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Encapsulation of Plant Growth-Promoting Bacteria in Poly(itaconic acid) Microspheres by Spray Drying

Vitaliy Kapishon, Marie-Josée Lorrain, Alfred C. W. Leung, Anne-Marie Gélinas, Meiqun Wu, Manon Sarrazin, Sabahudin Hrapovic, Antoine Pagé, Fanny Monteil-Rivera,* and Usha D. Hemraz*

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ABSTRACT: Poly(itaconic acid) (PIA) is a renewable, biobased, hydrophilic polymer of superabsorbent properties, with the potential to replace synthetic superabsorbent polymers in agricultural applications. Herein, we tested PIA for encapsulation of two model plant growth-promoting bacteria (PGPB), *Bacillus subtilis* and *Pseudomonas fluorescens*, using spray drying. Linear PIA was prepared, mixed with PGPB, and spray-dried to form microbe-loaded microspheres. The latter were analyzed for mass yield, moisture content, morphology, and microbial survival. Incubation of the microbes with PIA prior to spray drying showed no significant toxicity. The spray-dried microcapsules were about 1−10 μm in diameter, had a moisture content of about 11%, and contained a viable load of microbes (up to 1×10^8 cells/gram of product). The microspheres underwent instantaneous swelling-dissolution to release live bacteria. The CFU data showed a survival of about 79% for *B. subtilis* after spray drying. This work presents PIA as a safe, sustainable, and promising new encapsulation material for microbial inoculants.

KEYWORDS: *bioencapsulation, poly(itaconic acid), plant growth-promoting bacteria, spray drying*

1. INTRODUCTION

The use of microorganisms as a biocontrol and biofertilizer has been gaining commercial importance in the past two decades as part of a movement toward more organic and sustainable agriculture.[1,2] The application of these beneficial microorganisms has been proven to improve plant yields and offer natural crop protection against plant diseases and parasites while offering a renewable and greener alternative to conventional chemical pesticides and fertilizers.[1,2] A subclass of these microorganisms are already found in the plant rhizosphere and called plant growth-promoting bacteria (PGPB).[3] These PGPB are known to favor nitrogen fixation, solubilize and deliver other minerals and nutrients, secrete plant growth hormones and biopesticides, or improve soil structure and water retention.[4−6] PGPB formulations are delivered through several commonly used methods, including seed treatment, root-dipping, soil, and foliar applications. Despite the above-mentioned benefits, commercial application of PGPB is still at the early stage, with only a few variations of PGPB at the commercial stage.[7,8] Among the barriers hampering commercialization of PGPB are the higher technical handling and cost for production of PGPB formulations compared to those of agrochemicals, as well as the inconsistency of performances of PGPB formulations observed in the field despite high soil-plant colonization potential demonstrated under controlled conditions.[6,8,9] To have a chance to be successfully commercialized, PGPB formulations should at least match the high standards of agriproducts. In practice, it means PGPB formulations should give maximum crop yields while being affordable and easy to use, compatible with farmers' equipment, efficient under different field conditions and types of soil, able to help the survival of inoculated bacteria for the time needed by the plant, stable during storage, and safe for humans, animals, plants, and the environment.[8]

Bioencapsulation, i.e., putting a cell or a microorganism into a semipermeable membrane matrix, typically in the form of a microcapsule, is seen as a promising way to enhance the delivery of inoculants to plants. The main reasons for using bioencapsulation are to (1) provide a suitable microenvironment for the microbial strain(s), including physical or chemical protection over a prolonged period, in order to avoid a rapid decrease of the cell viability during storage, transport, and application, (2) protect the inoculants against potential abiotic stress in soil (pH, high temperature, high salinity, desiccation), (3) safeguard the inoculants against the better-adapted native soil microflora, and (4) ensure their gradual and prolonged release.[8−12] There are several reports describing encapsulation of PGPB in a biopolymer matrix for an improved microenvironment and cell residence.[9,13−16] The two main factors that dictate the overall stability and effectiveness of solid microbial inocula are the choice of material for encapsulation and the preparation technique. There is a wide range of encapsulation techniques that have been used to successfully immobilize PGPB on the bench-scale such as liposomes, complexation or cross-linking, coacervation, emulsions, and freeze-drying.[8,11] However, the most scalable and industrially

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relevant methods are spray drying, air drying, and extrusion methods.[8,14,17,18] Among those, spray drying is widely used in large-scale production of microbial formulations because it is economical and adaptable and produces excellent product quality.[11] As for the encapsulation matrix, research efforts have been focused on immobilizing microbes in simple natural materials (peat, clay, talc, char, or biochar) as well as more complex biopolymers including proteins and polysaccharides (alginate, starch, gum, agar, cellulose, and cellulose derivatives).[8,9,14,15,19−22]

While the use of natural biopolymers as the bioencapsulation matrix is advantageous in terms of biodegradability and biocompatibility, it can also be accompanied by challenges.[8,13] Materials prepared from natural biopolymers often lack chemical functionality, mechanical stability, and durability compared to their synthetic polymer counterparts. There is often a batch-to-batch variability with naturally sourced materials, as well as limited processability (e.g., aqueous solutions of alginate can only be processed up to 1−3% w/v because of high viscosity; the gelation of κ-carrageenans is temperature-dependent and the polymer and bacterial solutions have to be heated above a temperature of 50 °C).[23] In addition, there are limits to shelf life and postapplication stability as polysaccharides can often be a food source for foreign microorganisms. For example, alginate beads are easily colonized and utilized as a nutrient source by soil fungi.[15] While sodium alginate is the most common biopolymer used for encapsulation and can also be spray-dried, it is usually cross-linked in the presence of calcium ions.[15,24,25] Poly(itaconic acid) (PIA) is a biobased polyanionic polymer prepared from the polymerization of itaconic acid (IA),[26−28] a double-bond bearing dicarboxylic acid compound, which is mainly produced by microbial fermentation of corn starch and other sugars. PIA is a renewable alternative to compete with petroleum-based poly(acrylic acid) and polyacrylamide absorbent materials and industrial resins. It has a high swelling and water-absorbing capacity, as well as a very strong affinity for cationic metals. PIA is currently commercialized by Itaconix as a copolymer or as a partially neutralized sodium salt (with M_n values of 8000 g/mol or less) for cleaning, dispersing, painting, and coating applications for a number of industries, including household, food, cosmetics, and agriculture.[29] The synthesis of PIA from IA by free radical polymerization (FRP) in aqueous and organic solvents is well documented and produces a polymer with a backbone containing carbon−carbon bonds.[26−28] Although these are stable bonds, it has been claimed that PIA is biodegradable.[27,29]

In this study, we have used PIA, synthesized from the FRP of IA, to encapsulate two PGPB, *Bacillus subtilis* and *Pseudomonas fluorescens*, using spray drying to produce dry formulations containing active PGPB. The rationale for encapsulation of PGPB in PIA was to look at a polymeric alternative with better chemical stability, as compared to natural biopolymers[15,24] commonly used for bioencapsulation, as well as have an alternative to the commonly utilized sodium alginate. The benefits of addition of the vinyl polymer to a microbial inoculum formulation should be more pronounced in agricultural seed treatment applications, where several natural materials lack the desired physical properties for a durable and stable coat formation. The high chelation capacity of PIA also makes it useful for coformulation and codelivery of plant micronutrients such as zinc, iron, and magnesium.[26] The two selected model organisms are known to have beneficial effects on plants as biocontrol and growth enhancement agents[30] and serve as a good representation of two very different microbes: a Gram-positive, robust, and stress-tolerant spore-former (*B. subtilis*), and a Gram-negative, sensitive, non-spore-former (*P. fluorescens*). To the best of our knowledge, there have been no reports of utilizing PIA or PIA-enriched materials to immobilize PGPB or any other industrially/agriculturally relevant microorganisms. The resulting products were analyzed for yield, moisture content, capsule size, and morphology. The recovery of viable microbes from the microspheres was determined by CFU counting.

2. MATERIALS AND METHODS

2.1. Materials. IA, *tert*-butyl hydroperoxide (tBHP) (70% w/v in water), sodium alginate, maltodextrin, and calcium chloride were purchased from Sigma-Aldrich (Oakville, Canada or Milwaukee, WI). Two model PGPB, *P. fluorescens* (ATCC BAA477) and *B. subtilis* (ATCC23857) were purchased from American Type Culture Collection (ATCC).

2.2. Preparation of PIA. Half-neutralized PIA was synthesized by FRP with slight modifications to a previously published procedure.[27] In a typical experiment, IA (200 g) was dissolved in 100 mL of distilled water and stirred in an ice bath, to which NaOH (1.2 M, 50 mL) was added dropwise using a dropping funnel. tBHP (70% w/v in water, 5 mL) was charged into the flask, and the solution was purged with nitrogen for 15 min. The flask was then sealed and heated to 100 °C for 1 h. A viscous product was then precipitated in 1.5 L of cold acetone and isolated by centrifugation for 10 min at 3500 rpm. The pellet was then washed by three precipitation-centrifugation cycles from water to acetone, and the final product was dried overnight in a vacuum oven at room temperature. The final product PIA was obtained with about 60% gravimetric conversion after polymerization and purification. The chemical structure was confirmed by ^{1}H NMR and molecular weight distribution by aqueous gel permeation chromatography (aq-GPC). Encapsulation solutions were prepared by dissolving PIA (10 or 20 g) in 100 mL of distilled water the day before spray drying and stored at 4 °C.

2.3. Preparation of Microbial Cultures. The production of microbial cultures was performed in DASGIP Benchtop Bioreactors that could simultaneously accommodate four parallel 1 L flasks. For the fermentation of *B. subtilis* and *P. fluorescens*, 5% inocula were used for both runs (50 mL of an overnight seed culture) in tryptic soy broth (TSB) media and incubated at 28 °C (*B. subtilis*) and 30 °C (*P. fluorescens*) at 250 rpm for 16−24 h to create working cell banks. An additional carbon source (50% glucose, 15 g) was injected into each vessel before fermentation started. The process was fully controlled and monitored in terms of temperature, pH, dissolved oxygen (DO), optical density (OD_{600}), and off-gas. The pH was set to 7 and was controlled by two feeding lines containing sodium hydroxide (NaOH, 5 M) and phosphoric acid (H_3PO_4, 3 M), respectively. Dissolved oxygen was kept constant at 30%, by using a cascade of airflow (6−28 L/h) and agitation (300−1000 rpm). The optical density was measured at the beginning and the end of the 16 h fermentations. After fermentation, the cells were harvested by centrifugation at 4 °C, 5000*g* for 10 min, and washed with saline (0.85% NaCl). The cells were resuspended in saline and aliquoted into tubes to have ∼ 10^{11} CFU/pellet after recentrifugation. All pellet tubes were stored at −80 °C and used for subsequent experiments.

2.4. Encapsulation. The polymer solutions (10% or 20%) that were to be used for encapsulation of the PGPB were sterilized using a 0.45 μm stericup filter (or by autoclaving at 121 °C for 20 min). Frozen pellets of bacteria (∼10^{11} CFU/pellet of *B. subtilis* or *P. fluorescens*) were resuspended in 100 mL of an aqueous sterile encapsulation polymer solution. The cell/polymer solution was homogenized using a homogenizer Polytron PT 10−35 GT-D (Kinematica AG, Switzerland) at 3700 rpm for 2 min at room temperature and incubated at 28 °C in a 500 mL flask for 1 h with an

agitation of 150 rpm. The incubated encapsulation samples were stored at 4 °C after the end of the incubation until the time for spray drying. The samples were agitated and spray-dried through a Buchi Mini Spray Dryer B-290. The spray drying conditions were as follows:

For *B. subtilis*, the inlet temperature was 100 °C, the feed rate was 5 mL/min, and at a 90% aspirator (maximum was 35 m^3/h) and a 50 mm airflow. The outlet temperature was between 47 and 51 °C.

For *P. fluorescens,* the inlet temperature was 70 °C, the feed rate was 2.5 mL/min, and at a 90% aspirator (maximum was 35 m^3/h) and a 50 mm airflow. The outlet temperature was 38−42 °C.

The dried samples were weighed for product yield and stored at 4 °C overnight. The moisture content of the spray-dried samples was determined gravimetrically after drying for 24 h in an oven at 105 °C.

2.5. Enumeration of Microbes. Before spray drying, 1 mL of each solution (cells redispersed/incubated with the polymer) was taken and diluted in 9 mL of saline buffer to perform CFU counting. Serial dilutions (10^{-1}−10^{-7}) in sterile saline solution (0.85% NaCl) were prepared and then 0.1 mL of each dilution was plated onto TSA agar plates in triplicate. The CFUs were counted after incubation of plates at 28 °C for ∼ 24 h.

For the spray-dried samples, 1 g of the microcapsules was weighed and dissolved in 9 mL of sterile saline buffer, and the CFUs were counted. The cultures were homogenized in a 50 mL flask for 2 min at 3700 rpm and incubated at 28 °C for 1 h with agitation (150 rpm). Serial dilutions (10^{-1}−10^{-7}) of the bacteria cells released from the microcapsules were prepared in sterile saline solution (0.85% NaCl) and then 0.1 mL of each dilution was plated onto TSA agar plates in triplicate. The CFUs were counted after the incubation of plates at 28 °C for ∼ 24 h for microbes released after encapsulation. The survival of the microbes was calculated as a percentage using the logarithmic values of the CFU after spray drying over the logarithmic values of the CFU prior to spray drying.[31,32]

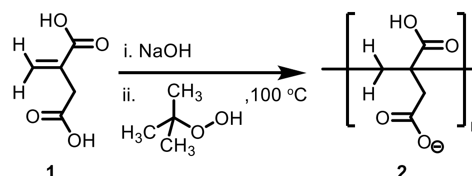
2.6. Material Characterization. The chemical structure of PIA was confirmed with ^{1}H NMR (D_2O) using a Bruker Avance 500 NMR spectrometer operating at a proton frequency of 500 MHz, equipped with a 5 mm PABBI Z-gradient probe. The NMR data are presented as follows: chemical shift δ (ppm), multiplicity, and integration. The following abbreviations were used to explain the multiplicities: s = singlet and m = multiplet. The ^{1}H NMR and ^{13}C NMR spectra were referenced using 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (TMS = 0.0) as the internal reference. The polymer molecular weight distribution was analyzed using an Agilent Infinity 1260 GPC instrument equipped with PL aquagel-OH Mixed L and PL aquagel-OH Mixed M columns. Conventional calibration was used with polyethylene oxide standards. A combination of sodium nitrate (0.2 M) and sodium dihydrogen phosphate (0.01 M) was used as an eluent at a flow rate of 1 mL/min and a sample concentration of 2 mg/mL. The columns and sensor were set to 40 °C.

Scanning electron microscopy (SEM) of spray-dried microcapsules was performed on a Hitachi S 3000N scanning electron microscope (Hitachi Scientific Instruments, Tokyo, Japan). A small amount of the sample was evenly distributed on a SEM sample stub covered with conductive carbon double-sided tape. The imaging was done in variable pressure (VP) mode using a backscattered electron (BSE) detector and in high vacuum (HV) mode using a secondary electron (SE) detector. In order to further improve the SEM resolution in HV mode, the negative effects due to the sample charging from such nonconducting samples were lowered by Au coating for 60 s using a Cressington sputter coater, model 108, Cressington Scientific Instruments Inc. Watford, UK. The microcapsules, as well as the bacteria, were also observed using a Keyence Digital (Optical) microscope VHX-1000 equipped with an RZ VH-Z100R x100-x1000 real zoom lens. For optical microscopy, samples were first visualized in a dry state, and then a drop of water was introduced at the edge of a coverslip while monitoring the microcapsules.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterization of PIA. Half-neutralized PIA **2** was synthesized by FRP of IA under aqueous conditions using tBHP as a free radical initiator by applying a process from a reported procedure,[27] but with modifications to favor the formation of PIA with high molecular weight (Scheme 1).

Scheme 1. Synthesis of PIA (2) by FRP of IA (1)



The resulting polymer was characterized by NMR, conducted in D_2O, and the resulting spectra were compared to those of IA (Figures 1A and S1−S3). The broad peak between 1.00 and 3.50 ppm corresponds to the protons from PIA. There is a slight overlap between the signal for the methylene protons on the backbone of PIA, which appears slightly downfield, and the two methylene protons on the side chains of the PIA repeat unit. The presence of the IA monomer was also detected in the PIA sample through the presence of three signals at 5.81, 5.33, and 3.11 ppm, indicative of an incomplete conversion. The integration of the PIA peaks (from 3.50 to 2.20 ppm minus approximate integration of peak c for IA) against the total integrals for both the monomer and the polymer peaks (peaks from 3.50 to 2.20 ppm plus peaks a and b) from the proton NMR spectrum was used to estimate the purity of the PIA sample obtained. A purity of about 88% was obtained for PIA despite the three precipitation/washing cycles conducted to remove the unreacted IA. No further attempts were made to purify the polymer as this would not be economically viable for a commercial application of PIA, and it was important to test the impact of the presence of IA in excess on the bacteria. GPC was used to obtain the molecular weight distribution of the PIA polymer (Figure 1B). A number average molecular weight (M_n) of ∼ 30,000 g/mol and a weight average molecular weight (M_w) of ∼ 40,000 g/mol were obtained for the synthesized PIA, resulting in a polydispersity index (PDI) of 1.3. The obtained molecular weights were higher than previously reported values (M_n = 5138−16,290 g/mol; M_w = 7,270−25,210).[28] The use of a higher initiator concentration (0.259 M) as well as the thorough washing of the product, which removed PIA of lower values, yielded higher molecular weight material with a narrower polydispersity.

3.2. Formulation and Spray Drying of Bacterium-Free PIA Mixtures. We first investigated the physical ability of PIA to form microspheres upon spray drying. A bacterium-free PIA solution (10% w/v in water) was spray-dried using two sets of conditions by varying the inlet temperatures (100 and 70 °C) and the feed rate (5 and 2.5 mL/min). In both cases, spray drying resulted in the formation of dry powders, which upon imaging using SEM showed PIA microspheres (Figure 2). A lower feed rate (2.5 mL/min) was required at the lower temperature (70 °C) to ensure complete drying. The key difference was that spray drying at the higher temperature and feed rate resulted in microspheres with a narrower size distribution (<5 μm, as opposed to <10 μm at lower temperature and feed rate). Spray drying at temperatures lower than 70 °C did not allow the successful formation of a dry powder.

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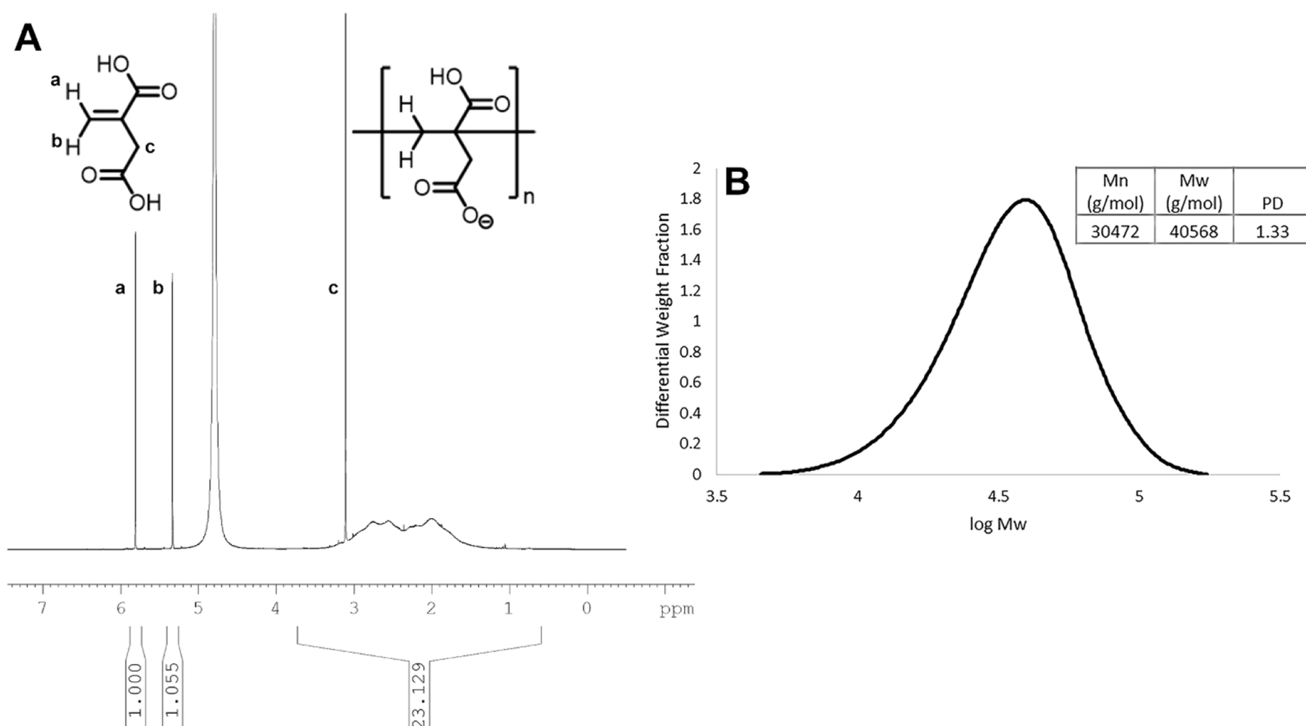

Figure 1. Chemical structure and molecular weight distribution of the PIA product as analyzed by ^{1}H NMR (A) and aq-GPC (B), respectively. The polymer purity was estimated to be ∼ 88% by using integrals from its proton NMR spectrum.

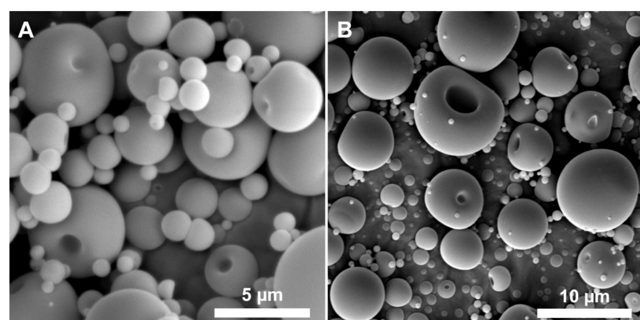

Figure 2. SEM images of dry powders of bacterium-free PIA microspheres produced by spray drying 10% w/v PIA solution under two conditions [(A) inlet temperature 100 °C, feed rate 5 mL/min; (B) inlet temperature 70 °C, feed rate 2.5 mL/min].

3.3. Optimization of Conditions for Encapsulation of Bacteria. Prior to conducting the encapsulation of the PGPB in the polymer matrices, it was important to investigate the effect of the temperature on the viability of the bacteria. While a temperature of at least 70 °C is required to form a dry, spray-dried PIA powder, an increase beyond the optimal temperature is detrimental to bacterial growth and will hamper bacterial survival. As such, the effect of temperature on both *B. subtilis* and *P. fluorescens* was investigated at four different temperatures (30, 50, 70, and 80 °C) over time (Figure 3). Initially and up to 1.5 h, there was not a significant difference in growth or survival for *B. subtilis* other than a slight increase in the growth at 30 and 50 °C. However, as the cultures were aged, a significant increase in growth was observed for *B. subtilis* at 30 and 50 °C, which implied that these temperatures were not irreversibly detrimental to the bacteria metabolism. Over time and at higher temperatures (70 and 80 °C), the cells did not grow. The initial growth observed for *B. subtilis* at 50 °C was not sustained beyond 4.5 h, and a decrease in absorbance was observed at 24 h. *P. fluorescens* was more sensitive to temperature and only displayed a growth profile at 30 °C. As such, it was important to limit the exposure of the bacteria to high temperatures during the spray drying process to maximize bacterial survival through adjustments to the inlet temperature, exposure time, flow rate, and solvent system.

3.4. Spray Drying and Characterization of Bacterium-Loaded PIA Microspheres. The encapsulation of the microbes was conducted in PIA matrices. Frozen pellets of bacteria were resuspended in filtered aqueous polymer solutions containing varying amounts of PIA (10 or 20% w/v), incubated, and subjected to spray drying. While *B. subtilis* was spray-dried at an inlet temperature of 100 °C and feed rate of 5 mL/min, a milder temperature of 70 °C was opted for *P. fluorescens* due to its temperature sensitivity. The microbe-loaded PIA microspheres were obtained as a fine, dry white powder upon spray drying. Table 1 summarizes the microbe-polymer formulations used for spray drying and their respective mass yield and moisture content after spray drying. Given that the spray-dried mass of bacteria was negligible compared to the mass of the polymer, the mass yield corresponded to the percentage of the spray-dried bacterium-loaded microspheres over the initial mass of the polymer used. In general, the use of a lower concentration of PIA was more efficient and resulted in a higher mass yield. The 10% PIA solution was less viscous than the 20% solution, leading to less instrument clogging and a smoother flow rate. Data for the PIA formulation were also compared to an alginate-based formulation (1.5% sodium alginate + 8.5% maltodextrin) that had previously been successfully used for encapsulation.[33] It was found that the PIA formulation had a higher moisture content than the alginate sample, likely due to PIA being more hydrophilic. There have been several reports which support the

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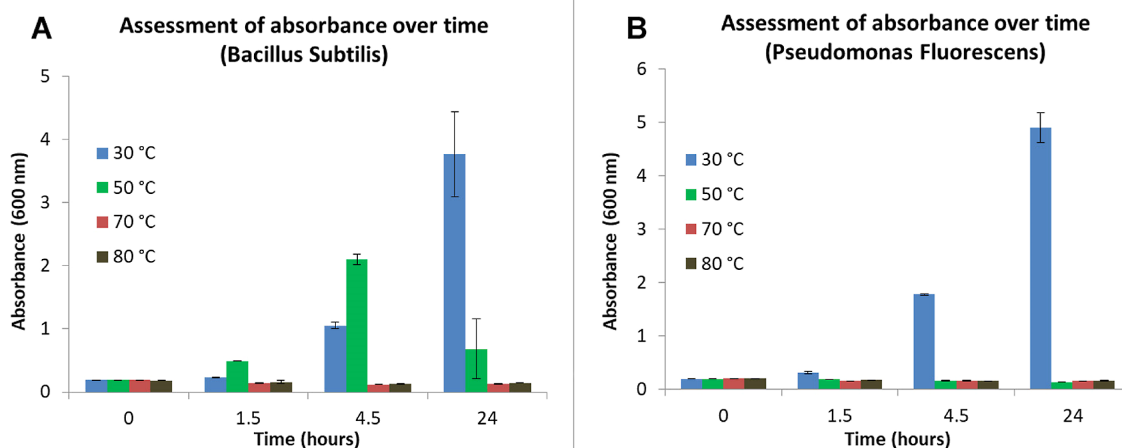

Figure 3. Effect of temperature (30, 50, 70, and 80 °C) on the growth of *B. subtilis* (A) and *P. fluorescens* (B) measured as cell turbidity over time. OD (at t = 0): 0.200 in TSB media.

Table 1. Matrix Compositions for Spray Drying and Their Respective Mass Yield and Moisture Content after Encapsulation of PGPB

polymer matrix	bacteria	inlet temp. (°C)	mass yield (%)	moisture content (%)
10% PIA	B. subtilis	100	62.1	11.8
10% PIA	B. subtilis	100	58.2	11.4
20% PIA	B. subtilis	100	44.5	11.4
1.5% alginate + 8.5% maltodextrin	B. subtilis	100	54.4	6.7
10% PIA	*P. fluorescens*	70	56.8	10.8
20% PIA	*P. fluorescens*	70	47.7	10.3

impact of residual moisture in improving microbial survival in microcapsules.[34−36]

The encapsulated *B. subtilis* was visualized using both optical and electron microscopy (Figure 4A−F). In general, the dried encapsulated *B. subtilis*-PIA particles appeared as spherical microspheres and had a concave morphology through SEM imaging (Figure 4B), likely due to dents formed during the drying process. The microbes were not visible in the microbe-loaded spray-dried powder. The addition of a small aliquot of water to the latter resulted in the instantaneous swelling to produce spherical hydrated microspheres, as observed by optical microscopy imaging in Figure 4C, which subsequently underwent dissolution to release motile bacteria, as observed in Figure 4D−F. Similarly, real-time observations under an optical microscope for *P. fluorescens* spray-dried in the PIA polymer showed swelling-dissolution and release of live bacteria (Figure 5).

3.5. Viable Cell Recovery from PIA Microspheres. Encapsulation by spray drying does not just provide a means to ensure that the microbes are viable, but it is also a cost-effective

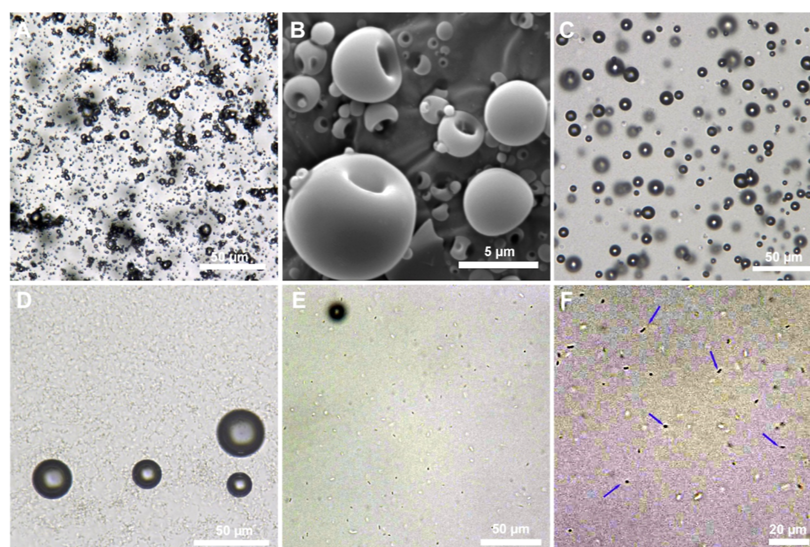

Figure 4. Microscopy images of *B. subtilis*-PIA microspheres, showing (A) spray-dried microbe-loaded PIA powder under an optical microscope, (B) SEM image of spray-dried microbe-loaded PIA powder, (C) optical microscopy image upon addition of a small amount of water to A to produce swollen microspheres, (D) optical microscopy image two min after addition of water with little to no microspheres left after the hydrated microbe-loaded microspheres burst to release a large number of motile bacteria, (E) five min after addition of water to A, showing a large number of motile bacteria released, and (F) magnified imaged of E, with blue arrows pointing toward the live bacteria.

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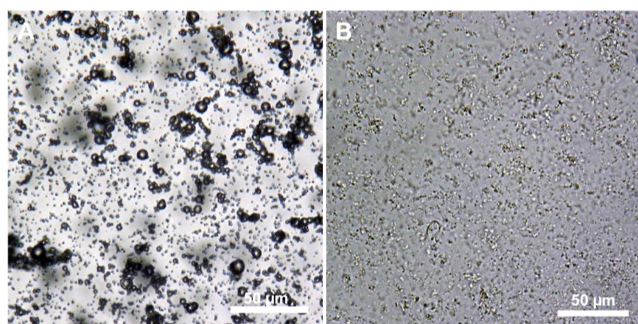

Figure 5. Optical microscopy images of *P. fluorescens* (A) encapsulated in PIA microspheres and (B) released from hydrated microbe-loaded microspheres.

method to produce a marketable product. Although PIA is a biobased polymer, it was important to confirm that the polymer solutions were not toxic to the bacteria. Our studies showed that there was a high level of biocompatibility when the two model bacteria were incubated in PIA solutions (Figure S4). The spray-dried bacteria-PIA powders were redispersed in saline buffer, serially diluted, and spread on agar plates for CFU counting the next day. The obtained CFU counts were compared to the microbial counts prior to the encapsulation process. The microbial counts for *B. subtilis* and *P. fluorescens* formulations are summarized in Table 2. For a better comparison, the survival rates have been calculated using log values instead of using the absolute values since the CFU values had large variations.[31,32] In general, there was a drop in the microbial count due to the high temperature that was used for spray drying. Yet, there were still a significant number of microbes that survived the spray drying process. For instance, for *B. subtilis* that was spray-dried at 100 °C in a 10% PIA polymer solution, the survival rate was found to be about 70% and up to 79%. These results were comparable to the CFU data obtained for the alginate and maltodextrin or the PIA and alginate formulations. Campos and co-workers encapsulated nitrogen-fixing bacteria by spray drying in sodium alginate/maltodextrin matrices to produce microcapsules with a 27% mass yield and 5−50 μm in size.[33] They also had bacterial survival greater than 76%, with the highest survival at 79% for the 1:14 sodium alginate/maltodextrin matrix. In their case, it was found that the presence of sodium alginate was crucial for protecting the bacteria and obtaining high survival. The absence of sodium alginate resulted in survival of less than 60%.[33] It is important to note that in our study a similar survival was obtained in the presence of PIA only and did not require any sodium alginate. A control experiment was conducted on *B. subtilis* in the absence of the PIA polymer at a lower temperature of 70 °C, and the bacterial survival was less than 48%. We speculate that there would have been a more pronounced drop in survivability of the microbes at a temperature of 100 °C. Increasing the concentration of PIA to 20% led to a lower microbial survival (46.8%), which could be due to the microbes being exposed to the elevated temperature for a longer time due to the higher solution viscosity. The storage stability and viability of the encapsulated microbes were investigated on a spray-dried *B. subtilis* using a 10% PIA solution, which was stored under refrigeration at 4 °C for an extended period. It was found that the encapsulated bacteria were viable with only a slight net loss in viability, with 83 and 78% of the *B. subtilis* surviving the storage in the absence of any additives at 3 and 6 months, respectively.

The *P. fluorescens* strain is an example of a non-spore-forming PGPB. As previously reported[37,38] and confirmed in our preliminary assessment of the effect of temperature on viability of the bacteria (Figure 3), it is more sensitive to heat than *B. subtilis*. The *P. fluorescens* cells were also spray-dried with different amounts of PIA for encapsulation. For the purpose of this work and to achieve reasonable cell recovery, the inlet temperature was deliberately decreased from 100 to 70 °C. This impacted the spray drying process, which had to be done at a feed rate reduced by half (2.5 mL/min) compared to the rate used for *B. subtilis* (5 mL/min). The spray-dried formulations with both 10 and 20% PIA showed viable cells, at a survival rate of about 50%. As expected, the overall survival of *P. fluorescens* was lower than what was observed for B. subtilis. *P. fluorescens* has been reported to have an optimal survival temperature of about 15 °C and a temperature as high as 37 °C can lead to a loss of survival by 50%.[37,39] These results support the idea that the PIA polymer provides protection to the microbes against high temperatures during the spray drying process, improving their survival. Looking toward a potential commercial use of PIA as a carrier of agricultural inocula, the observed "burst" release of bacteria at rehydration, as noted in Figures 4 and 5, could yield some stability and application challenges (e.g., moisture sensitivity and poor control of the release profile), which should be further investigated. For example, a possible solution could be the use of cross-linkers to prepare PIA-based superabsorbent hydrogels as recently reported by Choi and co-workers prior to mixing with PGPB.[40]

Table 2. Total CFU of PGPB in PIA and Other Polymer Matrices before Encapsulation by Spray Drying and upon Release after Spray Drying[a]

polymer matrix	bacteria	inlet temp. (°C)	CFU before spray drying	CFU after spray drying	survival (Log 10%)
control (No PIA)	B. subtilis	70	1.00×10^{12}	5.00×10^{5}	47.5
10% PIA	B. subtilis	100	5.13×10^{11}	4.43×10^{8}	73.8
10% PIA	B. subtilis	100	1.72×10^{10}	1.11×10^{8}	78.6
10% PIA	B. subtilis	100	3.70×10^{9}	5.47×10^{6}	70.4
20% PIA	B. subtilis	100	8.80×10^{8}	1.54×10^{4}	46.8
1.5% alginate + 8.5% maltodextrin	B. subtilis	100	1.70×10^{11}	4.82×10^{8}	77.3
10% PIA + 1% alginate	B. subtilis	100	3.30×10^{9}	5.00×10^{6}	70.4
10% PIA	*P. fluorescens*	70	1.14×10^{10}	7.08×10^{4}	48.2
20% PIA	*P. fluorescens*	70	8.73×10^{9}	1.55×10^{4}	52.0

[a]The total CFU was calculated using the CFU obtained for 1 g of the spray-dried sample multiplied by the total mass of spray-dried powder obtained from 100 mL of the polymer solution.

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3.6. Incorporating Volatile Solvents in PIA-Based Solutions. We investigated the use of volatile solvents as a way to reduce the spray drying temperature (Figures S5−S8). The effect of two alcohols, methanol and ethanol (with a boiling point of 65 and 78 °C, respectively), on *B. subtilis* was explored at different concentrations over time. An increase in temperature (from 30 to 70 °C) negatively impacted the survival of the microbes. At 70 °C, there were no significant differences in the survival of the bacteria as we increased the concentration of the alcohol from 0 to 20%. A similar trend was observed for both alcohols on *B. subtilis*. Next, we proceeded with the spray drying of *B. subtilis* in 5 and 10% ethanol and methanol, which resulted in microspheres of similar morphology as what was observed for the PIA solutions in the absence of any alcohol (Figure S9). Table 3 shows the microbe-polymer formulations in various percentages of ethanol and methanol and the bacterial survival after spray drying. A very slight decrease in the survival of the bacteria was observed with values ranging from about 70%, thus indicating that the presence of either alcohol did not improve the survival of bacteria during spray drying. It is important to note that despite the toxicity imparted by the alcohols, the bacteria managed to have a high viability. This, therefore, points out the versatility and efficacy of using PIA as an encapsulating material.

Table 3. Preparation of *B. subtilis* Microbe-PIA Formulations in Water-Alcohol Mixtures for Spray Drying and Their Survival after Spray Drying

polymer matrix	bacteria	CFU before spray drying	CFU after spray drying	survival (Log 10%)
10% PIA in water	B. subtilis	1.70×10^{10}	1.10×10^{8}	78.6
10% PIA + 5% EtOH	B. subtilis	1.03×10^{10}	3.21×10^{7}	75.0
10% PIA + 10% EtOH	B. subtilis	4.99×10^{9}	3.35×10^{6}	67.3
10% PIA + 5% MeOH	B. subtilis	8.82×10^{9}	1.27×10^{7}	71.4
10% PIA + 10% MeOH	B. subtilis	3.29×10^{9}	1.06×10^{7}	73.8

4. CONCLUSIONS AND FUTURE STUDIES

PIA was investigated as a carrier material for bioencapsulation of two plant-beneficial microbes, *B. subtilis* and *P. fluorescens*. Bioencapsulation was performed by spray drying the mixtures of microbes and polymers in aqueous media under two different spray drying conditions. All spray-dried formulations showed viable cells by CFU enumeration, and the PIA microspheres released bacteria instantaneously after rehydration, as observed by optical microscopy. There was no evidence of toxicity of the polymer during incubation of bacteria with the PIA polymer prior to spray-drying. The recovery of viable microbes was determined by CFU counting and had a survival of up to 79% for *B. subtilis*, which is comparable to survival rates previously reported for sodium alginate-based polymer matrices. This study presents PIA as a safe and sustainable new encapsulation material for microbial inoculants, therefore documenting for the first time the potential use of a synthetic biopolymer in the absence of sodium alginate as a biocompatible host matrix to deliver live bacteria for agricultural applications. Complementary work will be necessary to ensure a successful path toward commercialization. This includes (1) studying the fate and degradability of PIA in the soil environment, (2) exploring other scalable methods, such as extrusion or fluidized bed drying, for capsule production, (3) probing the effect of the physicochemical properties of the polymer, the method of particle fabrication, and the presence of additives on the efficiency of cell immobilization, cell survival, and controlled release from the beads more systematically, and (4) investigating the effect of the encapsulated cells on plant growth in controlled growth environments and in the field. In addition, testing the applicability of the encapsulating procedure to mixed cultures and increasing control on cell release by tuning PIA solubility in water through cross-linking with polyethylene glycol are two promising unexploited research directions that would be worth investigating.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.3c00356.

NMR of IA and PIA; biocompatibility of PIA with bacteria; effect of alcoholic PIA solutions on bacteria; and SEM images of spray-dried bacterium-loaded microspheres from alcoholic PIA solution (PDF)

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Notes

The authors declare no competing financial interest.

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