen fixing bacteria that were isolated from various soils in Europe (Ivanova et al, 2003).

In general, bio–fertilizers are applied as liquid inoculants, directly to the soil or to the seed (Bashan, 1998). Unfortunately, in this form, the inoculant must be applied immediately because it is not easy to handle. Indeed, it shows a quick and high decrease of bacteria viability during storage and transportation, i.e. inadequate for long–term conservation. In addition it presents a high risk of contamination and may result in a low survival of bacteria in the soil. Moreover, to insure a good colonisation of young roots, the inoculant should be delayed few days after planting which could lead to additional work for the farmer (Fages, 1992).

In the last few years, several new dry inoculant formulations for agriculture, by encapsulation with various polymers, followed by drying, have been proposed including polyacrylamide–based inoculants (Dommergues et all. 1979) and sodium alginate (Fages, 1990). Most of these inoculant carriers, which are delivered in powder form, permit the entrapment of living cells, their protection against various stresses during storage and their progressive release into the soil. But, despite their potential, these powders, as well as the liquid forms of inoculant are not widely applied today. This is mainly because they are expensive due to the new encapsulation technology, the polymer costs and other causes mentioned above. It is even reported that despite the improvement of inoculant viability compared

to liquid inoculum, the surviving rate during the processing of fine powder inoculant remains low, especially at the dehydration step (Paul, 1993) where the capsule size is not sufficient to provide a higher protective effect. It is also well known that handling and processing active powders is not easy, e.g. it is sometimes necessary to re–disperse the inoculant powder in water (reconstitution of the liquid form) before spreading in the field (additional work for the farmer).

The aim of this study was to develop a new form of dehydrated bio–fertilizer (big capsules) with *Azospirillum brasilense* that can overcome the main drawbacks of the liquid or powdered inoculum and favour their application in wheat and other cereal fields. This report presents the first part of this study that was entirely focussed on the formulation of the encapsulation matrix. This carrier was selected after optimisation of the encapsulation procedure, which consisted in assessing the maximum bacterial survival with respect to the polymer characteristics (low cost, biocompatible, biodegradable, with release properties that are temperature and water dependent), to the survival rate and to other processing requirements: – encapsulation technique (one step process, ability to form beads, low viscous solution) – drying (quick and safe) – storage (higher survival rate, capsules as resistant as cereal seeds). The target was to reach about 10^6 CFU/capsules, which is the adequate number of bacteria that can fulfil the need of a seed if considering one capsule per seed. The advantages and inconveniences of these new capsules were discussed.

MATERIALS AND METHODS

Strain. *Azospirillum brasilense* was used as the model micro–organism in this study. It was furnished from our partner Nicolaus Wirén at the University of Hohenheim, Stuttgart, Germany.

Media. The media used in this study was YEP. Its composition per liter was: Bactopeptone, 10g; Yeast extract, 5g; NaCl, 5g. The media were sterilized by autoclaving for 20 min at 121°C.

Preparation of the culture. The inoculum was prepared in a 10 ml Erlenmayer flask containing 9.8 ml of medium and 0.2 ml of pre–culture of *Azospirillum brasilense*. The flask was incubated overnight at 30°C, on a rotating shaker at 120 rpm. This culture was centrifuged for 10 min at 8.720 x g at 4°C. The cells were then washed thoroughly with 10 ml of 0.8% NaCl solution and re–suspended in 1 ml of 1% peptone solution.

Macroencapsulation process. The encapsulation matrix was a mix of sodium alginate (ALGOGEL 3001, SG 30–60, Degussa, France), standard cornstarch (ROQUETTE, France) and modified cornstarch (CLEARGUM CO 01, ROQUETTE, France). These polymers were sterilised separately as a dry powder in an autoclave at 121°C for 20 min before dispersion in distilled water. Sodium alginate was first dissolved in water for 30 minutes, followed by the addition of modified and standard starch. Then, the cells were added into 30 ml of encapsulating matrix solution and mixed homogeneously. The mixture was introduced in a syringe and placed on the encapsulation device (Figure 1) and extruded drop by drop through the needle (1.55 mm) by acting as a syringe pump at the rate of 120 ml/h. The whole experiment was performed under aseptic conditions in a laminar air flow hood. The drops fell directly in a 1.5% $CaCl_2$ solution for reticulation. After 30 minutes, the minimum time required for total reticulation, the microcapsules (about 5–6 mm diameter) were washed 3 times with sterile tap water prior to drying.

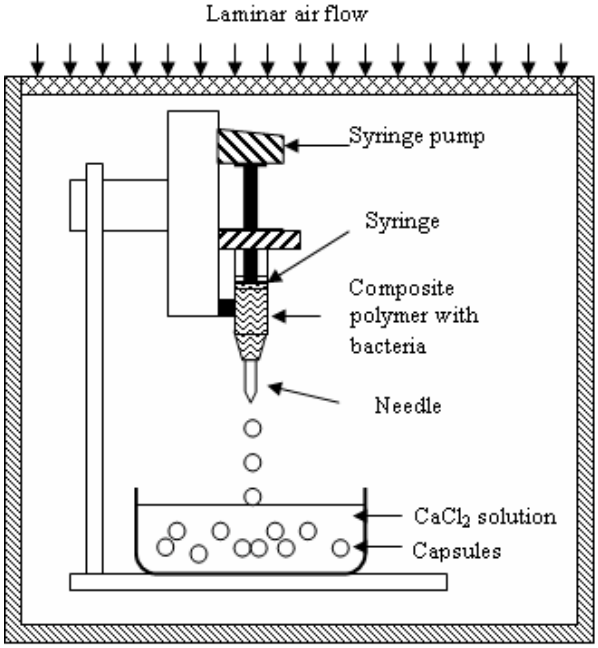

Figure 1. The encapsulation device in the laminar air flow hood

Drying. The wet macrocapsules were spread on a 10 mesh sieve and dried using two different techniques. The first was in an oven at 40°C, 35% RH. The second was by crossing the capsule bed with a dry air stream (5% RH) at room temperature (about 25°C) and at two air velocities (2 m/s, and 7 m/s).

Analysis

Figure 2 presents a summary of different types of analysis, which were performed at different encapsulation steps.

Viscosity. The viscosity of various polymer solutions or suspensions were determined using a viscometer (VT 500 MVDIN, HAAKE, Germany, temperature 20°C, shaar rate 0–700 s^{-1}).

Bead size measurements. The capsule diameter of both wet and dry capsules was determined with binoculars, which were connected with a video image analysis system (Leica, Germany).

Water content and water sorption isotherm. The water content was determined by the classical drying method where samples (approximately 10 g of

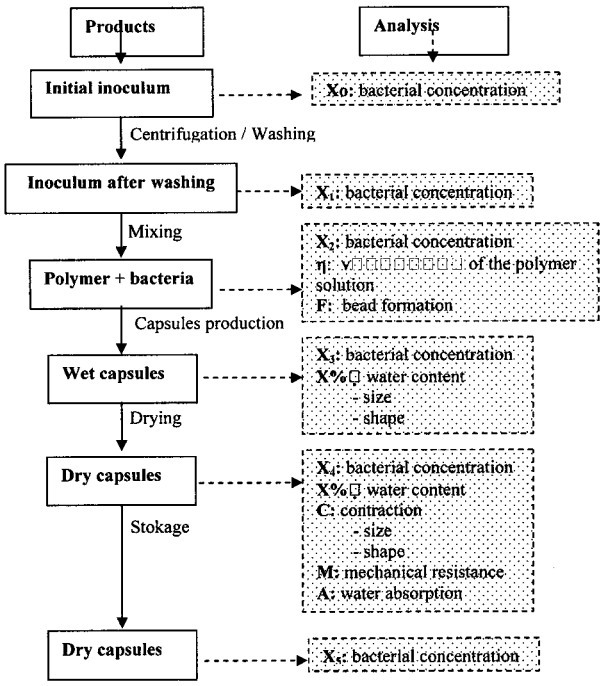

Figure 2. Analysis of the products at different encapsulation steps

capsules) were maintained at 100°C for 48 h in an oven. The water content in the wet basis was calculated as the percentage of mass loss during dehydration in the oven, and defined as $X(\%) = (P_i - P_f)/P_i$, where P_i is the initial mass of the wet capsules and Pf the final mass of the dry capsules. The water sorption isotherm is the quantity of water absorbed by dry capsules versus the relative humidity at a given temperature. For its determination, about 10 completely dry capsules were weighed and placed in hermetically closed chambers at controlled and various relative humidities using saturated solutions of salts, which provide different relative humidities at 25°C: LiCl – 11.4%; CH_3COOK – 22.6%; $MgCl_2$ – 31.3%; K_2CO_3 – 44.0%; NaBr – 59.0%; $CuCl_2$ – 68.0%; $BaCl_2$ – 90.7%. At equilibrium (after about 72 hours), the final capsule weight was measured and the water absorbed was calculated.

Mechanical resistance. The mechanical resistance of the capsules was measured with a Lloyd platform (LR 5K, Lloyd Instrument S.A. France), by uniaxial compression at a speed of 1mm/min. The experiment consisted in crushing one dried capsule, while recording the compression force. The mechanical resistance of the capsule was taken as the maximum force required to break or to split it.

Storage. After drying, capsule samples were placed under four different conditions (4°C, 96% RH; 4°C under vacuum; 25°C, 51% RH; 25°C, under vacuum) in order to study the effect of storage conditions on the viability of the bacteria in the capsules. Every month, 10 capsules, from each set, were collected, rehydrated and derecticulated to check the cell viability.

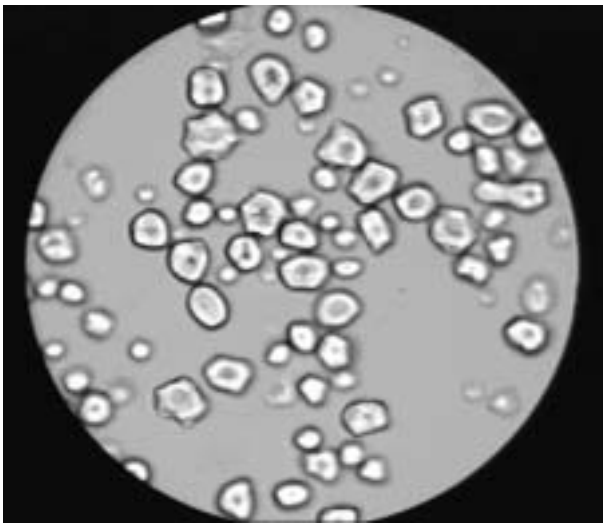

Figure 3. Microstructure of starch

Cell count. The cell concentration was determined during each step of the whole process from the bacteria culture to capsule storage in order to assess the viability. For all enumeration, it was decided to count only living cells. This was done by the classical method, which consisted in adequate dilution of the liquid inoculum, followed by plating in YEP agar. Then, after incubation at 30°C for 24 hours, the cell colonies were counted. The results were expressed as CFU/ml (colony forming units per ml). For the cell concentration in wet capsules the above method was applied but after derecticulation. The derecticulation consisted of suspending about 10 beads in a 10 ml solution of Na–tricitrate (10% w/w) under gentle shaking for 30 min. For dry capsules, rehydration with NaCl solution (0.8% w/w, pH=7) for 30 min is required before derecticulation and enumeration.

A problem arose while counting bacteria in the solution containing starch (an insoluble material). Indeed, the observation of such a solution under the microscope showed the presence of small starch globules (about 10 microns in size) (Figure 3). This starch globule can lead to wrong cell counting, i.e. the use of direct counting under a microscope using a hemocytometer such as the Thomas cell is prohibited in this case. That is why the cumbersome and time consuming plate counting technique was used. Even with this technique, adequate dilution was required to avoid the attachment of many bacteria on one spherical starch microstructure that would lead to one colony when they were plated. The result in Table 1, which presents a comparison of cell numbers in a saline solution, colloidal solution and dispersion, shows that with this precaution, the error in cell counting was negligible.

Behaviour of capsules in the soil. The objective of this experiment, which was performed in bottles containing sterile soil, was to estimate the release properties by assessing the behaviour of capsules in the

Table 1. Effect of micro-spheres on cell counting

	Sample	Cell concentration (CFU/ml)
1.	Saline solution	$3.49 \cdot 10^9 \pm 2.10^8$
2.	Colloidal solution (Alginate 2%)	$1.67 \cdot 10^9 \pm 3.10^8$
3.	Starch based solution (50% dry material)	$1.55 \cdot 10^9 \pm 5.10^8$

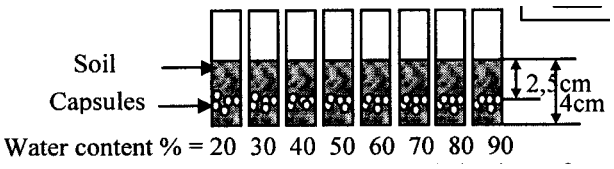

Figure 4. Experimental equipment to test the behaviour of capsules in the soil

soil at various humidities. Dry capsules were put in the soil, at 2 cm from the surface (usual depth of wheat seeds during sowing) and at various soil humidities (Figure 4). The capsules were observed and their diameter was measured every day.

RESULTS

Carrier formulation and optimisation

Alginate solution is the most often used polymer for the encapsulation of bacteria (Pareilleux, 1993). In the classical formulation, the dry matter in the initial encapsulating solution did not exceed 2% to fulfil the low viscosity required by the capsule production device. This led to high energy consumption to evaporate the 98% water and to final capsules with very small size. So, various formulations were prepared, analyzed and tested with the objective to select the matrix composite, which presented the highest dry matter and lowest viscosity of the initial solution, in order to obtain a maximum strength of the formed capsules and to reduce the contraction rate, the overall cost of the polymer and the drying step.

One of the cheapest polymers that can help to increase the dry matter without changing the viscosity too much is starch, but the addition of standard starch to the alginate solution could not lead to a stable solution, because of the sedimentation of starch microgranules. Indeed, the encapsulating solution, which is a solution of a given polymer (alginate, agarose, chitosan, karageen, etc) at low concentration in water, is colloidal. This type of solution is quite stable, at least in the laps of experimental time. But when an insoluble material, e.g. starch, was added to such solution at higher concentration, a dispersion was formed. This dispersion is not stable because of the sedimentation of starch microgranules and this was experienced during the study. To overcome this stability problem, an agitation system (not shown on the encapsulating device in Fig. 1) was adapted to the system in order to obtain a homogeneous solution and capsules. But this was not sufficient to solve the problem, so it was decided to add a small amount of an emulsifier (a modified starch) that seriously reduced the sedimentation. It may be seen from Table 2 that the addition of different starches allowed an increase of the initial dry matter (up to a total initial dry matter of 50%) with a very low increase of the viscosity.

Table 2. Some properties of the alginate–based matrix with various composition

Matrix	Composition (%)			Initial dry matter	Viscosity (Pa·s)	Beads formation
	Algi-nate	Modified starch	Corn starch			
Classical	2	0	0	2	0.12	Yes
Matrix 1	1.6	2.4	23	25	0.35	Yes
Matrix 2	1.6	2.4	36	40	0.37	Yes
Matrix 3	1.6	2.4	46	50	0.40	Yes
Matrix 4	3	2.4	44.6	50	0.57	Yes
Matrix 5	6	2.4	41.6	50	3.10	No

The mechanical resistance of a capsule is directly related to the storage conditions and to the choice of application technique in the field. One of the objectives was to make it as close as possible to the mechanical resistance of the wheat seed, which is about 135 N. In general, the mechanical resistance of the resulting beads decreases with increasing starch concentration (Table 3). This resistance was only 13 N for a capsule produced with a composite solution containing 1.6% alginate and 46.4% starch. This means that this formulation had to be upgraded to improve the resistance of the capsules and allow their storage under the same conditions as wheat seeds. Knowing that alginate possesses a high plastic effect, its concentration was increased from 1.6% to 6%. It was not possible to produce capsules with the encapsulation device using the 6% alginate based solution (Matrix 5, Table 3), because of the high viscosity of this formula. The 3% alginate solution leads to a higher and reasonable value of the mechanical resistance of 105 N (Matrix 4 of Table 3). These capsules were then selected,

Table 3. Characteristics of the capsules

Matrix	Mechanical Resistance	Size (mm)			Water content (%)
		Initial	Final	Contraction	
Classical	170 N	6	1	83%	7
Matrix 1	81 N	6	3.2	47%	7
Matrix 2	17 N	6	3.5	42%	7
Matrix 3	13 N	6	4	33%	7
Matrix 4	105 N	6	4	33%	7
Matrix 5	-	-	-	-	-

as a sort of compromise, for the further experiments, i.e. the final formula of the encapsulation solution was: – alginate (3%) – standard starch (44.6%) – modified starch (2.4%).

The use of a high solid content in the initial encapsulation solution decreases the contraction ratio, which is calculated as the difference between the diameters of the wet capsule and the dry capsule divided by the wet capsule diameter. It gives an indication of the size and the size reduction after drying. Just for comparison, for a wet capsule of 6 mm diameter, the final capsule size after drying is 1 mm for a 2% alginate solution and 4 mm for the 50% composite polymer solution, corresponding to a contraction ratio of respectively 83% and 33% (Table 3).

Drying of the capsules

Drying the capsules is one of the ways of improving the survival of bacteria during storage. The requirement for the stability of bacteria during storage is a water content less than 10% in the wet basis. Drying was performed by two different methods. With the first method (40°C in a oven at 35% RH), it appears that about 48 hours are required to dry the samples to a water content lover than 10% (Figure 5). This drying time was found to be too long to expect any industrial application, so a second drying system was chosen where a dried air stream (5% RH) crossed the capsule bed at room temperature (about 25°C). With this system only 3 hours were needed to reach this target at an air velocity of 2 m/s, and less than 1 hour at 7 m/s) (Figure 6). In all cases, the water content in the final dry capsules was about 7%, quite sufficient for saving the shelf life of bacteria.

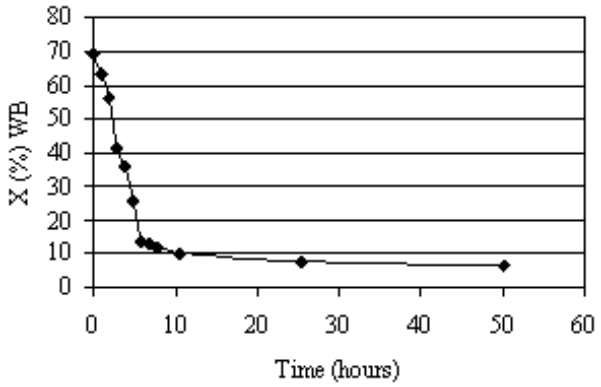

Figure 5. Water content in the capsules during air drying in the oven at 40°C

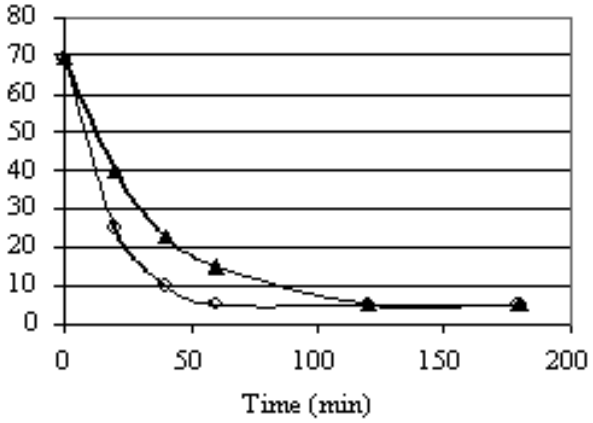

Figure 6. Water content in the capsules during drying at the rate: Δ – 2 m/s, O – 7 m/s,

These drying times are very short compared to the drying times reported to dry small alginate capsules with similar systems (18 to 96 hours; Fages, 1990), (11 to 30 hours; Paul et al, 1993). This difference is the consequence of one of the advantages of large size composite capsules. Indeed, the bed of big capsules, unlike the fine ones, is very porous allowing the air stream to be in contact with all the particles and a high and fast mass transfer of water during dehydration. Note that these large capsules were dried homogeneously without additional sequential stirring of the bed required with fine particles and they could even be fluidized easily. Finally, compared to the small exclusive alginate capsules, the cost of the drying step of big composite capsules is significantly reduced for two reasons: the high porosity of the capsule bed mentioned above and the relatively low amount of water to be removed (50% w/w of the composite capsules versus 98% w/w for the exclusive alginate capsules.

Capsule cost

Another advantage of using starch for increasing the total solid content in the initial encapsulation solution is the reduction of the overall cost of the final capsules. This can be seen in Table 4, which presents a comparison of the capsule cost regarding the polymer price for the two formulations: exclusive alginate and the composite polymer. This table shows that the cost of exclusive alginate capsules is 8 times the one of the capsules with composite polymer. The calculation in this table assumes complete drying of the capsules, i.e. a kg of dry polymer is required to produce a kg of dry capsules. The capsule cost was calculated using the following equation:

Table 4. Cost of capsules

Parameter		Type of capsules	
		Exclusively	Composite
Composition	Alginate	2	3
	Standard Starch	0	44.6
	Modified Starch	0	2.
Total cost for 1 kg of capsules		11.8 €	1.387 €

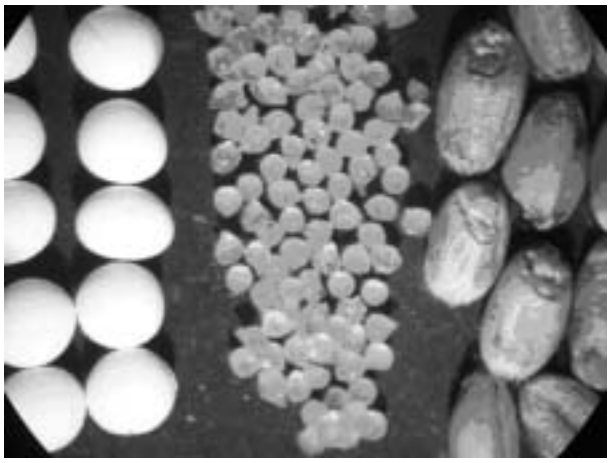

Figure 7. From left to right, the shape of the capsules with composite polymer, alginate, and wheat seeds

$$P = \frac{1}{DM} \sum C_i \cdot P_i$$

where: DM – is the concentration of initial dry material, C_i – the percentage of polymer in the solution, P_i – the price of the polymer.

The polymer costs per kg are: 11.8 € for alginate; 0.6 € standard starch; and 3.0 € for modified starch.

Some characteristics of the capsules

The shape and size. Figure 7 presents from left to right photographs of dry composite capsules, alginate capsules and wheat seeds. It can be seen that the size of the exclusive alginate capsules is too small, while the composite capsule size is quite close to that of the wheat seeds. The capsule shape is different to that of the wheat seed and is close to spheres.

Water absorption isotherm. Figure 8 presents the water sorption isotherm curve at 25°C of the composite capsules under different relative humidities of the air. It appears that the capsules absorb water with increasing relative humidity. When the relative humidity exceeds 65%, the capsules absorb more than 10% of water, i.e.

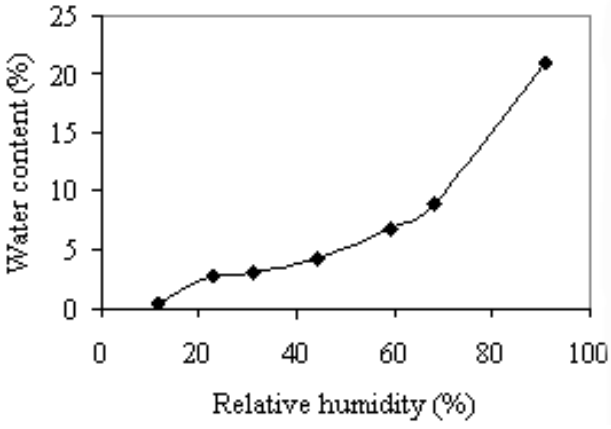

Figure 8. Water sorption of the composite capsules at 25°C

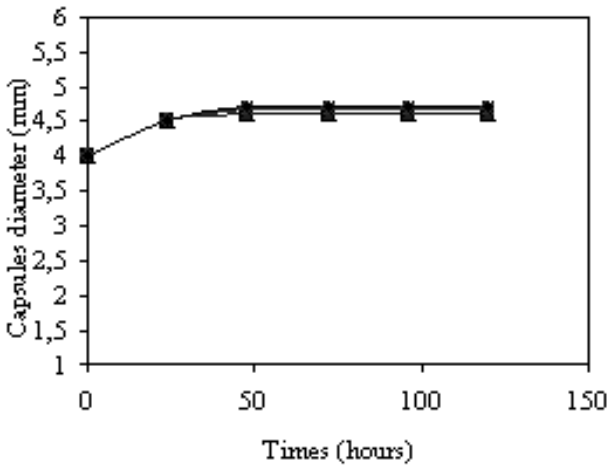

Figure 9. Swelling of the composite capsules in the soil at different humidity: –+– 20%; – –30%; –□–40%; –x–50%; –o– 60%; – – 70%; – – 80%; –■–90%

the relative humidity in the storage area should not exceed this critical limit (65%) to fulfil the requirements for the storage of dry bacteria (water content less than 10%).

Behaviour of beads in the soil

It was interesting to check the behaviour of macrobead carriers under soil conditions to establish their biodegradability and the length of their protecting effect on the encapsulated bacteria. The capsule diameter increases rapidly during the first 20 h in soil, due to swelling after the absorption of water, increasing from 4 mm to 4.7 mm as shown in Figure 9, after that it remains constant all the time. The final diameter after swelling could not reach the initial diameter of the wet capsules (6 mm) before drying, but the morphology (shape and structure) of the capsules was similar to that of the initial wet capsules. Note that the capsules absorbed water regardless of the soil humidity, indicating that these capsules require a very small amount of water to swell and, therefore, to develop the potential to deliver their active components.

After about three to four months the capsules were completely disintegrated in the soil, which is one of the principal objectives: biodegradability. This result indicates that the capsules can supply the plant with bacteria for the above period of time, assuming that the capsules behave like an incubator.

Viability of bacteria during the process

Encapsulation

The cell concentration was determined during each step of the encapsulation process from the bacteria culture, centrifugation, mixing of the culture with the polymer, transfer from the beaker to the encapsulating device, capsule production and drying. Figure 10 represents the total number of bacteria at

those different steps. It reveals that the total cell number did not change significantly during the first step of centrifugation and washing. It increased during the second step (mixing of the matrix with the bacteria), due to bacteria multiplication that can be explained by the presence of a high concentration of a carbon source (starch, alginate) in the medium. A slight decrease was observed during the transfer step due to losses of a small quantity of the matrix/bacteria mixture in the syringe and the preparation beaker. The same observation was made during bead production and this could be explained by the fact that some bacteria were lost in the $CaCl_2$ gelling solution (0.2%) and in the washing solution (0.07%). Finally the viable cell number decreased during drying and this decrease is normal, because some bacteria could not survive the dehydration stress, especially those at the top surface of the capsules. To summarise, from the cell preparation to the end of the drying step, the total number of viable bacteria did not change too much (about 2.109 CFU), corresponding to about 10^6 CFU/capsule.

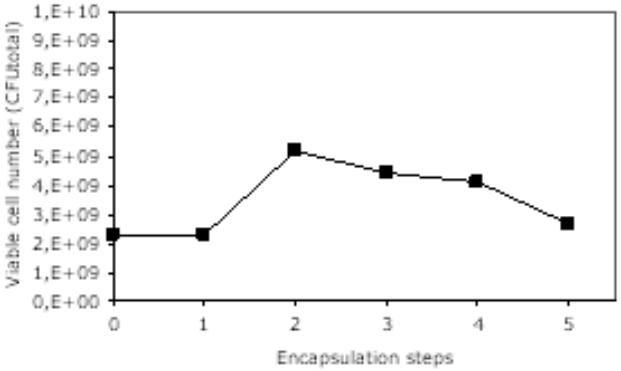

Figure 10. Number of bacteria during the encapsulation process

Drying

The survival percentages of the bacteria in the composite big capsules were more than 15% after drying with the two types of drying systems (Table 5). This can be seen from the table that the slow drying rate leads to a higher survival rate. These survival rates are very good for the two types of drying systems compared to the low survival rate reported during the drying of small alginate capsules (1 to 8.5%; Paul et al, 1993).

The reason of this high survival rate is probably the good protection that provides large capsules with a high solid content during drying. This protection effect was tested by a simple experiment: The total number of viable bacteria was measured initially in three different solutions containing *A. brasilense* (saline solution (0% polymer), a 2% alginate solution and a 50% composite polymer solution). Then after encapsulation and drying under the same conditions (40°C in the oven for 48 hours), this number was checked in a second time in the dried capsules formed by the three solutions. Since the saline solution could not form capsules, an equivalent volume of this solution was simply dried under the same conditions as capsules. The result in Table 6 shows that the higher the initial solid (polymer) content, the better the survival percentage. There is a combined effect of the solid content and the size of the final capsules for this protective effect. This phenomenon seems well known (Potts, 1994), but the mechanisms of this protection during dehydration are not well established, but it can be argued that the hiding capacity of the capsules with a higher initial solid content protect the bacteria cells against high osmotic pressure.

Table 5. Bacterial survival after drying

Type of drying	Total number of cells in the capsules (CFUtotal)		Survival (%)
	wet capsules	dry capsules	
40°C, in the oven	$9.00 \cdot 10^{11}$	$3.20 \cdot 10^{11}$	35.56
Dry air stream 25°C, 2 m/s	$9.00 \cdot 10^{11}$	$1.55 \cdot 10^{11}$	17.22
Dry air stream 25°C, 7 m/s	$9.00 \cdot 10^{11}$	$1.40 \cdot 10^{10}$	15.56

Table 6. Protective effect of the matrix on the shelf life of bacteria

Composition of the encapsulation polymer	Total number of cells (CFUtotal)		Survival (%)
	in the solution	after drying	
0.8% NaCl (control)	$3.53 \cdot 10^9$	$3.10 \cdot 10^5$	0.009
2% Alginate	$2.20 \cdot 10^9$	$3.80 \cdot 10^5$	0.020
3% Alginate + 44.6% standard starch + 2.4% modified starch	$3.10 \cdot 10^9$	$1.13 \cdot 10^9$	36.50

Storage

Figure 11 presents the viability of bacteria in the big composite capsules during 6 month storage under four different conditions. A decrease of the total number of viable cell was observed during the first seven days of storage under any conditions. This decrease can be explained by the presence of unstable forms of cells in capsules after drying. Indeed, after drying, all the bacteria were perhaps not in their stable form (the so–called C–form) where they are more resistant. The C–form formation is typical for *Azospirillum brasilense* and may occur during unfavourable conditions (drying, N–free condition). So, all the bacteria which did not succeed to transform themselves into the C–form during the drying process may have died at the beginning of storage and all the others (the stable ones) survived well and for a longer time since the total number of viable cells remained constant for 6 months. This result shows that this encapsulation approach is without any doubt a good method to extend the shelf life of the inoculum.

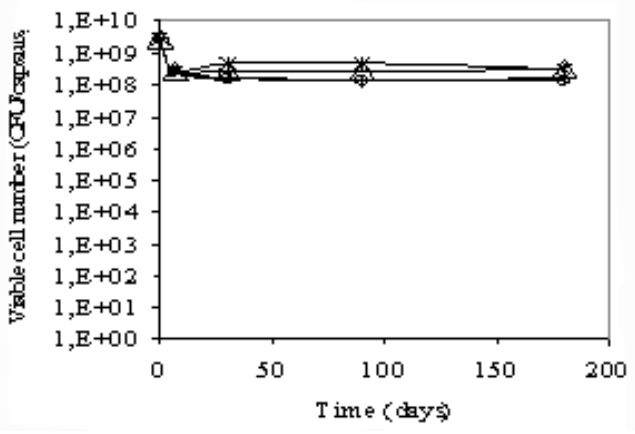

Figure 11. Viability of bacteria in the capsules after 6 months of storage at: –+– T = 25°C, RH = 51%; –Δ– T = 4°C, –O–RH = 96,9%; T = 25°C, vacuum; –– T = 4°C, vacuum

DISCUSSION

The first aspect of this discussion is the stability of the bacteria in the dried composite capsules. It was found that the total number of bacteria decreased quickly during the first seven days of storage after which it remained stable. This simply means that the capsules and, therefore, the encapsulated bacteria were not immediately in their stable form after encapsulation and drying. The first thing that can be incriminated here is the variation of the total cell number during the process, indicating that the bacteria vere submitted to a succession of favourable and unfavourable conditions. The second point is the variation of the water content between the final water content after drying and the water content during storage. Indeed, the drying parameters that preserve bacteria are: temperature <40°C and slow drying rate, but the problem is that the water content after drying is sometimes too low in such a way that during storage, where there is a higher relative humidity, there is water pick up, e.g., the water sorption isotherm (25°C) of the capsules shows that the water content of the capsules at the storage relative humidity (55%) is about 6.5% which is sufficient for the stability of bacteria, while the water content after drying was 7%. The two variations (of conditions and water content), mentioned above are known to cause the sequestration of bacteria that results in their weakness. It may be very difficult to process the bacteria without submitting them alternatively to successive favourable and unfavourable conditions, but it is possible to conduct the drying process in order to obtain to a final water content that can guarantee the stability of the capsules and the bacteria during storage. In this case the equilibrium water content is about 7%, and that explains the drying times which were used during these experiments, 48 hours in the oven at 40°C, 90 min with an air stream at 2 m/s and 40 min at 7 m/s (Figures 5 and 6).

The second aspect is the potential that these new composite capsules represent. It was shown through this investigation that using cheap polymer to increase the total solid content of the initial matrix solution presents great advantages in terms of processing time, overall encapsulating cost. It also results in big capsules which bring higher protection to the encapsulated bacteria during the process and during storage. The matrix that was used here to encapsulate *A. brasilense* can be applied to other types of inoculants. Their production by the dropping device is quite simple and can be easily scaled up, by multiplying the number of nozzles, to respond to the big demand in the agricultural domain. It is quite clear that working in aseptic conditions is not compatible with higher and cheap production rates. Indeed, working in aseptic conditions was done here to quantify under good conditions the viability of bacteria and now, experiments are in progress to show that this production can be done in clean and not aseptic conditions.

All these advantages are in favour of their acceptance by farmers and their application in the field. Indeed, these large size capsules can be handled and processed (storage, transportation, sowing) in the same way as wheat or cereal seeds because they are too close to them regarding their physical characteristics (size, mechanical resistance, hydroscopicity). The only weakness of this formulation, i.e. the point which is not completely clear is the number of capsules per seed. The present investigation was based on the "one capsule/one seed" assumption that in practice can only be done manually (not acceptable by the farmer) since there is no seed–drill that can sow one capsule with one seed at the same time. In addition, their distributions using actual drill–seed machines and their subsequent efficiency on the farm are not well known. These questions should find some answers by studying the release of bacteria in the soil, root colonisation by the bacteria and real trials on the farm.

CONCLUSION

A new bacteria carrier was been developed using a composite encapsulating polymer at an initial high solid content (50%). The use of these capsules can provide high bacterial numbers under field conditions, extension of the shelf life of the bacteria and their protection against soil environmental. These capsules, by their cost effectiveness and easy application, have the potential to convince the agrochemical industry to accept the application of microbial inoculants and, therefore, reduce or avoid the use of chemical fertilisers.

Perspective

Till now all the experiments were performed under sterile condition. For industrial application it is not possible to sterilise all materials and the apparatus. Our future investigations will be focused on working in clean conditions. The future work will include testing of the inoculum effect on plant growth (wheat); investigation of

the mechanism of plant root colonisation by the bacteria (release, mobility and viability in the soil); effect in the soil (i.e. their capacity to effectively fix nitrogen in the soil). Since the application of such capsules seems to generate additional investment, especially during sowing, the seed coating with polymer containing bacteria will also be studied as the ultimate technology that will reconciliate the farmers with these new bio fertilisers.

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Notes: The authors are solely responsible for this report and this does not represent the opinion of the European Community, which is therefore not responsible for any use that might be made of data appearing therein.

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IZVOD

ALGINATNE MIKROKAPSULE KAO NOSAČI U PROCESU PRODUKCIJE BIOĐUBRIVA

(Naučni rad)

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U ovom radu su dati preliminarni podaci za razvoj suvih kapsula za inkapsulaciju biođubriva koji bi trebalo da zameni klasične pesticide ili herbicide. Kapsula veličine 3–4 mm u prečniku sadrži imobilisanu bakteriju *Azospirillum* za fiksaciju azota. Metoda je modifikovana jer se pored alginata (3%) nalazi skrob-standardni (44.6%) kao i modifikofani skrob (2,4%), a inkapsulirana koncentracija ćelija je bila oko 10^6 ćelija/kapsuli. Razlog za razvoj imobilisanog biođubriva pored evidentnih ekoloških prednosti i visokog stepena inkapsulacije se pokazao kao bolji od tečnog ili čvrstog-liofoliziranog oblika.

Ključne reči: Enkapsulacija, Azot, Bakterija, Biođubrivo.