Formulating bacterial endophyte: Pre-conditioning of cells and the encapsulation in amimated pectin beads

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ABSTRACT

Despite the benefits of bacterial endophytes, recent studies on the mostly Gram-negative bacteria lack of regard for formulation strategies. The encapsulation into biopolymeric materials such as amimated pectins hydrogels is a suitable alternative. Here, this research aimed at supporting the capability of the plant growth-promoting bacteria Kosakonia radicincitans DSM16656T to endophytically colonize plant seedlings. In this approach, the pre-conditioned cells through osmoadaptation and hydroxyectoine accumulation were used. In general, pre-osmoadapted and hydroxyectoine-supplemented bacteria cells formulated in amimated pectin dried beads increased the endophytic activity by 10-fold. Moreover, plant promotion in radish plants enhanced by 18.9% and 20.7% for a dry matter of tuber and leaves. Confocal microscopy studies with GFP-tagged bacteria revealed that bacterial aggregates formed during the activation of beads play an essential role in early colonization stages. This research encourages the integration of fermentation and formulation strategies in a bioprocess engineering approach for exploiting endophytic bacteria.

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1. Introduction

Plant growth-promoting endophytic bacteria (PGPB)-based formulations are considered a suitable alternative to overcome issues caused by chemical fertilizer usage [1]. These bacteria offer substantial advantages for their interaction with hosts [2,3], in comparison to native superficially occurring bacteria that persist mainly in soil and root plants neighborhood. Bacterial endophytes are isolated from surface-disinfested plant tissue or extracted from the plant endosphere [4,5]. Since bacterial endophytes can enter and persist into plant tissue, giving to the host physiological advantages against biotic and abiotic stresses, research attention has surged during the last years [2,6].

Regular application targets for PGPB such as soil or phyllosphere, represent harsh environments, where conditions can fluctuate significantly at nanometer and micrometer levels [7,8]. These alterations include microbial interaction, temperature, UV radiation, free moisture, pH, organic matter content and nutrients supply. Gram-negative bacteria are generally less tolerant facing disturbing surroundings than Gram-positive bacteria, yeasts or filamentous fungi. The lower sensitivity to environmental changes owing to mainly the chemical composition of outer cellular layers [9–11]. Thus, shortly after bacteria inoculation into the soil without a proper carrier, the population declines rapidly for most PGPB species [12]. For Gram-negative bacteria, the ability to withstand abiotic stresses during technical formulation processing is a central problem that requires research efforts. More challenging is to conserve the biological activity and further ability to colonize the plant endosphere.

The potential of PGPB has primarily been acknowledged and well documented across the globe in the last few decades [1,13]. Hence, plant growth stimulation and yield improvements by endophytic bacteria were evident in laboratory, greenhouse and field levels in several host species [14,15]. Some of these studies carried out even under drought stress, nitrogen deficiency and excessive salinity [16].

A well-studied PGPB is Kosakonia radicincitans (syn. Enterobacter radicincitans) [17], which endophytic preferences in plant tissue were demonstrated [18]. K. radicincitans can stimulate growth in a range of plant hosts [18–20], solubilize inorganic
phosphate [21], fix atmospheric nitrogen and to produce phytohormones such as auxins and cytokinins [22,23]. However, despite the reported benefits, most of the investigations regarding PGPB, including *K. radicincitans*, used fresh cultivated cells previously to reveal beneficial effects on plants, without formulation proceedings, which could guarantee a long shelf life of the product and further reproducibility [19,24,25].

Previous studies with promising bacteria in agriculture suggested that salt-stressed cells and compatible solutes inclusion could potentiate their biological activity. For *Staphylococcus saprophyticus* (STI), the biofilm formation and exopolysaccharides (EPS) production increased along with NaCl concentration, enhancing its plant growth-promoting abilities [26]. A similar strategy was extended to endophytic bacterial, which studies revealed that pre-conditioning at high salinities and the accumulation of selected compatible solutes could drive the strengthening of bacterial phenotypes [27]. Recently, *K. radicincitans* upon osmoadaptation and hydroxyectoine accumulation increased its phosphorous solubilizing ability, and interestingly plant tissue colonization [28]. Hydroxyectoine also provided benefits on drying survival and endogenous metabolome shifting [29]. As outlined above, salt stress pre-conditioning mechanism along with an intracellular compatible solutes accumulation is a feasible alternative to strengthen endophytic bacteria cells, previously encapsulation process and application.

Encapsulation/immobilization within hydrogels is a sizeable emerging field in the pharmaceutics, nanotechnology, medicine, aquaculture, and cosmetics industries [12]. The strategy focuses on increasing the microorganism tolerance against unfavorable surroundings caused by biotic and abiotic factors, such as antagonists or dryness [30–32]. Indeed, the immobilization of microorganisms in polymeric materials provides them with several significant advantages over free-living suspensions. Thereby, this approach may offer an uninterrupted supply of nutrients without competing with other microbes, protection against environmental stress and longer shelf life in storage [33–35].

Pectins are a diverse family of biopolymers extracted from the plant cell wall, with an anionic polysaccharide backbone of α-1,4-linked α-D-galacturonic acids. Uronic acids integrate into the backbone carboxyl groups arepatically substituted by methyl esters and/or carboxamidate groups. Pectins differ according to their degree of esterification (DE) and/or degree of amidation (DA). Both parameters represent the percentage of carboxyl groups esterified and/or amidated, respectively [36]. Due to differences in DE and DA, the viscosity, gelling, mechanical properties, and stability of pectin are affected [37,38]. Pectins operate widely as emulsifiers, gelling agents, glazing agents, stabilizers, and/or thickeners in food, pharmaceutical and personal care products [39].

Amidated pectins can serve as scaffolding drug delivery systems, owing to their versatility targeting specific sites and releasing rates [40–42]. Nevertheless, considering the entrapment of microbes, the amidated pectin-based hydrogels as a biologicals carrier remain relatively unexplored. However, some studies suggested the advantages of this biopolymer for entrapping microbial cells. Indeed, amidated pectins provided favorable characteristics for encapsulating live bacteria, such as *Lactobacillus casei* supporting protection to gastric acids [43,44]. This biopolymer is also cytocompatible with B-16 melanoma cells and human blood [45]. Krell et al. (2018) co-encapsulated the fungal endophyte *Metarhizium brunneum* CB15 and cellulase in amidated pectin beads, increasing the endophytic capacity in potato plants. For bacteria immobilization, the incorporation of calcium salts to pectin matrices can enhance polysaccharides reactivity to the bacterial enzymatic arsenal, since many pectinases require calcium ions or are stimulated by their presence [46,47]. Regardless of these benefits, a detailed investigation on amidated pectin as an advantageous carrier for PGPB is so far missing.

This research aimed at determining the capability of amidated pectin dried beads as a delivery system for the bacterium endophyte *K. radicincitans*. Moreover, the ability of encapsulated pre-osmoadapted and hydroxyectoine-added cells to colonize the endosphere in radish plants was assessed.

2. Materials and methods

Hydroxyectoine was acquired from Sigma Aldrich (Cat: 70709, Sigma Aldrich Corporation, Germany). Amidated pectin references were provided by Herbstreith & Fox KG (Neuenburg/Wuertt, Germany). Details are given in Table 1. All other materials corresponded to analytical reagent grade and were used as received.

2.1. Bacterial endophyte and growth conditions

Bacterial endophyte *Kosakonia radicincitans* DSM16656 [Ref: 6554: Leibnitz-Institut DSMZ] was provided by the Leibnitz Institute of Vegetable and Ornamental Crops in Grossbeeren, Germany. Bacterial cells were maintained on glycerol (50% w/v) and ENDO agar (Merck, Darmstadt, Germany) stocks at ~80 °C. Liquid starter cultures were produced in standard nutrient broth (Merck, Darmstadt, Germany) at 30 °C and 190 rpm for 24 h.

Chemically growth medium (DM) was used composed by (g L-1): glycerol (15), yeast extract (8), K2HPO4 (2.74), KH2PO4 (1.31), MgSO4.7H2O (0.5), FeSO4.H2O (0.06), MnSO4 (0.01) at pH 7.4. Pre-conditioning of bacteria before the formulation step was ensured by amending DM with NaCl [1, 4%] to obtain water activities (aw) at 0.96 and 0.95, respectively (LabMaster-a w, Novasina AG, Lachen, Switzerland) [29,48].

Bacterial suspensions for encapsulation procedures were prepared as follows: DM (100 ml) was poured into 250 ml baffled Erlenmeyer flasks that were autoclaved at 121 °C, 1.5 atm, for 30 min. The initial inoculum concentration in the media was adjusted at 10⁶ cells ml⁻¹. Cultivation was carried out at 190 rpm in a rotary incubator at 30 °C (IKA KS 4000 ic control, Staufen, Germany). Hydroxyectoine was sterilized separately by filtration through a 0.2 µm membrane filter (Durapore® 0.2 µm PVDF, Millipore, Ireland). Afterward, cells harvesting was conducted at the exponential phase after 21 h (OD600 0.7-0.9) by centrifugation at 2352 g for 15 min (Mikro HT 200R, Hettich GmbH & Co. KG, Tuttingen, Germany). To prevent osmotic imbalance, the obtained pellet of bacteria was washed and centrifuged twice with a corresponded NaCl solution [1, 4%]. The bacterial cells were stored in the same NaCl solution adjusted at OD600 at 1.5 until use in the formulation assays. Previously to encapsulate the bacteria cells, the intracellular uptake of hydroxyectoine was confirmed. Briefly, 50 µL of concentrated biomass was extracted for quantitative evaluations with 570 µL of an extraction solution (methanol/chloroform/water 10:4:4, v/v) by intense shaking for 5 min followed by the inclusion of equal volumes (170 µL) of chloroform and water [29].
The hydrophilic top layer was analyzed by HPLC, using an EC 150/4.6 NUCLEODUR® 100-5 NH2-RP column and a UV-detector at 215 nm. The chromatographic separation was performed at a flow rate of 1 ml min⁻¹ at 30 °C, accompanied by a column heater and using a solvent gradient established between eluents A and B (80% ACN in HPLC water) [29,49].

2.2. Screening of pectin materials references as a nutrient source

Several pectin references with a range of galacturonic acid contents, esterification and amidation degrees were selected (Table 1). The pectins were screened by their compatibility and potential for serving as a nutrient source for K. radiicincitans cells. Briefly, 1 ml of 2% pectin material supplemented with 1% yeast extract was placed in a microtiter plate (MTP) for conducting cultivation (RoboLector-BioLector system, m2p-labs, Baesweiler, Germany). The dissolved oxygen tension and GFP signal intensity as parameters for detecting bacterial growth activity were selected. All BioLector tests were monitored online with a pO₂-optode (filter DO [Pst3] Ex (nm) = 520; Em = 600) and GFP filter (filter GFP Gemini [Pst3], Gain = 5, Ex (nm) = 470; Em = 525). The experiments were performed at 30 °C under constant agitation (1200 rpm, shaking diameter = 3 mm, orbital) in 48-well MTP-48-BO flower-plates, Lot No: 1711 (mp2-labs, Baesweiler, Germany) with a working volume of 1000 µl DM. Each treatment was replicated three times.

2.3. Encapsulation

Calcium amidated pectin hydrogel beads as immobilization support were investigated. Briefly, an encapsulation suspension was obtained by mixing ALM pectin solution [4% w/v Amid CU-L 066/10 (DE 24% and DA 24%)] in ultrapure water (Elix Advantage Water Purification System, Merck Millipore, Darmstadt, Germany) at 50% w/w. The pectin solution was supplemented with 14% w/w maltodextrin, 1% w/w sorbitol and 1% w/w monosodium glutamate. Amyloglucosidase (Panzym® HT 300, Novozymes A/S, Bagsværd, Denmark) was used as amylolytic enzyme for maltodextrin degradation at 0.5 AGU g⁻¹ of the matrix [50]. K. radiicincitans cells suspension for the osmoadaptation and hydroxy-pectin treatments were added into the matrix to a final concentration of 15% w/w (~ 8 × 10⁵ viable cells ml⁻¹), and after gently stirred for 5 min. For bead formation, the suspension was dripped into a sterile calcium gluconate-cross-linking solution (0.1 M) by using a syringe with a cannula (diameter 2.1 x 0.8 mm, Sterican, B. Braun Melsungen AG, Melsungen, Germany) [51]. The gelled beads cured in the calcium gluconate-cross-linking solution for at least 10 min. Beads were separated by filtration and washed with the corresponding NaCl solution [1%, 4%] to remove residual calcium gluconate. Beads were dried to low water content in a two-step drying process [51]. Briefly, beads were put in an oven at 30 °C for 24 h, and later in a desiccator filled with silica gel for another 24 h at room temperature to reach aw < 0.3 (LabMASTER-aw at 25 °C, Novasina AG, Lagen, Switzerland). 1 ml of free-living cells at 1.0 × 10⁷ CFU ml⁻¹ was used as drying process control. Four replicates composed each treatment. Bead’s diameter before and after drying with a digital image analyzer was assessed (Digitimizer image, MedCalc Software, Ostend, Belgium).

2.4. Encapsulation efficiency

The encapsulation efficiency or survival after entrapping K. radiicincitans cells in amidated pectin beads was carried out as followed: 10 beads were disintegrated in a solution containing 0.03 M citric acid and 0.05 M sodium carbonate (pH 7 ± 2) for 1 h in a rotatory shaker at 150 rpm [52]. After complete dissolution, the entrapped viable bacteria were counted by diluted samples and plated on standard nutrient agar media (Merck, Darmstadt, Germany), and incubated at 30 °C for 24 hours. Bacterial cells encapsulation efficiency (BEE) was calculated (Eq. (1)).

\[
\text{BEE (\%)} = \frac{\log_{10} N_1 - \log_{10} N_0}{\log_{10} N_1} \times 100
\]

(1)

Where \(N\) is the number of viable entrapped bacterial cells and \(N_0\) displays the free viable bacterial cells before encapsulation [53].

Similarly, encapsulation efficiency for sorbitol as a chemical parameter was determined. Here, the amount of sorbitol in the remaining calcium gluconate cross-linking solution was quantified. Briefly, after 10 min of hardening time, beads were separated and 1 ml of calcium gluconate solution was recovered, centrifuged at 21130 x g for 5 min (Mikro HT 20H), Hettich GmbH & Co. KG, Tuttlingen, Germany), and filtered through a 0.45 µm membrane filter. The concentration of sorbitol by HPLC (EC 150/4.6 NUCLIDUR® 100-5 NH2-RP column, RI detector) was determined. The chromatographic separation was performed at a flow rate of 1 ml min⁻¹ at 30 °C, controlled with a column heater, and using a solvent gradient established between eluents A and B (80% acetonitrile in HPLC water). The peak areas were integrated and compared with calibration curves constructed with sorbitol [0.2-20 mg ml⁻¹]. The entrapment efficiency (EE) for sorbitol was calculated (Eq. (2)).

\[
\text{EE (\%)} = \frac{\text{Total sorbitol added} - \text{amount of sorbitol in cross linker solution} \times 100}{\text{Total sorbitol added}}
\]

(2)

2.5. Plant growth promotion in radish by osmoadapted and encapsulated K. radiicincitans cells

The efficacy of amidated pectin beads as formulation alternative for the endophyte K. radiicincitans under glasshouse conditions was assessed. Moreover, the extended cross-effect of preconditioning by osmoadaptation and the inclusion of hydroxypectone was also addressed. Radish (R. sativus L. var. sativus) seeds of cultivar Rondar (F1 Hybrid; S & G GmbH, Kleve, Germany) were used as plant systems. Ten radish seeds were placed per pot, with ten pots per treatment, filled with 1.5 L of a 1:1 (v/v) quartz-sand soil mixture (Fruhstorfer Erde type T25: P₂O₅: 200-300 mg L⁻¹, Hawita Gruppe GmbH Vechta, Germany). Afterward, pots were randomly placed on trivets to avoid the transfer of bacteria [19]. The viable cells concentration in dried beads was adjusted by considering the higher desiccation tolerance encountered previously in cells upon salt stress and hydroxypectone addition (Fig. 51) [29]. Thus, K. radiicincitans inoculation with osmoadapted as well as bacteria cells charged with hydroxypectone was conducted by locating two dried beads with the same log unit of cells concentration (~2.0 × 10⁶ CFU/bead) under every single seed. The treatments were beads with osmoadapted bacteria cells at 1% NaCl, 4% NaCl and 4% NaCl with hydroxypectone [1 mM]. Besides, amidated pectin dried beads without the endophyte as a control for the formulation components were established. Native seed without any treatment as the absolute control was used. Free-living cells as traditional endophyte seed application treatment was also tested [19]. Seedlings were irrigated and fertilized manually with Hoagland solution (50 ml per day) [54]. Plants under natural light conditions were maintained. Temperature and humidity were recorded over the growth period, with an average temperature of 18 ± 2 °C and with an air humidity > 45% [28].

Plant sampling was conducted one-week post-planting from three different locations per plot. The seedlings were rinsed thoroughly with sterile water for removing soil with beads adhering to the roots. Further, samples were flash frozen prior to the isolation of
nucleic acid procedures. At this plant age, the root length with a digital image analyzer (Digimizer image, MedCalc Software, Ostend, Belgium) was measured. The plants were equally thinned to five plants per pot, avoiding space limitation during growing and tuber maturation stage. Final sampling at five weeks post-planting was carried out. Five radish plants from the center of each pot were harvested. The total fresh mass of tuber and leaves material and the tuber diameter of each plant were measured. The leaves were separated from roots, oven-dried at 60 °C for 4 days until constant weight, and dry weight of tubers and leaves were determined. The whole experiment was repeated twice.

2.6. Nucleic acid extraction and quantification of K. radicincitans in planta using qPCR

Bacterial DNA was extracted from approx. 50 mg lyophilized plant material using DNeasy plant mini kits (Qiagen, Hilden GmbH, Germany) according to the manufacturer’s instructions. The lysis of bacterial cells was ensured through adding 5 mm sterile metal beads and using a Retsch MM200 mechanical disruptor (Haan, Germany) at 30 rpm for 5 min. The quality and purity of DNA were determined with a NanoDrop (Thermo Fischer Scientific, Darmstadt, Germany). Quantitative real-time PCR (qPCR) measurements were carried out using an Advanced TM Universal SYBR® Green I dye Supermix system (Bio-Rad Laboratories, Hercules, CA, USA). K. radicincitans species-specific primer and plant TEF reference gene for in planta bacterial quantification were used [55]. The fold colonization of K. radicincitans entrapped cells in treated plants concerning to the reference gene and to the control plants was calculated and represented with the 2 −ΔΔCT method [56].

2.7. Encapsulated bacteria and radish seedlings interaction: GFP-tagged bacteria approach

An in vitro study was conducted to visualize the endophytic mode of action and the chemotactic performance of encapsulated bacteria cells within radish seedlings. As described in Witzel et al. (2017), electrocompetent bacterial cells were transformed with plasmid pMP4655 [57]. Single colonies of eGFP mutants of K. radicincitans grown on Luria-Bertani agar plus gentamycin (150 µg ml−1) were inoculated in 100 ml standard nutrient broth, and the encapsulation of bacteria followed the procedure above.

Three radish seeds and three amidated pectin beads containing immobilized GFP-labelled K. radicincitans (~ 1.0 × 10^7 CFU per bead) were located in a Petri dish with 20 ml of agar media (1% w/v). After four days of incubation at 30 ± 1 °C, GFP tagged bacteria activity inside beads and their chemotactic interaction with radish seedlings was detected by multispectral and kinetic fluorescence imaging (PSI Open FluorCam FC 800-0, PSI, Brno, Czech Republic). The following parameters for capturing images were used: Reflectance mode: Blue light source (447 nm) at 5% intensity, bandpass filter (440/40 nm), shutter at 2 milliseconds and sensitivity at 0%; Fluorescence mode: GFP bandpass filter (517/20 nm): Blue excitation light (447 nm) at 100% intensity, shutter at 300 milliseconds and sensitivity at 38%. Further, root colonization by immobilized bacterial was recorded with a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss Jena GmbH). Bacterial eGFP fluorescence signals were captured using argon laser excitation at 488 nm (BP505-550 180 filter, Plan Apo 63/1.4 oil lens), and root images were taken using bright-field settings [55,58].

2.8. Statistical analysis

Data were analyzed using the SPSS Statistics v.22 software (SPSS, Chicago, IL), and are presented as mean values ± standard deviations (SD). Data were checked for normality and homogeneity of variance using the Shapiro-Wilk and Bartlett test, respectively. Means were tested for significant differences by one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. Data from glasshouse experiments were subjected to Duncan post-hoc test. In this study, the level of significance was set at p < 0.05.

3. Results

3.1. Hydroxyectoine uptake by K. radicincitans

The accumulation of hydroxyectoine during the cultivation at high salinities previously to encapsulation was assessed with HPLC. In response to high salinity during the exponential growth phase in DM at 4% NaCl, bacterial cells amassed this osmolyte after 21 h at 235.09 ± 17.56 (n = 3) µmol g−1 dry weight cells. Osmolyte content in K. radicincitans cells increased over time, >500 µmol per gram of dry biomass at 24 h. No hydroxyectoine was detectable in cells grown in DM in the absence of salt.

3.2. Screening of pectin materials references as a nutrient source

Since the early days during plant growth are considered crucial to provide an open window to the pathogen-endophytes entrance, a rapid bead activation through water uptake and internal bacterial proliferation are essential [1]. Then, to assess the affinity of selected pectin materials to serve as a nutrient source for encapsulation procedures, a high-throughput microfermentation study was applied. Dissolved oxygen tension and GFP signal intensity curves demonstrated that pectin references with a degree of amidation (DA) favored DOT activity and further bacterial growth (F3, 17 = 59.21; p < 0.05). Noticeable, DA in combination with a high galacturonic acid content in pectin, posses a significant effect on kinetic growth and oxygen consumption during K. radicincitans cultivation (F5, 17 = 30.45; p < 0.05, Fig. 1 A–B). Thus, GFP intensity curves confirmed that the pectin amid references AU-L-063/10, CU-L065/10 and CU-L-066/10, are assimilated by K. radicincitans, which included the combination of DA and galacturonic acid content (DA/GA) at 15%/83%, 11%/89% and 24%/91% respectively (Fig. 1B). Interestingly, CU-L-066/10 showed the shortest lag phase among the biopolymers tested with ~6 h in comparison to 9.3 h required for AU-L-063/10. Yeast extract alone at 1% cannot support growth under the evaluated conditions.

3.3. Encapsulation efficiency

The utility of a biopolymer hydrogel for providing a delivery system relies on the entrapment effectiveness of the active ingredient and additives [33,59]. Thus, to look into the efficiency of amidated pectin beads with CU-L-066/10 for encapsulating K. radicincitans cells and formulation additives, the concentration of these parameters before and after the entrapment was determined. The initial cell count of K. radicincitans before beads preparation was 9.46 ± 0.28 log CFU ml−1. The encapsulation efficiency for bacteria cells using amidated pectin beads was 98.37 ± 1.39% (n = 4). Regarding sorbitol encapsulation efficiency, HPLC showed that 48 ± 1.39% (n = 4) of this polyol remains into the amidated pectin beads after the cross-linking reaction and further drying procedure. Beads diameter before and after drying were 3.38 ± 0.31 mm and 2.26 ± 0.26 mm, respectively (n=8). Drying survival results are shown in Fig. S1. The endophyte was quite sensitive to the drying process, since no viable cells from the free-living treatment after the desiccation procedure were recovered.
3. K. radicincitans plant colonization

In general, K. radicincitans cells encapsulated in dried amidopectin beads can colonize plant tissue. Thereby, bacterial cells could leave the beads and settle into the eight days seedlings (Fig. 4). Thus, regarding relative K. radicincitans gene copy number response, dried amidopectin beads containing pre-conditioned bacteria cells with 4% NaCl, significantly enhanced the plant tissue colonization, in comparison to non-pre-conditioned immobilized cells at 1% NaCl (F2, 11 = 2460.71; p < 0.05, Fig. 3). Consistent with biomass production of radish plants, the plant colonization was stronger with intracellular hydroxyectoine in K. radicincitans cells osmoadapted at 4% NaCl, which the roughly 10-fold DNA copy number increment was significantly under the evaluated conditions (F2, 15 = 10477.33; p < 0.0001, Fig. 3). Free-living cells were capable of colonizing early plant tissue copiously in comparison to entrapped cells, with values ~100 fold higher than encapsulated treatments (Fig. S3), indicating the role of rapid bacteria activation for settling. The effects of salt pre-conditioning and hydroxyectoine inclusion in free-living cells on endophytic ability were previously discussed [28].

In vitro assessments suggested that the immobilization in amidopectin beads allowed the successful endophytic colonization, through first the root adhesion to the round shape beads and further, the establishments of root hairs and secondary roots within the bead matrix (Fig. 4). The bacterial aggregates formation within the beads (Fig. 5A–B) along plant interaction is quite interesting, since these aggregates ~10 μm were also observed in both colonization stages, forming biofilms after seed spraying in either roots hairs and secondary roots (Figs. 5C and S4). These advantageous sites for colonizing are facilitated by the junction, where lateral root emerges through the endodermis, the cortex and the epidermis (Fig. S4).

In Fig. 6, the mapping of chemotaxis of encapsulated K. radicincitans cells was evident, since GFP fluorescence images demonstrated the activation of beads, the localization of high bacterial density and the affinity of cells movement towards radish seedlings roots.

4. Discussion

Exploitation and manipulation of beneficial bacterial endophytes can be a sustainable alternative to cope with the demanding eco-friendly and productive agriculture. The formulation of bacterial endophytes can enhance the bio-prospection of this emerging low-input strategy. Formulation approaches for endophytic bacteria for applying as plant growth stimulators or biocontrol agents were established. These strategies include weetable powders [60], pellets [61], gel-based inoculants [62] and foliar sprays [63]. However, few studies have dealt with the entrapment or encapsulation of endophytic bacteria as proper application technology, using mainly the coating of seed by a bacteria-calcium alginate mix [14,64]. Herein, this research extends the knowledge of manipulated cultivations and further cells encapsulation by ionic gelation as an alternative to formulate bacterial endophytes. Thus, the effectiveness of amidopectin as a nutrient source and entrapment biopolymer for K. radicincitans cells was established.

Though the evident capability of K. radicincitans to promote radish growth, the results suggest that integrate cells osmoadaption during the cultivation and the uptake of advantageous compatible solutes such hydroxyectoine are beneficial to the endophytic activity. Noteworthy, both environment variations during growth may lead to greater phenotypic plasticity [65]. Thereby, previous studies discussed the beneficial effects on bacteria endophytes caused by salt stress and the uptake of...
Fig. 2. Growth promotion in glasshouse radish plants inoculated with amidated pectin dried beads containing pre-conditioning K. radicincitans cells by osmoadaptation at 1% NaCl, 4% NaCl and 4% NaCl + hydroxyectoine [1 mM]. A) Fresh mass of tuber and leaves. Fresh tuber mass (F[4, 39] = 1.47, P = 0.2321); leaves fresh mass (F[4, 39] = 3.15, P = 0.0259). B) Dry mass of tuber and leaves. Dry tuber mass (F[4, 39] = 9.66, P < 0.0001) and dry leaves mass (F[4, 39] = 7.74, P = 0.001). C) Tuber diameter after 5 weeks of planting (F[4, 39] = 7.64, P = 0.0002). D) Glasshouse-grown radish plants inoculated with pre-conditioned K. radicincitans cells encapsulated in amidated pectin dried beads. Beads control (without bacterium), beads with pre-conditioned bacterial cells in DM at 4% NaCl and DM at 4% NaCl with the addition of hydroxyectoine [1 mM]. Different letters represent significant differences according to post hoc Dunnett’s test at p < 0.05 (means ± SD, n = 8).

Fig. 3. Accumulation of K. radicincitans DNA in inoculated radish plants with amidated pectin dried beads. Effect of pre-conditioning of K. radicincitans in culture media by osmoadaptation at 1% NaCl, 4% NaCl and 4% NaCl + hydroxyectoine [1 mM], on relative gene expression in plant tissue. Different letters above bars indicate significant differences of treatments according to Tukey post hoc test at p < 0.05, (means ± SD, n = 4).

Fig. 4. Interaction of encapsulated bacteria cells in amidated pectin dried beads with radish seedlings (gnotobiotic system), phase-contrast microscopy approach (EVOS™ XL core, Life Technologies). K. radicincitans colonizing root hairs and secondary root surface 4 dpi. Scale bar: 5000 μm.
hydroxyectoine, including metabolic reordering and enhancements of phosphatases activity [28,29]. Hydroxyectoine as osmolyte provides significant osmotic-stress relieving features, superior drying tolerance due to high glass-forming temperature, protein stabilization and water-binding, among others [66–68]. Despite the relatively high cost of hydroxyectoine, biotechnological applications are growing, which are facilitating by the advent of high-yielded optimized production processes and the ectoine genome-modified strains usage [69,70].

En trapping bacterial cells in amidated pectin beads is a promising alternative to support plant colonization. Moreover, immobilized bacterial cells in pectin-based beads endure the drying process in comparison to fresh free-living cells, indicating an advantage for bioproduction development. Thus, encapsulated bacterial cells in dried amidated pectin beads maintain the capability to promote growth in radish plants. The yields are comparable to Berger et al. [2015], who reported increments in radish weight in tuber and leaves from 20 to roughly 50%, using fresh cultivated cells applying either seed-inoculated or two-leaf sprayed plants. Outstanding, the results of the present study were achieved by applying dried beads, since it is clear that encapsulation of cells and drying intrinsically depressed motility, which is highly implied with the plant interaction and colonization. In addition, dried beads may require a longer establishment to swell and uptake water for the surroundings, providing pores within the gel-matrix, facilitating the increment of internal $a_w$ for K. radicincitans cells multiplication [71]. The latter supported the higher radish yields compared to free-living cell treatment. The free-living cells may have a longer time than entrapped cells on seeds surrounding to take advantage of the essential early colonization sites, during seed sprout and seedlings developing. These results indicate that rapid colonization by bacterial cells plays a key role in the final plant growth performance. Embedded cells require an additional step for sensing root exudates through the polymeric matrix. They must first take up water from the soil to release the cells from the beads.

The osmoadaptation as a sub-lethal pre-conditioning procedure along with osmolytes addition could modify the identity and functioning of metabolites that bacteria cells produce. These alterations favor the competitiveness and the establishment in the rhizosphere before colonizing the plant endosphere [28,29]. Hence, osmotic unbalanced can modify mechanisms of quorum sensing (QS), quenching (QQ), or extracellular polymeric substances (EPS). The effects occur during the biofilm formation and further signaling steps for endophytic establishment [72–74]. Therefore, small groups of cells aggregates and biofilm formation, mediated through QS, provide advantages to capitalize on favorable environments or withstand stressful conditions [73].

The successful bacterial establishment could also involve indirect mechanisms provided by the formulation such the additives into the bead. Pectin is one of the major components in plant cell walls, and it might serve as an environmental factor in the stimulation of bacterial biofilm formation, during plant colonization, mimicking natural conditions and triggering the bacterial enzymatic arsenal [75,76].

This study demonstrated that amidated pectins with a high content of galacturonic acid could provide an advantageous nutrient source for endophytic bacteria, supporting the early establishment in the soil. Indeed, K. radicincitans can encode for pectinases secretion and utilizes D-galactose precursor of D-galacturonic acid as a sole carbon source [58,77]. Moreover, other studies in spruce provide evidence of D-galacturonic acid and D-sorbitol utilization as a trait for contributing to the endophytic lifestyle and proliferation in highly reducing microsites in plants [78,79]. Besides, amidated pectin beads have also successfully used as a carrier for delivering fungi endophyte in potato plants [32].

Regarding the other components in the bead, monosodium glutamate has been demonstrated as a nutrient source for plant bio-stimulation, enhancing soil microbial activity and soil respiration [80]. Maltodextrine- amyloglucosidase combination included within the beads may boost an additional C-source for bacteria proliferation. Considering that bead components may modify the nutrient niche of root surfaces and subsequent soil microbiome, they could influence the switch of K. radicincitans from bead-rhizosphere to endophytic lifestyles. This effect could alter radish carbon metabolism, including secondary metabolites such as glucosinolates and inducing priming of defense responses [81–83].

In line with biomass production of radish plants, entrapped cells colonization ability increased with hydroxyectoine inclusion in K. radicincitans cultivation, in which the roughly 10-fold specific DNA copy number increment was significantly under the glasshouse conditions. Similar results with free-living cells were found [28]. This finding indicates that plant colonization improved by synergistic effects of pre-conditioned cells by osmoadaptation and formulation performance. The hydroxyectoine amassing during cultivation may support the endophyte persistence in plant tissue, since this osmolyte may confer protection from osmotic stress during biofilms formation [84]. The best of our knowledge, the current study is the first to deal with the pre-conditioning of cells and pectin-based beads as a formulation alternative for supporting endophytic performance.

This research provides further details on the colonization patterns of GFP-labeled K. radicincitans in radish seedlings. Thus, bacteria cells entrapped in pectin beads can colonize radish seedlings and show an endophytic lifestyle by two main pathways. First, through root hairs during the first stage of root development. In contact with beads, root hairs establish mainly nearby the frontiers of growth out the bead and eventually into the bead by multiple adhesions entrance events. In these zones, K. radicincitans cells are predominant planktonic cells and forming aggregates thereafter. Secondly, bacteria cells are capable of colonizing the secondary roots, either the region of cell maturation (the basis) or the root cap (the tip) reaching the cortical tissue. This mechanism occurs during the second stage of root development. Surprisingly, secondary roots penetrate the bead matrix, and they can establish into it. K. radicincitans aggregates embedded into the bead, proliferate and migrated through the capsule material, colonizing the secondary root. Usually, since groups of cells appeared at depths > 100 μm, the location of bacterial aggregates was not restricted to the bead borders, where the oxygen concentration could be higher [85]. The bacterial aggregates distribution suggested the facultative capability that allows bacterium the anoxic growth and the entire exploitation of bead structure. This feature could favor the proliferation through the redox gradient between anoxic sites and the microaerobic parenchymatic tissue environment [86]. Altogether, pectin hydrogels could consider as an in-vivo-like biofilm system, diffusion-limited, wherein bacterial endophytes growth exhibits central features of in-vivo biofilms showed during plant colonization.

Pectin as a biopolymer for entrapping bacteria could trigger the enzymatic arsenal of the bacterium. The early barrier encountered of this polysaccharide may mimic natural conditions and activate the plant cell wall-degrading apparatus. These mechanisms include the secretion of endopolygalacturonase and pectin esterases to degrade the backbone of α-D-galacturonic acid, which are considering as physiological traits in endophytes [87]. Besides, other important enzymes that K. radicincitans DSM16656 could encode and facilitate plant colonization are glycoside hydrolases (>127 proteins), α-glucosidases, mannosidases, galactosidases and glucanases [58,76,79].

The root exudates that diffuse to the neighborhood of the bead could be the driving force for entrapped K. radicincitans cells to
Fig. 5. Confocal laser scanning micrographs showing the inner colonization of radish seedlings by encapsulated K. radicincitans cells expressing eGFP. A. Bacterial aggregates formation inside and at the edge of amidated pectin bead [Sliced 20 μm, CM 1800 microtome (Leica Instruments, Nussloch, Germany)]. B. Amidated pectin–encapsulated GFP-tagged K. radicincitans cells colonizing root hairs by forming aggregates 4 dpi. C. K. radicincitans colonizing root hairs and secondary root epidermis at 4 dpi. In all cases were visualized the formation of bacterial aggregates and biofilms in plant tissue. Scale bar: 50 μm.
swim towards seedlings, since the fluorescence images showed higher GFP intensities in the bead’s boundary pointed to the radish roots. Indeed, the induction of chemotactic response of bacterial endophytes to root exudates was reported [88] and is presented as the first step for colonizing the rhizoplane region [89]. Although all beads containing bacteria endophyte showed chemotactic activity, the images of pre-osmoadapted cells with hydroxyectoine suggested that these cells might have advantages for sensing...
the roots exudates. Questions remain regarding this hypothesis, to delve into the capability of osmotic stress to modify the flagella apparatus, the bacteriocin such kosakonic, the metabolite profile and the quorum sensing in bacteria endophytes [86,90,91]. Noticeably, with this study, the use of multispectral–kinetic fluorescence imaging emerges as an applicable methodology for targeting analysis of GFP-labelled bacterial endophytes and plant host interaction.

5. Conclusions

This study showed that physiological modifications by osmotic stress, the accumulation of compatible solutes during cultivation, and the entrapment of these pre-conditioned cells in ammaded pectin dried beads enclosed a feasible strategy to improve bacterial endophyte-host interactions. For the first time, a successful endophytic activity of K. radiincisitans cells encapsulated in ammaded pectin dried beads (DA = 24%; GA = 91%) was demonstrated. Besides, their capability to proliferate as aggregates, to migrate through the biopolymer matrix and to promote radial growth under glasshouse conditions was elucidated. The phenotypic plasticity of K. radiincisitans DSM16656® triggered by osmoadaptation and providing exogenously hydroxyoetine during cultivation persists in entrapped cells for increasing plant bio-stimulation and endophytic performance. These findings advance inoculent technology for plant growth-promoting bacterial endophytes.

Funding

This research was funded by the University of Applied Sciences in Bielefeld, Leibnitz Institute of Vegetable and Ornamental Crops, Grossebener, ABITEP GmbH in Germany; Universidad Nacional, AGROASÍA and Mincienias (formerly Coiciencias) (Grant 642) in Colombia.

Declaration of Competing Interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

CRediT authorship contribution statement

Mauricio Cruz Barrera: Conceptualization, Investigation, Writing - original draft. Desiree Jacobs-Schoenwandt: Conceptualization, Writing - review & editing, Funding acquisition, Writing - original draft. Martha Isabel Gómez: Conceptualization, Funding acquisition, Writing - original draft. Juan Serrato: Investigation, Writing - review & editing, Writing - original draft. Silke Ruppel: Writing - original draft. Anant V Patel: Writing - original draft.

Acknowledgements

The authors want to thank Birgit Wernitz for technical assistance during greenhouse and qPCR experiments at IGZ. In addition, Dr. Jan Gräfe for his support during the FluorCam imaging capturing and Dr. Philip Albers during the CLSM analysis.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.ijbte.2020.e00463.

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