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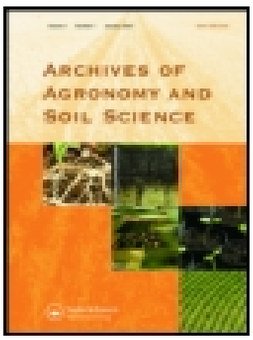

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Evaluation of chitosan and alginate immobilized *Methylobacterium oryzae* CBMB20 on tomato plant growth

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ABSTRACT

Methylobacterium oryzae CBMB20, a promising plant growth promoting bacteria (PGPB) and a biocontrol agent, was immobilized in different formulations such as wet chitosan, dry chitosan, wet alginate and dry alginate and were tested for tomato plant growth promotion. Chitosan solution (1.5%) with pH 5.5–6.0 and 90 min contact time was found optimal for immobilization. The chitosan formulations showed better entrapment efficiency and good degradability resistance apart from slow release of cells under prolonged incubation. Survivability of bacteria (80%) was observed in wet chitosan formulation even after 90 days of storage at 4°C. The spermosphere survival of bacteria was high in both dry and wet chitosan formulations applied soils even after 21 days under greenhouse conditions. While the alginate formulation degraded fully, partial degradation of chitosan formulation was observed even after 30 days, indicating its ability to support the survival of *M. oryzae* CBMB20 in soil. Plants inoculated with wet chitosan formulation registered 1.3 fold increase in the shoot and root length and dry weight compared to other treatments. Hence, chitosan formulation supporting better plant growth compared to alginate will be a better carrier for taking bacteria to the plant rhizosphere and thereby promote plant growth.

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Methylobacterium oryzae CBMB20; immobilization; spermosphere survival; entrapment efficiency; degradation

Introduction

Plant growth promoting bacteria (PGPB) are applied along with the seeds during sowing or in soil directly. This process requires a higher volume of inoculum for covering the entire area and allowing the bacteria to colonize the roots of the target plants. Inoculation of seeds is the most effective, economic and ideal way of taking the PGPB to the rhizosphere and soil (O'Callaghan 2016).

However, it becomes necessary to entrap and protect PGPB to sustain their population so that they remain for longer periods in the rhizosphere and agricultural soils. Formulations of different polymers have been recommended as a solution to protect PGPB inoculants against biotic and abiotic stresses, gradually release inoculants into the soil (Bashan et al. 2002; Liu et al. 2008; De-Bashan and Bashan 2010; Schoebitz et al. 2012) and help in storage and transportation of the

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PGPB to various areas (Bashan et al. 2014). Thus, a quality inoculant formulation containing an effective bacterial strain can imply the success of PGPB as inoculants in plants.

A good carrier material for PGPB inoculation is characterized by its mechanical stability, cost effectiveness, biodegradability, user and eco-friendliness, and availability (Liu et al. 2008). Chitosan is a natural, safe, inexpensive, biodegradable, polycationic biopolymer produced from chitin, the major constituent of arthropods exoskeleton and fungal cell walls, and the second renewable carbon source after lignocellulosic biomass (Kurita 2006). Chitosan was reported to be among the carrier materials for delivery of bioinoculants as plant growth promoter in agricultural fields (Chen et al. 2007; Costa et al. 2014; Joe et al. 2014; Dar et al. 2015). The porous nature of chitosan is attributed to support the growth and physiological activity of the immobilized microorganisms (Angelim et al. 2013). Additionally, it was reported to behave as a chelating agent for nutrients and minerals to make them available for uptake by plants (Ramírez et al. 2010), induce plant response to biotic and abiotic stress (Khan et al. 2003) and supports the growth of beneficial microorganisms (Yen and Mau 2007).

Microbial cell survival during and post-encapsulation is also governed by a number factors as reports indicate loss of cells up to 90% during the preparation and drying process (Schoebitz et al. 2012). Diffusion of hydrophilic molecules from the alginate matrix was correlated with various factors including growth media composition, strain and physiological state of cells, and process parameters used to prepare the formulations (Joe et al. 2012). Hence, optimization of each factor to ensure the best inoculant activity after drying and storage becomes necessary to obtain biodegradable and dried inoculants with a high living cell concentration (Schoebitz et al. 2012, 2013).

Methylobacterium oryzae CBMB20 isolated as an endophyte of rice is a promising plant growth promoting bacteria (PGPB) and a biocontrol agent with many beneficial traits including: a) nitrogen fixation; b) phosphate solubilization; c) siderophore production; d) phytohormone production; e) ACC deaminase production; and f) biological control of pathogens and diseases apart from being resistant to kanamycin (Madhaiyan et al. 2007; Joe et al. 2014; Yim et al. 2014). Hence, it is required to make proper formulations which can act as a proper bioinoculant and withstand various environmental stresses when applied in soil.

The objective of this study was to immobilize *M. oryzae* CBMB20 in chitosan and alginate under different conditions and evaluate the formulations for various properties including the strength, bacterial entrapment, survival, sustainable release of the bacteria over time, their degradation in soil, spermosphere colonization of the immobilized *M. orzyae* CBMB20, and their effect on tomato seed germination and growth under greenhouse conditions.

Materials and methods

Chitosan with high molecular weight and more than 75% degree of deacetylation was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO. USA). Alginate was purchased from Samchun Pure Chemical Co. (Sandan-ro, Gyoenggi-do, South Korea).

Preparation of chitosan-M. oryzae CBMB20 and alginate-M. oryzae CBMB20 formulations

Chitosan formulation was prepared following the procedure as described by Angelim et al. (2013). Actively growing cultures of *M. oryzae* CBMB20 (8.00 log Colony Forming Units (CFU) ml^{-1}) was mixed with different concentrations of chitosan solution (0.3–3.0% weight/volume (w/v) 3% gel prepared in 1% acetic acid) and subjected to agitation in a rotary shaker at 150 revolutions per minute (rpm) for 3 h at room temperature. Chitosan-biomass mixture (25 ml) was slowly dropped into 1% (w/v) tripolyphosphate (pH 9.0) by using 10 ml syringe to obtain uniform size beads. The beads were incubated in tripolyphosphate solution for hardening (3 h) at room temperature. After hardening, the beads were collected and washed in 0.15 M K_2HPO_4 (pH 8.0) solution three times. In parallel, alginate formulations were prepared by using 2% (w/v) alginate solution at the volume

ratio of 1:4 and incubated in a rotary shaker at 150 rpm for 60 min. 25 ml of alginate-biomass mixture was dropped into 0.1 M $CaCl_2$ using a 10 ml syringe to obtain alginate beads. The alginate beads in $CaCl_2$ solution were incubated for hardening (30 minutes) at room temperature. After hardening, the beads were collected and washed three times with saline solution (Bashan et al. 2002). Both the formulations were stored at 4°C until further use.

Characterization of chitosan and alginate formulations

The size of the bead formulations containing bacteria and polymer was measured and the mean values of 10 bead samples were recorded. The entrapment efficiency and the immobilized viable cells in the beads was measured by crushing 1 g of bead after preparation and immersing the same in 10 ml of 0.1 M acetic acid (pH 4.0) for chitosan and 0.1 M sodium citrate (pH 6.0) for alginate followed by stirring for 60 minutes at 100 rpm at room temperature. After that, they were serially diluted with 0.1% (w/v) peptone solution and spread on nutrient agar plates. The CFU were counted after 72 h of incubation at 30°C. The entrapment efficiency was calculated using the following formula:

$$\text{Entrapment efficiency}(\%) = \frac{\log \text{CFU of immobilized cells in beads}}{\log \text{CFU of total cells}} \times 100$$

To check the cell viability, both the formulations were stored at 4°C and 30°C for 3 months. The number of viable cells in the formulations was measured at different intervals of storage periods (0, 10, 30, 60 and 90 days).

To study the release of cells from the formulations, one gram of beads were kept in 10 ml of sterile saline solution and incubated at 30°C for 240 h. 100 µL of samples were taken at 0, 24, 144, 240 h and the number of viable cells in the solution was measured by serial dilution method (He et al. 2015). All the experiments were conducted in triplicate.

Both the chitosan and alginate formulations were analysed using scanning electron microscopy (SEM). The samples for SEM were prepared as mentioned by Bashan et al. (2002). A 5% volume/volume (v/v) glutaraldehyde solution was used to fix the beads for 5 h, and then washed several times with 1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer at pH 7.2. It was again fixed with 1% (w/v) osmium tetroxide (OsO_4). Subsequently, 1 M HEPES buffer was used to wash the beads several times and the dehydration was made in increasing concentration of ethanol at 4°C, which is as follow: 25% (v/v) for 10 min, 50% (v/v) for 30 min, 70% (v/v) for 10 h, and 100% (v/v) for 60 min. Finally, the beads were air-dried in a clean bench. The beads were mounted on a stub using double-sided tape and gold coated (20mM) in a sputter coater. The image was visualized and captured using scanning electron microscope (SEM) at voltage 3.0 kV (Hitachi S-2500C, Hitachi Co., Japan).

All data were normalized and subjected to analysis of variance (ANOVA). Significant differences among means were tested with least significant difference (LSD) test at $P < 0.05$ using SAS Version 9.1.3 service pack 4 (designed by SAS Institute Inc., Cary, North Carolina, USA).

Degradability of chitosan and alginate formulations in soil

Both chitosan and alginate formulations were evaluated for their degradability in soil (Bashan et al. 2002). Eight treatments (T1- wet chitosan + CBMB20; T2- wet chitosan; T3- dry chitosan + CBMB20; T4- dry chitosan; T5- wet alginate + CBMB20; T6- wet alginate; T7- dry alginate + CBMB20 and T8- dry alginate) were arranged in pots in a completely randomized design with three replicates in each treatment. 40 beads per treatment were placed in 5 × 5 cm mesh nylon bags (10–20 µm) and buried 5 cm below the soil surface in the greenhouse condition and incubated for 30 days. The soil in the pot was kept slightly below water saturation level by adding water as necessary. The bags

were pulled out from the soil every 3 days and the beads were examined under a stereoscopic microscope. The following indices were used to score the biodegradation of beads: 0 = beads not degraded; 1 = little degradation with small holes and deformations in the bead structure and 2 = completely degraded or beads were absent from the bag.

Spermosphere colonization of M. oryzae CBMB20

Five treatments (T1 – CBMB20 free cells; T2- wet chitosan formulation with CBMB20; T3- dry chitosan formulations with CBMB20; T4 – wet alginate formulations with CBMB20 and T5- dry alginate formulations with CBMB20) were arranged in a completely randomized design with three replicates and maintained under greenhouse condition. One gram of seeds mixed with equal amount of wet and dry forms of chitosan and alginate formulations as well as free cells were sown in pots containing 0.5 g autoclaved field soils. The soil bacterial population was determined before application i.e. at 0th day. Spermosphere colonization of *M. oryzae* CBMB20 was analyzed after 7, 14 and 21 days (Emmert et al. 1998). Rhizosphere soil (1 g) along with one seedling were taken and kept in 10 ml of phosphate buffer for 30 min at 150 rpm. The soil suspension was serially diluted and plated on nutrient agar with kanamycin and the colony forming units were scored.

Tomato seed germination

Tomato seeds (cv: YeoreumMujeokHeukchima; Mirae Seed Co.) were surface sterilized using 2% NaOCl for 30 seconds followed by 70% ethanol for 1 min and washed 3–4 times with sterile distilled water (Sauer and Burroughs 1986). Six treatments (T1 – control; T2 – wet chitosan formulation with CBMB20; T3 – dry chitosan with CBMB20; T4 – wet alginate with CBMB20; T5 – dry alginate CBMB20 and T6 – CBMB20 free cells) were arranged in three replicates. Both the formulations and free cells (equivalent to 5.30–5.47 log CFU g^{-1} seeds) were mixed with surface sterilized seeds and kept for 4 h (Madhaiyan et al. 2007). Ten seeds per treatment were placed in triplicates on moist sterilized filter paper (Whatman No.2) kept in Petridish and incubated for 6 days (144 h) at 28 °C with a cycle of 12 h of dark followed by 12 h of light (18 µmol m^{-2} s^{-2}) in a plant growth chamber (DS 54 GLP, DASOL Scientific Co., Ltd., South Korea). Seeds without any treatment served as control. The percentage of seed germination was checked at 24 h interval.

Inoculation of immobilized M. oryzae CBMB20 on tomato germination and plant growth

The effect of chitosan and alginate formulations on tomato plant growth was evaluated under greenhouse condition. Pots were filled with field soil (Chungbuk National University, South Korea). The chemical properties of the dry soil used are as follows: pH 6.12; 0.65 ds m^{-1} electrical conductivity (EC); 1.28g kg^{-1} of organic matter; 1.53 cmol (p+) cation exchange capacity (CEC); 0.03% total N; 31.34 mg kg^{-1} of available P_2O_5; 0.3 cmol $kg^{-1}$of K; 0.21 cmol kg^{-1} of Ca^{2+}; 0.44 cmol kg^{-1} of Mg and 0.11 cmol kg^{-1} of Na respectively. Ten treatments (T1- control; T2- wet chitosan with CBMB20; T3- wet chitosan; T4- wet alginate with CBMB20; T5- wet alginate; T6- dry chitosan with CBMB20; T7- dry chitosan; T8- dry alginate with CBMB20; T9- dry alginate and T10- free cells of CBMB20) were arranged in a completely randomized design with three replicates in each treatment. Tomato seeds (1 g) were mixed with each of the the formulations by keeping the level of inoculum equivalent to 5.47 log CFU g^{-1} and 5.30 log CFU g^{-1} of wet and dry formulation respectively alongwith 10 ml of 1% (w/v) carboxymethyl cellulose (CMC) solution (Kanjanamaneesathian et al. 2000). Seeds were sown in seedling trays and grown for 15 days. The same procedure was repeated with the seedlings at 15 days after sowing (DAS). The soils were placed in pots and were saturated with water before sowing. Water was added only when needed to keep the pots moist without exceeding the water holding capacity. Shoot length, root length and dry weight of root and shoots were measured at 30 DAS (Lichtenthaler and Buschmann 2001).

Statistical analysis

All data were normalized and subjected to analysis of variance (ANOVA). Significant differences among means were tested with least significance difference (LSD) test at $P < 0.05$ using SAS Version 9.1.3 service pack 4 (designed by SAS Institute Inc., Cary, North Carolina, USA) for all data in the experiments.

Results and discussion

The use of bacterial inoculants have contributed to increased agronomic efficacy by reducing production cost and use of chemical fertilizers (De Souza et al. 2015). Cell immobilization in a biodegradable polymer matrix is a crucial process for the proper functioning of formulations in field conditions (Bashan et al. 2014; Bashan 1998). Formulations based on alginate and chitosan were prepared using *M. oryzae* CBMB20, a pink pigmented facultative methylotroph and a promising bioinoculant (Madhaiyan et al. 2007; Yim et al. 2014), evaluated and tested for their efficiency on the growth of tomato plants.

Immobilization of M. oryzae CBMB20 on chitosan and alginate

Chitosan, a hydrophilic polysaccharide derived from chitin, is a well-known biopolymer studied in various fields including drug-delivery, bio-remediation, biocontrol and nanotechnology (Chen et al. 2012; Angelim et al. 2013).

Chitosan has been reported as being a potential broad-spectrum antimicrobial agent (Goy et al. 2009). Hence, the effect of chitosan on *M. oryzae* CBMB20 was studied by inoculating them in Ammonium Mineral Salts (AMS) medium containing chitosan for 72 hours. The viability of cells decreased with an increase in chitosan concentration. The relative survival of *M. oryzae* CBMB20 decreased when the medium was supplemented with 2% chitosan. There was no adverse impact observed on the growth and survival of *M. oryzae* CBMB20 up to 1.5% of chitosan. Hence, chitosan concentrations lesser than 1.5% was used in formulation studies (Figure 1(a)).

When the chitosan bead formulations were tested for stability and mechanical strength, an increase in the size was noticed with an increase in chitosan concentration from 0.3 to 1.0% (w/v), and the size stabilized (1.70 and 1.77 mm) at higher chitosan concentrations from 1.2% and 3.0% (w/v) (Figure 1(b)). While lower concentration resulted in very soft beads with low mechanical strength as also observed by Boyaval and Goulet 1988, higher concentrations above 2.0% resulted in hard beads hindering the chance of cell diffusion into the polymer (Figure 1). Higher concentrations of chitosan also reduced the diffusion of substrate, gas and metabolic substrates along with lower degradability (Mi et al. 2002; Chen et al. 2012).

The effect of chitosan concentration on entrapment efficiency has the trend similar to the effect on the size of chitosan concentration. The entrapment efficiency of chitosan formulations increased up to 80% in chitosan concentrations from 0.9 to 1.5% (w/v). Lower chitosan concentrations were not suitable to generate an adequate matrix to hold the microbial cells in the formulation. As the optimal concentrations of chitosan for immobilization was 1.5% (1.71 mm), the same was used for the further studies. Simultaneously, it was also observed that the entrapment efficiency of chitosan formulations was relatively constant over the pH range 3.0 – 7.0 with the entrapment efficiencies between 73.2 and 84.5% depending on the pH range of the chitosan solution with maximum entrapment efficiency at pH 5.5 and 6.0 (Figure 2(a)) which is in agreement with an earlier study on *Acetobacter* (Chen et al. 2012). This was attributed to the electrostatic interaction between the negatively charged bacterial cell wall and the positively charged amine group on the chitosan molecule dissolved in lower pH (Chatterjee et al. 2007). Lower pH of the chitosan solution was also reported to alter the physiological properties of microbial cells (Chen et al. 2012) which was

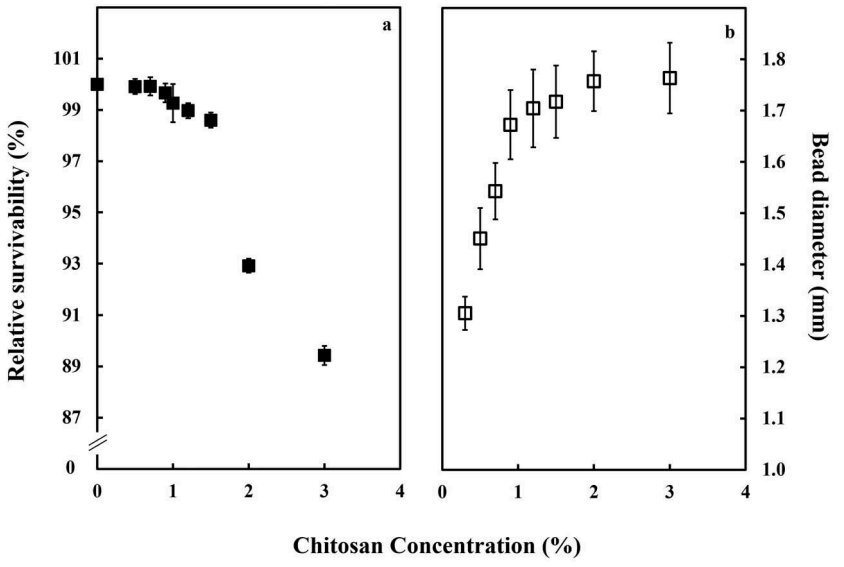

Figure 1. (a) Survival (%) of *M. oryzae* CBMB20 in different chitosan formulations (0.5 – 3%). (b) Diameter of different chitosan formulations (0.5 – 3%).

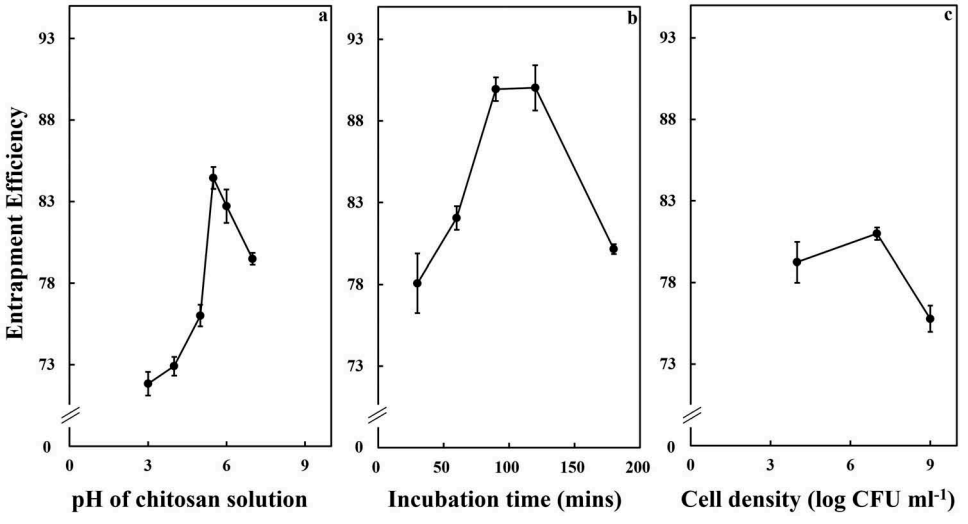

Figure 2. The entrapment efficiency of chitosan beads with varying (a) pH of the chitosan solution, (b) incubation time with *M. oryzae* CBMB20 cell culture and (c) density of *M. oryzae* CBMB20 cells.

evident in this study where acidic pH (pH 4.0–5.0) significantly decreased the number of bacterial cells and bacterial entrapments.

The entrapment efficiency reached the maximum when bacterial cells were kept for 90–120 minutes in optimized pH and chitosan solution improving the chances of bacterial cell binding and attachment (Figure 2(b)). Prolonged exposure to chitosan solution was reported to reduce the number of bacterial cells (Chen et al. 2012; Li et al. 2013). Among the different bacterial cell population studied (4–9 log CFU ml^{-1}), the entrapment efficiency was high between 4 and 7 log CFU ml^{-1} (79.25 and 80.90%) (Figure 2(c)). Cell leakage and weak mechanical strength of

formulations were reported when increased population of immobilized cells were used (Ha et al. 2008). About 10% entrapment efficiency was observed when stationary phase bacterial culture was used for formulation preparation. Based on all the observations, 1.5% chitosan concentration at pH 5.5 was optimum for immobilization of stationary phase (96 h of bacterial culture) *M. oryzae* CBMB20 at an initial loading rate of 7.00 log CFU ml^{-1} and a contact time of 90 min.

The alginate formulation was prepared using 2% (w/v) alginate solution and 60 min incubation with an initial cell density and growth phase of inoculum similar to that used in chitosan immobilization. When compared, the size of chitosan formulations were more compact and smaller (1.93 mm) than alginate formulations (2.75 mm), with higher entrapment efficiency.

Scanning Electron Microscopy analysis showed successful entrapment of the bacteria in both chitosan and alginate formulations (Figure 3(a,b)). While the outer layer of chitosan formulations appeared wrinkled and fractured (Figure 3(a)), the alginate formulations appeared smooth. Chitosan formulations have been reported to possess porous surface owing to its physical properties (Angelim et al. 2013; Costa et al. 2014).

Results on the survival of *M. oryzae* CBMB20 in both wet and dry chitosan and alginate formulations stored at two different temperatures (4°C and 30°C) were comparable (Figure 4). Survival of *M. oryzae* CBMB20 in both chitosan and alginate formulations stored at both the temperatures was greater than free suspension cells even after 90 days. As expected, the survival

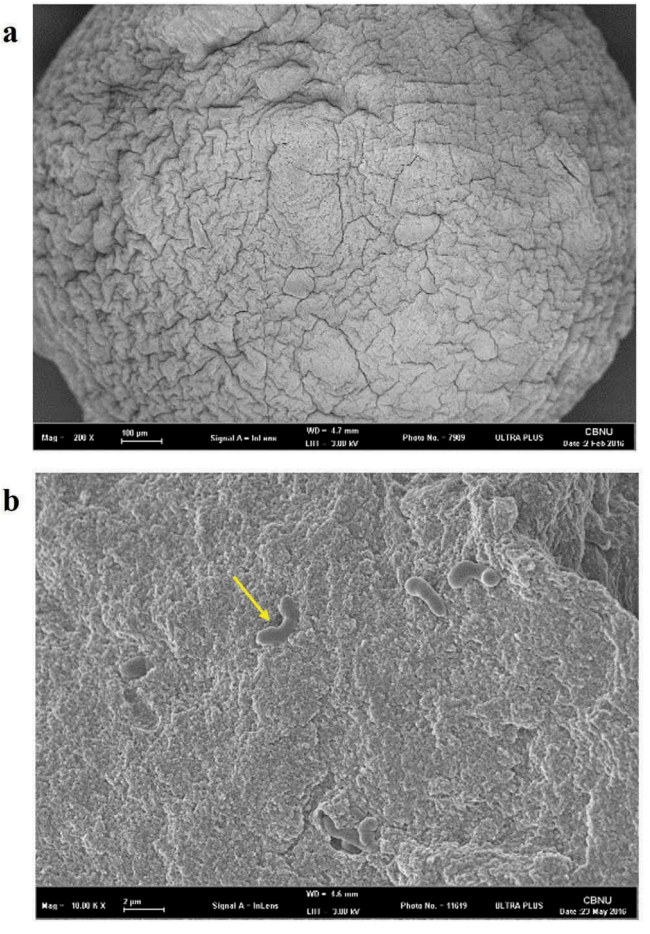

Figure 3. Scanning electron micrograph of chitosan beads immobilized with *M. oryzae* CBMB20 (a) surface of the beads and (b) bulk view which shows the immobilized cells of CBMB20, the yellow arrow shows one of the CBMB20 bacterial cells.

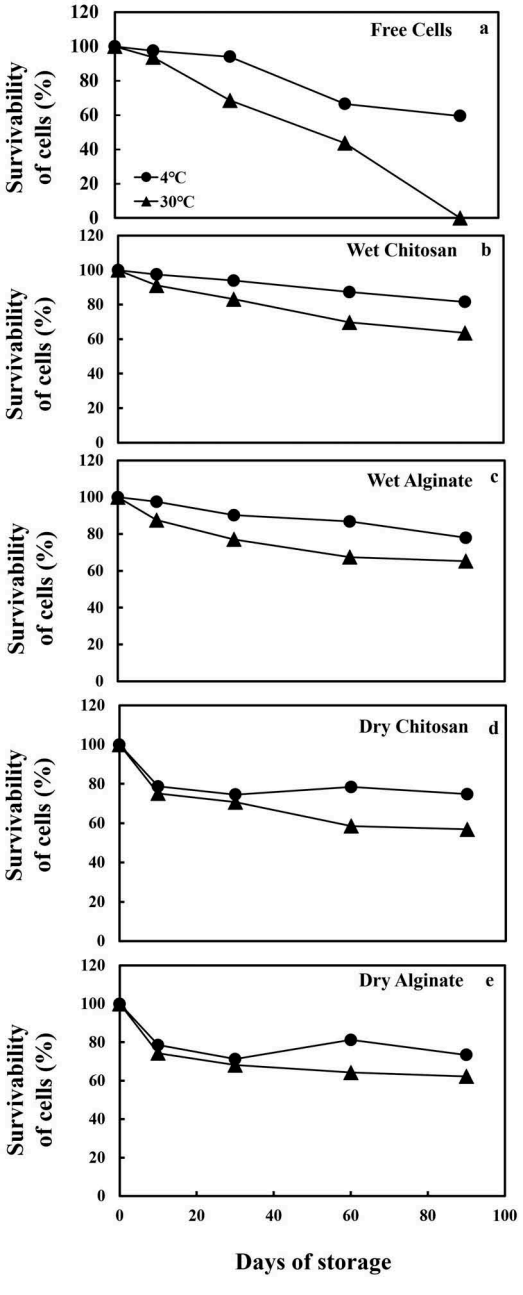

Figure 4. Survival of (a) free cells of CBMB20. (b) Wet chitosan immobilized CBMB20. (c) Wet alginate immobilized CBMB20. (d) Dry chitosan immobilized CBMB20. (e) Dry alginate immobilized CBMB20 subjected to prolonged storage at 4°C and 30°C.

of *M. oryzae* CBMB20 in the dry formulation of chitosan and alginate was lower than the wet formulation and free suspension cells because of the drying process. Nevertheless, the dry formulation supported the survival of immobilized cells even after 60 days of storage. Prolonged storage of free cells is reported as a viable option (Schoebitz et al. 2013). In this study also, the population decreased drastically from 68.6% to 43.6% during 30 and 60 days with cell viability getting lost completely at 90 days.

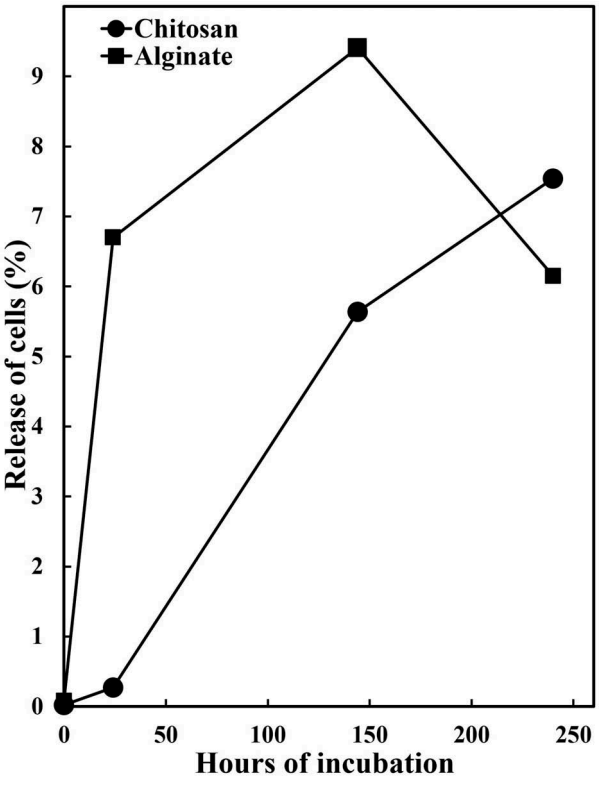

Figure 5. Release of *M. oryzae* CBMB20 cells in saline solution.

Formulations protect the living cells of inoculants from various environmental stresses and release bacterial cells into the soil in a gradual but prolonged manner (Bashan et al. 2002; De-Bashan and Bashan 2010; Schoebitz et al. 2012). Studies have shown that the entrapment efficiency and release behaviour of cells from chitosan-polyphosphate depended on various parameters including the chitosan concentration, mixing ratio, stirring speed, molecular weight and degree of deacetylation of the commercial chitosan production (Sinha et al. 2004; Wu et al. 2012; Yuan et al. 2018). In contrast, alginate a polymer prone to swelling easily gets affected by chelating agents (Sargus-Patino 2013). Even though both chitosan and alginate formulations were intact, their integrity was checked for release of immobilized bacteria in saline solution at 30°C. Both the formulation held microbes intact for 72 h in saline solution at 30°C. After 144 h the release of the microbes started from both the formulations (Figure 5). But the release in chitosan formulations was lesser (5.64%) than alginate formulations (9.41%). However, the release of cells was similar after 240 h of incubation in both the formulations. From the results it was evident that about 10% of immobilized *M. oryzae* CBMB20 could be released into aqueous environments within 300 hours of incubation. The strong mechanical strength, high molecular weight and degree of deacetylation of chitosan makes it an efficient carrier material for the sustainable release of bacteria over a longer period.

Degradability of chitosan and alginate formulations in soils

Degradability of the polymer is referred to weight loss of matrix during prolonged incubation times in nature and open conditions (He et al. 2015). Degradation of the formulated beads in

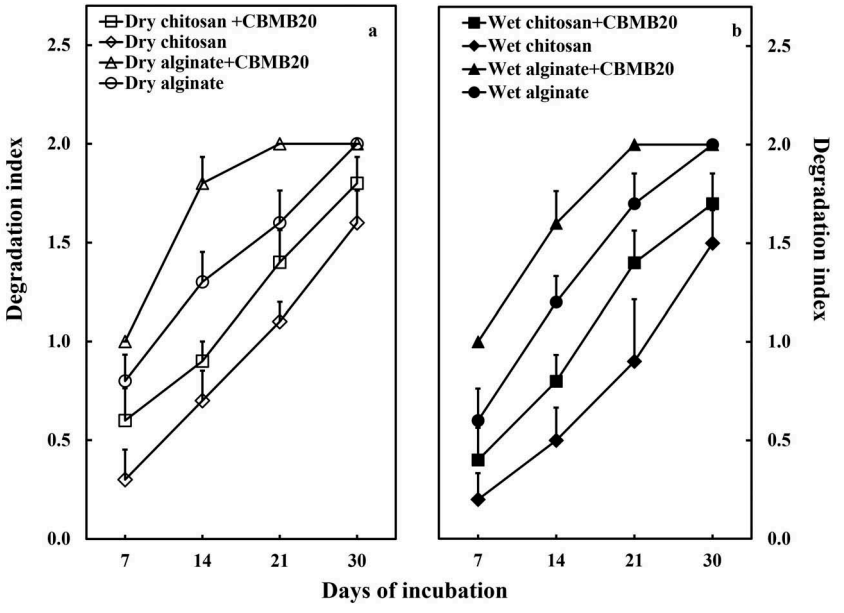

Figure 6. Degradability of chitosan and alginate formulations containing CBMB20.

soil was observed for 30 days in greenhouse conditions and recorded on a scale of 2.0, being the score for full degradation. While the degradation of both chitosan and alginate formulations were negligible on 7th day after the application, it was significantly greater on the 14th day in wet and dry alginate immobilized *M. oryzae* CBMB20 (Figure 6). Both the wet and dry alginate formulations were fully degraded within 30 days after inoculation. On the other hand, a slow degradation trend was observed in both wet and dry chitosan immobilized *M. oryzae* CBMB20, the property attributed to the recalcitrant nature of chitosan in pH>6.5 (George and Abraham 2006).

Spermosphere survival of M. oryzae CBMB20 inoculated through chitosan and alginate formulations

The spermosphere represents a short-lived, rapidly changing, and microbiologically dynamic zone of soil surrounding a germinating seed. It is analogous to the rhizosphere, being established largely by the carbon compounds released into the soil once the seed begins to hydrate (Nelson 2004). Understanding of bacterial survival in the spermosphere plays an important role to establish the plant-microbe interaction (Emmert et al. 1998). While the survival of *M. oryzae* CBMB20 in wet and dry chitosan formulations was 4.37 and 4.35 log CFU g^{-1} soil, the wet and dry alginate formulations recorded 3.69 and 3.79 log CFU g^{-1} soil in the tomato rhizosphere after 21 DAS (Figure 7). The population of free cells dramatically decreased from 6.19 log CFU g^{-1} soil on 7 DAS to 2.95 log CFU g^{-1} of soil on 21 with immobilization offering up to 1.5fold enhanced survival of the inoculated bacteria. While this could be attributed to the protective nature of carrier materials in maintaining the population, the possibility of bacteria growth inside the chitosan formulations during the disintegration of beads, resulting in the rapid adaptation of bacteria to the environment cannot be ruled out (Angelim et al. 2013).

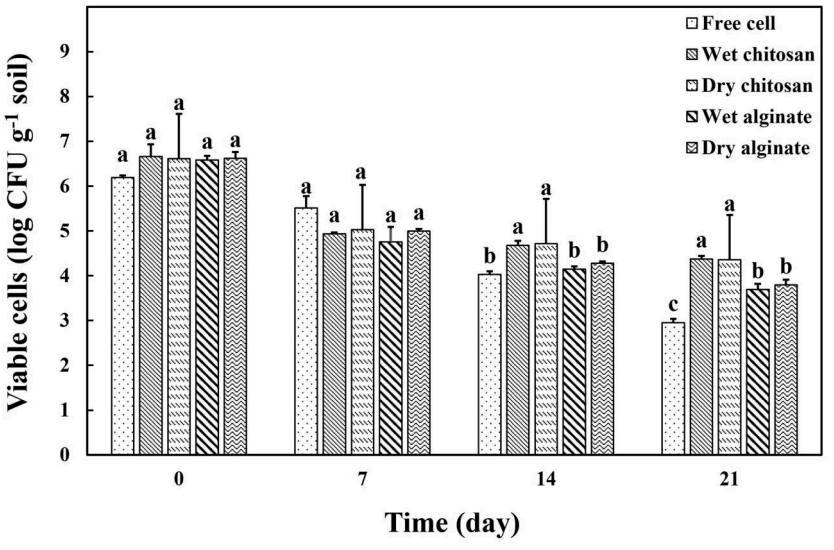

Figure 7. Spermosphere survival of chitosan and alginate immobilized CBMB20 cells.

Effect of chitosan and alginate formulations on tomato seed germination and plant growth

A number of studies have reported significant beneficial impact of immobilized plant growth promoting bacterial bioinoculants on plant growth and productivity under laboratory, greenhouse and field conditions (Raja et al. 2003; Young et al. 2006; El-Fattah et al. 2013). In this study, the wet chitosan formulation significantly enhanced seed germination (97.33%) when compared to control and free cell suspension inoculation (82.67% and 93.33%) respectively. This was also reflected in the increased shoot length, root length, and plant dry weight observed in seedlings treated with immobilized *M. oryzae*. The shoot length was: Wet chitosan + CBMB20 > Dry chitosan + CBMB20 > Wet alginate + CBMB20 > Wet chitosan > Dry alginate + CBMB20 > Dry chitosan > Wet alginate > Free cell > Dry alginate > Control. Whereas the root length differed little from the shoot length like Wet chitosan + CBMB20 > Dry chitosan + CBMB20 > Wet alginate + CBMB20 > Dry alginate + CBMB20 > Wet chitosan > Wet alginate > Free cell > Dry alginate > Dry chitosan > Control. In both cases, *M. oryzae* CBMB20 immobilized wet chitosan formulation followed by wet chitosan alone provided better results than other formulations. When the dry weight was analysed, the order of increase was different from the shoot and root length. The order was Wet chitosan + CBMB20 > Dry chitosan + CBMB20 > Wet alginate + CBMB20 > Dry alginate + CBMB20 > Wet chitosan > Free cell > Wet alginate > Control > Dry chitosan > Dry alginate. Interestingly, the control plants showed better dry weight than Dry chitosan and Dry alginate inoculated plants (Table 1). The chlorophyll a and carotenoid contents were also better in plants inoculated with wet chitosan immobilized with *M. oryzae* CBMB20 with free cells registering higher chlorophyll b content. Soil treatment with chitosan alone has also been shown to improve the growth parameters like plant height, fresh and dry plant weight and shoot and root area in beans (Sheikha and Malki 2011). The results observed in this study on the use of both chitosan alone and the formulation of chitosan with *M. oryzae* CBMB20 were encouraging and established them as plant growth promoters. This is the first report evaluating the efficiency of chitosan formulation containing *M. oryzae* CBMB20 on the growth of tomato plants under greenhouse condition apart from another solitary report on *Bacillus* sp. immobilized chitosan promoting the growth of pearl millet (Raja et al. 2003).

Table 1. Tomato plant growth parameters (shoot length, root length, shoot dry weight and root dry weight) after 30 days of chitosan and alginate immobilized CBMB20 inoculation.

	Plant growth (cm)			Plant dry weight (g plant^{-1})	
Treatments	Shoot length	Root length	Shoot	Root	Total dry weight
Control	43.23 ± 1.88^{f}	15.1 ± 1.55^{f}	6.6 ± 0.17^{d}	4.23 ± 0.11^{c}	10.83 ± 0.37^{e}
Wet chitosan + CBMB20	59.53 ± 0.15^{a}	31.46 ± 0.22^{a}	8.73 ± 0.06^{a}	6.49 ± 0.21^{a}	15.22 ± 0.17^{a}
Wet chitosan	53.18 ± 0.05^{cd}	24.69 ± 0.21^{d}	7.29 ± 0.03^{c}	5.3 ± 0.24^{b}	12.59 ± 0.12^{cd}
Wet alginate + CBMB20	55.6 ± 0.08^{bc}	28.98 ± 0.13^{b}	8.06 ± 0.09^{b}	6.26 ± 0.13^{a}	13.4 ± 0.34^{b}
Wet alginate	51.13 ± 0.63^{de}	23.36 ± 0.16^{d}	7.14 ± 0.16^{c}	4.87 ± 0.25^{bc}	12.01 ± 0.23^{d}
Dry chitosan + CBMB20	56.3 ± 0.10^{b}	29.42 ± 0.05^{b}	7.96 ± 0.37^{b}	5.33 ± 0.07^{b}	14.22 ± 0.26^{b}
Dry chitosan	51.5 ± 0.86^{de}	19.18 ± 0.18^{e}	6.46 ± 0.15^{d}	4.31 ± 0.26^{c}	10.77 ± 0.2^{e}
Dry alginate + CBMB20	53.13 ± 0.7^{cd}	26.95 ± 0.29^{c}	7.89 ± 0.19^{b}	4.74 ± 0.38b^{c}	12.63 ± 0.08^{cd}
Dry alginate	45.2 ± 0.85^{f}	19.24 ± 0.28^{e}	6.52 ± 0.19^{d}	4.01 ± 0.28^{c}	10.53 ± 0.54^{e}
Free cell	49.27 ± 0.55^{e}	23.3 ± 0.36^{d}	7.65 ± 0.08^{bc}	4.77 ± 0.37^{bc}	12.41 ± 0.20^{d}

Each value represents mean ± S.E (standard error) of three replications; letters show differences between treatments according to LSD test ($P \leq 0.05$).

Conclusions

This study demonstrates the preparation of chitosan and alginate based formulation of *M. oryzae* CBMB20. Chitosan solution (1.5%) and 90 min contact time was found optimal for immobilization of stationary phase (96 h) *M. oryzae* CBMB20 cells, with an initial loading rate of 7.00 log CFU ml^{-1}. Superiority of chitosan formulation was established through its entrapment efficiency, slow degradability and its spermosphere colonization. The chitosan immobilized *M. oryzae* CBMB20 promoted tomato seed germination and plant growth under greenhouse conditions. This formulation holds potential for further large scale application in agricultural fields.

Disclosure statement

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