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A novel, green, low-cost chitosan-starch hydrogel as potential delivery system for plant growth-promoting bacteria

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ABSTRACT

The study examines the use of macrobeads for the controlled-release of bacteria. Macrobeads were prepared by an easy dripping-technique using 20/80 wt/wt chitosan-starch blends and sodium tripolyphosphate as cross-linking agent. The resulting polymeric matrix was examined by SEM, XRD, TGA, and solid-RMN. The swelling-equilibrium, thermal behaviour, crystallinity, and size of macrobeads were affected by the autoclave-sterilization. The diameter of the sterilized xerogel was *c.a.* 1.6 mm. The results suggested that ionotropic-gelation and neutralization were the mechanisms underlying hydrogel formation. Plant growth-promoting bacteria (PGPB) were loaded into macrobeads separately or co-inoculated. Bacteria loaded macrobeads were dried and stored. Bacteria survived at least 12 months in orders of 10^9 CFU of *A. brasilense*/g and 10^8 CFU of *P. fluorescens*/g. Bacterial release in sterile saline solution tended to a super Case-II transport mechanism. Polymeric-matrix release efficiently both PGPB in natural soils, which uncovers their potential for the formulation of novel and improved biofertilizers.

1. Introduction

The complex needs of sustainable agriculture require new actions to counteract climate changes and the environmental risks of intensive agriculture production. The increasing demand for food has led to excessive application of chemical fertilizers and pesticides to attain maximum yields of crops. As a result of these practices, a large number of environmental problems are increasingly being raised, like eutrophication, soil acidification, water contamination and soil salinization (Leip et al., 2015; Li et al., 2013; Savci, 2012). Bio-fertilization should be an alternative practice to contribute to sustainable agriculture and to the recovery of degraded soils. Biofertilizers are based on living microorganisms that can be applied to seeds or soil, where bacteria colonize the rhizosphere and promote plant growth (Brahmaprakash & Sahu, 2012; Vassilev et al., 2015). Thus, they are collectively called plant growth-promoting bacteria (PGPB). PGPB associate with many plant species and are commonly present in many natural environments (Bashan, de-Bashan, Prabhu, & Hernandez, 2014; Mishra & Arora, 2016). They promote growth by providing nutrients to the plant and by controlling pathogenic fungi or bacteria in some cases (Bashan et al., 2014; Mishra & Arora, 2016). The new tendencies of green crop production points to the gradual reduction in the use of chemicals without affecting yield or quality of the crops. In this context PGPB serve important functions in sustainable agriculture as substitutes or supplements to chemical fertilizers (Bashan et al., 2014; Gupta & Dikshit, 2010).

Azospirillum is one of the most studied genera of PGPB, commercially available as inoculant for crop production in several countries, including Argentina, Mexico, India, Italy, and France (Bashan & De-Bashan, 2010). Great amount of evidence gathered from decades of research proved the positive impact of *Azospirillum* spp. inoculation on yield crops such as wheat and maize (Díaz-Zorita, Canigia, Bravo, Berger, & Satorre, 2015). In Argentina, strain Az39 of *Azospirillum brasilense* is used for the formulation of all *Azospirillum*-based commercial inoculants (Cassán et al., 2015). On the other hand, fluorescent *Pseudomonas* spp. have an outstanding capacity to biocontrol phytopathogens and are usually capable of solubilizing phosphate (Lucy, Reed, & Glick, 2004). In particular, *Pseudomonas fluorescens* ZME4 has been isolated from inner tissues of maize and display strong PGPB qualities (Maroniche, Rubio, Consiglio, & Perticari, 2016). Both *A.*

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brasilense and P. fluorescens can stimulate each other when cultivated together (Pagnussat et al., 2016) and are increasingly employed in combined inoculants as biofertilizers (Valverde, Gonzalez Anta, & Ferraris, 2015).

To be applied, PGPB must be supported in peats or in liquid formulations. Some of them must be added with chemicals, like bacterial protectors or gums, to achieve bacterial adhesion to the seeds (Bashan & De-Bashan, 2010; Bashan et al., 2014). Surfactants and various additives are usually applied to improve storage stability and solubility of liquid inoculants (Mishra & Arora, 2016). Liquid formulations are prepared in water or in organic oils, easy to produce, low cost. They can be applied directly to seed or soil (Bashan et al., 2014). The main disadvantage of the application in liquid carriers is the uneven distribution of bacteria on the seeds (Covarrubias, De-Bashan, Moreno, & Bashan, 2012). Peat used as carrier is composed of complex organic matter. Although it is an inexpensive material, it has many problems in maintaining bacterial viability (Fallik & Okon, 1996). Peat mines are situated in preserved environments where extraction is forbidden. Natural peat deposits are inexistent in some countries (Albareda, Rodríguez-Navarro, Camacho, & Temprano, 2008). In most countries, there are no regulations of the level of contaminants in the most commonly used non-sterile peat preparations (Mishra & Arora, 2016). For this reason, alternative materials should be employed to maintain the quality and efficiency of the bacterial inoculum and reduce production costs and negative environmental impacts (Fernandes, Rohr, de Oliveira, Xavier, & Rumjanek, 2009; Herrmann & Lesueur, 2013). In both liquid or peat based inoculants, the bacteria encounter difficulties in colonizing the roots or seeds, and show poor survival (Bashan et al., 2014). They are susceptible to other soil bacterial competitors, to predation by soil micro-fauna, or to environmental stresses (Fallik & Okon, 1996).

PGPB immobilization has been widely investigated in agriculture as an inert carrier that allows for protection, gradual release, and long periods of storage without losing the viability of microorganisms. There are many preparation techniques to improve the survival of microbial inoculants that include emulsion, spray drying, solvent extraction/evaporation, coacervation, ionic gelation, and extrusion (Bashan et al., 2014). Formulations obtained by these techniques are normally based on alginate or a mixture thereof with other biopolymers and organic substances. Nevertheless, the survival of microorganisms in the carrier or when applied to seeds decay in soil (Bashan et al., 2014; Cortes Patiño & Bonilla, 2015). Xavier, Holloway, and Leggett (2004) showed that since 1980, most of the studies on supported PGPB represented less than 1% of the scientific articles on microorganisms (Xavier et al., 2004). However, the inoculant market needs to develop and commercialize new inexpensive supported biofertilizers that could be more effective and stable over time (Herrmann & Lesueur, 2013).

Chitosan (CS) is a natural polycationic polymer (polysaccharide), biocompatible, biodegradable, nontoxic, simple to handle, consisting of (1,4)-linked 2-amino-deoxy-β-d-glucan. It is the second most abundant natural biopolymer on earth, and can be obtained by alkaline deacetylation of chitin, a component of shrimp or crab shells (Elsabee & Abdou, 2013; Perez & Francois, 2016). However, CS is an expensive biopolymer for industrial application. A way to reduce the cost of the final product is to mix it with other inexpensive polymers like starch (ST) (Perez & Francois, 2016). Starch is also a natural, abundant, nontoxic and biodegradable polysaccharide. Therefore, this biocompatible polymer can be used blended with CS as support in immobilizing PGPB (Maiti, Ray, & Mitra, 2012; Perez & Francois, 2016). Solubility of CS in aqueous solutions is attained via protonation of its amine groups in acidic environments. Some ionic crosslinking reagents have been used to prepare a physical hydrogel (Elsabee & Abdou, 2013). Sodium tripolyphosphate (STPP) is a non-toxic multivalent anion that can form crosslinks by ionic interaction between its negatively charged counter ion and the protonated amino groups of CS (Bugnicourt & Ladavière, 2016; Perez & Francois, 2016). The crosslinking density between STPP and CS can be controlled by the charge density of STPP, because it depends on the solution pH. STPP can be dissolved in water and generate hydroxyl (OH^-) and tripolyphosphoric ($P_3O_5^-$) ions. The use of CS/ST blends could allow to prepare a much lower cost supported biofertilizer. Previous results showed that this combination form a miscible gel that contributes to a favourable hydrogel formation (Perez & Francois, 2016).

The degradation rate of the polymeric matrix is directly related to its composition, the preparation method, and the biological activity of the microorganisms. These aspects affect the controlled release properties of the beds and their degradability (Bashan, De-Bashan, & Prabhu, 2016; Cortes Patiño & Bonilla, 2015). This work is based on the hypothesis that a polymeric CS/ST matrix loaded with one or more PGPB is capable of progressive releasing viable cells in sterile water and non-sterile soil, providing an improvement condition for storage and a convenient and economical material for the formulation of biofertilizers. This work aims at obtaining macrobeads that are biodegradable, inexpensive, and easy to prepare on the base of a hydrogel prepared with ionotropic crosslinking of CS/ST mixtures. It also aims at determining the feasibility of using these macrobeads for the support of controlled release biofertilizers containing *A. brasilense* Az39 and P. *fluorescens* ZME4 singly or in combination. To achieve these objectives, the effects of sterilization on macrobeads particle size, structure, thermal properties, and equilibrium degree of swelling were evaluated. Also, it was determined the viability of bacteria in the beds along one-year period of storage, and their controlled release in water or soil, either when immobilized as single species or co-immobilized.

2. Materials and methods

2.1. Materials

Chitosan (CS) of medium molecular weight (81% degree of deacetylation), native potato starch (ST), ethyl alcohol (≥99.5% purity), acetone (≥99.9% purity), glutaraldehyde solution (25% in water) and sodium tripolyphosphate (STPP) (85% purity) were purchased from Sigma-Aldrich (USA). Lactic acid (85% wt/wt) and sodium hydroxide (NaOH) were purchased from Cicarelli (Argentina). Potassium hydroxide (> 85% purity), sodium chloride (99.5% purity) and DL-malic acid (≥99.5% purity) were purchased from Biopack (Argentina). Beef peptone and yeast extract were purchased from Britania (Argentina). Dipotassium hydrogen phosphate was purchased from Merck (Germany). All reagents were of analytical grade and used as received.

2.2. Preparation methods of chitosan-starch macrobeads

The procedure to obtain the CS/ST macrobeads by ionotropic gelation using STPP was reported by Perez and Francois (2016) as follows: a 3% wt/wt CS solution was prepared by dissolving CS powder in an aqueous solution of lactic acid (1% v/v) with mechanical stirring (Perez & Francois, 2016). The potato ST gel was prepared by heating an 8% wt/v potato ST solution in deionized water with constant magnetic stirring. Gelatinization was achieved at 76 °C in a boiling distilled water bath. The crosslinking solution was prepared by dissolving STPP in distilled water to produce a final concentration of 1% wt/v (pH 8.6). The blends were prepared by mixing the solution of CS and the ST gel with mechanical stirring for 1 h.

After obtained, blend with a CS/ST mass ratio 20/80, was kept at room temperature for 30 min, then used to prepare macrobeads by a dripping technique. The CS/ST blend was dripped through plastic tips 2–3 mm in diameter into the STPP solution. Crosslinking was achieved during 2 h of continuous stirring at room temperature. Some macrobeads were prepared dripping the polymeric blend onto a 2 M NaOH aqueous solution (pH 10) to evaluate the chitosan coagulation process. Polymeric matrices were kept for 2 h into the alkaline solution. The macrobeads were removed from the STPP or NaOH solutions and

extensively washed with distilled water. To ensure complete cleaning, after a drying process at 40 °C, the macrobeads were rehydrated for 3 h under magnetic stirring and dried at 40 °C for 48 h. Finally, CS/ST polymeric matrices were sterilized by autoclaving (121 °C, 1 atm) for 20 min and dried at 40 °C for 24 h.

2.3. Characterization

2.3.1. Nuclear magnetic resonance (NMR)

High-resolution ^{31}P solid-state spectra were recorded using the ramp $^{1}H-^{31}P$ and the combined techniques of proton dipolar decoupling (DD), magic angle spinning (MAS) and cross-polarization (CP). Experiments were performed at room temperature in a Bruker Avance II-300 spectrometer equipped with a 4-mm MAS probe. Glycine was used as an external reference for the ^{31}P spectra, and the Hartmann − Hahn matching condition was set in the cross-polarization experiments ^{31}P spectra. The recycling time was 4 s. Different contact times during CP were employed in the range of 200–1500 μs for ^{31}P spectra.

2.3.2. X-ray diffraction (XRD)

XRD patterns were obtained using a Rigaku diffractometer with Bragg Brentano geometry and CuK_α radiation ($\lambda = 0.1542$ nm, 40 kV, 20 mA) in the range of $2\theta = 5–50$ º at a scanning rate of 1°/min and a scan step of 0.05°. The chart speed was set to 5°/min. Measurements were performed at ambient conditions.

2.3.3. Thermogravimetric analysis (TGA)

Thermal degradation processes were investigated using a Shimadzu TGA-50 (Japan). Measurements were carried out by heating the sample from 20 to 600 °C under an inert atmosphere maintained by injecting N_2 at a flow rate of 30 mL/min, with a heating rate of 10 °C/min and using a sample weight of approximately 10 mg.

2.3.4. Scanning electron microscopy (SEM)

The morphology of the prepared materials (CS/ST macrobeads sterilized and non-sterilized) sputter-coated with gold was examined with a Karl Zeiss Supra 40 SEM (Germany). The surface morphology was examined with a field emission gun operated at 3 kV. The micrographs were taken at magnifications between 70 and 25,000×. After bacteria immobilization, samples were step-wise dehydrated in ethanol (series from 50% to 100%) and then in acetone (100%), critical-point dried and coated with gold. SEM micrographs were obtained with a Philips XL 30 Scanning Electron Microscope.

2.3.5. Equilibrium swelling property of CS/ST macrobeads

A known weight of polymeric matrix was dried to a constant value and then immersed in distilled water at room temperature. The experimental equilibrium swelling degree (Q_e) was calculated as:

$$Q_e = \frac{wt_e}{wt_o} x\ 100 \quad (1)$$

where, wt_e is the weight of the swelled macrobeads at equilibrium and wt_o is the weight of the dry polymeric matrices before the swelling process.

2.3.6. Bacterial strains and culture conditions

Azospirillum brasilense Az39 (Az39) and *Pseudomonas fluorescens* ZME4 (ZME4) strains were cultivated at 30 °C with orbital shaking at 100 rpm in 30 mL of Luria Bertani broth (LB) consisting of 10 g/L tryptone, 5 g/L beef extract, and 10 g/L NaCl. When cultures reached $OD_{600} = 1$ for ZME4 and $OD_{600} = 1,5$ for Az39 (10^9 CFU/mL in both cases, corresponding to late exponential growth phase), the cells were collected by centrifugation at 5000 g for 10 min and carefully resuspended in 1 mL of the same spent medium cell free by filtration through 0.22 μm millipore filter. These were the inocula of each species to be immobilized. Bacterial CFU in the inocula were enumerated by serial dilutions with sterilized distilled water and plating on Agar Congo Red (ACR) consisting of D,L-malic acid 5 g/L, K_2HPO_4 0.5 g/L, $MgSO_4$ $7H_2O$ 0.2 g/L, NaCl 0.1 g/L, yield extract 0.5 g/L, KOH 4.7 g/L, $FeCl_3$ $6H_2O$ 0.015 g/L, Agar 20 g/L, pH 6.8 or LB for Az39 and ZME4, respectively, using the drop plate method (Herigstad, Hamilton, & Heersink, 2001). For this, 10 μL of each dilution was seeded in triplicated as a drop on each agar media. Plates were incubated at 30 °C for 1 day for ZME4 or during 2 days for Az39. Colonies of each drop were counted, averaged and multiplied by the dilution factor to obtain CFU/mL.

Antibiotic-resistant variants of *A. brasilense* Az39 (Az39f) and *P. fluorescens* ZME4 (ZME4e) were used to determine CFU values of trapped and free bacteria during their release in natural non-sterile soil. Strain Az39f is a natural rifampicin-resistant variant that was selected by spreading 0.1 mL of an Az39 overnight broth culture onto ACR medium plate containing rifampicin in a concentration gradient from 0 to 100 μg/mL. Normal phenotype growing colonies in the zone of higher antibiotic concentration were streaked in ACR medium containing 100 μg/mL of rifampicin and then sub-cultured in nutrient broth (NB) without antibiotic. To confirm the stability of the resistance, after five additional rounds of sub-cultures without antibiotics, CFU were counted in ACR medium with or without 50 μg/mL rifampicin. To obtain ZME4e strain, a gentamicin resistance determinant was integrated into ZME4 chromosome using the miniTn7 plasmid pME3280a (Zuber et al., 2003). To accomplish this, strain ZME4 was transformed with pME3280a and pUX-BF13 helper plasmid (Bao, Lies, Fu, & Roberts, 1991) by electroporation and recombinant clones were recovered in LB agar medium containing 12.5 μg/mL of gentamicin. Chromosomal integration of the miniTn7 transposon was confirmed by PCR.

2.3.7. Cells immobilization

Suspensions of each bacterial species were adjusted to a concentration of 10^{10} CFU/mL and immobilized by immersing 10 g of sterilized CS/ST macrobeads in a weight of inocula of Az39 or ZME4 corresponding to xerogel equilibrium swelling. For the simultaneous loading of both strains, the macrobeads were soaked with a mixed inocula containing 0.5×10^{10} cells of each species in the same way. The macrobeads were allowed to absorb all the inoculum and then dried at 30 °C for 3 days. All the operations were done in sterile conditions. Macrobeads loaded with bacteria were stored for one-year at ambient conditions (20–25 °C, 70–80 relative humidity %) inside glass flasks sealed with common paper, in order to be not hermetic. All the experiments were carried out in triplicate.

2.3.8. Bacterial survival quantification

During the storage period, viable bacteria of each strain were quantified measuring CFU/g DW of macrobeads at various time intervals. Briefly, 20 mg of dried macrobeads containing the immobilized bacteria were immersed in 1 mL of sterile distilled water and maintained at 30 °C for 2 h in a rotary shaker at 100 rpm. Then, the hydrated macrobeads were completely crushed with a pestle and vortexed at maximum speed for 10 s. The obtained homogenates were used to estimate the CFU/g DW of macrobeads by the drop plating method, as described in 2.3.6 section. In the case of macrobeads loaded with both bacterial species, serial dilutions of Az39 were plated in ACR and incubated at 37 °C, and serial dilutions of ZME4 were plated in LB and incubated at 30 °C. All the experiment was carried out in triplicate.

2.3.9. Cells release kinetics in sterile water

The cumulative release of Az39, ZME4 or Az39 + ZME4 from macrobeads was determined at various time intervals using the following procedure: 20 mg of macrobeads were immersed in 2 mL of sterile saline solution (0.9% NaCl) in plastic tubes at room temperature during 17 days. Aliquots of 0.1 mL were removed from the middle part of the tube at different interval times and the CFU present in the solution were

determined as described in Section 2.3.6. All the experiments were carried out in triplicate.

2.3.10. Cells release in soil

The release kinetics of Az39f, ZME4e, and their combination from CS/ST macrobeads were examined under conditions of constant soil moisture. Soil samples were collected from the superficial 10 cm- horizon of a Typical *Argiudol*, free of vegetation, from fields in National Agricultural Technology Institute (INTA), Balcarce, Argentina. A mixture of 10 mg of dried macrobeads and 1 g of non-sterilized soil was kept for 30 days at 20 °C. At specific times during incubation, macrobeads were recovered from the soil, washed, and the swelling ratio was determined. The macrobeads were removed and transferred into 10 mL of sterile distilled water and CFU counts were estimated as described in Section 2.3.6. The CFU of released bacteria in soil were determined by the plate count method. LB agar supplemented with gentamycin (12.5 μg/mL), or ACR supplemented with rifampicin (50 μg/mL) were used for ZME4e or Az39f, respectively. All the experiments were carried out in triplicate under sterile conditions.

3. Results and discussion

3.1. NMR assays

In order to analyze the influence of the sterilization process on the ionic crosslinking of the biopolymers, NMR was conducted using sterilized and non-sterilized macrobeads. Fig. 1 shows the ^{31}P spectra of sterilized and non-sterilized CS/ST macrobeads. It was possible to observe three peaks when non-sterilized macrobeads are tested. The sample showed a peak at 2.37 ppm, which was attributed to end chain phosphate group (Barbi et al., 2015; We, nslow, Fiske, & Mueller, 1999). The presence of characteristic peaks referring to triphosphate specie were attributed to α terminal phosphorous (−10.27 ppm) and middle phosphorous (−22.53 ppm) atoms indicating the presence of tripolyphosphate units in macrobeads structure (Sacco et al., 2014). The obtained spectrum confirmed an ionic binding between CS and STPP in non-sterilized macrobeads These results were in agreement with the CP-MAS ^{31}P-NMR spectrum obtained for crosslinked CS using STPP reported by Sacco et al. (2014).

NMR spectrum obtained with sterilized CS/ST macrobeads presents only two peaks at 2.37 and −10.27 ppm because the peak at −22.53 ppm disappeared indicating the rupture of ionic binding between the crosslinker and the CS (Fig. 1). Spectrum recorded showed a substantial change in the intensity of these peaks compared to the spectrum belonging to non-sterilized macrobeads. There was a significant intensity increase (2.37 ppm) or decrease (−10.27 ppm) of these two peaks, respectively. Therefore, the sterilized material became less crosslinked than the non-sterilized one (Jen & Shelef, 1986; Palmeira-de-Oliveira et al., 2011).

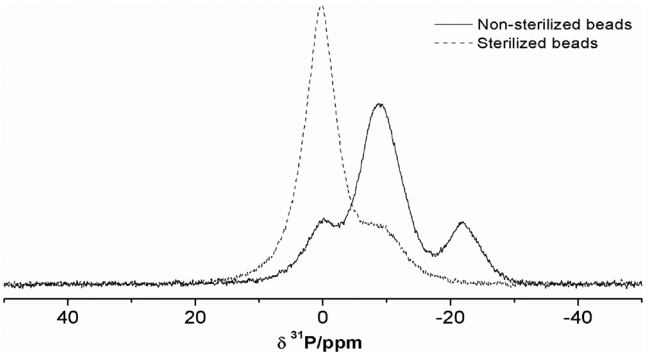

Fig. 1. ^{31}P-NMR spectra of non-sterilized and sterilized CS/ST xerogels crosslinking with STPP.

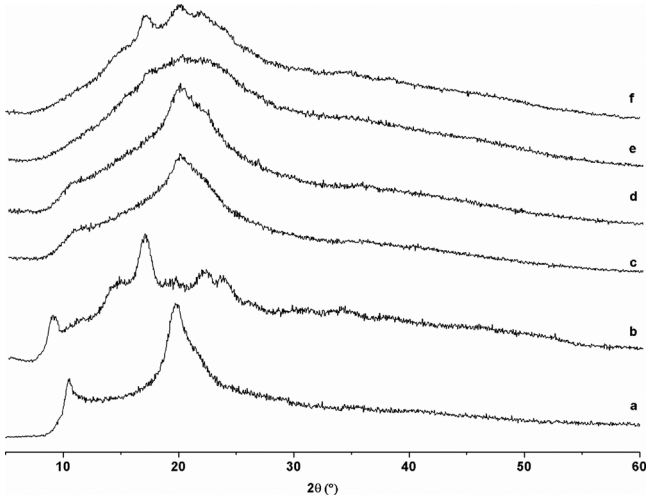

Fig. 2. X-ray powder diffraction patterns of neat chitosan (a), neat potato starch (b), non-sterilized CS/ST polymeric matrix prepared using NaOH (c), sterilized CS/ST polymeric matrix prepared using NaOH (d), non-sterilized CS/ST polymeric matrix prepared using STPP (e), sterilized CS/ST polymeric matrix prepared using STPP (f).

3.2. XRD studies

X-ray diffractograms of CS powder, ST potato powder, and sterilized and non-sterilized CS/ST polymeric matrix prepared using NaOH or STPP are shown in Fig. 2. CS is a semi-crystalline polymer with two main diffraction peaks (10.55 and 19.70°) observed in its X-ray diffraction pattern (Fig. 2(a)) as it was already reported by Perez and Francois (2016) (Perez & Francois, 2016). The potato ST powder showed a typical B-type diffraction pattern with reflection peaks located at 5.75, 9.47, 17.09, 20.03, 23.02 and 26.55° (Fig. 2(b)) (McPherson, 1999; Perez & Francois, 2016).

Sterilization effects on the structure of CS/ST polymeric matrices prepared with NaOH was also studied (Fig. 2(c)). The use of NaOH produced a coacervation-phase inversion due to the neutralization of positive charges of CS and the polymer precipitation. The formed macrobeads had an irregular shape and most of them collapsed after drying. Non-sterilized material showed a good compatibility between CS and ST related to physical interactions between both biopolymers macromolecules (Pelissari, Grossmann, Yamashita, & Pineda, 2009; Perez & Francois, 2016). The same phenomenon had already been observed by other researches (Pelissari et al., 2009; Tuhin et al., 2012). The diffractogram indicated a crystallinity decrease respect to neat biopolymers because the CS and ST characteristic peaks, associated with its semi-crystalline structure, disappeared. The OH groups existing in ST and CS structure and the amino groups belonging to CS polymeric chain might interact through hydrogen bonding (Pelissari et al., 2009; Perez & Francois, 2016). These inter-macromolecular interactions inhibit the formation of ordered alignment of polymer chains reducing the crystallinity of the CS/ST macrobeads structure. A similar crystallinity modification due to physical crosslinking was also observed by other research groups (Bourtoom & Chinnan, 2008; Perez & Francois, 2016; Xu, Kim, Hanna, & Nag, 2005). Furthermore, the sterilization process produced a crystallinity increase of these macrobeads (Fig. 2(d)) because the autoclaving temperature might have affected the structure by disrupting the inter-macromolecular interactions between CS and ST.

The heat treatment associated with sterilization, also promoted the increase of crystallinity in sterilized ionically crosslinked macrobeads (Fig. 2(f)). The obtained diffractogram had three new peaks (17.12 and 19.70 and 24.19°), compared to the non-sterilized macrobeads pattern (Fig. 2(e)). Either inter-macromolecular hydrogen bonding or ionic

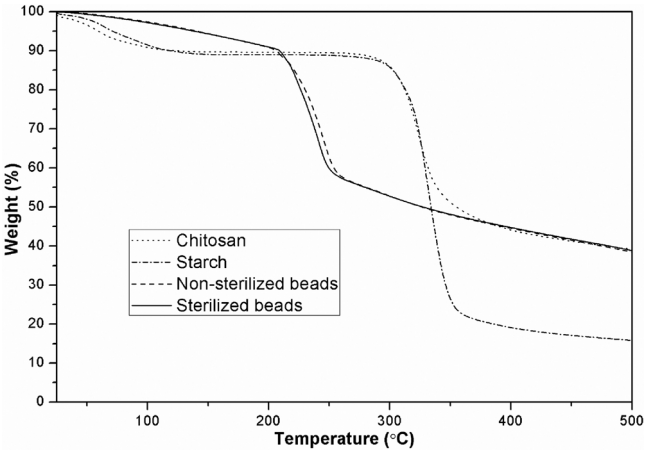

Fig. 3. TGA Thermograms of chitosan power, starch potato power, non-sterilized and sterilized chitosan/starch macrobeads.

crosslinking might be affected by the autoclaving temperature, explaining part of the observed results.

3.3. Thermal analysis

Thermogravimetric analysis curves of CS, ST, non-sterilized and sterilized macrobeads obtained using STPP are shown in Fig. 3. From the TGA results, it was possible to obtain the mass loss of neat biopolymers due to water evaporation which occurred in a range between 25 and 150 °C (Pelissari et al., 2009; Perez & Francois, 2016; Tuhin et al., 2012). TGA curves of CS and ST exhibit an onset of weight loss at 337.22 °C for CS and 324.45 °C for ST, which can be attributed to depolymerization of polysaccharides chains. Similar degradation behavior of these biopolymers in powder have been reported (Pelissari et al., 2009; Perez & Francois, 2016; Tuhin et al., 2012).

CS/ST macrobeads formation caused a shift on the onset of weight loss to a lower value (245.42 °C). The registered difference in the maximum thermal degradation temperature was related to the amorphization of the obtained polymeric material (Bourtoom & Chinnan, 2008; Perez & Francois, 2016; Xu et al., 2005), as already was discussed for XRD results. Sterilization induced changes in the final matrix structure associated with the decrease of ionic crosslinking density and of intermolecular interactions between both biopolymers, reducing the maximum degradation temperature to 240.81 °C, in agreement with NMR and XRD results.

3.4. SEM analysis

Ionically crosslinked macrobeads were round and homogeneous in shape. The sterilization process produced a significant reduction in the average particle size from 2.97 ± 0.07 to 1.82 ± 0.04 mm approximately (Fig. 4(a) and (b)). RMN, XRD and TGA results indicated that heat applied when sterilization affected the crosslinking density of hydrogel network, producing in turn a decrease in the size of macrobeads. According to TG results and considering that a temperature higher than 300 °C is required for depolymerization and decomposition of CS and ST, it was dismissed the possibility of the polymer degradation at the applied sterilization temperature (Perez & Francois, 2016). Serp, Mueller, von Stockar, and Marison (2002) also reported a size modification of alginate macrobeads after a thermal treatment. They obtained a 23% diameter reduction after a treatment at 130 °C for 20 min. They indicated that the shrinkage of the macrobeads was due to re-arrangements of the alginate chains coupled with loss of water (Serp et al., 2002).

The surface of sterilized and non-sterilized polymeric matrices was smooth and the morphology of the materials did not show any significant change. Micrographies belonging to ionically crosslinked macrobeads showed some holes and fractures (Fig. 4) probably caused by the dripping technique used (Perez & Francois, 2016). In contrast, CS/ST macrobeads prepared with NaOH were brittle and easily destroyed after the drying process (data not shown).

Due to significant physico-chemical differences between non-sterilized and sterilized ionically crosslinked CS/ST macrobeads, the effect of thermal treatment was studied on the swelling behavior (the ratio of swollen to dry mass).

Sterilized macrobeads showed a much higher equilibrium degree of swelling (235%) with respect to non-sterilized ones (143%). The sterilization process would produce a decrease in the number of intermacromolecular hydrogen bonding and ionic crosslinking, leading to a significant increase in equilibrium swelling capacity. This behavior would make the sterilized polymeric matrix appropriate to be loaded with PGPB and used as a controlled release system.

3.5. Bacteria survival time, and bacteria release behavior

3.5.1. Morphology of immobilized PGPB

The surface of the macrobeads, as well as their interior, was populated with Az39, ZME4 and Az39 + ZME4 as seen in the SEM micrographs (Fig. 5). Both, Az39 and ZME4 cells immobilized as single species adhered to the surface of the macrobeads and formed agglomerates and multilayer biofilms. Amorphous material resembling extracellular polymeric matrix auto-secreted by biofilms could be observed on the surface of macrobeads loaded with Az39 (Flemming & Wingender, 2010). Some cells were seen interconnected to each other by fibrous material that resembled fimbriae. This characteristic was not seen in ZME4 cells. Curiously, when both microorganisms were co-immobilized, they attached to the macrobead surface as highly ordered monolayers, with patches of aggregates (Fig. 5). Moreover, inside the xerogel Az39 and ZME4 tended to agglomerate forming thick biofilms in the holes or channels of the macrobeads (Fig. 5).

3.5.2. Bacteria survival

The total number of viable bacteria was measured immediately after immobilization and before drying (day 0) being in the order of 10^{10} CFU/g of swelling hydrogel for all the cases. As shown in Fig. 6, this number decreased to *c.a.* x10^9 CFU/g 15 days after drying. Cell survival after the drying process is a critical step that decreases viability. Creus, Sueldo, and Barassi (1996) demonstrated that wheat seeds imbibed in a suspension of 10^8 *A. brasilense* cells/mL, could maintain *c.a.* 10^7 viable cells after air-flow dried at 30 °C, showing that bacterial number decreased one order of magnitude when seeds were dried up to 15% humidity (Creus et al., 1996).

After one year-storage the viability of Az39 immobilized separately or together with ZME4 in dried CS/ST macrobeads remained constant at *c.a.* 10^9 CFU/g (Fig. 6). In contrast, ZME4 showed a gradual decline in cell survival during storage period. After 60 days of storage, ZME4 count was reduced to 10^8 CFU/g and then decreased to *c.a.* 10^7 CFU/g at the end of storage (Fig. 6). Clearly, survival would depend on the adaptive nature of each microorganism (Bashan, 1986). Our results emphasized the better adaptability of *Azospirillum* over *Pseudomonas* to cope with stressful dry conditions (Chowdhury et al., 2007; García et al., 2017). Paul, Fages, Blanc, Goma, and Pareilleux (1993) demonstrated that a large percentage of bacteria were destroyed during the drying of polymeric macrobeads prepared from alginate (Paul et al., 1993). Different percentage of bacteria survival during the drying have been reported by other researchers (He et al., 2015; Wu, Guo, Qin, & Li, 2012; Wu, He, Chen, Han, & Li, 2014). Even when a decrease in the number of viable cells of both strains was observed in this work, the overall decrease in viability could be considered low for the period of one-year and thus suitable for biofertilizer formulations.

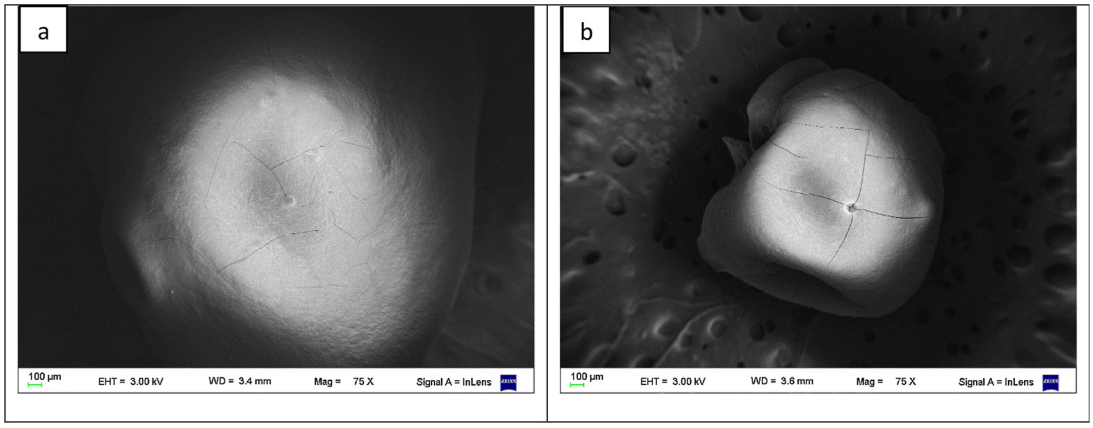

Fig. 4. Scanning electron micrographs of non-sterilized (a) and sterilized (b) CS/ST macrobeads are shown.

3.5.3. Bacteria release behaviour

3.5.3.1. Cells release kinetics in sterile water. The release behavior of bacteria in sterile saline solution from hydrogels is shown in Fig. 7. After being immerse in saline solution for 1 h, macrobeads containing Az39, ZME4 (immobilized separately), Az39 and ZME4 (co-immobilized) released more than 10^3 CFU/g of hydrogel in all cases. Macrobeads loaded with ZME4 reached 10^7 CFU/mL in the first 6 days and continued releasing cells for a period of 17 days (Fig. 7). The release of Az39 from mono-species or combined species macrobeads were similar, reaching 10^5 CFU/g 6 days after immersion. Interestingly, co-immobilized bacteria were released faster than individually immobilized ones, during the whole period for ZME4 and until day 4 for Az39. These results confirmed that the prepared macrobeads had suitable properties for releasing bacteria.

When macrobeads are immersed in an aqueous solution, liquid penetrate into the polymer matrix, causing its swelling. The transport mechanism contributes to the rate and extent of cells liberation out of the polymeric matrix (Peppas & Sahlin, 1989; Ritger & Peppas, 1987). The release mechanism was determined using the Eq. (2):

$$\frac{M_t}{M_\infty} = kt^n \tag{2}$$

where M_t/M_∞ represent the number of cells released by the hydrogel at time t and at equilibrium, k is a constant depending on kinetic characteristics and experimental conditions, and n is the kinetic exponent describing the controlling release transport mechanism.

M_∞ and k were included in k', and Eq. (3) was used to fit the data:

$$M_t = k't^n \tag{3}$$

The release profiles of bacteria from the macrobeads were fitted with the Ritger–Peppas equation. For a spherical matrix, if n = 0.43, the release controlling mechanism is the diffusion (Fickian); non-Fickian release or anomalous transport if 0.43 < n < 0.85; Case II transport or zero-order release if n = 0.85 where the release rate is constant and controlled by polymer relaxation, and super Case II transport if n > 0.85 when the release is erosion-controlled. The obtained release exponent n, for all the samples displayed super Case II transport (n > 0.85) as shown in Table 1. This indicated that polymer relaxation properties controlled the cell release and determined the

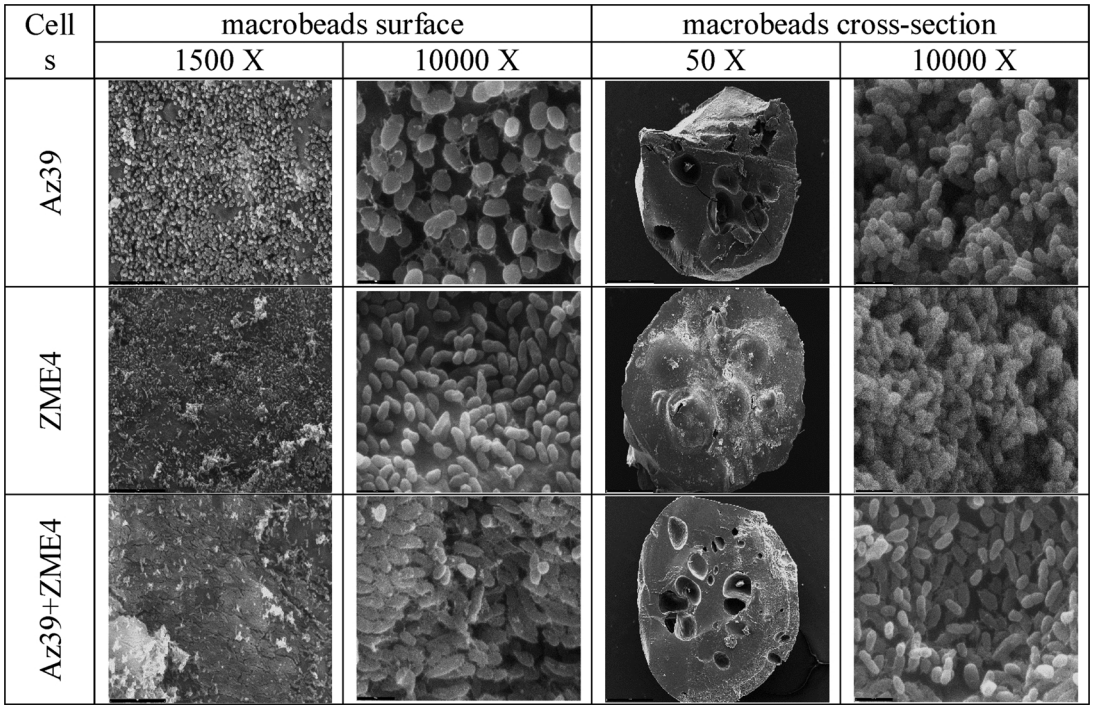

Fig. 5. SEM micrographies of the bacteria morphology on macrobeads surface (a) and cross-section (b) near macrobead center. Magnified by 50 X, 1500 X and 10,000 X.

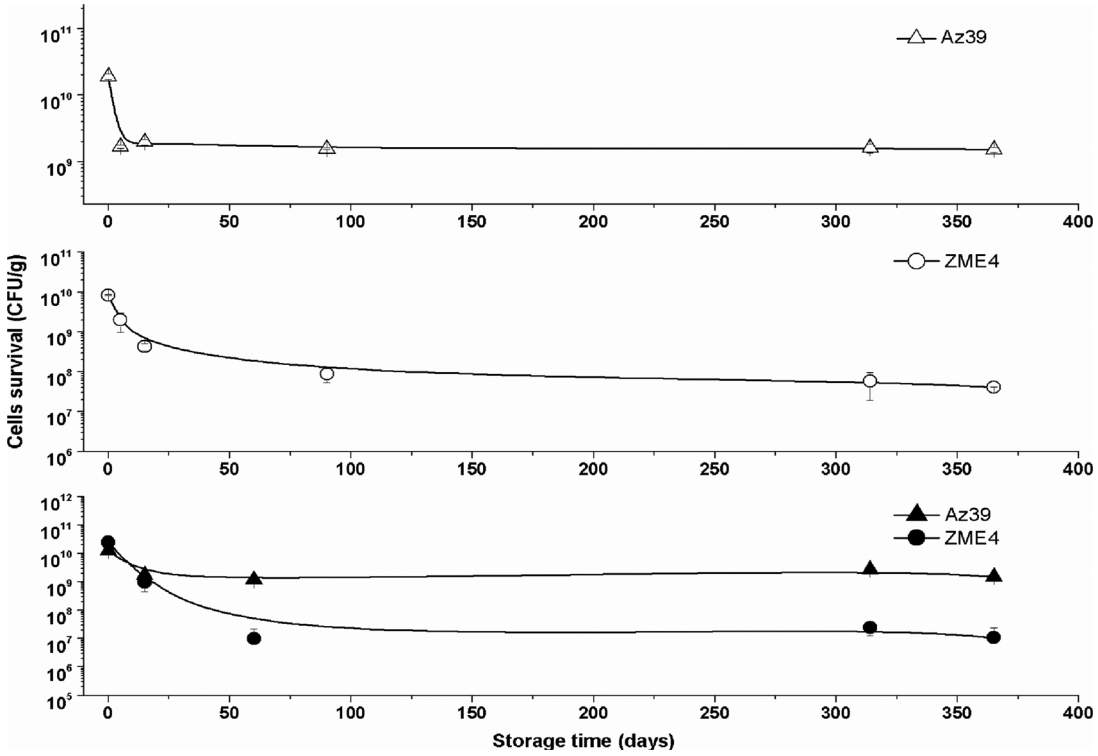

Fig. 6. Survival of *Azospirillum brasilense* Az39, *Pseudomonas fluorescens* ZME4 separately (open symbol) and in combination (closet symbol) in dry CS/ST macrobeads.

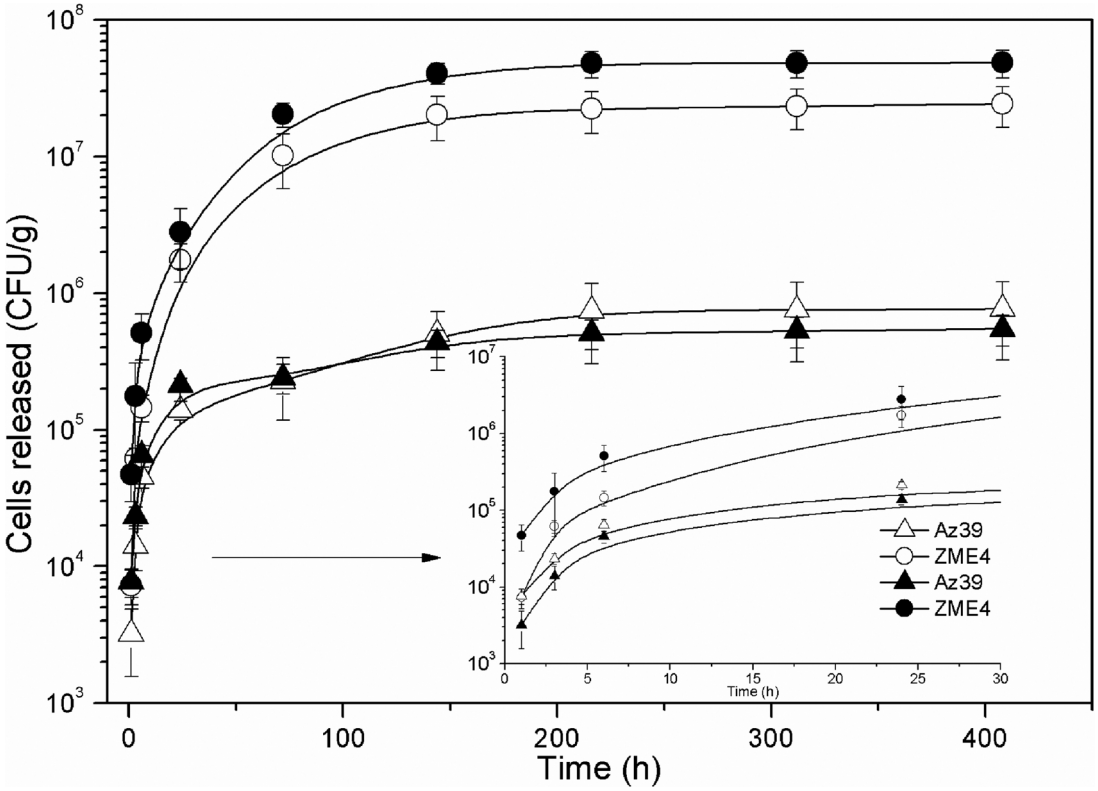

Fig. 7. Kinetics release of *Azospirillum brasilense* Az39, *Pseudomonas fluorescens* ZME4 separately (open symbols) and jointly (closed symbols) from prepared CS/ST.

release rate. He et al. (2015), Wu et al. (2012), and Wu et al. (2014) prepared a biofertilizer supported on alginate/clay, alginate/starch/clay and alginate/starch and studied the release mechanism of different types of bacteria. They reported a similar controlling mechanism ($n > 0.85$, super Case II transport) as in our study (He et al., 2015; Wu et al., 2012, 2014).

The results obtained here suggested that the hydrogel structure and its absorbing properties were crucial for controlling the release

Table 1
Bacterial release kinetics data for CS/ST hydrogel.

Bacteria	Diffusional exponent n for cells transport	k'	R^2	Transport mechanism
Az39	0.93 ± 0.10	7316.03 ± 2316.37	0.9868	Super Case-II transport
ZME4	1.75 ± 0.05	6647.80 ± 1134.63	0.9998	Super Case-II transport
Az39f	0.94 ± 0.07	10768.29 ± 2272.08	0.9943	Super Case-II transport
ZME4e	1.26 ± 0.03	52038.01 ± 5259.79	0.9995	Super Case-II transport

Experimental data were fitted to the Ritger–Peppas equation (Eq. (3)).

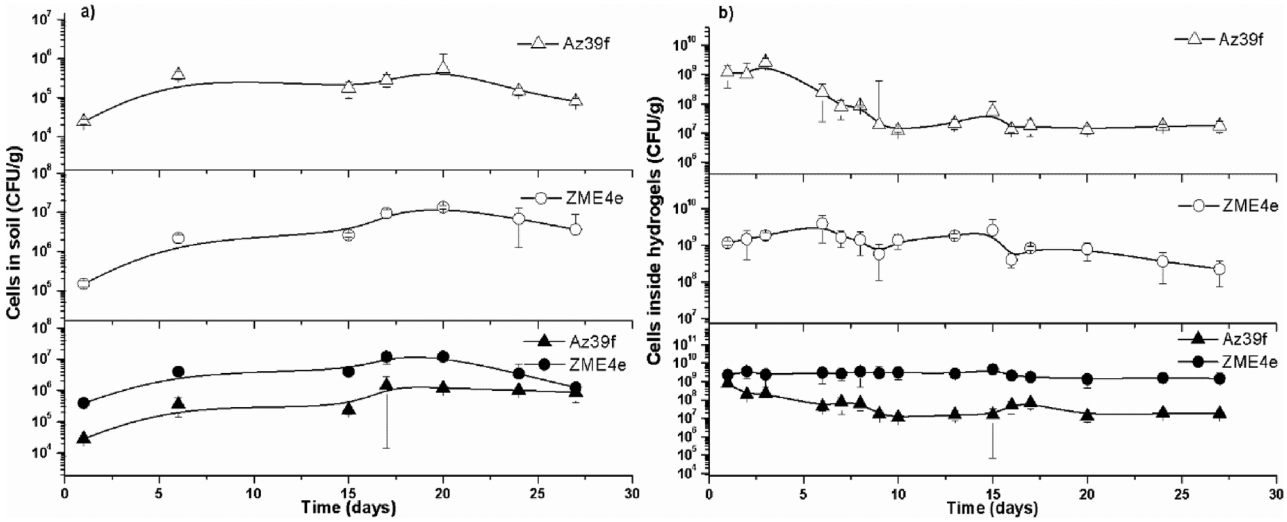

Fig. 8. Release of loaded bacteria in natural soil (a) and viable bacteria inside CS/ST macrobeads (b).

behavior of the PGPB. The release rate was fast and prolonged. These are two required properties for a polymeric matrix to be a good biofertilizer delivery system.

3.5.3.2. Cells release in soil. The amount of bacteria remaining inside the macrobeads after applied into the soil is shown in Fig. 8(a). High concentrations of viable cells were verified inside the macrobeads one day after sowing them into non-sterile soil (10^9 CFU/g). A rapid increase in the CFU/g was observed for individually loaded species (Az39f and ZME4e) after the first day. This might be attributed to the rehydration of cells and consequent the restoration of cell division. On the other hand, this phenomenon was not observed when both strains were co-immobilized. In this case, the greater number of bacteria would have created a competition among species derived from nutrient limitation. Nevertheless, after 28 days the number of cells was still high ($> 10^7$ UFC/g).

On the other hand, the kinetics of the CFU counts in soil showed an opposite shape at least to day 20 Fig. 8(a), then it remained near constant Fig. 8(b). It could be noticed that bacteria released from the macrobeads should migrate through the soil and compete with the native microflora already present there. Az39f, ZME4e both from single species or combined loaded macrobeads, grew rapidly and progressively in soil after one day. A high rate of liberation from CRBs was tested with average values of 10^6 (Az39f and Az39f when co-immobilized with ZME4e) and 10^8 CFU/g (ZME4e and ZME4e when co-immobilized with Az39f). Bacterial concentration increased progressively during the first 20 days and then began to decrease after 24 days. The release rate of ZME4e form macrobeads was much faster than Az39f (both, immobilized separately or co-immobilized)

The polymeric material prepared and tested as biofertilizer support entails two main advantages. First, the supporting material would provide protection for beneficial bacteria against adverse environmental factors occurring in the rhizosphere where bacteria encounter harsh conditions and have to compete with native microflora. Second, the rapid initial release would enable the fast colonization of roots, and then, the subsequent gradual and prolonged release of cells would boost and allow long-term biofertilizing effects.

Scaling-up procedures and characterization of technical reagent-grade material are the next steps for their commercialization. The available data on the present procedure suggest that a new approach in the field of sustainable agriculture can be considered: the concept of a controlled release of biofertilizers.

4. Conclusion

A biodegradable and biocompatible ionically crosslinked hydrogel based on a chitosan-starch blend was developed and its suitability as bacteria carrier was stablished.

Sterilization produced a decrease of the hydrogen bond or ionic interactions depending on the macrobeads preparation method. As a consequence, a decrease in the polymeric matrix size and an increase in the equilibrium swelling degree were observed.

The dried hydrogels loaded with *A. brasilense* and/or *P. fluorescens* could be stored at room temperature and humidity for a long period while preserving the viability of bacteria. The recovery of bacteria from macrobeads was in the order of 10^9 and 10^8 CFU. g^{-1} for *A. brasilense* and *P. fluorescent*, respectively after 1 year of storage. Immobilized bacteria adhered to the surface and the channels of the macrobeads and formed agglomerates and multilayer biofilms. Cell release from the chitosan-starch macrobeads in sterile water was gradual and exhibited a super Case II transport mechanism ($n > 0.85$), meaning that the chain relaxation was the controlling release mechanism. Release in natural soil was also gradual with a concomitant decrease of bacterial number associated with the beads. Survival and release kinetics differed between both bacterial species, indicating that physiological characteristics of the loaded bacteria influence the final behavior of the plausible biofertilizer. Given that the polymeric matrix loaded with one or more PGPB was capable of progressive releasing viable cells in sterile water

and non-sterile soil, it shows great biotechnological potential as a convenient and economical material for the formulation of controlled-release biofertilizers for crops.

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