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REVIEW ARTICLE

Bioencapsulation of microbial inoculants for better soil–plant fertilization. A review

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Abstract Plant fertilization is a major issue in the context of increasing population and food risk, higher cost of fertilizers, and low target efficiency of traditional mineral fertilization practices. Alternatively, application of microbial inoculants to the soil can enhance the uptake of nutrients by plants and increase the efficiency of mineral fertilizers and manures. Encapsulation methods involve covering and protecting the microorganisms. Encapsulation of bacterial cells has been challenged and used mainly in the agricultural industry using processes, such as spray drying, interfacial polymerization, or cross-linking. Here, we review techniques for microbial inoculants and their benefits for sustainable agriculture. Techniques include fluidized bed, extrusion, molecular inclusion, coacervation, liposomes, ionic or inverse gelation, and oil-entrapped emulsion. Major topics discussed are formulation of microbial inoculants, conventional inoculants, bioencapsulation materials, bioencapsulation techniques, and future trends. We found that (1) conventional inoculant does not provide adequate protection for microorganisms. (2) Bioencapsulation improves the protection and controlled release of bacteria. (3) Sodium alginate is one of the most used products for the bioencapsulation of microorganisms. (4) The bioencapsulation of microbial inoculants is performed with the incorporation of an active ingredient into a matrix followed by a mechanical operation, and finally stabilization by a chemical or physical–chemical process. (5) Spray-drying process works on a continuous basis, low operating cost, and high quality of capsules in good yield, although the high temperature used in the process is not very appropriate for encapsulating non-spore-forming bacteria. 6) Fluid-bed process is a promising encapsulation technique for large-scale production in agricultural industry. (7) Ionic gelation is currently the most adequate method found to encapsulate bacteria. (8) Some advantages and drawbacks are found for each technique; therefore, the selection of suitable bioencapsulation method will depend on bacteria strain, cost, processing conditions, and handling.

Keywords Immobilization · Microencapsulation · Spray drying · Cross-linking · Coacervation · Carrier material · Shelf-life · PGPR/PGPB

Contents

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1 Introduction

Immobilization and encapsulation of bacterial cells has been widely used in agriculture, pharmaceutical, food, and other industries to achieve a protective structure or a capsule allowing immobilization, protection, release, and functionalization of active ingredients. Therefore, less exposed to adverse environmental factors thanks to encapsulation, tends to stabilize cells, potentially enhancing their viability and stability in the production, storage, and handling of cultures and also confers additional protection during rehydration (Kim et al. 1996).

Rhizobacteria are free-living bacteria isolated from agricultural lands and crop plants, including traditional isolations from cereals and grasses (Bashan and de-Bashan 2005; Park et al. 2005; Lugtenberg and Kamilova 2009; Schoebitz et al. 2009). They are often labeled as plant growth-promoting rhizobacteria (PGPR), which colonize the surface of the root or intercellular spaces of the host plant, frequently improving root establishment. In this regard, PGPR have a potential role in developing sustainable agriculture for crop production (Bashan et al. 2004; Rivera-Cruz et al. 2008). These mechanisms lead to plant growth promotion of diverse nature such as nonsymbiotic nitrogen fixation (Pedraza 2008), phosphate solubilization (Rodriguez et al. 2006; Zaidi et al. 2009), and production of various phytohormones improving root growth, water absorption, and nutrients (Dobbelaere et al. 2001; Spaepen et al. 2007). Nevertheless, colonization of plant roots by direct inoculation of free PGPR cells into the soil is not easy because it is susceptible to environmental variations, such as soil conditions, fluctuation of pH and temperature, humidity, protozoa predation, and salt stress (Wu et al. 2012). This unpredictability of the PGPR inoculation success on plants is mainly due to quality of formulations of inoculants containing an effective bacterial strain and determines the success or failure of a biological agent. Cell survival can be improved by immobilizing them into biodegradable carriers (Fig. 1), being formulation the industrial art of converting a promising PGPR strain in a commercial inoculant product (Bashan 1998). Peat and clay inoculants present a high chemical variability and low cells survival on storage conditions (Cassidy et al. 1996). Therefore, improving the cell survival during storage period to ensure good protection of microbial inoculants in soil is a major goal to obtain a positive response on plant inoculation (Vassilev et al. 2001). Inoculation

Fig. 1 Immobilization of microbial cells in calcium alginate beads by cross-linking technique

strategies include application of formulations aimed at providing a protective niche together with the provision of nutrients sources (Rekha et al. 2007) and appropriate conditions for transport, handling, and storage (Bashan et al. 2002).

Commercial inoculants were first introduced in 1896 using cultures of *Rhizobium*, which were grown in flat glass bottles containing only a small amount of gelatin medium (Smith 1992). Other early inoculants carriers and formulations utilized were liquids, sterilized soil, and rhizobia dried on cotton (Smith 1997). In 1897, Bayer released on the German market a product made of bacteria grain which was based on the species *Bacillus subtilis*. In 1958, 10^7 ha were inoculated using two species, *Azotobacter chroococcum* and *Bacillus megaterium* var *phosphaticum* in the Soviet Union (Lemanceau 1992).

Recently, the yield generated by microbial inoculants has increased from 50 to 70 % of the total area tested finding over 2 million ha of the species *B. subtilis* applied in the USA. Despite these results, the soil inoculation continues to be an experimental field in various countries with different plants and soil conditions (Lemanceau 1992). The outcomes achieved on inoculation of PGPR in different regions of the world under different crop types and soil conditions evidenced a significant increase in crop yields (Okon and Labandera-Gonzalez 1994; Fuentes-Ramirez and Caballero-Mellado 2005; Diaz-Zorita and Fernandez-Canigia 2009)

2 Formulation of microbial inoculants

Inoculants carriers have been used to improve effectiveness by supplying nutrients, protection from desiccation, and slow cells release. PGPR works optimally under laboratory and greenhouse conditions. However, formulating microorganisms into a product and applying is really difficult under field conditions (Stephens and Rask, 2000). The success of using microbial inoculants introduced into soil requires the

survival of an adequate numbers of bacteria reaching suitable habitats where they can thrive (Heijnen and Van Veen 1991). For these reasons, formulation is a key factor in the success of microbial inoculants.

The carrier is usually a convenient and economical material, which is able to release slowly viable cells in high-quality physiological condition, containing one or more beneficial bacteria (Bashan 1998). There are a number of alternative carriers and formulations for delivery of bacteria: talc (Kloepper and Schroth 1981), vermiculite, perlite (Temprano et al. 2002), polyacrylamide (Dommergues et al. 1979), carrageenan (Cassidy et al. 1996), alginate (Bashan 1986), alginate–starch (Schoebitz et al. 2012), alginate–humic acid (Young et al. 2006), and powder formulations (Amiet-Charpentier et al. 1999; Denton et al. 2009), although the most practical carriers used worldwide on commercial crops are peats (Rose et al. 2011), liquids (Albareda et al. 2008; Diaz-Zorita and Fernandez-Canigia 2009), and clays (Goss et al. 2003).

3 Conventional inoculants

Peats are the most commonly used carriers to inoculate seed legumes with rhizobia (Denton et al. 2009). Peat is a complex organic material with a high variability. This particular situation affects on the quality of the final product, stability during storage, and survival of microorganisms in the final product since microbiological contamination decreases the shelf life of the inoculants (Bashan 1998). Furthermore, peat formulations are not able to ensure high cells density and after 6 months of storage a reduction on cells viability is observed (Fallik and Okon 1996).

Liquid formulations are inoculants, which use broth cultures mainly in water, but also in mineral or organic oils. The seeds are either dipped into the inoculant before sowing, or an applicator evenly sprays the liquid inoculant on the seeds (Bashan, 1998). Liquid formulations simplify production and application for the farmers and may have some advantages since they use low-cost material and are easily attainable by small producers (Singleton et al. 2002; Albareda et al. 2008). Liquid inoculants allow contacting directly seeds and microorganisms and consequently increasing the survival of bacteria on plant roots. However, bacterial survival rates on liquid formulations decrease because this technique does not provide a protective environment for microorganisms and the number of bacteria distributed in each seed is quite heterogeneous. In addition, microorganisms are not sufficiently protected against environmental conditions and contamination during storage, transport, and application into the soil (Bashan et al. 2002). The use of liquid inoculants mainly requires a correct storage, without losing their efficiency and cell viability. A stable population of rhizobia on liquid formulations can be stored for 3 months (Albareda et al. 2008); whereas, PGPR on liquid formulation could show a decrease on the number of living cells (Bashan et al. 2002; Haggag and Singer 2012).

Clays are widely used as microbial inoculants and have a long history of usage in various agricultural formulations applied as granules, suspensions, and powder. Clays can act as a desiccant providing excellent storage for dried inoculants due to large surface area, pore size distribution, and total porosity. Besides, water can be controlled to provide moisture for biologically active formulations (Goss et al. 2003). In addition, clays absorb or distribute dispersing and suspending agents. Clays inoculants carriers increased survival levels of rhizobia in soil for 60 days, being these beneficial effects recorded the result of the creation of protective microhabitats accessible to the bacteria but inaccessible to predators (Heijnen and Van Veen 1991; Heijnen et al. 1992).

4 Bioencapsulation

The principle of rhizobacteria bioencapsulation is to protect the microorganisms introduced into the soil and to ensure a gradual and prolonged release (Bashan 1986; Kim et al. 2012). The degradation rate of the encapsulation matrix will have a direct relation with the biological activity of the soil microorganisms. The dried capsules can be stored at room temperature for a long period presenting a favorable environment for bacteria and reducing the risk of decreased survival. These inoculants can be improved by incorporating essential nutrients for bacterial growth, transforming the capsules in bioreactors, which are capable of increasing the number of encapsulated bacteria inoculated into the soil. Bacterial inoculants have solved many problems associated with traditional peat inoculants, which originate great variability in peat quality (Deaker et al. 2004). Numerous advantages related to the bioencapsulation of rhizobacteria are found, for instance, controlled release of bacteria into the soil, protection of microorganisms in the soil against biotic and abiotic stresses, and contamination reduction during storage and transport.

4.1 Bioencapsulation materials

Natural and synthetic polymers have been used in the bioencapsulation of rhizobacteria. Hydrogels extracted from seaweed, such as alginate, carrageenan, agar-agar, and agarose, considered as natural, are formed by polymerization or cross-linking (Table 1). Also, certain synthetic polymers have been used for bioencapsulation of living cells, such as polyacrylamides, polystyrene, and polyurethane (Trevors et al. 1992; Cassidy et al. 1996). Gums and proteins are

Table 1 Immobilization techniques and carrier materials used on bioencapsulation of microbial inoculant to increase plants performance

Plant	Technique	Formulation material	Microorganism	Reference
Soybean	Gelation	Polyacrylamide	*Rhizobium japonicum*	Dommergues et al. (1979)
Wheat	Cross-linking	Alginate and skim milk	*Azospirillum brasilense*	Bashan (1986)
Wheat	Cross-linking	Alginate, skim milk, and clay	*Pseudomonas fluorescens*	van Elsas et al. (1992)
Onion	Cross-linking	Agar	*Enterobacter* sp.	Vassilev et al. (1997)
Wheat	Cross-linking	Alginate and skim milk	*A. brasilense* and *P. fluorescens*	Bashan and Gonzalez (1999)
Lettuce	Cross-linking	Alginate and skim milk	*Enterobacter* sp.	Vassileva et al. (1999)
Sugar beet	Cross-linking	Alginate	*P. fluorescens*	Russo et al. (2001)
Tomato	Cross-linking	Alginate, starch, and talc	*Streptomyces* sp.	Sabaratnam and Traquai (2002)
Cattle pasture	Cross-linking	Alginate and wheat bran	*Beauveria bassiana*	Bextine and Thorvilson (2002)
Lettuce	Cross-linking	Alginate and humic acid	*Bacillus subtilis*	Young et al. (2006)
Tomato	Cross-linking	Alginate	*A. brasilense* and *Chlorella sorokiniana*	Yabur et al. (2007)
Lettuce	Cross-linking	Alginate and humic acid	*Pseudomonas putida* and *B. subtilis*	Rekha et al. (2007)
Wheat	Cross-linking	Alginate	*B. subtilis* and *Pseudomonas corrugata*	Trivedi and Pandey (2008)
Legume trees	Cross-linking	Alginate	*A. brasilense* and *Bacillus pumilus*	Bashan et al. (2009)
Alfalfa	Emulsion	Canola oil and xanthan gum	*Sinorhizobium meliloti*	John et al. (2010)
Cotton seed	Cross-linking	Alginate	*Klebsiella oxytoca*	Wu et al. (2011)
Sorghum	Cross-linking	Alginate	*A. brasilense* and *C. sorokiniana*	Trejo et al. (2012)

frequently used as protective materials to cells, although they usually turn out to be more expensive.

Carbohydrates such as starches, maltodextrins, corn syrup solid, acacia gums, and so on are used extensively in spray-dried encapsulations (Reineccius 1991). Among the properties of these materials, their low viscosity at high solids contents and good solubility, which are desirable in an encapsulation agent is pointed out.

Starch and products derived from it such as maltodextrins and β-cyclodextrins have been widely used to encapsulate other type of compounds, such as flavors, being the subject of extensive studies (Reineccius 1991; Goubet et al. 1998; López et al. 2012). Sodium alginate is one of the most commonly used products for the bioencapsulation of microorganisms. The resulting inoculum is used for various purposes: the immobilization of bacteria (Bashan 1986; Bashan et al. 2002), fermentation and application of biological control agents (Bashan and Holguin 1994), or biostimulants for plant growth (Bashan and Levanony 1990; Schoebitz et al. 2012).

Sodium alginate is produced by brown algae, such as *Macrocystis pyrifera*, *Laminaria digitata*, *Laminaria hyperborean*, and *Eklonia cava*. Alginate production is not exclusive to seaweed. Indeed, there are some bacteria able to produce extracellular alginate. An example is *Azotobacter vinelandii* (Nunez et al. 2000) and several *Pseudomonas* strains (Fett et al. 1986, 1989).

Alginates are linear macromolecules comprising two monomers linked by alpha 1–4: β-acid and D-mannuronic acid to α-L-guluronic acid having a molecular weight between 20,000 and 200,000 Da. The properties of alginate are variable according to the origin of the seaweed and the manufacturing process. For instance, in relation to their molecular weight, alginates will have different solubility properties and complexation with calcium. The alginate solution is mixed with the cell culture and is extruded into a solution of $CaCl_2$ in concentrations from 0.05 to 0.1 M. The residence time of the ball in this solution for the complete gelation is around 20–30 min (Cassidy et al. 1996; Fig. 2).

4.2 Survival of rhizobacteria encapsulated in alginate

The alginate matrix protects cells from mechanical stress and limits their mortality during prolonged storage (Young et al. 2006). After 1 year of storage at room temperature, the survival rate of *Azospirillum lipoferum* immobilized in alginate beads dry was 10^{10} CFU g^{-1}. The concentration in this situation was higher than the conventional microbial inoculants (Fages 1992; Trejo et al. 2012).

After 3 years on storage conditions at 4 °C, the survival rate of *B. subtilis* and *Pseudomonas corrugata* immobilized in sodium alginate beads was 10^8 CFU g^{-1}. Furthermore, both rhizobacteria did not lose their ability to increase the plant growth (Trivedi and Pandey 2008). The survival of strain *Azospirillum brasilense* Cd and *P. fluorescens* 313 immobilized in alginate beads after 14 years on storage conditions was 10^5–10^6 CFU g^{-1}. After this long period of storage, rhizobacteria did not lose their ability to stimulate growth of wheat plants. This research showed that rhizobacteria could survive in the alginate inoculant over long periods (Bashan and Gonzalez 1999).

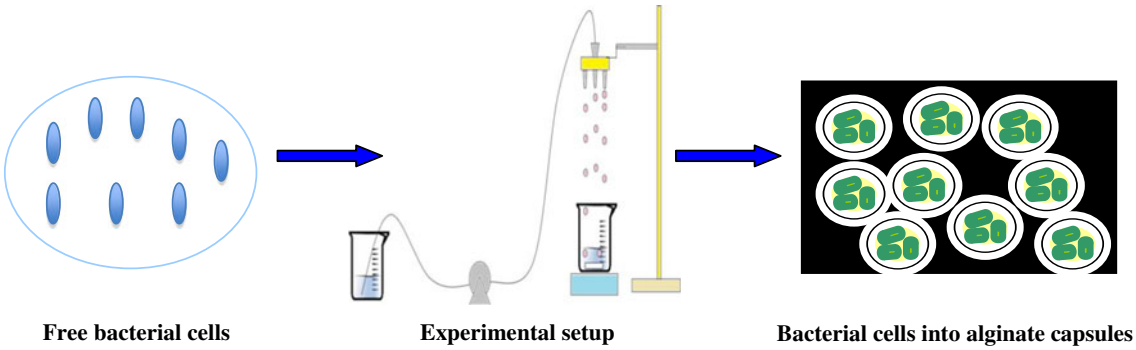

Fig. 2 Schematic diagrams of encapsulated bacterial cells and making a protective shell around the microorganisms

4.3 Starch filler in alginate beads

Calcium alginate beads are structured as a loose network filled with a large quantity of water (Nussinovitch 2010). Thus, using alginate alone for cell bioencapsulation would not adequately protect cells during the drying process and results in slightly distorted beads. Filler materials, such as starch, may be added to the formulation to increase the dry matter in the beads, improving mechanical resistance and allowing for a progressive release of cells into the soil (Bashan et al. 2002). The matrix failed to protect cells during drying as a two log decrease in *A. brasilense* cell number, due to alginate beads contained around 97–98 % of water (Bashan 1986). Using starch in the bioencapsulation, the water content was reduced to 65 % and significantly improved cell survival (Schoebitz et al. 2012). In addition, it is necessary to take into account that starch is an inexpensive material for bioencapsulation purposes and it is one of the most abundant natural biopolymers (Hickman 1999). It is used as additives and adhesives (John et al. 2011) and also as a filler and carbon source (Tal et al. 1999; Schoebitz et al. 2012). Previous studies on probiotic carriers have revealed a protective effect of starch due to cell adhesion to granules (Wang et al. 1999; O'Riordan et al. 2001a, 2001b). 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 (Schoebitz et al. 2012; Fig. 3).

5 Bioencapsulation techniques

Different methods are used to encapsulate bacterial cells, pointing out physical processes, such as spray drying, spray chilling/cooling, extrusion, or fluidized bed; chemical processes like co-crystallization, molecular inclusion, or interfacial polymerization; and also physicochemical processes, such as coacervation, liposomes, and gelation/inverse gelation (Madene et al. 2006; Fig. 4). Table 2 summarizes the main advantages and disadvantages of these methods.

Most of these techniques described in this section have been widely used for probiotics in alimentary industries or medical field; however, they are not often applied for PGPR and therefore should be reconsidered in the agricultural field. Currently, the number of microbial inoculant research projects has been intensified in the agricultural industry, caused mainly by the reduction and restriction of chemical fertilizers (Marra et al. 2012).

The bioencapsulation of microbial inoculants is performed in three stages. The first step involves the incorporation of an active ingredient into a matrix (liquid or solid). The second stage is a mechanical operation that involves making dispersion or spraying a solution onto solid particles under mechanical stirring, and the last step consists of a stabilization by a chemical process of polymerization or by a physical–chemical processes (gelation and coacervation) or physical (evaporation and solidification) on a droplet or pellet formed during the second stage. New techniques of encapsulation continue to emerge for developing formulations and processes to manage the improvement of capsule properties and characteristics.

5.1 Physical processes

5.1.1 Spray drying

Spray drying is a commercial process which is widely used in large-scale production of microbial formulation, is economical and adaptable, and produces an excellent quality of the product. This method involves the dispersion of the bacterial cells in a carrier material forming an emulsion or dispersion, with homogenization of the liquid followed by atomization and spraying of the mixture into a hot chamber (Watanabe et al. 2002) leading to evaporation of the solvent and consequently the development of microcapsules.

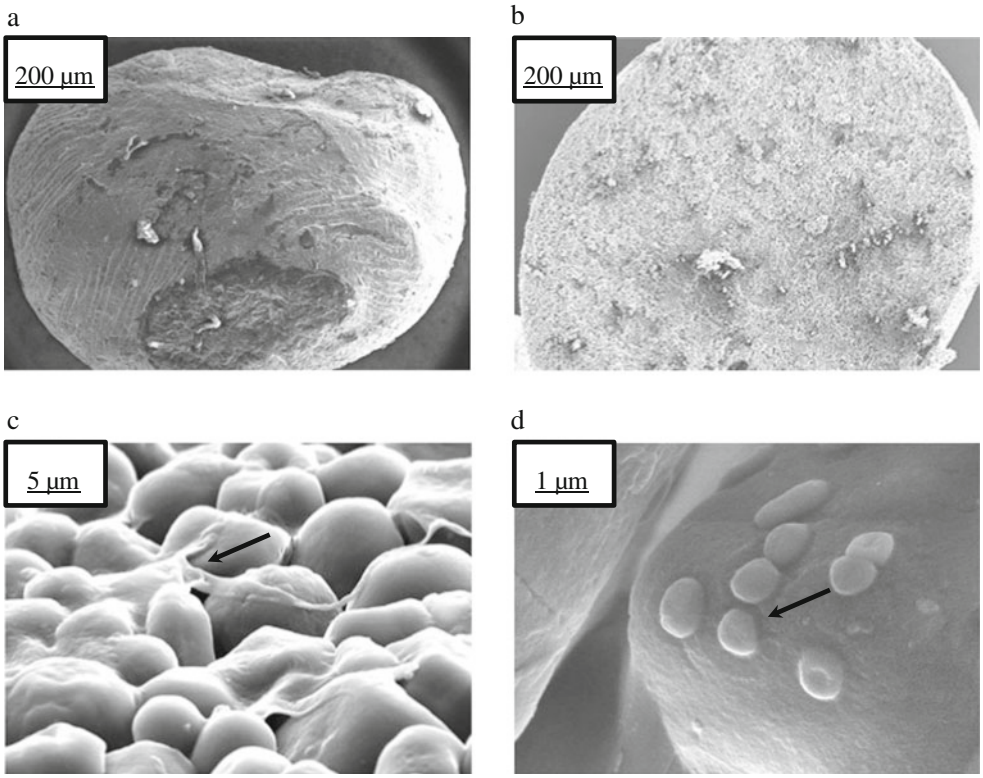

Fig. 3 Scanning electron microscope pictures of alginate-starch bead: **a** alginate-starch bead; **b** transverse section of a alginate-starch bead; **c** *arrow* shows distribution of alginate and starch granules on the surface of dried beads; **d** and *arrow* shows *Raoultella terrigena* TFi08 immobilized and adhered to the surface of starch granules

Optimal wall materials for this technique include substances with high solubility in water, low viscosity at high concentration, effective emulsification and film-forming characteristics, and efficient drying properties (Reineccius 1988). The main advantages of the spray-drying process is to manage on a continuous basis, low operating cost, high quality of capsules in good yield, also rapid solubility of the capsules, small size and high stability capsules. However, it is important to take into account some disadvantages of the spray drying, such as the high temperature used in the process which may not be suitable for encapsulating bacterial cultures, the lack of uniformity in the microcapsules produced, or the limitation in the choice of wall material. In addition, this process produces very fine powder that needs further processing and is inadequate for heat-sensitive material (Risch 1995). Nevertheless, appropriate modification and control of the processing conditions (inlet and the outlet temperatures) achieve viable encapsulated cultures of the required particle size distribution.

At an inlet temperature of 100 °C and low outlet temperature of 45 °C, *Bifidobacterium* cells were encapsulated satisfactorily to produce microspheres with gelatinized modified starch as a coating material (O'Riordan et al. 2001a). In this study, spray drying was found to be a valuable process for encapsulating these bacteria.

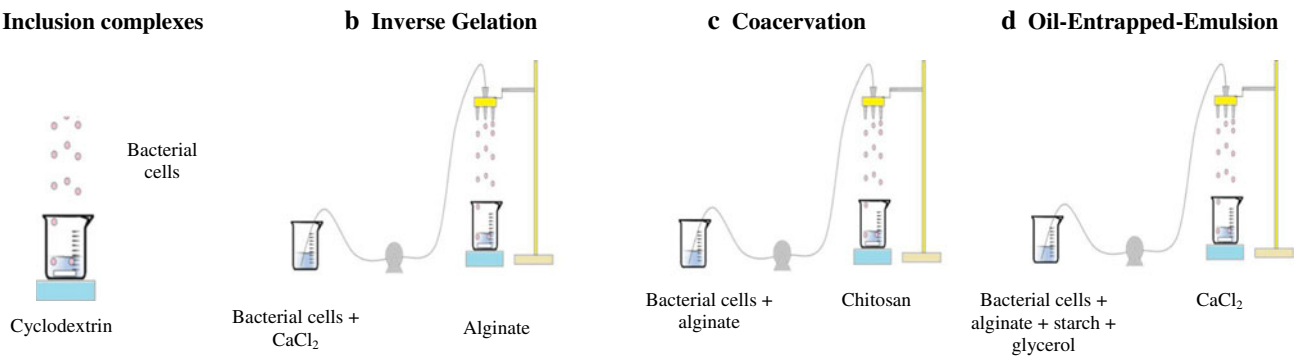

Fig. 4 The schematic diagrams of the experimental set-up corresponding to **a** inclusion complexes: cyclodextrins form a cavity in which bacteria are located; **b** inverse gelation: bacterial cells and calcium chloride are dropped into alginate solution; **c** coacervation: bacterial cells and alginate form a coacervate with chitosan solution; and **d** oil-entrapped emulsion: bacterial cells, alginate, starch, and glycerol are dropped into calcium chloride

Table 2 Comparison of physical, chemical, and physical–chemical encapsulation techniques for microbial inoculant

	Spray drying	Fluidized bed	Extrusion	Molecular inclusion	Interfacial polymerization	Coacervation	Liposomes	Ionic gelation	Inverse gelation	Oil-entrapped emulsion
Core	Beads	Capsules	Either	Beads	Capsules	Capsules	Capsules	Beads	Capsules	Beads
<500 μ	Yes	No	No	Yes	Yes	No	Yes	No	No	No
Water soluble	Yes	Yes	Yes	Poorly	No	Without cross-linking	No	No	No	No
Uniform	No	Yes	Yes	On molecular scale	Very	Thin corners	Yes	Yes	Yes	Yes
Time for processes	10–30 s	10 min–1 h	10 min–1 h	Minutes	10 min–2 h	12–16 h	Minutes	1–2 h	1–2 h	1–2 h
Relative cost	Very low	Low	Medium	Very high	Low-medium	High	Very high	Medium	Medium	Medium
Advantage	Fast and inexpensive	Flexible and inexpensive	Loading capacity, biocompatible, and stability	Selective trapping	Well-developed process and large batches	Insoluble wall, impermeable to hydrophobic molecules	Tiny particles and biocompatible	Biocompatible	Biocompatible	Biocompatible
Disadvantages	Possible dust and high temperature	Coating fine particles	Diffusion and release	Very low loading	Core wettability and few wall materials	Aggregation, core wettability, and few wall materials	Few wall materials, very thin walls, and low loading	Few wall materials and process control	Few wall materials and process control	Process control and slow release
Useful for	Thermal resistance cells	Irregular particles and uniform walls	Enzymes and vitamins	Flavor	Hydrophobic drops (<20 μ) and completely insoluble walls	Hydrophobic drops (<20 μ)	Capsules <2 μ	Cells, enzymes, and suspensions	Cells, enzymes, and suspensions	Cells, enzymes, and suspensions

Some reports indicated that survival of bacteria during spray drying decreased with increasing inlet temperature (Mauriello et al. 1999) due to inlet temperatures of above 60 °C resulting in poor drying and the humid product often accumulated in the cyclone. Other bacterial strains have also been reported to lose viability at higher inlet temperatures (Gardiner et al. 2000; Golowczyc et al. 2010).

On the other hand, other authors (Amiet-Charpentier et al. 1998a, 1999) studied the microencapsulation of the rhizobacteria *Pseudomonas fluorescens* using the spray-drying technique, focusing on obtaining living bacteria for a minimum of 5 or 6 months, when the inlet temperature was at 60 °C, there was bacterial survival and was estimated around 10^7 CFU g^{-1} of powder. From these studies, a relationship between bacteria cells, beads, and residual moisture was observed taking out to be around 25 % of residual moisture as the most excellent conditions.

In spite of this, the spray-drying technology is not considered as a good cell immobilization technique due to a high mortality resulting from simultaneous dehydration and high temperature inactivation of microorganisms like non-spore-forming bacteria (Picot and Lacroix 2003), such as *Rhizobium* (John et al. 2011). For example, Amiet-Charpentier et al. (1998a) found 100 % of cells mortality of *Pseudomonas* strains at the end of the drying process, when the inlet was at a temperature above 80 °C.

5.1.2 Fluidized bed

Fluid bed spray coating is a process used to avoid some problems of the spray-drying technique. In this process, the particles to be coated are fluidized with hot air at the coating chamber. After that, the coating material is sprayed through a nozzle onto the particles and film formation is started out, followed by a sequence of wetting and drying stages (Fig. 5). The small droplets of the sprayed liquid reach onto the particles surface and blind them together. The solvent is evaporated by the hot air and the coating material remains on the particles (Jacquot and Pernetti 2003).

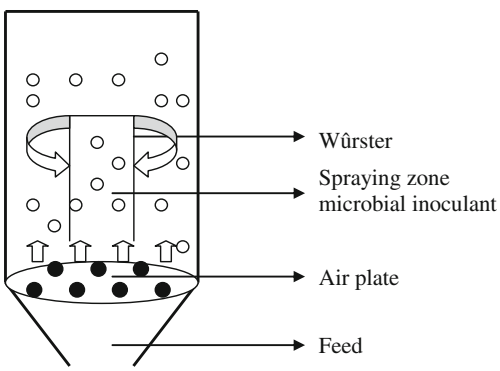

Fig. 5 Fluid bed drying, in this process the particles to be coated are fluidized with hot air at the coating chamber

The advantages of this technology is to allow specific particle size distribution, low porosities (Uhlemann and Mörl 2000), also high drying rates, smaller flow area, high thermal efficiency, lower capital and maintenance costs, and an ease control.

Solid particles extremely tiny to coat properly in a coating pan (particles below 1–2 mm in diameter) will be coated by fluidizing them and slowly spraying a coating onto the moving fluidized particles. This method works on particles down to 100–150 μ, although unusual conditions should be taken into account to avoid aggregation of the fewest quantity of coated particles (Sparks and Jacobs 1999).

Wurster fluid bed device introduced a change of this method by using an open cylinder, which is hanging above the fluidization plate and where the fluidization air fed goes to the center region of the fluidization plate, carrying the particles upward through the cylinder. The particles are not fluidized in this region but are simply being sent out while they desiccate. This deviation gives superior control of the recirculation action of the particles (Prata et al. 2012; Fig. 5).

Fluidized-bed drying is generally considered less stressful for drying cells than spray-drying technology; it involves less extreme water loss and temperature gradients (Larena et al. 2003; Morgan et al. 2006). In spite of being this technique more advantageous, the application of fluidized-bed technology for coating some inoculants is not common. Herridge and Roughley (1974) compared seed pelleted using a fluidized bed with the conventional rotating drum technique and observed that fluidized bed produced a firm pellet, but the survival of the inoculum was low, possibly due to the air temperature occasionally reaching 35 °C. For this reason, the conditions required to obtain capsules of high integrity may compromise viability of the microbial inoculant (Deaker et al. 2004).

5.1.3 Extrusion

The microencapsulation by extrusion involves the change of an emulsion of the active material and wall material under high pressure. The bacterial cells are dispersed in a matrix polymer at high temperature (around 110 °C). The main advantage of the extrusion method is the stability against oxidation since carbohydrate matrices in the glassy state have excellent barrier properties and consequently extrusion turns out to be a convenient process even though it has very low loading (Gouin 2004). On the other hand, process parameters and diffusion of cells from extruded carbohydrates is enhanced by structural defects such as crakes, thin wall, or pores formed during or after processing (Wampler 1992).

Production of beads can either be accomplished by multi-nozzle system, rotating disc atomizers, or by the jet-cutting

technique. Centrifugal system using either a multi-nozzle system or a rotatory disc has also been developed for the mass production of microcapsules (Heinzen 2002).

The centrifugal extrusion technique represents a liquid coextrusion process utilizing a nozzle consisting of concentric orifices located on the outer circumferences of a rotating cylinder (Schalmeus 1995). This liquid or core material is pumped through the inner orifice and a liquid shell material through the outer orifice creating a co-extruded rod of core material delimited by shell material. As the system rotates, the extruded rod breaks up into droplets to form capsules.

5.2 Chemical processes

5.2.1 Molecular inclusion

The inclusion complexes are defined as the result of interactions between compounds where a smaller guest molecule suits into the lattice formed by the other (Godshall 1997). The main example of molecular inclusion is cyclodextrins, which are enzymatically modified starch molecules (Fig. 6). The inner hydrophobic cavity of β-cyclodextrin and its molecular dimensions allow total or partial inclusion of a wide range of compounds. The central cavity of the molecule is quite hydrophobic, whereas its external surface has a hydrophilic character. This conformation is basically responsible for the physicochemical properties of cyclodextrins (Shieh and Hedges 1996).

According to Goubet et al. (1998), the retention can be influenced by the molecular weight and shape, steric hindrance, chemical functionality, polarity, and volatility of the core material. The presence of water or high temperature is required to release guest molecules once complexed (Reineccius et al. 2002). On the other hand, the main disadvantages are that cyclodextrins are relatively expensive;

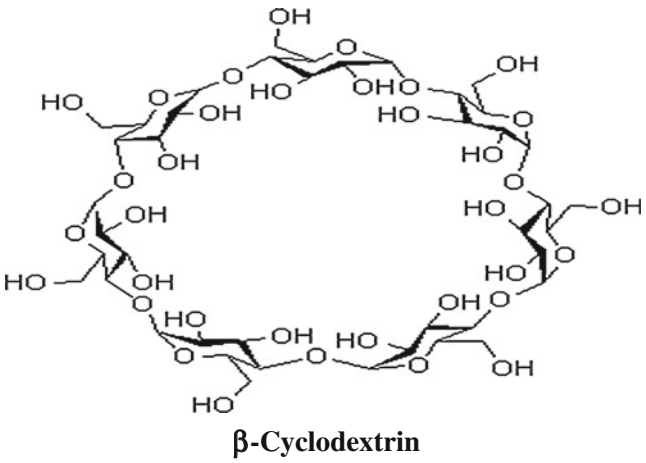

Fig. 6 Matrix used for molecular inclusion, the inner hydrophobic cavity of β-cyclodextrin, and its molecular dimensions allow total or partial inclusion of a wide range of compounds

Gouin (2004) suggested that the cost of cyclodextrins would never be lower than $6/kg, and they present a very low loading. Besides, the immobilization of bacteria could turn out to be complicated due to a size effect. Even though studies about rhizobacteria in inclusion complexes are not often, it is used in the medical field and it could be a promising method (Huang et al. 1999).

5.2.2 Interfacial polymerization

Interfacial polymerization technique involves the formation of an emulsion with an aqueous suspension of the cells as the discontinuous phase and an organic solvent as the continuous phase. The droplet containing the cells and the reaction is set off when a biocompatible reagent, soluble in the continuous organic phase, is added to the emulsion. This process allows obtaining a high active loading (up to 90 %) but involves high pH and toxic chemicals such as sebacoyl chloride (Yeo et al. 2001). For instance, microcapsules can be produced by dripping an alginate suspension (polyanion) in a chitosan solution (polycation), where the blend of alginate and cells is dripped in a solution of chitosan (acetic acid 1 % at pH 4) with continuous stirring.

Synthetic polymers such as nylon or cross-linked polyethyleneimine membranes are unsuitable for live cell immobilization (Larisch et al. 1994) due to the toxicity of the reagents involved in the process. Rao et al. (1989) reported a microencapsulating procedure for *Bifidobacterium pseudolongum* which involved mixing the bacterial cells with starch and suspending the powder in light paraffin oil.

Chitosan, a water-soluble polymer (pH<6) has been used to microencapsulate *Lactococcus lactis* (Groboillot et al. 1993). In this study, a cross-linked chitosan membrane was formed by emulsification/interfacial polymerization using biocompatible reagents with oil-soluble cross-linking agents at low concentrations to minimize cell contact. Nevertheless, the anti-bacterial property of chitosan can limit its use as coating material in encapsulation (Sudarshan et al. 1992; Kong et al. 2010).

L. lactis cells were encapsulated within gelatin membrane cross-linked with toluene-2,4-diisocyanate at an oil/water interface. Reagent toxicity was avoided by the use of vegetable or silicone oil as a dispersant, and by minimizing cell exposure to the water-insoluble cross-linking agent during membrane formation (Hyndman et al. 1993). Larisch et al. (1994) observed a toxic effect mediated by solvents and reagents when *L. lactis* subspp. Cremoris was encapsulated within poly-L-lysine (PLL) membranes formed on alginate microspheres.

Some studies using monomers that are nontoxic for the microorganisms have been carried out in order to increase their productivity in fermentations with relative success (Groboillot et al. 1994; Hyndman et al. 1993).

5.3 Physicochemical processes

5.3.1 Coacervation

Coacervation is a phenomenon of forming a liquid rich in polymer phase in equilibrium with another liquid phase called coacervate (Korus 2001). Generally, the core material used in the coacervation must be compatible with the polymer and has to be insoluble in the coacervation medium. A core material is dispersed in an aqueous solution of one or more polymers; whereas in the aqueous phase, it can change pH, ionic strength, and temperature to induce the formation of a second polymer that becomes the wall material. Coacervation can be simple or complex. Simple coacervation is the result of the interaction of a dissolved polymer with a low molecular substance (for example, gelatin with alcohol or sodium sulfate). Complex coacervation occurs through the interaction of two polymers whose macromolecules bear opposite charges and is achieved by lowering the pH of a solution containing two polymers, one of which increases positive charges as a result of the change (high-isoelectric-point gelatin), and the other one has only negative charges (e.g., Arabic gum) (Bungenberg de Jong 1949).

Optimization of wall material concentration in the emulsification and coacervation process is difficult because the concentration required to obtain a fine emulsion may be different to the concentration needed to increase the yield of microcapsules (Nakagawa et al. 2004). Other limitations are evaporation, dissolution of active compound into the processing solvent, and oxidation of product (Flores et al. 1992).

Therefore, the coacervation method is efficient but expensive and has important limitations. Amiet-Charpentier et al. (1998b) showed that it is possible to obtain a polymer-containing rhizobacteria by complex coacervation.

5.3.2 Liposomes

Liposomes are microcapsules-like structures where the coating or outer membrane consists of one or more hydrated bilayers surrounding or encapsulating an active material in the internal compartment (Immordino et al. 2006; Fig. 7). For example, phospholipids may be used to form a spherical bilayer surrounding a nonpolar substance, such as a steroid. Currently, this process is expensive and low loading therefore must be considered only at laboratory scale.

5.3.3 Ionic gelation

A drop of aqueous solution or suspension containing the active material and sodium alginate is dropped into a solution of calcium chloride to form the capsules (Lim and Sun 1980). When the drop reaches the calcium chloride solution, a membrane of calcium alginate forms instantaneously,

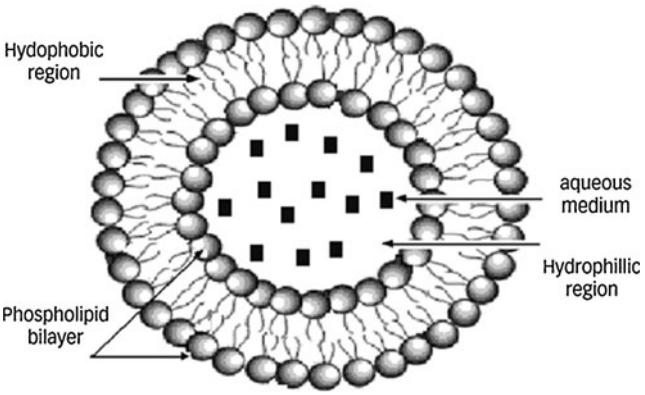

Fig. 7 Liposome structure where the coating or outer membrane consists of one or more hydrated bilayers encapsulating compounds in the internal compartment

maintaining the drop shape in this aqueous/aqueous system. Calcium diffuses in gelling the entire drop, which is then placed in a solution of a polycation that displaces the calcium from the outer surface, forming a permanent membrane. This capsule is then placed in sodium citrate, which slowly solubilizes the calcium through formation of the soluble citrate complex, ungelling the internal portion of the drop. By controlling the molecular weights of the reactants and the times of the reaction, the thickness and size of the permanent wall can be controlled over a wide range.

The conventional method produces calcium alginate beads through ionic gelation by dropping an alginate solution into a calcium chloride solution. The main advantage of gel immobilization is the biocompatibility, although scaling up is difficult and the beads are often porous to cells (Lacroix et al. 1990). Nevertheless, Covarrubias et al. (2012) recently have shown that alginate beads keep some cells inside the beads and prevent outside microbes from entering inside the beads, even under extreme conditions. Roy et al. (1987) presented alginate- containing lactic acid bacteria, which tended to be liquefied by lactic acid. To reduce mass transfer effects, PLL membrane coating of alginate beads followed by liquefaction of the alginate core was reported (Lim 1983); however, leakage of cultures from the matrix was still observed with PLL-alginate beads (Champagne et al. 1992). A variation of this method is to add other materials, such as starch to improve this process to encapsulate rhizobacteria, as is described by Schoebitz et al. (2012) where the matrix solution is prepared by mixing alginate and starch to improve the survival of rhizobacteria species by encapsulating in alginate beads, which allows the stable production of dried beads containing a high cellular concentration.

Sheu and Marshall (1993) reported an emulsion method to encapsulate lactobacilli in calcium alginate gels. In this method, the bacterial cells were mixed with sodium alginate solution and one part of this mixture was added dropwise to

vegetable oil containing an emulsifier such as Tween 80. Calcium chloride solution was then added to this emulsion until the water-in-oil-emulsion was broken. Similar procedures were used to encapsulate several kinds of bacteria, such as *Bacillus bifidum*, *Bacillus infantis*, and *Bacillus longum* (Gobbeti et al. 1997; Khalil and Mansour 1998; Kebary et al. 1998).

5.3.4 Inverse gelation microcapsules by using alginate

Inverse gelation deals with dropping a calcium suspension in an alginate solution. The conventional method to produce calcium alginate beads through ionic gelation is by dropping an alginate solution into a calcium chloride solution. If the procedure is inversed, that is to say, calcium chloride solution dropped into an alginate solution, aqueous-core calcium alginate capsules are produced (Koyama and Seki 2004; Sasaki et al. 2008). By diffusion in the alginate solution, calcium will gelify the alginate and form a membrane around the droplets. The calcium suspension consists in calcium chloride solution dispersing bacterial cells and this emulsion finally drips into alginate solution (Abang et al. 2012; López et al. 2012). A variation of inverse gelation is performed by using modified starch, which is added in calcium chloride solution, or even starch with alginate solution to increase the solid content in the membrane.

Abang et al. (2012) studied the effects of process variables on the physical properties of capsules produced by the inverse gelation method. In this study, alginate was used to form the capsule membrane and three different methods of incorporating the calcium source in oil were tested. The process variables examined were sodium alginate concentration, calcium chloride concentration, and curing time while physical properties of the capsules investigated were membrane thickness and elastic modulus.

This methodology was used by Jankowski et al. (1997) to develop biocompatible capsules consisting of a liquid core with calcium alginate membranes for encapsulating lactic acid bacteria.

5.3.5 Beads by oil-entrapped emulsion

Beads are formed by dripping an alginate solution (containing a dispersion of cells and glycerol) into a calcium solution. Diffusion of the calcium in alginate droplets leads to their gelation (López et al. 2012). Starch and glycerol are added to the formulation to increase the solid content and solve one of the drawbacks of alginate beads, that is to say, decrease porosity since glycerol also increased the viscosity and consequently improved the stability of emulsion.

This method appears to be a promising technology for storing and delivering microorganisms since glycerol could improve both cell viability and controlled release.

6 Conclusions and future trends

This review brings information, opening up the potential for succeeding in the encapsulation of microbial inoculants for agricultural purposes, showing the advantages of using them over liquid inoculants, peat, and clay.

The advances in this field have been presented featuring bioencapsulation materials and techniques used for immobilization and bioencapsulation of rhizobacteria. Nevertheless, conventional microbial inoculants are not able to ensure high cell viability during formulation, storage, and inoculated in the soil. Conventional inoculants need to be stored at room temperatures avoiding extreme temperature oscillation, and the shelf life of liquid inoculants on storage conditions is very short and their viability decrease for one or two logs. The use of liquid inoculants is not able to offer protection to rhizobacteria against biotic and abiotic soil stresses, such as pH, moisture, temperature oscillation, and protozoa depredation. Instead, bioencapsulation provides a niche where rhizobacteria are protected from the soil stress. Furthermore, the liquid inoculum, after being introduced into the soil has an instantaneous and very fast release and the rhizobacteria are delivered only in the initial moment of the plant growth. Alternatively, the encapsulated inoculants confer a gradual cells release that achieves long-term fertilizing effects.

Apart from the comparison between capsules and liquid inoculants, one of the most expensive steps is the fermentation since requires to prepare a huge volume of water and broth and sterile medium. However, each capsule into the soil, may act as a mini-bioreactor increasing the concentration of initial viable cells. This can reduce costs associated with the multiplication of bacteria. Furthermore, if there are several capsules around the rhizosphere soil, each capsule may act as an independent unit, which increases the cells concentration enhancing the roots colonization and the microbial inoculants efficacy on the field. The choice of an appropriate technique of bioencapsulation will depend on the properties of the bacteria, the degree of stability required during storage and processing and also the production cost.

Microencapsulation by spray drying is an economical and flexible way to encapsulate bacteria although the temperature used could turn out to be the critical point. In addition, fluid-bed process is also becoming a promising encapsulation technique for microbial inoculant large-scale production to be applied in agricultural industry. However, ionic gelation is currently the most adequate method found to encapsulate rhizobacteria. Alginate-starch beads are able to load a high cells concentration at 10^9 CFU g^{-1} with an average diameter of bead at more than 4 mm. However, the diameters of beads/capsules have a huge variation of diameter (1 μ to 6 mm) that depend on the encapsulation techniques, bead matrix composition, and the internal diameter of the diffusers. From the farmers and agricultural industry point of view, the

ideal diameter of the beads should be similar to the seed used. Because, in this way, the process of sowing and the introduction of microbial inoculants can be performed at the same time and using the same seed drill. Additionally, the beads and seeds are introduced close into the soil favoring the effectiveness of the microbial inoculants.

The inconvenience of bioencapsulation technology must not be ignored. Many of the bioencapsulation devices are confectioned to produce beads in laboratory conditions and very small scale. This allows testing encapsulated formulations only in growth chambers and greenhouses conditions. To produce a large amount of inoculant, trials on large fields are required to use innovative bioencapsulation devices. GeniaLab developed the jet cutter technology in Germany. This technology allows the production of beads with a high viscosity involving the fluid transfer through a needle. It is cut into segments evenly with the use of a rotating cutting wheel. The diameter of the beads is conditioned by the speed of rotation of the cutting wheel and the mass of fluid passing through the cutting wheel. Using the jet cutter technology, it is possible to scale-up the production of beads to expand the use of encapsulated microbial inoculants in agriculture. In the future, the developments of new formulation procedures will be determined by the demand of the industry translating the studies from laboratory scale to industrial production.

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