Research paper

Parameters influencing adsorption of *Paraburkholderia phytofirmans* PsJN onto bentonite, silica and talc for microbial inoculants

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ABSTRACT

The aim of this study was to evaluate the mineral carriers bentonite, silica and talc as potential supports for immobilization of the plant growth promoting bacterium *Paraburkholderia phytofirmans* PsJN, and determine the factors influencing bacterial adsorption to provide stable and efficient microbial inoculants for use in the field. Results reveal that adsorption of PsJN depends on pH, the number of immobilized cells decreasing from pH 5.5 to 9. Zeta potential measurements indicated that the surface charge of the carrier had certain, but not major influence on bacteria immobilization. The amount of Mg^{2+} contained in the carrier was a key feature, determining the extent of immobilization of PsJN in buffer (talc > bentonite > silica). Moreover, we evaluated the hydrophobicity and its influence on adsorption of PsJN by measuring the contact angle and the number of adsorbed bacterial cells. Highest number of bacterial cells was found on talc, the most hydrophobic material of the three tested ones (bentonite: 3.8×10^9 CFU g^{-1}; silica: 3.0×10^9 CFU g^{-1}; talc: 1.4×10^{10} CFU g^{-1}). By contrast, similar immobilization capacity was observed on the three materials, when bacteria culturing and bacteria adsorption were performed in a single step. This might be related to the fact that during culturing biofilm is formed as a result of clonal growth of initially attached bacteria, rather than the recruitment of planktonic cells.
Altogether, the important factors for adsorption in buffer (pH 5.5) appeared to be mainly the electrostatic and hydrophobic interactions.

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1. Introduction

Microbial biofertilizers and biocontrol agents are promising alternatives to agrochemicals in sustainable agriculture; however the lack of effective formulations is a major limitation for their application in fields. To maximize the chances of inoculation success, the formulation of an inoculant should combine at least three fundamental and essential characteristics: supporting the growth of the intended microorganisms, providing viable microbial cells in good physiological condition for an acceptable period of time and deliver enough microorganisms at the time of inoculation to reach a threshold number of bacteria that is usually required to obtain a plant response. In addition, bacteria must survive in soil, compete with adapted microflora and withstand predation by soil microfauna (Bashan et al., 2014). In this regard, it is necessary to develop novel conveyance systems which provide suitable microenvironments and physical protection against harsh biological conditions to prevent rapid decline of introduced bacteria.

Recently, some advanced technologies have been developed for the effective storage, transportation and enhanced efficiency of formulations by encapsulating cells in biocompatible polymers like alginate and acacia gum. The principle of this technique lies in the entrapment of cells within a shell or capsule that protects, isolates and releases gradually the microorganism of interest, though many of the encapsulation technologies require special equipment, long preparation times and high production cost (John et al., 2011).

Alternatively, cell adsorption on solid carriers, mostly mineral particles, is applied to bring plant-growth promoting bacteria (PGPB) to the field. For example, Albareda et al. (2008) used perlite, attapulgite, sepiolite and amorphous silica for immobilization of *Sinhorhizobium fredii* and *Bradyrhizobium japonicum* achieving 10^9–10^{10} CFU g^{-1} and showed that those materials can be used as carriers for rhizobia. Especially perlite gave good results in terms of long survival and seed yield. Likewise, Jiang et al. (2007) reported the immobilization of *Pseudomonas putida*, a bioremediation and biocontrol agent, on montmorillonite, kaolinite and goethite yielding 10^{10} CFU g^{-1} (Albareda et al., 2008; Jiang et al., 2007). This method is simple, inexpensive and has minor influence on physiological activities (Li et al., 2014).

The process of immobilization involves the transport of cells from the bulk phase to the surface of the support, followed by adhesion of cells, and subsequent settlement at the support surface. The initial attachment in general can be evoked by either unspecific or specific interactions. The latter ones involve proteins that bind at the interacting surfaces. Among the non-covalent unspecific interactions such as

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electrostatic and van der Waals forces, hydrophobic interactions are considered the strongest ones in biological systems (Harimawan et al., 2011). Consequently, the physicochemical properties of cells and carriers, and the chemical nature of the environment are assumed to play a major role in immobilization processes. In a review on bacterial adhesion Hori and Matsumoto (2010) state that from those physicochemical interactions adhesion cannot be fully explained. Besides, adhesion is also mediated by the presence of cell lipopolysaccharides and appendages such as fibrils, fimbriae or flagella. Species, strains and even individual cells may differ in their surface properties and hence adhesion to surfaces as well (Busscher et al., 2008).

Our aim was to evaluate different minerals as potential supports for immobilization of PGPB and elucidate the impact of their physicochemical and morphological properties on the mechanism of bacteria adsorption. We took particular note of bentonite, silica and talc which have different chemical composition and completely different morphology. In brief, we studied in detail the process of bacteria immobilization and investigated whether the contact time, type of carrier, its surface charge and hydrophobicity influences bacteria adsorption. As model we used *Paraburkholderia phytofirmans* PsJN, which is among the best studied plant-growth promoting bacteria. It colonizes the rhizosphere and endosphere, and promotes growth, and enhances abiotic and biotic stress tolerance in a variety of crops and vegetables (Mitter and Petric, 2013).

2. Materials and methods

2.1. Bacteria strain and culture conditions

P. phytofirmans PsJN::*gfp*2x, a genetically modified variant of strain PsJN expressing the green fluorescent protein (GFP) and antibiotic resistance to kanamycin to ensure selective detection, was grown in liquid Luria-Bertani (LB) (Sambrook et al., 1989) containing kanamycin (25 mg/L). Bacteria were grown to stationary phase for 72 h as shake culture incubating at 200 rpm at 28 °C. Bacteria were harvested by centrifugation at 4500 rpm and 4 °C during 10 min and resuspended in sterile 0.9% NaCl or corresponding adsorption buffer.

2.2. Carrier characteristics

The inorganic carriers used in this study were two phylosilicates: bentonite (BentonitMED; Zeolith-Bentonit-Versand, Germany), talc (Luzenac MAS T5; Imerystalc, Germany); and a hydrophilic precipitated silica (Sipernat 22S; Evonik, Germany).

BentonitMED (particle size ca. 16 μm, $\rho = 2.6$ g cm^{-3}) is a powder consisting of over 95% montmorillonite (major elements: 58% SiO_2, 5.2% MgO and 17% Al_2O_3) which forms gel-like films. Luzenac MAS T5 is a chemically inert mineral containing 46.5% SiO_2, 30% MgO and 11% Al_2O_3 as major elements with a median particle size (d_{50}) of 11.0 μm and a density (ρ) of 2.8 g cm^{-3}. Sipernat 22S (particle size (d_{50}) = 13.5 μm, $\rho = 2.1$ g cm^{-3}) contains 98% SiO_2. BentonitMED and Luzenac MAS T5 (10% aqueous suspension) have alkaline pH of 9, while Sipernat 22S (5% aqueous suspension) has a pH of 6.5 (theoretical values). The pH value of carriers was measured with a Seven pH-meter (Mettler Toledo, Switzerland).

No additional treatments were applied to the carriers.

2.3. Adsorption of P. phytofirmans PsJN gfp::2x onto inorganic carriers

2.3.1. Adsorption buffers

P. phytofirmans PsJN::*gfp*2x was adsorbed onto bentonite, silica and talc as follows: 100 mL of bacteria suspension ($\approx 10^8$ CFU mL^{-1}) were added to 1 g of each carrier. The mixtures prepared in this way were incubated at 28 °C with shaking at 200 rpm for 1 h. The following adsorption buffers were used: pH 5.5 (12.15 g/L CH_3COONa, 0.64 g/L CH_3COOH), pH 6.5 (8.22 g/L K_2HPO_4, 8.45 g/L NaH_2PO_4) pH 7 (4.68 g/L

K_2HPO_4, 16.37 g/L NaH_2PO_4), pH 8 (0.64 g/L K_2HPO_4, 25.26 g/L NaH_2PO_4), pH 9 (7.4 g/L $Na_2B_4O_7$, 38 g/L H_3BO_3).

2.3.2. One-step protocol

P. phytofirmans PsJN was pre-grown on LB agar containing kanamycin (25 mg/L) for 48 h at 28 °C. The biomass from the plates was then suspended in sterile 0.9% NaCl solution; the concentration of the bacteria was approximately 10^8 viable bacterial cells per milliliter. 1 mL of cell suspension was inoculated in flasks containing 100 mL of LB broth (pH 5.5). An amount of 1.0 g of carrier was added to each flask. The flasks were incubated at 28 °C with stirring (200 rpm) during 72 h.

2.3.3. Washing

After incubation the pH value was measured with a Seven pH-meter (Mettler Toledo, Switzerland). At the end of each experiment the mineral particles were washed three times with 10 mL adsorption buffer or 0.9% NaCl with the aim to separate unattached bacteria from the fraction containing mineral powder and attached bacteria. Separation was accomplished by letting carrier sink to the bottom of the falcon tube (short centrifugation 1000 rpm – 5 min – 4 °C). Unabsorbed bacteria floating in the supernatant were discarded. The controls were treated accordingly except that they were not inoculated with bacteria.

2.4. Viable cell count

Number of immobilized PsJN cells was estimated as colony forming units (CFU) as reported by Hrenovic et al. (2008), with some modification. In brief, each carrier was placed into a tube containing 10 mL of sterile 0.9% NaCl, crushed with a sterile glass rod and dispersed by shaking (2500 rpm/10 min). Suspensions of immobilized bacteria prepared in this way were serially diluted (10^{-1}–10^{-5}). Volumes of 10 μL were spotted (drop method) onto LB agar containing 25 mg/L kanamycin. After incubation at 28 °C for 72 h bacterial colonies were counted and reported as CFU per gram dry carrier. At the same time, the CFU in the supernatant was assessed in order to determine the number of free cells per mL of inoculum. All the measurements were done in triplicate.

2.5. Scanning electron microscopy (SEM)

A continuous layer of carrier particles was fixed on double sided adhesive carbon discs (Leit tabs). Scanning electron microscopy (Hitachi TM-3030 tabletop microscope) was performed to confirm the immobilization of bacterial cells onto bentonite, silica and talc.

2.6. Zeta potential of carriers

For measurements of zeta potential 2 mg of carrier material were dispersed in 10 mL of 0.001 M adsorption buffer (pH 5.5, pH 6.6, pH 7, pH 8 and pH 9) by shaking. Zeta potentials were measured using the Zetasizer Nano ZS (Malvern Instruments, UK).

2.7. Contact angle measurements

Bacterial surfaces for measuring contact angle were prepared by dropping 10 μL of bacteria suspension on glass slides and drying at room temperature for 16 h. Carrier surfaces were prepared by collecting carrier particles on a double sided tape. Double sided tapes with a continuous layer of carrier were mounted on glass slides. Then the contact angle (θ) of a 4 μL drop of MiliQ water with the bacterial or carrier surface was measured with a goniometric eyepiece CAM 101 (KSV, Helsinki, Finland) and determined by the Attension Theta Software version 4.1.9.8 (Biolin Scientific, Stockholm, Sweden). Each reported contact angle is the mean of at least three independent measurements.

2.8. Atomic force microscopy (AFM)

A continuous layer of carrier particles was fixed on double sided adhesive carbon discs (Leit tabs). The AFM NanoWizard II® (JPK Instruments AG, Germany) was operated in the intermittent contact mode with an aluminum coated Point Probe Plus Silicon (PPCC-NHR) (Nanosensors, Switzerland) featuring a resonance frequency of 300 Hz. Height images were recorded at randomly selected sites on the samples, from which the root mean square roughness (rms) was calculated. Root mean squared roughness is the standard deviation of the distribution surface heights, a sensitive measure of deviations from the mean line.

2.9. Statistics

Statistical analyses were carried out using GraphPad PRISM version 5.00 for Windows (GraphPad Software, San Diego, California, USA). The numbers of bacterial CFU were logarithmically transformed beforehand to normalize distribution and to equalize variances of the measured parameters. The comparisons between samples were done using one-way analysis of variance (ANOVA) and post-hoc Bonferroni test for pair-wise comparisons. The comparisons between samples of immobilization in LB broth and immobilization in buffer (pH 5.5) were done using independent two samples two-tailed t-test (unequal variances). The correlation between variables was estimated by Pearson correlation analysis. Statistical decisions were made at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Characteristics of the selected carriers

The inorganic materials tested in this study, bentonite, silica and talc, were carefully chosen as potential carriers for PGPB for the following reasons: they are naturally occurring, chemically inert and harmless to plants and animals. Further, they are all of similar particle size (ranging from 11 to 16 μm), but different chemical composition, which renders them especially suitable for analysis of the various parameters without the need to consider potential effects resulting from different particles sizes. In addition, particle morphology is completely different ranging from spherical silica particles to flat-layered talc structures which allowed us to study the impact of particle morphology on bacterial adhesion including porosity, surface area and the ability to adsorb water. Figures of merit for the three tested mineral particles bentonite, silica and talc are summarized in Table 1. From the table it is obvious that porosity, ability to adsorb water, hydrophilicity and surface roughness are quite similar for both bentonite and silica, while at the same time completely different for talc.

Bentonite and talc are 2:1 phyllosilicates, whose layered structure are comprised of an octahedral alumina and magnesium hydroxide sheet, respectively, sandwiched between two tetrahedral silica sheets. In bentonite, the silicon ion and the aluminum ions often undergo isomorphic substitutions, which is the main cause of hydration and swelling of bentonite. In contrast, talc does not swell in water because of the absence of isomorphic substitutions and it is considered hydrophobic due to the presence of siloxane groups at the faces. Both, bentonite and talc are negatively charged. The permanent negative charges on the basal planes (caused by isomorphic substitutions) and the hydroxyl groups at broken edges contribute to the surface charge of bentonite. In talc, negative charges at the edges result from the rupture of the ionic/covalent bonds existing between the layers. Hydrophilic fumed silica consists of primary particles of SiO_2 which are linked together to larger agglomerates. These agglomerates form a highly porous sponge-like structure, which adsorbs liquid into the pores. The porous structure of the hydrophilic fumed silica allows absorbing three times their weight in liquid. The negative surface charge of silica is due to the presence of silanol groups.

The different morphology of the three tested carriers is shown in Fig. 1. Bentonite had tendency to aggregate and form larger particles (20 μm) as indicated with white squares in Fig. 1a. Silica particles were individually distributed and showed rather homogeneous particle size. Micrographs reveal that the particle size of talc was very heterogeneous ranging from particles as small as 0.4 μm–50 μm ($d_{50} = 11$ μm). AFM images corroborated with SEM microscopy (Fig. 2) and provided information about the different roughness and ultrastructure of the carrier surface. As a consequence of the different morphologies, surfaces present distinct roughness values. Bentonite and silica presented rough surface (bentonite, rms = 203 ± 45 nm; silica, rms = 231 ± 39 nm) and a highly porous structure with increased surface area. The surface of talc (rms = 87 ± 31 nm) was smooth with perfect basal cleavage. Methods of cell immobilization onto surfaces are based on physical and chemical adsorption. For physical adsorption processes the specific surface area (SSA) of a certain material (total surface area per unit of mass) is a critical property, it is determined by the particle size distribution and surface roughness. The SSA of bentonite is 400–600 $m^2\ g^{-1}$, silica features an SSA of 190 $m^2\ g^{-1}$ and talc of 6 $m^2\ g^{-1}$ (values according to the data sheet). The specific surface area of bentonite of 400–600 $m^2\ g^{-1}$ consists of the interlayer surface plus the external surface. In the case of silica there is no interlayer surface, while in the case of talc the measured surface is the external surface as the interlayer surface is not accessible. This indicates that SSA values might be only compared for talc and silica. Greater SSA means closer interaction between different molecules, therefore, we expected largest values of specific surface area to lead to highest yields of immobilized bacteria on the carrier surface. Furthermore, carriers presented different density values ($\rho_{silica} = 2.1$ g cm^{-3}, $\rho_{bentotnie} = 2.6$ g cm^{-3}, $\rho_{talc} = 2.8$ g cm^{-3}). Low particle densities could facilitate buoyancy of the particles in the

Table 1
Chemical and physical characteristics of bentonite, silica and talc.

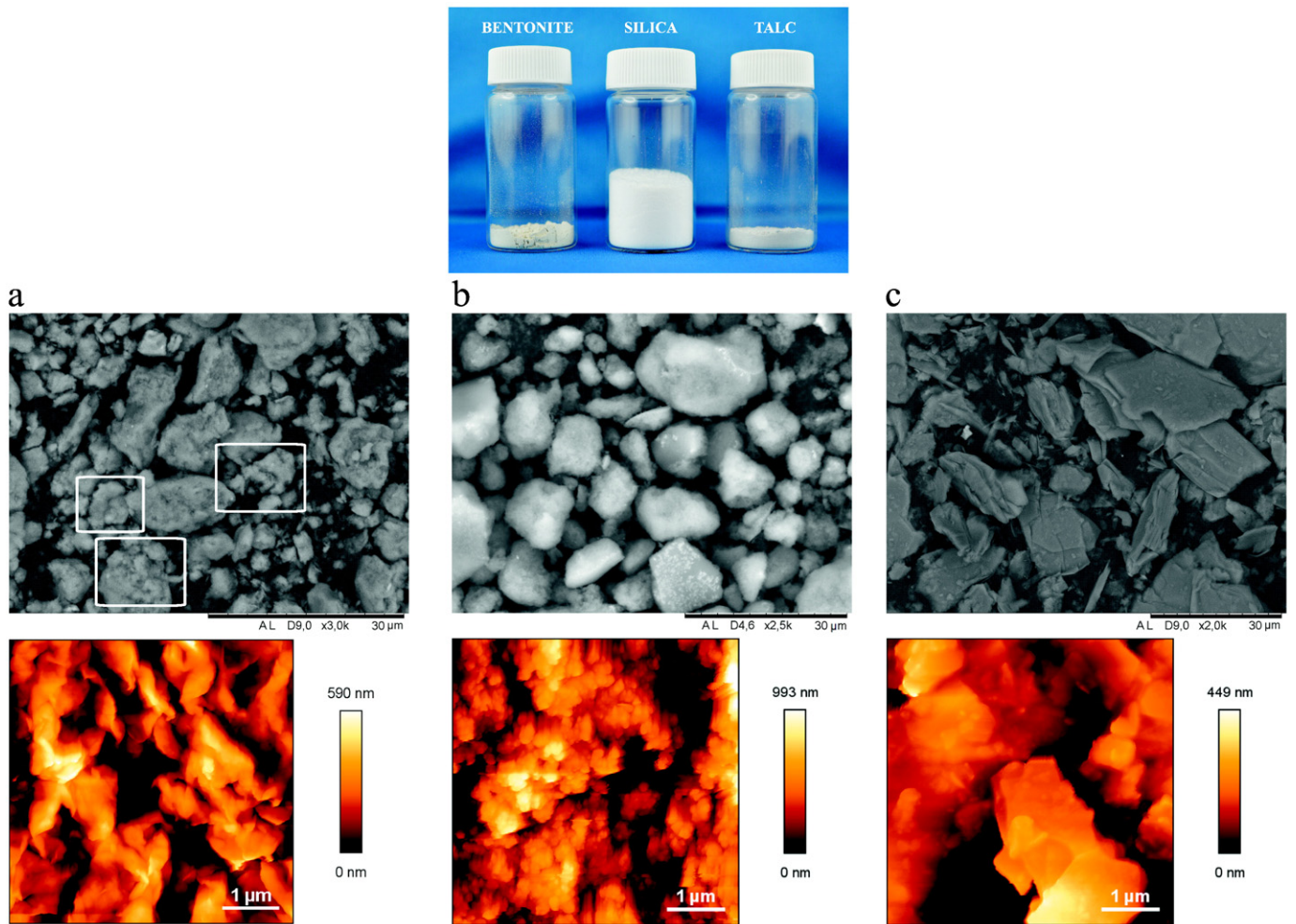|	Bentonite	Silica	Talc
Formula	$(Na, K)_4CaAl_6Si_{30}O_{72}\cdot24\ H_2O$	SiO_2	$Mg_3Si_4O_{10}(OH)_2$
Chemical composition (main components)	58% SiO_2 5.2% MgO 17% Al_2O_3 3.2% Fe_2O_3	98% SiO_2	46.5% SiO_2 30% MgO 11% Al_2O_3
Size (d_{50}) (μm)	16	13.5	11
Shape	Irregular spheres	Regular spheres	Layered plates
Porosity	Yes	Yes	No
Ability to adsorb water	Yes	Yes	No
Surface charge	Negative	Negative	Negative
Contact angle (°)	15.07 ± 4.69	$<10.00 \pm 0.00$	71.92 ± 10.43
Root mean square roughness (nm)	203 ± 45	231 ± 39	87 ± 31
Specific surface area ($m^2\ g^{-1}$)	400–600	190	6
Density (g cm^{-3})	2.6	2.1	2.8

Fig. 1. Photo images (on the top), SEM images (in the middle) and AFM images (at the bottom) showing a) bentonite, b) silica and c) talc. Bentonite aggregates are framed by the white squares.

bacterial suspension; assisting their dispersion and allowing the cells to establish more effective contact with the carrier surfaces. Interestingly, the SSA did not correspond with the amount of immobilized PsJN (Table 2), since talc displayed 10 times stronger ability to adsorb cells than silica despite about 30 times lower SSA. Bentonite and silica performed both in similar manner. Obviously, the specific surface area is only one of multiple factors contributing to bacterial adhesion on mineral particles. Furthermore, it is not known to what extent the SSA is

available to the bacteria, since their size is about 1–2 μm and much of the surface could be in pores of smaller diameter.

3.2. Immobilization of bacteria on inorganic carriers

The process of bacteria immobilization by adsorption onto mineral carriers is influenced by various factors, such as the chemical composition of the carriers and physicochemical interactions. However, it is
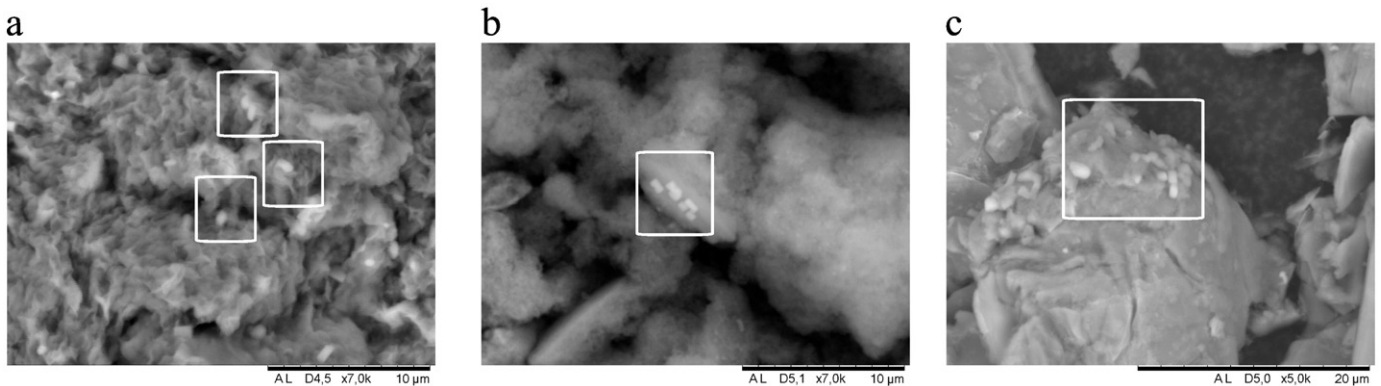

Fig. 2. SEM observation of mineral carriers after adsorption of *P. phytofirmans* PsJN::*gfp*2x for 1 h showing a) single bacterial cells settled onto the rough surface of bentonite, or lines/groups of bacteria adsorbed on b) silica matrix or c) talc outer surfaces. Cells of *P. phytofirmans* PsJN are framed by the white squares.

Table 2
Immobilization of *P. phytofirmans* PsJN onto bentonite, silica and talc after 1 h incubation under different pH conditions. Mean values of viable cells of three replicates and standard deviation are presented.

Parameter	Type of carrier	pH 5.5	pH 6.5	pH 7	pH 8	pH 9
Immobilized cells (log CFU g^{-1})	Bentonite	9.56 ± 0.15	8.17 ± 0.28	8.78 ± 0.12	7.97 ± 0.33	7.17 ± 0.04
	Silica	9.48 ± 0.04	8.44 ± 0.23	8.81 ± 0.25	7.59 ± 0.07	7.04 ± 0.15
	Talc	10.15 ± 0.03 A,B	9.57 ± 0.06 A,B	9.79 ± 0.08 A,B	8.77 ± 0.10 A,B	8.50 ± 0.07 A,B
Planktonic cells (log CFU mL^{-1})	Bentonite	8.73 ± 0.03	8.49 ± 0.05	8.56 ± 0.02	8.41 ± 0.03	8.41 ± 0.05
	Silica	8.72 ± 0.07	8.51 ± 0.11	8.59 ± 0.01	8.35 ± 0.06	8.48 ± 0.04
	Talc	8.22 ± 0.21 A,B	7.54 ± 0.30 A,B	7.46 ± 0.20 A,B	7.72 ± 0.04 A,B	8.18 ± 0.11 A,B
Zeta potential (mV)	Bentonite	−34.60 ± 0.59	−36.37 ± 0.94	−37.50 ± 0.25	−42.27 ± 0.90	−34.63 ± 0.40
	Silica	−31.13 ± 0.78	−32.07 ± 0.90	−37.23 ± 0.68	−43.50 ± 0.64	−37.10 ± 0.99
	Talc	−43.83 ± 0.75	−50.90 ± 1.50	−55.90 ± 2.08	−58.87 ± 1.58	−50.10 ± 0.57
pH after incubation	Bentonite	5.62 ± 0.03	6.71 ± 0.07	7.24 ± 0.08	8.28 ± 0.02	9.07 ± 0.03
	Silica	5.49 ± 0.01	6.98 ± 0.08	7.26 ± 0.03	8.06 ± 0.07	8.94 ± 0.01
	Talc	5.50 ± 0.03	6.74 ± 0.04	7.23 ± 0.01	8.28 ± 0.02	9.04 ± 0.02

Significant values (A): compared to bentonite.
Significant values (B): compared to silica.

not yet well understood which factors determine the efficiency of bacterial adsorption. Therefore, in order to elucidate which parameters govern the adhesion of PsJN on betonite, silica and talc we investigated the immobilization process in function of the contact time, pH, type of carrier, surface charge and hydrophobicity.

3.2.1. Immobilization of P. phytofirmans PsJN is pH dependent

Bentonite, silica and talc were incubated with bacterial suspension (10^8 CFU mL^{-1}) for 1 h. 10 times more bacteria were immobilized on talc than on bentonite and silica. Surprisingly, incubation times could be reduced to 15 min achieving the same immobilization capacity. Such fast deposition might be attributed to the fact that *P. phytofirmans* PsJN is motile by a single polar flagellum (Frommel et al., 1991), which is primarily used for swimming in liquid media. Our hypothesis is based on flagellar-mediated chemotaxis and motility, which might enable the planktonic cells in the suspension to move fast toward the mineral surface and overcome the repulsive forces existing between the cells and the mineral (Pratt and Kolter, 1998). Moreover, flagella are also important in swimming along the adsorption surfaces until an appropriate location for initial contact is found and enable attached bacteria to spread along the surface. In fact, swimming bacteria display a remarkable tendency to slip in close contact with surfaces once they reach them owing to hydrodynamic interactions (Bechinger et al., 2016). In addition, it has been also suggested that flagella could take part in physical adhesion to surfaces by overcoming possible repulsive forces existing between cells and surfaces (Garrett et al., 2008). Besides bacterial motility, initial transport of microorganisms toward a surface also occurs through Brownian motion, through sedimentation of the microorganisms in the aqueous environment, or through liquid flow. Thus, all these mechanisms together contribute to the fast deposition of *P. phytofirmans* on surfaces, with self-propulsion allowing for the more efficient explorations of the environment.

Adsorption of *P. phytofirmans* PsJN onto bentonite, silica and talc depended on the pH of the bacterial suspension, i.e. at lower pH values, more cells absorbed onto the carriers (Table 2, Fig. 3a). The highest
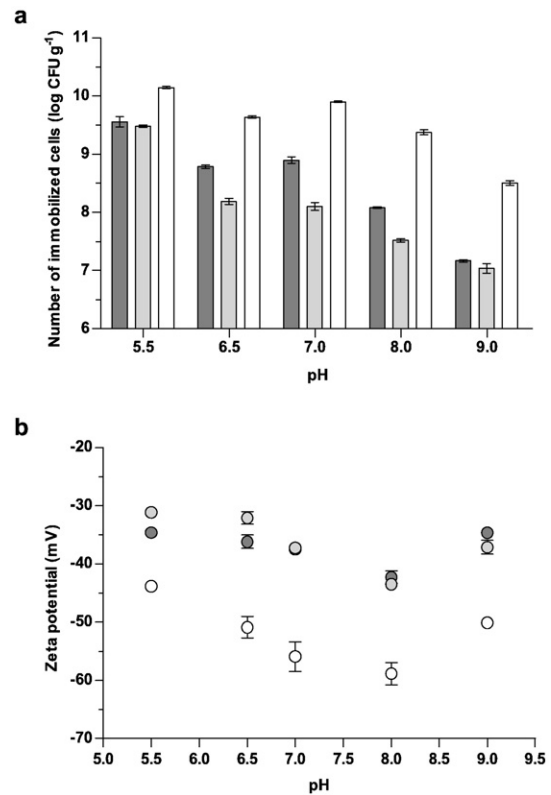

Fig. 3. Performance of *P. phytofirmans* PsJN onto bentonite (green), silica (red) and talc (blue): a) number of immobilized cells and b) zeta potential as function of pH value of the adsorption buffer. Mean values and standard deviations are presented.

number of immobilized cells was achieved when bacteria were incubated with bentonite, silica and talc at pH 5.5. Differences in the number of adsorbed bacteria were significant in the pH range of 5.5–9. The correlation between the number of immobilized bacteria and the pH of the adsorption buffer was negatively significant (r = −0.894 for bentonite, r = −0.964 for talc and r = −0.979 for silica), thus increasing the pH of the adsorption buffer led to a significant decrease in the number of immobilized bacteria.

In fact, zeta potential measurements revealed that the amount of cells adsorbed on the carriers depended on their surface charge, which is determined by the pH (Table 2, Fig. 3b). Bentonite, silica and talc are all negatively charged within the pH range of 5.5–9. Although the zeta potential of bentonite and silica was significantly more positive than the zeta potential of talc (i.e. at pH 5.5, −34.60 mV for bentonite, −31.13 mV for silica and −43.83 mV for talc), which might be attributed to a higher proton deficiency in talc than in bentonite or silica. At pH above neutrality bentonite and silica presented similar zeta potentials (i.e. at pH 7, −37.50 mV and −37.23 mV, respectively). Zeta potential values of all carriers were reduced at higher pH conditions showing changes in values of about 7–15 mV. However, the zeta potential became more positive (ca. 6–8 mV) when raising the pH up to 9 probably occasioned by the compression of the electrical double layer. Changes in pH do not only determine the surface charge as a result of ionization, but they also influence selective dissolution of the surface ions, which induce rearrangement of the electrical double layer, so shifting the zeta potential values (Uskoković, 2012). Under physiological conditions, bacterial cell walls are negatively charged due to functional groups such as carboxylates present in lipoproteins and lipopolysaccharides at the exterior surface of cell walls (Li et al., 2014). Thus, electrostatics interactions in adhesion on carriers are mostly repulsive.

The decrease of pH of the adsorption buffer induced an increase in the surface charge of the carriers, probably decreased the repulsive forces and enhanced the immobilization of bacteria by favoring the initial attachment of the cells. Similar observations were made by Jiang et al. (2007) and Kubota et al. (2008), who found that adsorption of bacterial cells onto zeolite was most efficient at acidic pH due to increased surface charge and thus reduced electric repulsion between bacteria and the carriers. However, when all, bentonite, silica and talc samples were considered, the decrease in charge density correlated negatively with the increase in the number of immobilized cells (r = −0.325, p = 0.053). For example, the zeta potential of bentonite and silica was significantly more positive than that of talc, but the immobilization rates were significantly lower. This might be due to other features controlling adsorption like the different Mg^{2+} content and hydrophobicity of the carriers. Namely, talc contains 30% of MgO, while bentonite contains 5.2% and silica does not contain any, suggesting that the Mg^{2+} can help to achieve high cell densities of immobilized cells. This observation is consistent with previous research, which showed that the immobilization rate increased remarkably when increasing the concentration of Mg^{2+} in zeolites or clay carriers (Hrenovic et al., 2005; Jiang et al., 2007). The promotive effect of cations on bacteria adsorption could be explained by the fact that cations tend to suppress the formation of diffuse electrical double layers around particles, leading to increased contact between bacteria and mineral surfaces (Gordon and Millero, 1984; Hermansson, 1999; Pethica et al., 1980; Zita and Hermansson, 1994). This indicates that the zeta potential might have some influence on the bacterial immobilization, but the primary factor is the type of material, which was already suggested by Hrenovic et al. (2009) in a study on immobilization of *Actinobacter junii* on clinoptilolite and bentonite. Rao et al. (1993) also suggested that electrostatic interactions are not the primary factors, but surface heterogeneities (defined by surface roughness, non-uniform charge distribution and size distribution). Similar deduction was obtained in a study on immobilization of *A. junii* on surfactant-modified zeolites (Hrenovic et al., 2008).

3.2.2. Immobilization is dependent on surface hydrophobicity

The repulsive electrostatic interactions between negatively charged solid surfaces and bacteria in the process of bacteria adsorption have to be overcome by attractive van der Waals, hydrophobic and specific interactions (van Merode et al., 2006). In order to investigate the effect of hydrophobic interactions on immobilization of PsJN on bentonite, talc and silica we determined the contact angle of water with the carrier surface as a measure for hydrophobicity and correlated this parameter with the number of immobilized cells.

All carriers showed positive affinity to water and were considered hydrophilic, since the contact angles were <90° (Förch et al., 2009). Increasing contact angle means decrease in affinity to water, thus talc resulted to be significantly more hydrophobic ($\theta = 71.92 \pm 10.43°$) than bentonite ($\theta = 15.07 \pm 4.69°$) and silica ($\theta = <10.00°$). The relationship between roughness and wettability was defined already in 1936 by Wenzel (1936) who stated that adding surface roughness will enhance the wettability caused by the chemistry of the surface. This was indeed observed in the present study, as bentonite (rms = 203 ± 45 nm) and silica (rms = 231 ± 39 nm) were significantly rougher and therefore more hydrophilic than talc (rms = 87 ± 31 nm).

The number of immobilized bacteria showed a significantly positive correlation (r = 0.999) with the contact angle of water and the carrier surface and significantly negative correlation with carrier surface roughness (r = −0.994). These results indicate that one of the most important factors for adsorption is the hydrophobic interactions as it was previously suggested by Kubota et al. (2008). Therefore, hydrophobicity might be a good parameter to predict adsorption of bacterial species.

In 2014, Liu et al. (2004) evidenced that when both bacteria and support are hydrophobic, microbial adsorption is highly facilitated. In contrast, if both bacteria and support are hydrophilic microbial adhesion would proceed with difficulty. For hydrophobic groups on the microbial cell walls to interact with the surfaces, adsorbed water must be displaced. This process, which allows for hydrophobic bond formation, involves unfolds of bacterial surface structures or rotation of the bacteria to face the surface with their most favorable site (Busscher et al., 2010).

P. phytofirmans PsJN::*gfp*2x showed a water contact angle of 39.23 ± 3.33° and therefore it was considered hydrophilic (Daffonchio et al., 1995; van Loosdrecht et al., 1987). Thus, when PsJN interacts with highly hydrated hydrophilic surfaces water removal is energetically unfavorable and may be difficult to overcome by counteracting attractive interactions. Hence, the diminished number of adsorbed cells on bentonite and silica, which are highly hydrophilic and adsorb huge quantities of water. The increased hydrophobicity of talc enhanced the possibility of PsJN binding to the surface, as removal of interfacial water between the interacting surfaces was more favorable.

Additionally, it has been described that flagella play an important role in surface adhesion, beyond their role in cell motility (Friedlander, 2014). On hydrophilic surfaces, flagella adhere loosely and the tethered filaments continue to vibrate and avoid additional attachment after adsorption of an initial layer. On hydrophobic surfaces however, flagella are tightly bound onto the surface after initial attachment, by increasing the van-der Waals attractions between flagellar proteins and the surface. As flagella become more and more tightly bound onto the surface, the area which they occupy is decreased allowing additional cells to gain access and adsorb to the surface. Hence, the higher number of attached bacteria on talc than on bentonite and silica.

3.2.3. Immobilization of P. phytofirmans PsJN during growth in LB broth

Once the composition of the liquid formulation is established, the experiments should be directed to pilot and full scale studies. To check the feasibility of immobilization at industrial level and simplify the whole adsorption process, we performed bacteria culturing and bacteria adsorption in a single step testing adsorption of *P. phytofirmans* PsJN in LB broth compared to adsorption in acetate buffer.

Interestingly and in contrast to results previously obtained in adsorption buffer (pH 5.5) (bentonite: 3.8×10^9; silica: 3.0×10^9; talc: 1.4×10^{10}) the highest number of immobilized cells in LB broth was achieved on silica, though the difference is small (bentonite: 1.1×10^9; silica: 4.7×10^9; talc: 1×10^9) (Table 3), especially when considering that analysis by drop test allows for determining the order of magnitude of viable cells rather than exact numbers. Apparently, the efficiency of bacteria adsorption is influenced by the immobilization protocol. Similar immobilization capacity on silica, bentonite and talc in medium might be related to the fact that during culturing biofilm is formed as a result of clonal growth of initially attached bacteria, rather than the recruitment of planktonic cells. In that case surface characteristics of the carrier particles play a minor role in the adsorption process.

The initial pH of the broth (pH 5.5) was considerably increased after incubation with the carriers (ca. 2 pH units) likewise the pH in the positive control reactor containing solely the pure culture of *P. phytofirmans* PsJN (6.60 ± 0.02 pH units). The pH values in the negative control reactors (non-inoculated with bacteria) remained constant during incubation. This indicates that pH was increasing due to the metabolic activity of bacteria.

Immobilization of bacteria in the culture medium could simplify the immobilization procedure and in addition, nutrients and secondary metabolites might be added to the broth to increase product shelf-life. However, the risk of contamination, i.e. the propagation of other microorganisms present in the pores or on the surface of the carriers during the immobilization process is higher in culture medium than in buffer due to the presence of readily available nutrients at optimal growth conditions. In our particular case, non-inoculated controls showed few contaminations ($<10^3$ CFU g^{-1}), probably due to the presence of contaminants (e.g. yeast) on the particles that were not eliminated during the sterilization process (steam sterilization). No contaminations were found in the reactors containing solely the pure culture of *P. phytofirmans* PsJN, which evidenced that in fact some residual contaminants could survive on the carrier surfaces even after the application of steam sterilization. When immobilization was performed during bacterial growth, the carriers were incubated in a nutritionally rich medium for an extended period of time (72 h), giving rise to parallel growth of undesirable microorganisms. Unlike, when immobilization was performed in buffer, the suspension was devoid of nutrients and carrier-bacteria interaction was only allowed for a 1 h, impeding proliferation of contaminants.

Contaminations with other bacteria would negatively affect the quality of the formulation, its stability during storage, and the survival of microorganisms in the final product since microbial contaminations decrease the shelf life of the inoculants (Bashan, 1998). Moreover, many countries, including Australia, the Netherlands, Rwanda, Thailand and Russia (Marufu et al., 1995; Smith, 1992), legislate requirements for minimum populations of target organisms and contaminants per unit weight of product. For instance, in Australia contaminants must number less than 10^7 CFU per gram of peat while in France there must to be no contamination throughout storage (Catroux, 1991; Lupwayi et al., 2000). Contaminant-free inoculants can be achieved using nuclear radiation for sterilization of carriers (e.g. γ-radiation). However, this kind of sterilization processes implies elevated cost and time, and skilled personnel. In consequence, performance in culture broth was discarded as a feasible practice for immobilizing PsJN on carriers unless suitable sterilization methods, whereas immobilization in buffer was considered a promising approach to bring PsJN to the field.

4. Conclusions

In adsorption buffer talc is the most appropriate mineral carrier of PsJN among the tested ones, most probably because of its higher content in Mg^{2+} and greater hydrophobicity. Both help to overcome repulsive forces between PsJN and the mineral surface. Our experimental results show that adsorption of PsJN depends on the pH and consequently on the surface charge of the carrier. However, zeta potential measurements reveal that surface charge has certain, but not major influence on immobilization. Other parameters influencing adsorption are SSA and surface roughness. Altogether, results of our studies indicate that properties of bacterial cells (hydrophilicity) and mineral surfaces (charge, hydrophobicity, shape, porosity, Mg^{2+} content) play important roles in their interaction. Owing to contaminations, immobilization of PsJN at time of culturing is not recommended. On the other hand, immobilization in buffer was considered a promising approach to provide stable mineral-based inoculants for use in the field.

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Table 3

Comparison of *P. phytofirmans* PsJN immobilized onto bentonite, silica and talc in LB broth and acetate buffer (pH 5.5). Mean values of three replicates and standard deviation are presented.

Parameter	Type of carrier	LB broth	Buffer
Immobilized cells (log CFU g^{-1})	Bentonite	9.00 ± 0.16^C	9.56 ± 0.15
	Silica	9.67 ± 0.07^C	9.48 ± 0.04^A
	Talc	$8.86 \pm 0.44^{B,C}$	$10.15 \pm 0.03^{A,B}$
Planktonic cells (log CFU mL^{-1})	Bentonite	9.58 ± 0.07^C	8.73 ± 0.03
	Silica	9.46 ± 0.03^C	8.87 ± 0.01
	Talc	9.14 ± 0.46	8.22 ± 0.21
pH after incubation	Bentonite	7.14 ± 0.64^C	5.62 ± 0.03
	Silica	7.71 ± 0.07^C	5.49 ± 0.01
	Talc	7.63 ± 0.02^C	$5.50 \pm 0.03^{A,B}$

Significant values (A): compared to bentonite.
Significant values (B): compared to silica.
Significant values (C): compared to buffer, pH 5.5.

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