Bioencapsulation Research Group

Bioencapsulation Innovations

May 2016

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EDITORIAL

ENCAPSULATION IN AGRICULTURE/AQUACULTURE

Hydrocolloid beads can be utilized to encapsulate microorganisms, agricultural chemicals, genes, exogenous DNA, seeds, and other agents for agriculture and aquaculture. In the last few decades, increasing attention has been paid to beads from a range of materials and in various sizes appropriate for the immobilization of microorganisms, enzymes, antibodies, etc. The main rationale for using such beads is their ability to immobilize organisms or proteins for an extended period, after which the products of the entrapped living material can be collected. An additional and significant goal of agriculture is inoculation of seedlings and plants with beads containing fungal inoculum. Another simple, quantitative experimental methodology was used to jointly immobilize plant growth-promoting bacteria with green microalgae. This approach allowed adjacent interactions while avoiding external interference from contaminating bacteria. A very significant application of using polymers for bioencapsulation in agriculture is the controlled release of agricultural chemicals. These can include fungicides, germicides, growth regulators, herbicides, insect diets as well as insecticides. Numerous considerations are involved in fine-tuning a polymer for a specific system, including price, seasonal conditions, desired release rate, duration and simplicity of formulation, and application. The ultimate benefit of controlled-release formulations is that less chemical is used for a particular time interval, consequently lowering its effect on non-target species and limiting leaching, volatilization, and degradation.

Numerous natural and synthetic polymers are utilized for controlled-release purposes in agriculture. Natural polymers include carboxymethylcellulose (CMC), gelatin, gum arabic, starch and arabinogalactan. Synthetic polymers include polyvinyl alcohol, polystyrene, polyacrylamide and polyvinyl chloride, among others. Physical systems that integrate agricultural chemicals include microencapsulation, physical blends, dispersion in plastics or rubbers, laminates, hollow fibers and membranes. Special biotechnological applications for agricultural/aquaculture uses include gene-delivery systems by means of beads. One unique application of polymers in agriculture is the use of superabsorbent polymers. These water-absorbing preparations hold promise for use in planting and growth development, and in arid lands or regions where less frequent watering is desired along with full development of plants grown in such treated soil. Another use of encapsulation is the preparation of artificial seeds by encapsulating somatic embryos on specific media as well as seed coating.

In this newsletter, we have tried to include a variety of developments in, and novel applications of bioencapsulation. Two articles deal with aquaculture: the first describes the larvicultural development of Artemia bioencapsulation. This method provides a natural and cost-effective source of essential fatty acids to enrich Artemia, with potential benefit for marine fish larvae. Another novel approach in aquaculture and water treatment is the use of freeze-dried alginate beads with embedded denitrifiers and a carbon source to achieve sustained denitrification activity over a prolonged period of time when utilized for nitrate removal in freshwater and marine aquariums. This novel method may offer an attractive alternative to current methods for nitrate removal. Another contribution discusses the distribution of microorganisms in a carrier. This is a most important issue, particularly due to the differences between alginate and other carriers. A different use of dried cellular carriers is as a barrier to ultraviolet radiation, where the protected immobilized microorganisms can be sprayed directly on plants or on the ground. Another novel approach presented herein is carbon-dioxide-releasing formulations that are paving the way for novel attract-and-kill approaches based on botanical or microbial insecticides, as well as immobilization of soil-beneficial microorganisms and organisms.

As guest editor, I am certain that you will find the contributions novel, interesting and informative, in keeping with the consistently outstanding quality of the Bioencapsulation Innovations newsletter.

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CALENDAR

PROGRAM 2016

May

EUDRAGIT/ EUDRAGUARD : New products & technologies for oral pharmaceutical and nutraceutical targeted delivery
May 24, 2016
Paris, France
https://e-lab.eudragit.com/symposium/eudragit_eudraguard_advanced_workshop/

8th Training School on Microencapsulation
May 30 - June 2, 2016
Cork, Ireland
http://bioencapsulation.net/2016_Cork

July

11th International Symposium on Polyelectrolyte
June 27-30, 2016
Moscow, Russia
www.isp2016.org

COSM'ING - A Cosmetic Ingredients & Biotechnology International Event
June 29th → July 1st, 2016
Saint-Malo, France
https://www.b2match.eu/cosming2016

CRS annual meeting July 17-20, 2016
Seattle, Washington, USA
http://www.controlledreleasesociety.org/meetings/annual/

August

Sort course on Advances in Tissue Engineering
August 10 - 13, 2016
Houston, TX, USA
http://tissue.rice.edu

2nd International Conference on Therapeutic Drug Monitoring and Toxicogenomics
August 25-26, 2016
Philadelphia, USA
http://toxicogenomics.conferenceseries.com

September

22th Bratislava International Conference on Macromolecules: Polymers with Tailored Architecture and Properties - BIMac2016
September 6-9, 2016
Bratislava Slovakia
http://polymer.sav.sk/bimac/

Pellets and Micropellets for oral solid dosage forms
September 20-22, 2016
Binzen Germany
http://www.ttc-binzen.de/cm/index.php?id=1077

24th International Conference on Bioencapsulation
September 22-24, 2016
Lisbon, Portugal
http://bioencapsulation.net/2016_Lisbon

September

4Th conference on innovation in drug delivery: site-specific drug delivery
25-28 September, 2016
Antibes-Juan-les-Pins, France
http://idd2016.sciencesconf.org

October

32ème édition du Club Emulsion
Octobre 3-4, 2016
Castres, France
http://www.clubemulsion2016.org/

Introduction to Microencapsulation Workshop
Octobre 3-4, 2016
San Antonio, TX, UWA
http://www.swri.org/9what/events/courses/microencapsulation/registration.htm

November

Special session organized by BRG
Targeted Technologies for Sustainable Food Systems
28-30 November 2016
Vienna, Austria
http://www.effostconference.com/special-sessions.asp

December

Small Particle Formation
March 11- 12 , 2016
San Diego,CA, USA
http://nanoparticles.org/courses/SanDiego-2016Courses.htm

FUTURE CONFERENCES AND EVENTS SUPPORTED BY THE BRG

8TH TRAINING SCHOOL ON MICROENCAPSULATION

Cork, Ireland

May 30 - June 2, 2016

60 participants

12 Lectures by experts

8 pratical demonstrations

More information

http://bioencapsulation.net/2016_Cork

24TH INTERNATIONAL CONFERENCE ON BIOENCAPSULATION

Lisbon, Portugal

September 22 - 24, 2016

125 to 150 participants

40 oral presentations

up to 80 posters

More information

http://bioencapsulation.net/2016_Lisbon/

THEY SUPPORTED BRG IN BEING EXHIBITORS DURING THE 23TH INTERNATIONAL CONFERENCE ON BIOENCAPSULATION

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ARTICLE

ENCAPSULATED FORMULATIONS FOR MICROORGANISMS IN AGRICULTURE AND THE ENVIRONMENT

Bashan, Y., L. E. de-Bashan – The Bashan Institute of Science, Alabama, USA and CIBNOR, Mexico

INTRODUCTION

Inoculation of plants with microorganisms to enhance crop yields or native plants has been practiced for several decades now (Bashan et al., 2014; Calvo et al., 2014). Two factors predominate in the success of inoculation: effectiveness of the bacterial isolate and the technology of application. Technologies of microorganism encapsulation in these areas are experimental, and no commercial encapsulation products currently exist (Nussinovitch, 2010). The first formulation of encapsulation with alginate, which is the most common experimental polymer in agriculture, is already 30 years old (Bashan, 1986). Technologies of encapsulation related to agricultural and environmental fields are lagging behind the advances in pharmaceutics and nanotechnology. Consequently, the main goal of encapsulation in agricultural and environmental fields is to incorporate it into commercial products and to make the products acceptable to farmers as a routine practice, rather than creating new approaches or polymers. This should largely consist of an adaptation of current techniques to current agricultural and environmental needs.

MACROBEAD INOCULANTS

In 2016, alginate derivatives, frequently combined with adhesives, nutrients, surfactants, stabilizers, dispersal materials, bulk materials and cryo-protectants, are the preferred experimental polymers for most encapsulations of microorganisms for agricultural and environmental uses. Alginate formulations are currently used for the application of biological control agents, bacterial growth promoters, mycorrhizal fungi, and mushroom cultivation. The advantages of alginate formulations for these purposes are their nontoxic nature, biodegradability, availability at low cost (US$2 per kg for a Chinese product), slow release of the entrapped microorganisms into the soil—which can be accomplished by variations in the polymeric structure, and approval for human use by the US Food and Drug Administration.

While alginate formulations may have solved many of the difficulties associated with other agricultural inoculants, application of macro-alginate beads as inoculants has two unsolved disadvantages:

1. an additional treatment during sowing is needed by the grower, even if the inoculant is planted by seeding machines;
2. microorganisms released from the inoculant need to migrate through the soil to the plants.

Under agricultural practices, when beads are loosely mixed with seeds and sown together, the beads might land up to several millimeters or even few centimeters away from the plant. The bacteria released from the beads must move through the soil, facing competition with and predation by the native microflora. Consequently, the future of macrobeads in agriculture is uncertain.

MICRO-ALGINATE BEADS

The microbead concept (50–200 µm or even smaller) was developed to overcome the two difficulties of macrobeads (Bashan et al., 2002; Figure 1). The idea is that if the beads are small enough, yet still capable of encapsulating a sufficient number of bacteria, it would be possible to produce a "powder-like" formulation. At the seed-handling facility, the seeds are coated with this "bead powder" and sold to the farmer as "improved seeds". Today, seeds coated with fertilizers, fungicides, or hormones are commonplace and universally accepted by most farmers. In developed countries with large-scale agricultural practices, pre-coating seeds with microbead inoculants would eliminate the need for an additional expensive field treatment and provide

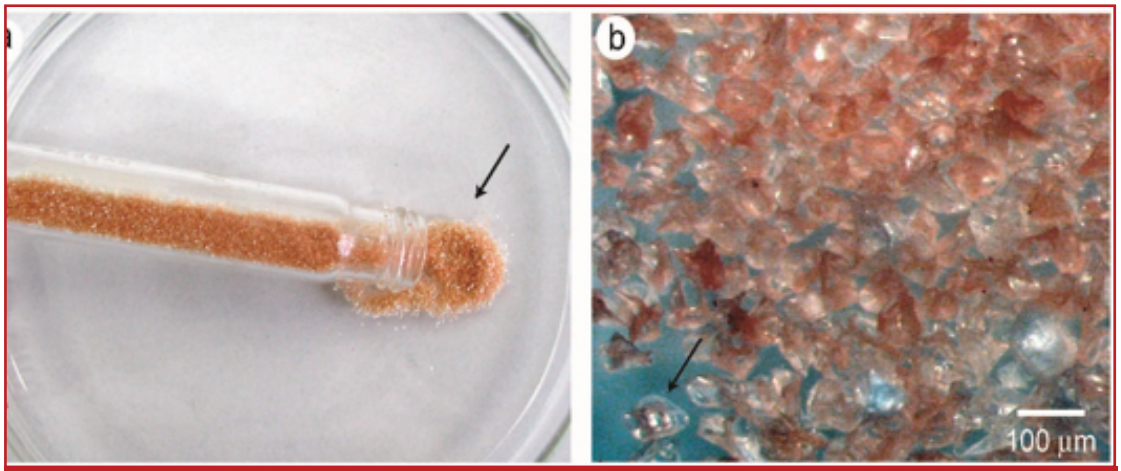

Figure 1. (a) Dry alginate microbead inoculant containing the plant growth-promoting bacterium Azospirillum brasilense currently being used in agriculture and the environment. (b) Single microbead (arrow).

the ultimate convenience and incentive to farmers. Application of formulations in micro-alginate beads to inoculate plants in the soil has been successful on several occasions (de-Bashan et al., 2002) .

FUTURE IMPROVEMENTS IN MICRO-ALGINATE BEADS

Currently, food, pharmacology, nanotechnology, and cosmetics represent much larger research fields for the use of encapsulation than its uses on land. Consequently, several technical improvements derived from these fields to make the polymer more suitable for the encapsulation of biological materials have been proposed. Although these encapsulations are unrelated to plant inoculants, they may provide insights for future developments. Based on experiments over the last three decades, alginate seems to be the most promising polymer. However, because of the relatively limited published research data on alginate beads related to land use, and even if the material is currently inexpensive compared to all other polymers, it is premature to predict whether alginate will displace traditional inoculants in the crop inoculation industry or will remain in the domain of industrial and environmental microbiology.

INOCULANTS WITH OTHER POLYMERIC MATERIALS

Ironically, although commercial alginate preparations are not yet available for agriculture, several other polymers that are used in industrial and environmental microbiology may serve as substitutes when the microorganism fails to adapt to alginate preparations. All of these materials are experimental and use both macro- and microbead formulations. They include chitosan, carboxymethylcellulose–starch, ethylcellulose, modified starch, and commercial film-forming "methacrylic acid copolymer". However, because very limited information is available on these potential carriers, it is impossible to predict their future as vehicles for bacterial inoculants.

DRIED POLYMERIC CARRIERS

A main bottleneck in the production of any inoculant for agricultural and environmental improvements is shelf life, which should be increased rather than maintaining a high bacterial count because the number of bacteria eventually decreases during storage. From commercial and agricultural standpoints, longer survival of bacteria in polymeric preparations makes dry formulations extremely attractive. Such experimental formulations exist and have a shelf life of 1 to 14 years, superior, by far, to any existing commercial microbial inoculant.

CONCLUSIONS AND PERSPECTIVES

Even though numerous experimental results indicate that this is perhaps the future of inoculants, no encapsulated formulation of microorganisms has obtained industrial approval. Immobilization of microorganisms is a large emerging field in pharmaceuticals, nanotechnology, medicine, aquaculture, and cosmetics (Bioencapsulation Innovations, http://bioencapsulation.net). Many different and efficient immobilization techniques have been developed for these fields, but almost none of these technologies has been tested in the inoculant field apart from direct polymerization of beads from a few polymers. Many of these emerging technologies from other fields merit testing in the agricultural inoculant industry.

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Prof. Yoav Bashan is the President of The Bashan Foundation, the President of The Bashan Institute of Science (USA), the Group Leader of Environmental Microbiology at the Northwestern Center for Biological Research (Mexico) and is also affiliated with Auburn University (USA). He obtained his doctoral degree from the Hebrew University of Jerusalem in Israel. His main field of research is the interactions among plant growth-promoting bacteria and plants in agricultural and environmental settings, mostly using immobilization techniques. Prof. Bashan has published over 360 scientific works, 211 of which have been published in peer-reviewed journals with impact factors. He has obtained over 14,700 citations of his studies; H-index: 62; i-index: 182.

ARTICLE

MARINE FISH LARVICULTURE AND ARTEMIA BIOENCAPSULATION

Gajardo, G. – Laboratorio de Genética, Acuicultura & Biodiversidad, Universidad de Los Lagos, Osorno, Chile

Acevedo, F. – Scientific and Technological Bioresource Nucleus (BIOREN), Universidad de La Frontera, Temuco, Chile

INTRODUCTION

Marine fish larviculture and bioencapsulation are intimately related, because overcoming the high mortality observed during larval rearing in captivity (larviculture) requires proper nutrition via an artificial live diet. This is particularly true during metamorphosis, when the fish larva undergoes radical molecular, biochemical, physiological and morphological changes. The brine shrimp *Artemia* is commonly used as a convenient and cost-effective replacement for marine plankton. This salt-loving extremophile is the sole macroscopic inhabitant adapted to the harsh conditions of hypersaline lakes worldwide (Fig. 1).

Artemia, however, coexists with a high diversity of microorganisms with great biotechnological potential that contribute to its survival. Its surprising life cycle evolved to cope with these stressful conditions (Fig. 2) (Gajardo and Beardmore, 2012), and it has been creatively exploited for larviculture development.

As artificial live prey often lack essential nutrients, such as fatty acids, that are common in the highly diverse natural food web (Dhont et al., 2013), *Artemia* needs to be enriched to provide a balanced diet to the larvae.

Figure 1. Hypersaline lake in Atacama Desert, Chile, the ecosystem where the brine shrimp *Artemia* lives and coexists with a great microbial biodiversity. The area is a nesting ground for flamingos which are natural *Artemia* dispersers (from Gajardo and Beardmore, 2012).

This is done through the bioencapsulation process, which takes advantage of the ability of *Artemia* nauplii, instar II (48 h posthatch), to filter particles of less than 28 µm. Enrichment products exist in different forms—as unicellular organisms, microencapsulated, emulsified or self-emulsifying concentrates, microparticulated products or a combination of these. However, since larval nutritional requirements are species-specific, the most appropriate enrichment for larval development in a given species needs to be established and tested. *Artemia* is the perfect biological vector to test and deliver relevant nutrients to the larva, such as essential fatty acids, micronutrients (vitamins, pigments, sterols, antioxidants, enzymes, immunostimulants), and novel nutrients (lysine, iodine and selenium). *Artemia* also delivers therapeutic substances (vaccines, probiotics) that help control the microbial community in larval-rearing tanks, another source of larval mortality (Dhont et al., 2013). The *Artemia*–bacteria coexistence in the wild, in laboratory-reared animals and in the gut microbiome (Quiroz et al., 2015) has brought about alternative anti-infective strategies for aquaculture rearing. This is due to the discovery of bacteria with probiotic capabilities, stemming from their ability to synthesize ß-poly-hydroxybutyrate (PHB) (Baruah et al., 2015). Such PHB-producing bacteria help control the microbial community in larval-rearing tanks, and can be delivered to the larvae via *Artemia*. The host–pathogen relationship has been well studied with the gnobiotic *Artemia*, an ideal system that eliminates interference caused by the highly diverse bacterial community of natural (real) systems.

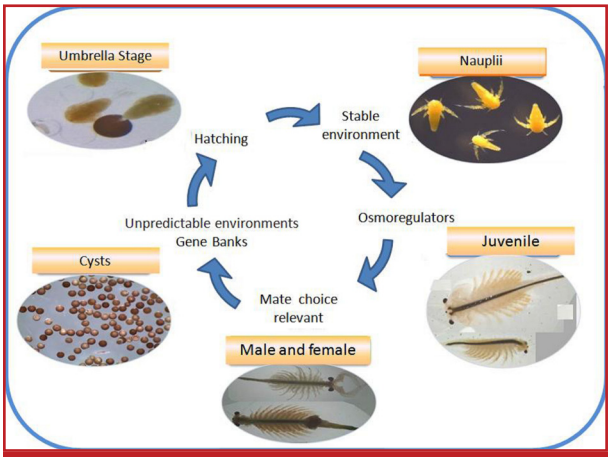

Figure 2. The life cycle of the salt-loving extremophile *Artemia* has been exploited for larviculture development. Cysts produced under stressful conditions are the exchange coin in the aquaculture business. Cysts are commercialized vacuum-canned. After 24 h of hydration, swimming nauplii hatch, and after 48 h (instar II) they are ready to be enriched and given as a convenient live food to the marine fish larvae (from Gajardo and Beardmore, 2012).

This note highlights two benefits for larviculture development of *Artemia* bioencapsulation: (1) the opportunity to test new aquafeed ingredients aimed to increase aquaculture sustainability. In particular, the use of oil bodies (OBs) extracted from the rapeseed *Brassica napus* as an alternative to fish oil; (2) isolation of PHB and/or PHB-producing bacteria from the *Artemia* gut microbiome of laboratory-reared animals as a way of controlling bacterial communities and, therefore, fish larval mortality in rearing tanks.

MATERIAL & METHODS

OBs from the rapeseed *B. napus* are intracellular lipid-rich organelles that are easily extracted from seeds, and can be used to enrich *Artemia* for later testing in marine fish larvae. OBs have also proven to be safe and efficient delivery carriers of bioactive molecules for food and/or pharmaceutical purposes, such as astaxanthin (Acevedo et al., 2014), a pigment required to increase the quality of fish flesh. To bioencapsulate them, hatched *Artemia* nauplii were distributed

in conical tanks containing artificial seawater (35 ppt, at 25 °C, with aeration and light), and their uptake of OBs was monitored. Temporal samples of nauplii were checked by the rapid Oil Red O qualitative lipid-assessment technique and by confocal microscopy using AxioVision (Zeiss, Germany). To isolate PHB-producing bacteria, a protocol for micro-dissecting the *Artemia* gut under a stereoscopic microscope was developed (Quiroz et al., 2015). The isolated bacterial strains were tested for growth in different culture media, and the successfully growing strain was tested for presence of PHB by means of spectrophotometry (200–300 nm) using pure PHB (Sigma) as a control. The PHB-producing strain was identified by 16S rDNA gene partial sequencing homology.

RESULTS & DISCUSSION

The spherical shape and size (0.5 to 2.0 µm in diameter) of OBs (Fig. 3) and their oil content which is rich in monounsaturated fatty acids (such as 18:1n-9) and saturated fatty acids (such as 16:0 n-3) make them suitable for ingestion by *Artemia*.

Preliminary trials showed that nauplii take up and store OBs for longer than 24 h of enrichment (Fig. 4), during which time the nauplii increased their basal fatty acid composition 1.6-fold. After 72 h, enriched *Artemia* nauplii still conserved (1.3-fold) the ingested components.

A culturable PHB-producing bacterium identified as *Vibrio* sp. strain HS1 (100% sequence homology) was isolated from the gut of laboratory-reared *Artemia* individuals. This is an interesting result because different types of vibriosis need to be combated in aquaculture hatcheries. This strain accumulates 706 mg PHB per kilogram dry culture (about 70% of cell dry weight). This is not an uncommon result as the *Vibrio* strain M11, closely related to *Vibrio natriegens*, concentrates about 41% of its cell dry weight as PHB.

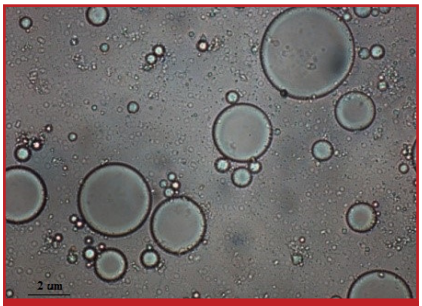

Figure 3. Oil bodies isolated from *Brassica napus* seeds. Light microscopy using a 100× oil- immersion objective. The image was captured with a coupled camera (Micropublisher 3.3 RTV) and processed using Qcapture Pro 6.0 software.

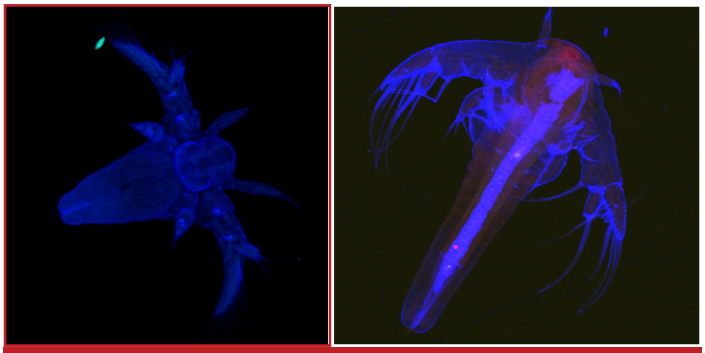

Figure 4. (a) Non-enriched *Artemia*. (b) *Artemia* enriched with oil bodies, after 72 h (laser scanning microscopy, FV 1000 Fluoview, Olympus Europa GMBH, Germany, 100× oil immersion objective).

CONCLUSIONS & PERSPECTIVES

The live dietary *Artemia* can bioencapsulate different microparticles, such as the lipid-rich OBs extracted from the rapeseed *B. napus*. These are a natural and cost-effective source of essential fatty acids to enrich *Artemia*, and future studies should verify their benefit for marine fish larvae. *Artemia* is also a source of probiotic bacteria that offer alternative and environmentally friendly anti-infective treatments for aquaculture operations.

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Gonzalo Gajardo is Professor of genetics applied to aquaculture and biodiversity at University of Los Lagos. As a British Council postdoctoral scholar at Swansea University, Wales, UK, and as visiting researcher in Ghent University (Laboratory of Aquaculture & Artemia Reference Center), he became interested in the brine shrimp Artemia as a model extremophile for the study of adaptation and speciation, but also on its applied side as one the most used live diet for the larviculture of marine fish and crustaceans. In this regard he has concentrated in adaptation-related traits of interest for aquaculture using the New World species from the Atacama Desert, one of the driest in the world (North of Chile), and Laguna Amarga in Torres del Paine (South of Chile). These two magnificent touristic spots and extreme ecosystems serve as natural laboratories for monitoring climatic change, but are also the source of biotechnological metabolities and pigments either from Artemia or the associated bacteria.

ARTICLE

CELLULAR SOLID CARRIERS PROTECT BIOCONTROL AGENTS AGAINST UV LIGHT

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INTRODUCTION

Current developments in biotechnology have led to the isolation of numerous microorganisms for the improvement of plant protection and crop growth in agriculture. However, achievements with a microorganism in vitro do not guarantee its successful use in the field (Bashan, 1998). Soil microorganisms in general, and biocontrol agents in particular, are very sensitive to ultraviolet (UV) light. The direct use of antagonistic antibiotics derived from biocontrol agents is also limited by their UV sensitivity (Van Pee and Ligon, 2000). Immobilization of microorganisms in dried alginate carriers has been suggested to decrease the deleterious effects of UV radiation on the usefulness of the microorganism preparations.

Even though the literature contains numerous reports dealing with gel beads, alginate beads in particular (Nussinovitch, 1997, 2003), studies on the usage of dried hydrocolloid carriers are less common. Freeze-drying gel beads results in a low-density dried cellular product that includes many fused open and closed cells. These create the cell wall which influences the bead's physical properties and immobilization ability. The packaging of biocontrol microorganisms into cellular solids was developed as a means to reduce loss caused by exposure to environmental UV radiation. The term cellular solid originates from the word cell, i.e. a small compartment, an enclosed space. Connecting the solid edges is a simple way to fill space with unique structures, which can be observed in many natural instances, e.g. wood, cork, and natural sponge (Figure 1). The unique mechanical properties of cellular solids are shown in Figure 2. Our goal was to evaluate and correlate the properties of dried cellular solids to their ability to protect against UV radiation.

Figure 1 : Sponge – a natural cellular solid.

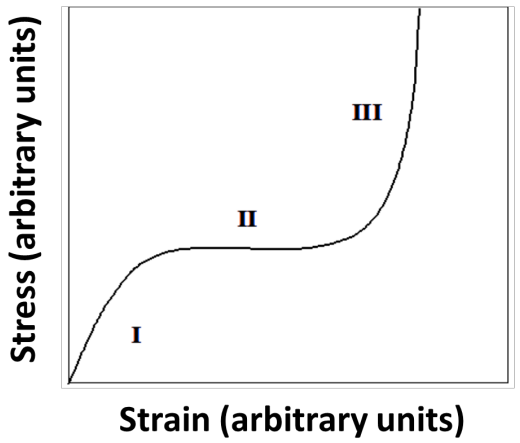

Figure 2. Schematic representation of a typical compressive stress–strain relationship of a cellular solid. (I) Deformation of the original matrix; (II) densification; (III) compaction of the collapsed cell wall material.

MATERIAL AND METHODS

The bacterium *Pantoea agglomerans* and the fungus *Trichoderma harzianum* were used as model microorganisms for entrapment. Alginate (2% w/w) was dissolved in distilled water. Glycerol (30% w/w) was added to the alginate solution as a cryo-protectant. For the alginate-filler bead preparation, a colloidal chitin suspension, bentonite or kaolin was added to the alginate and alginate–glycerol solutions to a final concentration of 0.5% (w/w). All mixtures were sterilized by autoclaving and the bacteria or fungal spores were then added at a 1:9 volumetric ratio to the different sterile alginate solutions. Those final mixtures were dripped into a 1% (w/w) sterile solution of calcium chloride and stirred for 30 min. A spontaneous cross-linking reaction produced spherical beads with a diameter of 2.9 to 3.6 mm, containing either 10^7 colony-forming units/bead (fungi) or 10^9 bacteria/bead. The beads were removed from the calcium chloride solution and washed twice with sterile distilled water. They were then freeze-dried and the carriers containing the fillers were subjected to 254 nm UV subtype C (UVC) radiation.

RESULTS AND DISCUSSION

The effects of UV radiation on the structural properties of cellular solids are of great interest, and various types of natural and artificial cellular solids have been studied to determine the radiation's penetration

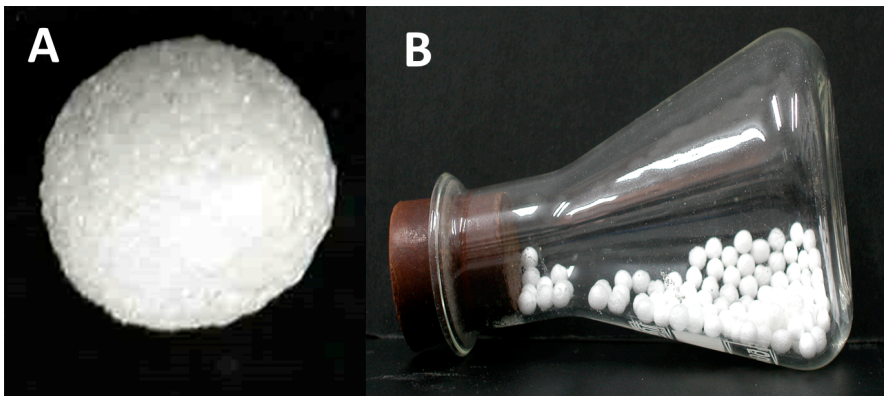

Figure 3. (A) Dried cellular solid carrier including biocontrol agent. (B) Numerous carriers in a container.

depth and transmission properties. UV radiation penetrates the surface of wood to a depth of approximately 75 mm and causes photodegradation of lignin in the wood's surface. Penetration depths of 40 to 400 mm have been found for various leaves, depending on leaf type and age, and UV wavelength. In the spectral range of 115 to 350 nm, the transmission through regular microporous polyethylene terephthalate membranes (an artificial cellular solid) depends mainly on pore size and density. Immobilization of cells in freeze-dried alginate–glycerol beads (Figure 3 and Figure 4) resulted in greater survival after UVC irradiation than that in a suspension of free cells. Adding chitin, bentonite or kaolin as filler to the alginate–glycerol formulation significantly increased bacterial survival (Zohar-Perez et al., 2003), with immobilization in the alginate–glycerol–kaolin beads resulting in the highest survival rates. The transmissive properties of the dried hydrocolloid cellular solid had a major influence on the amount of protection afforded by the cell carrier. Dried alginate matrix (control) transmitted an average 7.2% of the UV radiation. Filler incorporation into the matrix significantly reduced UV transmission: alginate with kaolin, bentonite or chitin transmitted an average of 0.15, 0.38 and 3.4% of the radiation, respectively. In addition, filler inclusion had a considerable effect on the bead's average wall thickness, resulting in a 1.5- to 3-fold increase relative to beads based solely on alginate.

Figure 4. Cross section of hydrocolloid-based carrier including bentonite, glycerol and immobilized microorganisms.

CONCLUSIONS AND PERSPECTIVES

A controlled natural cellular structure, to serve as a carrier for biocontrol agents or other biological purposes, was achieved by freeze-drying alginate-based gel beads or gels produced from water-soluble polymers. Results suggest that the degree of protection of entrapped microorganisms against UVC radiation is determined by the UV-transmission properties of the dried matrix and the cellular solid's structure. It was concluded that for maximum protection against UVC-radiation-induced cell loss, biocontrol microorganisms should be immobilized in alginate–glycerol beads containing kaolin (Zohar-Perez et al., 2003).

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ARTICLE

IMMOBILIZATION OF SOIL-BENEFICIAL MICROORGANISMS

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INTRODUCTION

The development of microbial inoculants is on the rise due to recognition of the deleterious effects on the environment of excessive application of chemical fertilizers. Sustainable agricultural production can be achieved by emphasizing the use of plant growth-promoting rhizobacteria as microbial inoculants (Schoebitz et al., 2013a). In general, microorganisms promote plant growth in three different ways: by synthesizing hormones for the plants, by facilitating the uptake of nutrients from the soil, and by lessening or preventing plant diseases. Encapsulation of microorganisms has been widely used in agriculture to obtain a surrounding structure that allows for protection, release and functionalization of the microorganisms. In fact, encapsulation tends to stabilize cells, providing less exposure to abiotic and biotic stresses and potentially enhancing their viability and stability during production, storage and handling, while also conferring additional protection during rehydration.

Numerous studies have focused on developing different encapsulation techniques to improve the survival of microbial inoculants. Encapsulation of microorganisms is one of the newest and most efficient technologies. For instance, encapsulation via dripping technique has been employed for the protection of cells, enabling better survival in soil after inoculation (Schoebitz et al., 2012). Encapsulated cells can be released into the target soil in a slow and controlled manner, providing greater long-term effectiveness. Nevertheless, since the 1980s, most studies on beneficial soil microorganisms have focused on bacterial genetics and physiology, and research on inoculant formulations represents less than 1% of the scientific articles on microorganisms.

PRINCIPLE OF INOCULANT FORMULATION

The principle of rhizobacterial inoculant encapsulation is to protect the microorganisms introduced into the soil and to ensure gradual and prolonged release. The degradation rate of the encapsulation matrix is directly related to the biological activity of the microorganisms; in fact, the quicker the matrix degradation, the lower the protection and biological activity of the microorganisms. Thus, encapsulation and storage at room temperature allows for a long storage period, as it provides a favourable environment for bacteria.. These inoculants can be improved by incorporating essential nutrients for bacterial growth, thereby transforming the capsules into bioreactors that are capable of increasing the number of encapsulated bacteria inoculated in the soil.

SPRAY DRYING

Spray drying, which is a dehydration process, is an immobilization technique used for the production of microbial inoculants that can be incorporated into agricultural systems. It has been demonstrated that heat-induced cellular damage is primarily associated with changes in the physical state of the membrane. In addition to the carrier–microorganism interaction, the impact of the spray-drying process is associated with processing parameters (inlet and outlet air temperature, feed flow rate, residence time in the drying chamber, design parameters of the drying chamber, and temperature of the drying medium) and the biology of the bacteria. In fact, Campos et al. (2014) obtained 91% bacterial survival for Enterobacter sp. immobilized using sodium alginate and maltodextrin as wall materials (2:13 w/w), a feed flow rate of 73 m^3/h, and inlet and outlet temperatures of 100 °C and 65 °C, respectively. In contrast, Schoebitz et al. (2013b) found lower cell survival after encapsulation of microorganisms such as Serratia sp. using only maltodextrin as the wall material, and inlet and outlet temperatures of 145 °C and 90 °C, respectively. In this sense, the strain type, temperature of the drying process, and formulation of the polymer mixture used as a vehicle are important parameters for the encapsulation of microorganisms to obtain a successful microbial inoculant.

Figure 1. Immobilization of microbial inoculants in calcium–alginate beads by ionic gelation technique (beads 2–3 mm diameter).

FLUIDIZED BED

The fluid-bed technique involves drying, cooling and coating of particulate materials for a wide range of heat-sensitive products. In fact, fluid-bed spray coating can avoid some of the problems associated with the spray-drying technique. In fluidized-bed drying, the particles to be coated are fluidized by hot air. The coating material is then sprayed through a nozzle onto the particles; film formation is initiated, followed by a sequence of wetting and drying stages. The small droplets of the sprayed liquid reach the particle surface, join with it, and the solvent is evaporated by hot air while the coating material remains on the particles. This technology offers a number of advantages. It allows specificity in particle size distribution and yields low porosities of the granules, while it also presents high drying rates, smaller flow area, high thermal efficiency and simple operation. Fluidized-bed drying performs better than spray drying because it is considered less stressful for drying microbial cells than spray-drying technology. In addition, it involves less extreme water loss and temperature gradients.

IONIC GELATION

This established method produces calcium–alginate beads (Fig. 1) through ionic gelation by dropping an alginate

solution into a calcium chloride solution. The main advantage of gel encapsulation is the biocompatibility, although scaling up is difficult and the beads are often porous to cells. A variation of this method is to add other material, such as starch, to improve the process of encapsulating rhizobacteria, as described by Schoebitz et al. (2012). In this method, matrix solution is prepared by mixing alginate and starch to improve the survival of the rhizobacterial species encapsulated in the alginate beads. This allows the stable production of dried beads that contain a high concentration of cells.

Table 1. Number of viable A. brasilense and R. terrigena cells encapsulated in alginate-starch beads. Cells were cultivated and immobilized in the logarithmic or stationary growth phase (CFU=colony forming unit)

bacteria	growth phase	initial culture (total CFU)	dry beads (total CFU)	yield (%)
Azospirillum brasilense	logarithmic	$1.3x10^{10}$	$3.7x10^{7}$	0.3
	stationary	$3.3x10^{10}$	$3.4x10^{9}$	10.4
Raoultella terrigena	logarithmic	$4.3x10^{11}$	$2.6x10^{10}$	6.1
	stationary	$2.5x10^{11}$	$1.1x10^{10}$	4.2

Drying is the most critical step for cell survival in encapsulation methods. This was confirmed by Schoebitz et al. (2012), who showed that a large proportion of cells are destroyed during dehydration (Table 1). In fact, cell mortality during the drying of encapsulated cells has been recognized as a critical point of the encapsulation. Nevertheless, the survival of dehydrated cells depends on various factors, such as: drying conditions, microorganism species, adjuvant used and culture conditions. It has been determined that drying kinetics is of particular importance for cell survival, showing a detrimental effect of fast drying. Improving cell survival during encapsulation is not a simple procedure. It depends on various factors: growth medium composition, strain and physiological state of the cells, and processing parameters. Each factor should be optimized to ensure the best inoculant activity after drying and during inoculant storage.

Figure 2. Wheat roots inoculated with beneficial soil bacteria immobilized in alginate-starch beads.

CONCLUSION AND PERSPECTIVES

Advances in microbial inoculant formulations have been presented, featuring encapsulation materials and techniques used for introducing beneficial microorganisms into the soil and near the plant root (Fig. 2). Nevertheless, conventional microbial inoculants cannot ensure high cell viability during the formulation process. Conventional inoculants need to be stored at room temperature to avoid extreme temperature oscillations, because the shelf life of liquid inoculants under storage conditions is very short and their viability decreases by one or two log. The use of liquid inoculants does not protect microorganisms against soil stresses.

The use of carrier materials to improve cell survival could be one of the key factors to success in microorganism formulation. However, even though there are different encapsulation techniques, no formulation of microorganisms has been developed by the microbial inoculant industry.

Encapsulation represents a wide area of research for the food and pharmaceutical industries. In fact, different encapsulation methods have been developed to serve different purposes. Nevertheless, almost no method has been evaluated for the production of microbial inoculants. Many of these promising technologies, which are used in other fields, warrant evaluation under field conditions to improve the development and quality of microbial formulations for successful inoculation.

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ARTICLE

BIOENCAPSULATION FOR NITRATE REMOVAL IN AQUARIUMS

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INTRODUCTION

Nitrate accumulation in aquariums

Since ammonia is highly toxic to aquatic organisms, its removal from aquarium water is of primary concern in any freshwater or marine aquarium setup. Such removal is usually accomplished by immobilizing nitrifying bacteria on substrates with a large surface area. These substrates are either situated within the aquarium (e.g. gravel) or in external filters, through which the aquarium water is recirculated. Under fully aerobic conditions, nitrifying bacteria convert ammonia via nitrite to nitrate. This latter ion is far less toxic than ammonia and is usually not actively removed.

The booming interest in the aquarium hobby has led to the introduction of new exotic ornamental fish species which are difficult to maintain in aquariums with water of inferior quality, including that with high nitrate levels. Whereas elevated nitrate levels are generally tolerated by «bread and butter» aquarium fish, invertebrates and some of the more exotic aquarium fish, such as those originating from soft water (i.e., water low in minerals), are unable to propagate or grow in water containing high nitrate concentrations (Camargo et al., 2005; Griffitts, 2007). Additional problems associated with elevated nitrate levels in aquariums are:

1. nitrate stimulates undesired algal growth on the walls of the aquarium (fouling);
2. nitrate reduction leads to accumulation of the highly toxic nitrite ion;
3. water exchange, the most common practice for controlling nitrate concentrations, in marine aquariums comes at a price, as salt is lost and has to be replenished.

Removal of nitrate in aquariums

A few commercial devices are available for nitrate removal. The most common devices are based on induction of nitrate-reducing microorganisms (denitrifiers). Denitrifying bacteria are facultative anaerobic microorganisms capable of nitrate respiration in the absence of oxygen. Most denitrifiers are heterotrophic organisms, meaning that they require an organic carbon source for energy and carbon supply. When conditions are right, i.e., available nitrate and organic carbon and absence of oxygen, these organisms reduce nitrate to elemental nitrogen gas (N_2).

Denitrifying filters are based on the concept of immobilizing the denitrifiers on the surface of insoluble carriers through physical or physico-chemical bonding. Often sand, plastic or ceramic particles are used for this purpose. Suitable carbon sources are supplied with the influent water and water flow is set at a rate which secures anoxic conditions within the filter. This fixed-film process for denitrification is successfully used in the treatment of nitrate-contaminated urban and agricultural wastewater, but its use in aquariums is problematic. Adsorbed microorganisms, immobilized by weak hydrogen bonds or by electrostatic interactions with the carrier (fixed-film processes), are easily washed from the support carriers into the aquarium water, resulting in microbial pollution. Furthermore, any excess organic matter, not utilized within the filter, will add to the microbial pollution in the aquarium water.

Moreover, unlike filters used for ammonia removal, the performance of denitrifying filters is often erratic and their successful operation relies, to a large extent, on the skills and experience of the operator. Specifically, it is often difficult to treat large volumes of aquarium water while maintaining the required anoxic conditions. Furthermore, dosing the filters with a carbon source (often an alcohol or a sugar) is difficult. Too much carbon will lead to a break-through of carbon in the effluent and, consequently, cause undesired bacterial growth in the aquarium water, whereas too little carbon will lead to incomplete denitrification and an accumulation of toxic nitrite, an intermediate in this process.

MATERIAL & METHODS

Alginate (2.0%, w/w) (Keltone LV, San Diego, CA; mol. wt. 70 000–80 000, 61% w/w mannuronic acid and 39%

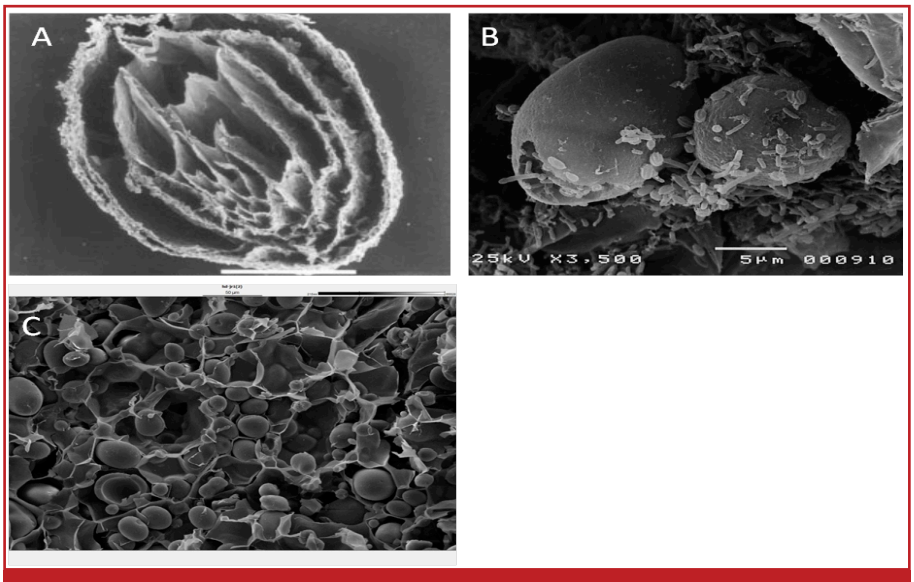

Figure 1. Electron micrographs of freeze-dried alginate beads showing a spongy appearance (A), and with bacterial colonization on the embedded starch granules (B). These granules serve as bridges between the walls of the alginate matrix (C).

w/w guluronic acid content) was dissolved in double-distilled water. The solution, mixed with a magnetic stirrer for 24 h at room temperature, was strained through a 100-mesh (US) nylon sieve to remove the remaining particles. The final pH of the alginate solution was 6.8. A potato-starch suspension (Sigma, St. Louis, MO) was added to the alginate solution at a final concentration of 2% (w/w). This solution was mixed with an axenic culture of *Pseudomonas* sp. containing 107 colony-forming units (CFU)/ml, in a proportion of one part bacterial suspension to nine parts alginate–starch solution (w/w), giving a final concentration of *Pseudomonas* sp. in the solution of about 106 CFU/g. This final solution (alginate–starch–bacteria) was dropped into a 0.5% (w/w) solution of calcium chloride (Sigma). A spontaneous cross-linking reaction occurred, resulting in spherical beads with an average diameter of about 3 mm. Some of these beads were stored at -80°C for about 24 h before freeze-drying (Repp Sublimator Model 15RSRC-X, Repp Industries, Gardiner, NY). Freeze-drying was carried out at -50°C and a pressure of 11 x 10^6 bar.

RESULTS & DISCUSSION

Washout of immobilized organisms can be significantly reduced by their entrapment within a matrix. The freeze-dried alginate beads containing entrapped denitrifiers (Tal et al. 1997, 1999) exhibited physical properties similar to those of sponge-like matrices (Figure 1A). In addition to serving as a carbon source for the entrapped denitrifiers (Figure 1B), starch granules strengthened the beads by serving as bridges between the matrix walls (Figure 1C). The porous nature of the freeze-dried bead allowed rapid release of nitrogen gas without physical damage to the bead's matrix. Encapsulation of the denitrifiers in the hydrocolloid beads revealed various advantages. Firstly, this entrapment method, as compared to other immobilization methods, significantly reduced carbon washout from the filter as the carbon source is incorporated within the entrapment complex. Secondly, as by means of this configuration both bacteria and carbon are entrapped in close vicinity, metabolic activity of the entrapped microorganisms is high. This leads to rapid depletion of oxygen within the entrapment complex and consequently, to conditions allowing efficient nitrate removal. Thirdly, as compared to synthetic encapsulation agents, this natural hydrocolloid does not pose an environmental hazard when discharged.

Figure 2. A prototype filter for nitrate removal in aquariums based on encapsulation of denitrifiers.

Using this entrapment concept for nitrate removal in both freshwater and marine aquariums (Figure 2), it was found that actively denitrifying beads could be maintained for a period of up to 4 months (Tal et al., 2003).

CONCLUSION AND PERSPECTIVES

Freeze-dried alginate beads with embedded denitrifiers and starch sustained denitrification activity over a prolonged period of time when used for nitrate removal in freshwater and marine aquariums. This novel method may offer an attractive alternative to current methods for nitrate removal.

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ARTICLE

DISTRIBUTION OF IMMOBILIZED MICROORGANISMS WITHIN HYDROCOLLOID BEADS

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INTRODUCTION

Gel and dried hydrocolloid beads can be used for many biotechnological purposes, such as water denitrification, matrices for the immobilization of denitrifying isolates, carriers of bacteria or spores for biological control of soil-borne root diseases, and carriers of gram-positive lactic acid bacteria starter cultures involved in dairy and food fermentation (Nussinovitch, 2010).

Immobilization refers to the inhibition of free cell movement by natural or simulated means. Cell-immobilization technology provides a number of benefits over inoculation with free cells. The applicable formulation should provide beneficial characteristics for the inoculant, such as long shelf life, proper survival at its destination, and satisfactory cell density and performance (Nussinovitch, 2003). Cell immobilization offers advantages for food-processing, as well as biotechnological and agricultural production. Specific uses include biotransformation, organic acid fermentation and conversion, amino acid and oligosaccharide production, water denitrification, and biomedical applications of microencapsulation, such as in building multilayer capsules for transplantation or to treat diabetes (Bashan et al., 2014; Zohar-Perez et al., 2005; Zvitov et al., 2004).

Water-soluble polymeric materials (gums) such as agar, carrageenan, alginate, low-methoxy pectin, gellan, chitosan, and blends of xanthan and locust bean gum, among many others, are commonly used for such applications. Each and every one of these materials has been used to manufacture natural-based carriers for encapsulation of microbial and fungal cells in the food, biotechnology, and agriculture industries (Bashan et al., 2014; Nussinovitch, 2010; Zohar-Perez et al., 2005).

Technique development and optimization require an enhanced understanding of the initial cell localization in the applied product. When cells are suspended in a carrier solution, they are distributed in a homogeneous, isotropic manner. It is assumed that after entrapment of the cells, they are dispersed homogeneously in the beads that entrap them as well. In fact, many references emphasize this initial state of homogeneous distribution, which is taken for granted. The purpose of this work was to explore whether this a priori statement is accurate.

MATERIAL AND METHODS

Immobilization of Microorganisms: Two genetically modified microorganisms (a kanamycin-resistant mutant of *Escherichia coli*, and a hygromycin-B-resistant mutant of *Trichoderma asperellum*) were mixed in an alginate (2% w/w)–glycerol (30%, w/w) solution and dripped into a 1% (w/w) solution of calcium chloride. A spontaneous cross-linking reaction produced spherical gel beads with an average diameter of 4.1 ± 0.2 mm, containing ~10^9 bacterial cell/bead or ~10^7 spore/bead. To create dry alginate beads, the beads were freeze-dried, resulting in dried beads with an average diameter of 3.0 ± 0.1 mm. The bacteria were also added to an agarose (2%)–glycerol (30%) solution. The agarose beads were produced by dripping the final mixture into distilled water, resulting in spherical beads with an average diameter of 4.2 ± 0.2 mm, containing ~10^9 cell/bead.

Confocal Microscopy and Image Analysis: Cell distribution in the beads was determined using confocal laser-scanning microscopy (CLSM) with a He-Ne laser. Inner ~0.5-mm slices taken from the center of the agarose- and alginate-gel and freeze-dried beads were scanned. Digital image analysis of the CLSM optical thin sections was performed with Zeiss 3D LSM software to determine cell distribution in the final images. This program determines the ratio (R) of the total volume of all fluorescent regions to the volume of the image sequence; these data were used to reveal cell concentration at different bead depths.

Electron Micrographs: To study the freeze-dried beads' structure, scanning electron microscopy (SEM) was performed with a Jeol JSM 35C SEM (Tokyo, Japan).

RESULTS AND DISCUSSION

Confocal microscopy was used to study the mode in which the cells are dispersed in the beads. Despite the assumption that after their entrapment, cells are distributed uniformly in the beads that entrap them, in this study we demonstrate that with some

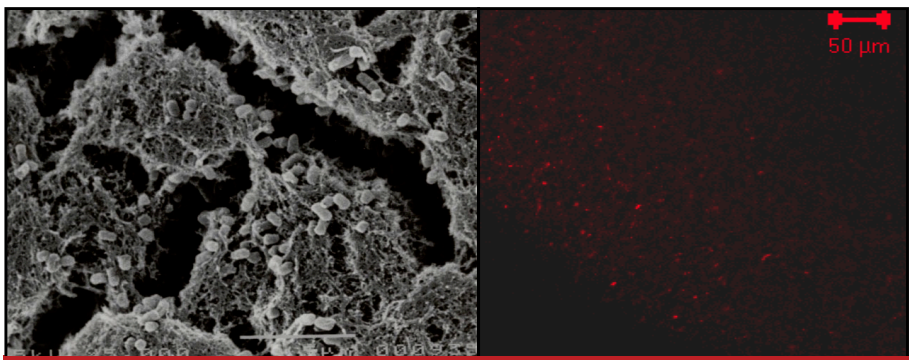

Figure 1. Typical electron micrograph (left; bar 500 µm) and confocal micrograph (right; bar 50 µm) of *Escherichia coli* distribution within alginate beads.

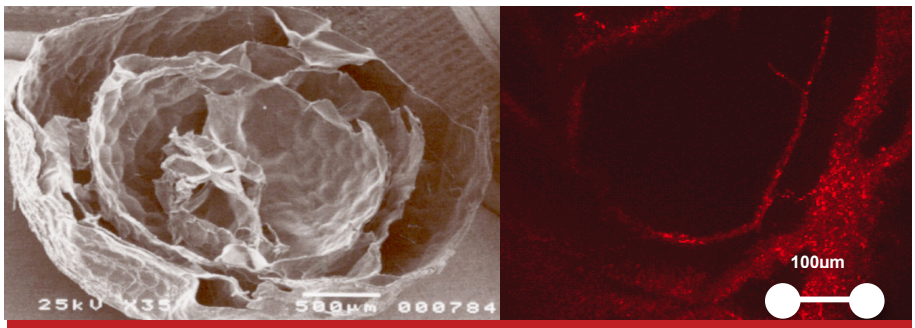

Figure 2. Electron micrograph of halved freeze-dried alginate bead (left) and typical confocal micrograph (right) of *Escherichia coli* distribution within alginate freeze-dried bead.

immobilization techniques, this a priori assumption is not accurate.

Escherichia coli distribution in alginate-gel beads was found to be non-homogeneous. A descending gradient of dispersal of the bacteria toward the alginate-gel bead's center was observed in confocal micrographs (Figure 1). Freeze-dehydration of alginate beads resulted in a porous structure and the microorganisms were found scattered along the pore walls (Figure 2). Drying of the alginate beads made this gradient distribution even more distinct due to the beads' contraction during the drying process. A similar nonhomogeneous distribution of *Trichoderma asperellum* spores was observed in the alginate-gel beads. From these observations, it appears that microorganisms with different sizes and properties behave similarly in alginate beads, suggesting that their distribution is due to the mechanism of bead creation.

In alginate, a spontaneous cross-linking reaction occurs on its surface, followed by a process that depends on the rate of calcium-ion diffusion into the formed bead, overcoming the resistance of the formed calcium–alginate layers that contract further with time. Accordingly, a more constricted network is created on the gel bead's exterior than at its core. Thus, despite the homogeneous cell dispersal in the original alginate drop (viscous solution) that fell into the cross-linking solution, stronger contraction at the bead's exterior results in a higher cell concentration in that area. Freeze-drying of the resultant bead changes its structure but preserves the original higher cell concentration near the surface.

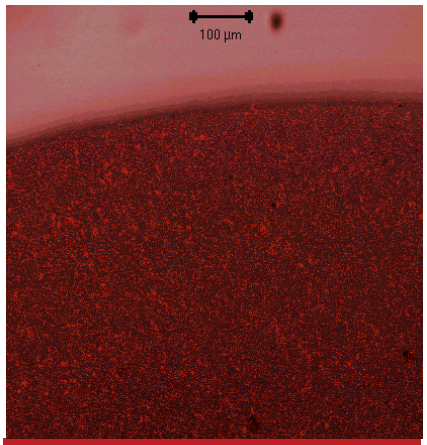

Figure 3. Typical confocal micrograph of *Escherichia coli* distribution within agarose bead.

To further examine this concept, bacterial distribution was studied in agarose beads, which are created with a different gelation mechanism that involves only minor contraction. In fact, cell distribution in the agarose beads was uniform (Figure 3). For alginate beads including *Escherichia coli* or *Trichoderma asperellum*, the calculated R was 7.2 and 5.1% near the bead's surface, respectively, compared to 1.5 and 1.9%, respectively, at the bead center. For the agarose bead, no significant differences were detected at the different spots, and thus the distribution kilooks consistent.

CONCLUSIONS AND PERSPECTIVES

Cell distribution in the beads that entrap them depends on bead formation, rather than on the type of entrapped microorganism. Taking this parameter into consideration provides the researcher with a useful tool for creating a tailor-made carrier of microorganisms for a predetermined operation, thereby increasing the probability of success. For example, when a product is designed for immediate use, a contracted cell carrier such as alginate is preferred.

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ARTICLE

CO_2-RELEASING CO-FORMULATIONS

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INTRODUCTION

CO_2 is a known attractant for many soil-dwelling pests, such as western corn rootworm and wireworm, which cause considerable economic damage in different agricultural production systems. Artificial CO_2-emitting sources may lure larvae away from plants and reduce damage in the field, especially when combined with a killing agent using attract-and-kill strategies (Figure 1).

Our research aims at developing CO_2-releasing insecticidal co-formulations based on baker's yeast and starch as the CO_2 source, entomopathogenic fungi or neem extract as the biological insecticide and a biocompatible material for encapsulation. To enable application at planting while still providing sufficient CO_2 emissions at hatching, the formulations have to release CO_2 over a period of several weeks. Our approach requires a carbohydrate source that can be encapsulated in a biocompatible material and utilized by baker's yeast. Carbohydrates, such as mono- or disaccharides, which can be metabolized by baker's yeast, cannot be encapsulated and retained by methods that are suitable for the encapsulation of microbial biological control agents (Vemmer and Patel, 2013), due to their low molecular weight. On the other hand, polysaccharides such as starch, which have sufficient molecular weight for encapsulation, cannot be metabolized by baker's yeast because the yeast lacks the amylase activity required to break down the starch molecules. We hypothesized

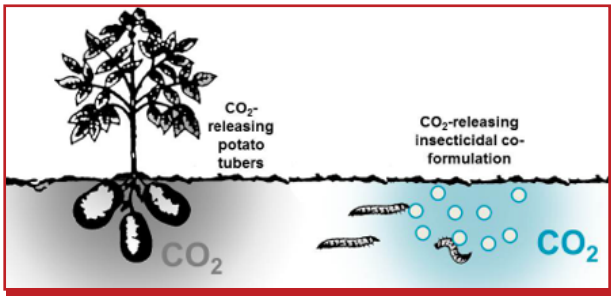

Figure 1: Scheme showing the orientation of soil-dwelling larvae toward CO2 gradients generated by an attract-and-kill formulation.

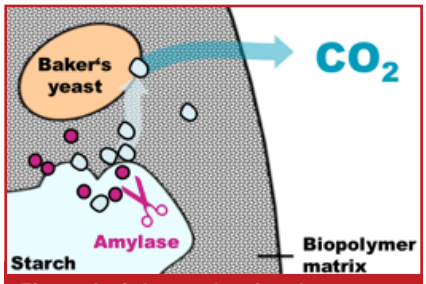

Figure 2: Scheme showing the degradation mechanism in the biopolymer matrix. Starch is degraded by enzymes and subsequently metabolized to CO_2 by baker's yeast.

that co-encapsulation of starch-degrading enzymes, either contained in a technical enzyme preparation or produced by a second micro-organism, e.g., the entomo-pathogenic fungus Beauveria bassiana, in combination with baker's yeast and maize starch could be a potential approach to develop long-lasting CO_2-releasing formulations (Figure 2).

MATERIAL AND METHODS

Freshly harvested baker's yeast biomass and optional additives, such as sterile maize starch, freshly harvested biomass of B. bassiana (mycelium and spores) or NeemAzal® technical (Trifolio-M GmbH), or their combination, were suspended in 2% (weight/weight) Na-alginate. The suspension was dripped with a syringe through a drain tube (Ø 0.90 x 40 mm) into sterile 2% (weight/volume) $CaCl_2$ solution where the droplets solidified by ionic gelation, forming a hydrogel.

CO_2 was quantified using a Carbon Dioxide Meter with pump-aspirated sampling (Vaisala CARBOCAP® GM70). Moist beads (10 g) were placed in perforated boxes filled with soil (mixture of Fruhstorfer Erde Type P and sand at a weight ratio of 1:2, 40% (weight/weight) humidity, 22 °C). CO_2 concentrations were determined by inserting a drain tube connected with the pump into the soil (8 cm–10 cm depth). The data are presented as differences between the actual CO_2 concentration in the soil and the CO_2 concentration of a negative control (moist Ca-alginate beads without active ingredients).

Scanning electron microscopy (SEM) was performed using a Hitachi S-450. Beads were prepared with supercritical drying and cutting followed by sputtering with gold.

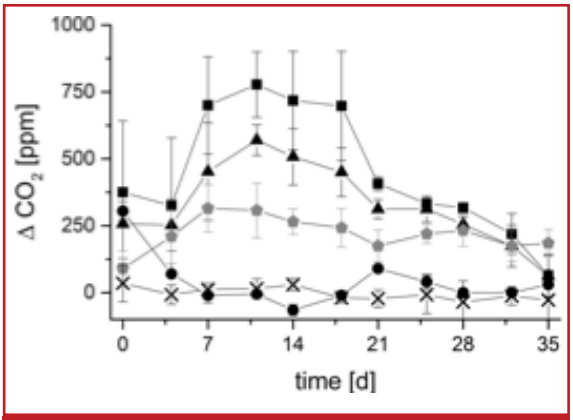

Figure 3: Δ CO_2 concentrations in soil from moist pure alginate beads (×), moist beads containing 16.7% baker's yeast (●), 16.7% baker's yeast biomass and 20% maize starch (▲), 16.7% baker's yeast biomass, 20% maize starch and 2% B. bassiana biomass (■) and maize starch (●). ΔCO2 concentrations in soil differ significantly between bead compositions (n = 4, F = 52.89; df = 7, 24; P < 0.0001).

RESULTS & DISCUSSION

Synergistic metabolic interactions of baker's yeast and B. bassiana inducing the conversion of maize starch to CO_2 resulted in high and long-lasting CO_2 concentrations in the soil (Vemmer et al., 2016). Formulations containing individual components or combinations thereof led to lower CO_2 concentrations (Figure 3). Interestingly, elevated CO_2 concentrations were observed for formulations without a co-encapsulated amylase source as well (Figure

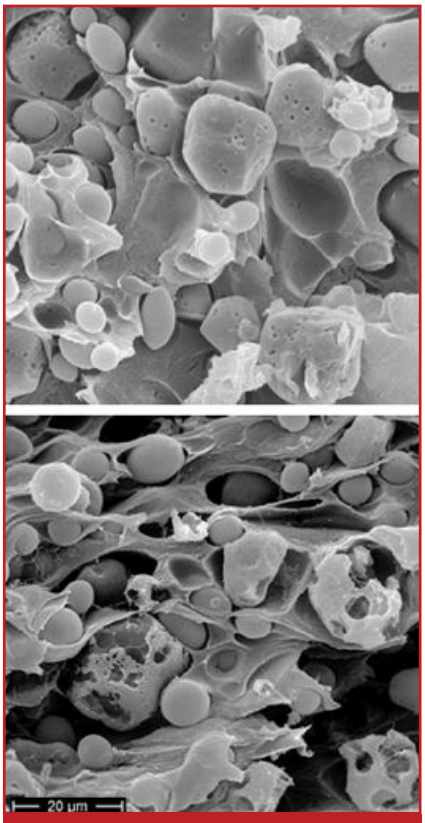

Figure 4: Scanning electron microscopy pictures of degrading crystalline starch co-encapsulated with baker's yeast and a technical amylase preparation in an alginate matrix before (above) and after (below) 1 week of incubation at 25 °C under 1100-fold magnification (© Pascal Humbert)

3). This can be explained by the presence of soil microorganisms, which produce exoenzymes with amylase activity and are therefore able to degrade the starch molecules to create a sugar-enriched environment.

To eliminate reliance on soil microorganisms or co-encapsulated microorganisms, a technical amylase preparation was added to the beads. In comparison to the first approach, where amylase was produced little by little, the degradation of starch to sugar molecules by the amylase started immediately. Thus, sugar molecules were directly available for conversion into CO_2 by the baker's yeast. At the beginning, CO_2 concentrations increased sharply (more than 1000 ppm) and then decreased gradually? (data not shown).

Figure 4 shows very clearly the starch degradation in the alginate matrix. At the beginning (day 0), the granule surface of the starch exhibited small holes which became bigger over time (7 days), resulting in significant erosion of the granules.

Several laboratory experiments with our partners from the University of Göttingen (headed by Prof. Stefan Vidal) revealed that our formulations are attractive for western corn rootworm and wireworm (data not shown).

Field trials have already shown initial success. For example, in 2015, wireworm damage on potatoes was significantly reduced in two fields by applying attract-and-kill beads with NeemAzal® technical (Trifolio-M GmbH) as the insecticidal component (Figure 5).

CONCLUSIONS & PERSPECTIVES

Our CO_2-releasing formulations pave the way for novel attract-and-kill approaches based on botanical or microbial insecticides. Co-encapsulation of starch and an amylase source is a simple way to provide a substrate for baker's yeast which does not diffuse out of the bead matrix and which can be converted to CO_2. Co-encapsulated entomopathogenic fungi will act as both amylase source and biocontrol agent.

Future work will focus on in-depth investigation of internal and external factors influencing the bead's performance and up-scaling of the formulation and drying processes toward product commercialization.

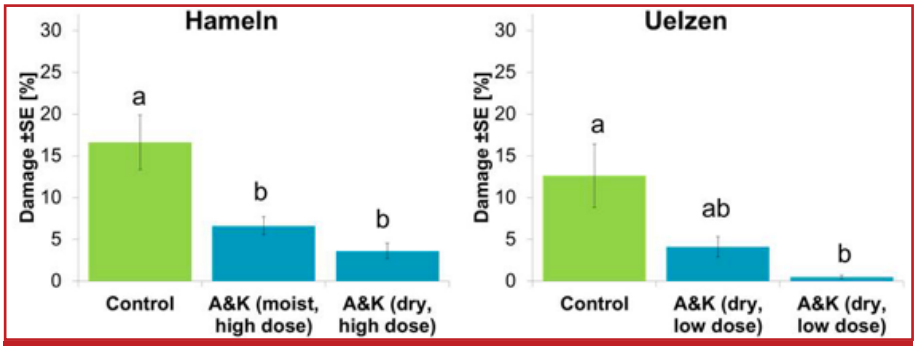

Figure 5: Wireworm damage on potatoes in two fields (Hameln and Uelzen) in Germany. High dose = 13.2 kg/ha; low dose = 0.6 kg/ha Azadirachtin A. Test design: 4 ridges of 7 m, harvest of the 2 middle rows, 8 repetitions, randomized block trial. Scoring: EPPO (damage [%]), harvest of 100 tuber/plot, (data kindly provided by Frauke Mävers, Univ. of Göttingen).

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PHD THESIS

PhD Thesis at Chemical Engineering Laboratory of Toulouse, France

The thesis is part of a collaborative project between 3 French laboratories (L2C at Monpellier, GEPEA at Saint Nazaire and the LGC of Toulouse. It concerns le development of a new technology of encapsulation, patented by L2C. The candidate will be in carve of developing the single drop method developed at L2C to a lab scale while GEPEA will transpose the technology to a large scale.

The tasks to be performed at LGC will include:

- To determine and characterize the mechanisms of interactions between droplets at the different stage of the micro encapsulation process,
- To develop the continuous production of the capsules based on the droplet deformation in a centrifuge system
- To model the process using Computational Fluid Dynamics using a specific code developed by LGC in collaboration with an other laboratory, IMFT.

The candidate must have a strong training in process engineering, an enthusiasm for experimental work and modeling. The salary is fixed to 1960 Đ (before social and medical care taxes). The thesis will start in October 2016.

For any more information or candidature, please contact
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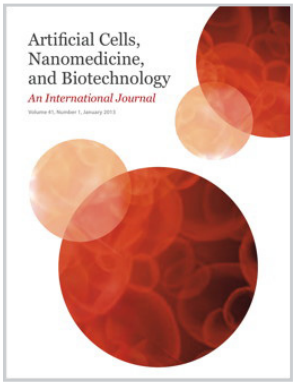

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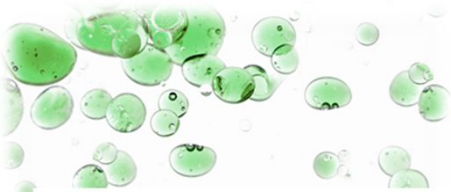

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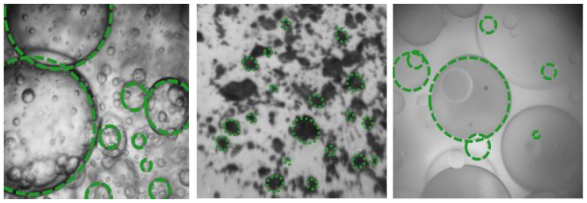
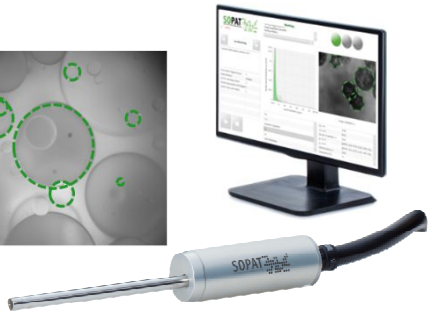
SOPAT has developed the perfect solution to monitor and control the particle size distribution of encapsulation products. Our in-situ probes can be applied to most production technologies such as spray drying, fluidized bed granulation, polymerization, etc.

Our technology consists of a photo-optic probe with integrated intelligent image analysis. Its fast algorithms facilitate process control in real-time. This enables the user to optimize process quality control and helps to save costs.

The measurement range of our in-situ probes is roughly between 1 and 10000 microns.





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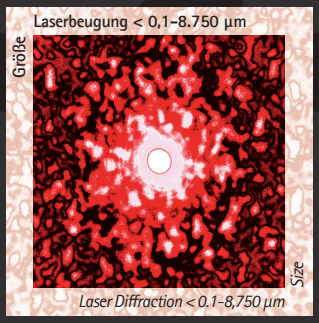
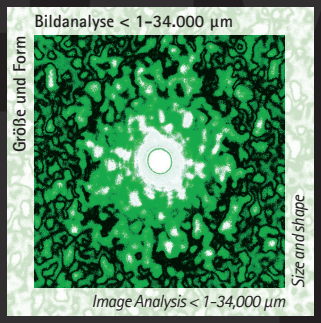
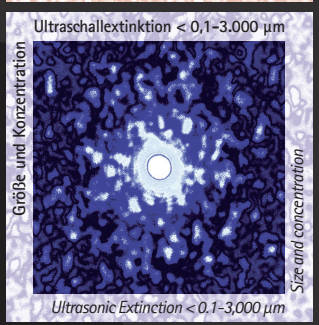
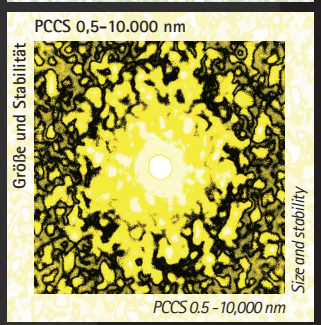
PARTIKELMESSTECHNIK
PARTICLE MEASUREMENT









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