Research Article

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Sclerotium oryzae biocontrol in flooded rice fields with floating microcarrier technology: The effect of chitosan molecular weight

Morelli N Matias,[a,b*] Sponton Osvaldo,[c,d] Leonardi J Rodrigo,[a,b] Santiago G Liliana[c,d] and Heinrich M Josue[b]

Abstract

BACKGROUND: Biocontrol strategies are of significant concern for their application in crops. Various green practices have been designed, but almost all of them had delivery constraints. In particular, to design biocontrol strategies against *Sclerotium oryzae* in flooded rice fields, the active agent should be retained on the plant leaves by spreading application, nevertheless the direct application onto the water produces the biocontrol agent dilution. An effective delivery model was needed. This work aimed to evaluate the effects of chitosan molecular weight on the formation of positively charged *Pseudomonas fluorescens*–chitosan complex as a floating microcarrier against *Sclerotium oryzae*. To this end, three different sizes of chitosan [molecular weights (MWs) 20 000, 250 000, and 1 250 000 g mol^{-1}] at different pH values (4, 6, and 7) were tested. The electrostatic interaction was analyzed through ζ-potential measurement. An adjustment of the experimental values was carried out for making predictions. The bacteria antifungal activity into the carrier with different chitosan MWs was analyzed.

RESULTS: Our results suggest that it is possible to form a bacteria–chitosan complex with a net positive charge under condition that improve bacteria incorporation to the microcarrier technology without harming bacteria viability and antifungal activity. Thus, high chitosan MW (1 250 000 g mol^{-1}) at pH 6 is preferable for microcarrier technology.

CONCLUSION: Our findings provide relevant information about bacteria–chitosan interaction and may be useful in biocontrol programs that involved these two components as well as situations in which bacteria adsorption to an anionic carrier or anionic surface is desirable.

Supporting information may be found in the online version of this article.

Keywords: ζ-potential; chitosan; *Pseudomonas fluorescens*; molecular weight; carrier

1 INTRODUCTION

Chitin, found in the shell of crustaceans, the cuticles of insects, and the cell walls of fungi, is the second most abundant biopolymer in nature.[1] Chitosan is derived from partial *N*-deacetylation of chitin.[2] The structure of chitosan corresponds to series of copolymers of $\beta(1\rightarrow4)$-linked glucosamine and *N*-acetyl-glucosamine. Its protonation constant (pK_a) value is around 6.5, thus chitosan has a high positive charge at acidic pH values.[3] Most chitosans are insoluble in water but become soluble in solutions with pH below their pK_a.[4,5]

At low pH, the majority of the amino groups, at the C-2 position of the glucosamine unit, will be protonated. In addition, it most likely that this cationic charge density enables reaction with the anionic surface of the lipopolysaccharide (LPS) leaflet of Gram-negative bacteria, as described for other polycationic agents and with anionic peptidoglycans in Gram-positive bacteria.[6] Also, Kovačević *et al*. demonstrated that the extent of adhered bacteria mostly depends on the type of terminating polyelectrolyte layer. In polyelectrolyte multilayers terminating with a positively charged layer, bacterial adhesion was more pronounced than was the case when the polyelectrolyte layer was negatively charged.[7] Chitosan deacetylation and pH determine chitosan charge density and therefore determine chitosan interactions

* *Correspondence to: MN Matias, Instituto de Desarrollo Tecnológico para la Industria Química, Consejo Nacional de Investigaciones Científicas y Técnicas – Universidad Nacional del Litoral, S3000 Santa Fe, Argentina. E-mail: matias.n.morelli@gmail.com*

a *Instituto de Desarrollo Tecnológico para la Industria Química, Consejo Nacional de Investigaciones Científicas y Técnicas – Universidad Nacional del Litoral, Santa Fe, Argentina*

b *Grupo de Innovación en Ingeniería de Bioprocesos – Facultad de Bioquímica y Ciencias Biológicas – Universidad Nacional del Litoral, Ciudad Universitaria (Paraje El Pozo), Santa Fe, Argentina*

c *Área de Biocoloides y Nanotecnología, Facultad de Ingeniería Química – Universidad Nacional del Litoral, Santa Fe, Argentina*

d *Área de Biocoloides y Nanotecnología, Instituto de Tecnología de Alimentos, Facultad de Ingeniería Química de la Universidad Nacional del Litoral, Santa Fe, Argentina*

with bacteria surfaces (the extent to which chitosan is positively charged).[8] Chitosan molecular weight (MW) is another variable that may influence the degree of interactions.[9] Chitosan pK_a shows a slight decrease from 6.51 to 6.39 when the molecular weight changes from 1 370 000 to 60 000 g mol^{-1}.[10]

Natural surfaces are negatively charged at ambient pH values and the electrostatic interaction with bacteria surfaces is, as a rule, repulsive. Especially at low ionic strength, say ≤0.001 M, long-range DLVO-type electrostatic repulsion dominates over van der Waals attraction.[11]The formation of a complex between bacteria and positively charged chitosan could therefore have important implications in bacteria interactions with natural surfaces.[12–16]

Despite this potential application, chitosan has been reported as an antibacterial agent. Factors including chitosan MW, chitosan DD, and individual microbial structural characteristics have been reported to influence the antibacterial activity of chitosan. In general, the antibacterial activity of chitosan increases with its DD and positive charge.[17–20] Acidic pH conditions and higher temperature are also favorable for chitosan antibacterial activity.[21-24] However, the effect of chitosan MW on antibacterial activity is still unclear and some contradictory results have been obtained.[25–32] For *Pseudomonas fluorescens* strains, several studies have been focused on the determination of chitosan minimum inhibitory concentration (CS-MIC) for different conditions.[31,33–35]

In our previous research, chitosan was proposed to improve *Pseudomonas fluorescens* adsorption onto a floating anionic microcarrier where the target is the air–water interface, as in the case of the treatment of rice stem root caused by *Sclerotium oryzae*.[36] With this application technology, a rising number of bacteria was achieved at the superficial couch where the fungal infection occurs. Despite the good results, further knowledge about the interaction between the bacteria and the chitosan is necessary.

Considering this previous work, the goal of this research was to study the impact of three chitosan (MWs 20 000, 2 500 000, and 1 250 000 g mol^{-1}) at different pH values (4, 6, and 7) on the formation of a *P. fluorescens*–chitosan complex with a net positive charge, which improves bacteria incorporation in microcarrier technology, without harming bacteria viability and antifungal activity.

1.1 Theoretical aspects

1.1.1 ζ potential

The electrostatic charge of small particles, such as cells, cannot be ascertained directly. It can be determined indirectly through ζ potential measurement.[37] The ζ potential is the difference in electric potential between that at the slipping plane and that of the bulk liquid. The slipping plane is the abstract plane in the vicinity of the liquid/solid interface where liquid starts to slide relative to the surface under influence of shear stress.[38] When a layer of macromolecules is adsorbed on the particle's surface, it shifts the slipping plane further from the surface and alters the ζ potential. The ζ potential change can be followed by electrokinetic methods. The electrokinetic method most frequently employed to measure the ζ potential change is particle electrophoresis.[37] Under an electric field, the particle velocity is a function of the particle surface charge, any adsorbed layer at the interface, nature, and the composition of the surrounding suspension medium. This technique can be used as a method to evaluate polymer–particle adsorption and has been used by many researchers.[39]

1.1.2 Polymer–particle adsorption

When an anionic particle (cells) and polycationic polymer (chitosan) are mixed, the polymer can be adsorbed, through electrostatic interactions, in different target points on the particle surface and can take different configurations. After polymer adsorption, polymer chains are extended and start to settle on the particle surface until the equilibrium configuration is achieved. This phenomenon is called reconfiguration. Extended polymer chains, before the reconfiguration phenomena, occupy less particle surface than chains that have already taken the equilibrium configuration. The adsorption and reconfiguration kinetics determine the total polymer chains adsorbed in a particle.

Equations (1)–(3) are used to describe the adsorption phenomenon. Polymer adsorption time is related to the fraction of total polymer adsorbed (f), the concentration of particles (n_1, n_2), and the rate adsorption coefficient (k_{ads}). The magnitude of the rate coefficient is related to the hydrodynamic sizes of the particles (a_1, a_2). This relationship depends on the solution viscosity (η) and temperature (T) without share stress, and on the mixing regime with shear stress (G). k in Eqn (2) represents the Boltzmann constant.[40]

$$\text{Adsorption time}: \quad t_{ads} = -[\ln(1-f)/k_{ads} n_1 n_2 \,] \tag{1}$$

$$\text{Diffusion}: \quad k_{ads} = 2kT/3\eta\left[(a_1+a_2)^2/a_1 a_2\right] \tag{2}$$

$$\text{Shear}: \quad k_{ads} = 4/3G(a_1+a_2)^3 \tag{3}$$

Ogura *et al*. found that 3% and 90% w/v solutions of chitosan (10% DD) in aqueous 10% w/v acetic acid showed a swirl-like (fingerprint) pattern under a polarizing microscope when sheared between glasses plates.[41] These results suggest that chitosan represents a family of rigid polymers regardless of deacetylation degree, and consequently this polymer needs a lot of time to achieve the equilibrium configuration. It is expected that the time for reconfiguration of the rigid polymer chitosan increases with its MW. The final factor is closely related to the total polymer chains adsorbed per particle and this influences the number of positive charges on the cell surfaces and therefore the binding sites that can interact with an anionic surface.

2 MATERIAL AND METHODS

2.1 *P. fluorescens* culture

P. fluorescens M1C strain was isolated from rice soil in Entre Rios, Argentina. It was preserved in an ultra-freezer at −80 °C. To obtain the *P. fluorescens* culture, it was allowed to grow in King Broth (20 mL) in an Erlenmeyer flask for 10 h in a culture chamber (28 °C, 150 rpm). Then an inoculum (20 mL) was used to inoculate King Broth (230 mL) in an Erlenmeyer flask (1 L). This inoculum was grown again with agitation in the culture chamber until the stationary phase was reached (28 °C, 150 rpm).

2.2 Chitosan

Chitosans were purchased from Glentham Life Sciences (Glentham Life Sciences, Unit 5 Leafield Way, Corsham SN13 9SW, Reino Unido, UK). The data sheet provided by Glentham Life Sciences showed the following information: chitosan average molecular weights (1 250 000, 250 000, and 20 000 g mol^{-1}), chitosan DD (90.21%; 90, 95% and 90, 27%) and viscosities (742 cps; 42, 23 cps and 4.84 cps).

2.3 Sample preparation

2.3.1 Bacteria samples

An aliquot of *P. fluorescens* culture was centrifuged and the cells were suspended in NaCl (10 mM) solution to achieve the desired

concentration (0.20% w/v). This concentration was selected to avoid interparticle scattering. The lower molarity of the suspended solution was used to prevent the interference between the electrical field and the presence of ions. This helped us to obtain more reliable data. The pH value was adjusted with NaOH (1 M) and HCl (1 M) to 2, 3, 4, 5, 6, and 7.

2.3.2 *Bacteria–chitosan samples*

Chitosan was dissolved in NaCl (10 mM) solution at different concentrations (0.0005%, 0.001%, 0.01%, and 0.05% w/v). The chitosan solutions were adjusted with HCl/NaOH 1 M to pH 4, 6, and 7. The last solution was agitated to improve the disentanglement of the polymer chains.

Then an aliquot of *P. fluorescens* culture was centrifuged and the cells were suspended in NaCl (10 mM) to achieve the desired concentration (0.20%, 0.15%, 0.07% w/v).

The chitosan solution was transferred to a falcon tube (50 mL) and an equal volume of culture cells was added. Again, the pH was adjusted with NaOH (1 M) and HCl (1 M) to pH 4, 6, and 7. The resultant solution was stirred in an orbital shaker (300 rpm, 10 min).

2.4 ζ potential determination

A ζ sizer Nano ZS (Malvern Instruments, Malvern, UK) was used to measure the ζ potential, which is determined from electrophoretic mobility (μ) based on Smoluchowski's formula (see Eqn (4)). This approximation holds well for particles larger than about 0.2 μm dispersed in electrolytes containing more than 10^{-3} M salt. One milliliter of each sample was deposited onto a Malvern polystyrene U-shaped cell. The cell was introduced onto the equipment and the measurement made.

$$V_{ep}/E = \mu = \zeta\psi\varepsilon_0\pi/\eta \tag{4}$$

where V_{ep} is particle velocity, E is the electric field strength, ε_o is the permittivity of the free space, ψ is the dielectric constant of the dispersion medium, and η is the viscosity medium (η of water is 0.001 Pa·s).[42]

The viscosity and dielectric constant of the suspending medium needed in the calculation, were approximated by values for water. Measurements were performed at 25 °C. Three replicate analyses were performed and the results were expressed in mV as the mean values. The standard deviation of the mean usually did not exceed 5 mV.

2.5 ζ potential data analysis

2.5.1 *Bacteria samples*

The *P. fluorescens* M1C pK_a value was quantified through Eqn (5):

$$pK_a = pH_{1/2\zeta_{max}} + 0.4343(F\zeta_{max}/2RT) \tag{5}$$

$pH(1/2\zeta_{max})$ and ζ_{max} are the parameters. The second term [containing Faraday's constant (F), the universal gas constant (R), and the absolute temperature (T)] is related to the ionic strength. Equation (5) can be applied to ζ potentials less than 25 mV.[43,44]

Equation (6) was used to calculate the parameters. The MATLAB built-in function lsqcurvefit was employed for the experimental results adjustment.

$$\zeta = \zeta_{max} pH^{\alpha} / \left(pH^{\alpha} + pH_{1/2\zeta_{max}}{}^{\alpha} \right) \tag{6}$$

2.5.2 *Bacteria–chitosan samples*

Equation (7) was used for experimental data collected from bacteria–chitosan sample analysis.

$$\begin{aligned} \zeta &= (\zeta_{max}\sigma^{\alpha}/\sigma^{\alpha} + k^{\alpha}) + \zeta_{0,pH} \\ \zeta_{Sat} &= \zeta_{max} + \zeta_{0,pH} \end{aligned} \tag{7}$$

where ζ is the net ζ potential, $\zeta_{0,pH}$ is the cell ζ potential without chitosan, and σ is the chitosan:bacteria ratio (g chitosan/g bacteria). The fitting parameters are ζ_{max} [directly related to the saturation point (SP) or ζ potential at the top of the curve], k (the curve inflection point), and α (a measure of the width and steepness of the sigmoidal curve). ζ_{Sat} is the ζ potential value at the SP and $\zeta_{0,pH}$ is the ζ potential at the start point when the chitosan concentration is equal to zero. The MATLAB built-in function lsqcurvefit was used to calculate the parameters and make predictions on SP and charge neutralization point (CNP).

2.6 Antifungal analysis

2.6.1 *Microcarrier preparation*

The microcarrier preparation comprises an oil-in-water (O/W) interface (1:1). The oil phase is sunflower oil and the emulsion is stabilized with lecithin (10% w/w). The mixture was homogenized (1 min) at the maximum speed of the blender (Waring Commercial, Orlando, FL, USA).

The bacteria–chitosan complex with different chitosan MWs was obtained as described previously (see section 2.3.2). The bacteria initial concentration was 1×10^9 UFC mL^{-1} and the chitosan initial concentration was 0.05% w/v (0.16 g chitosan per gram of bacteria). The complex was mixed 1:1 with the O/W emulsion. The pH value was adjusted to 6.00.

2.6.2 *Antifungal* in vitro *assay*

An ecological medium composed of agar-agar (15 g L^{-1}) and rice bran (10 g L^{-1}) was used. Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) of microcarrier solutions with different chitosan MWs were prepared. Serial dilutions of *P. fluorescens* suspensions without microcarrier also were prepared. A treatment with sterile water was used as a (+) control. An aliquot (100 μL) of each solution was transferred to the center of different Petri plates and dispersed on them. Then an inoculum of *Sclrotium oryzae* (a mycelia disc ranging from 4 to 5 mm) was transferred to the center of each plate.

The plates were incubated in a light/dark chamber at 25 °C. The mycelia growth was measured until the (+) Petri plate was fully colonized. Three replicate analyses were performed and the results were expressed in cm as the mean values. The standard deviation of the mean usually did not exceed 0.2 cm.

3 RESULTS AND DISCUSSION

3.1 *P. fluorescens* ζ potential determination

Figure 1 shows the ζ potential of *P. fluorescens* M1C *vs* pH value. As it can be seen, the ζ potential was negative for all pH evaluated. At pH 2 the ζ potential was −0.884 mV. As can be seen, the negative charge increased with increasing pH value. At pH 7 the ζ potential rose to −21.40 mV.

The ζ potential value at neutral pH values is in accordance with other reports where it is equal to −19.8 mV at pH 6 and −23.5 mV at pH 7 for *P. fluorescens* (ATCC 700830) and *P. fluorescens* P6a, respectively.[17,45]

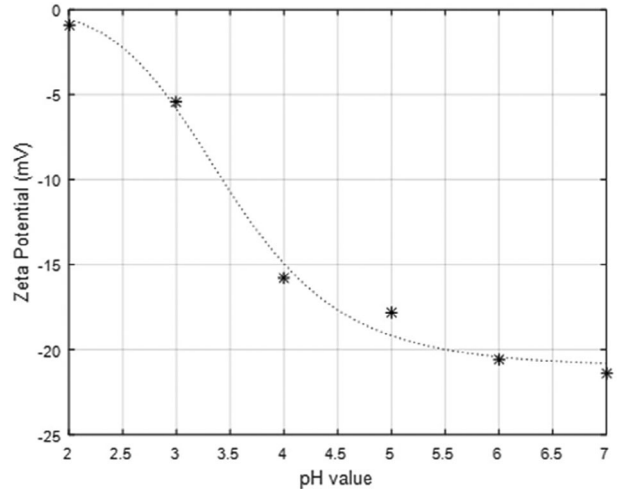

Figure 1. ζ potential *vs* pH value of *Pseudomonas fluorescens* M1C.

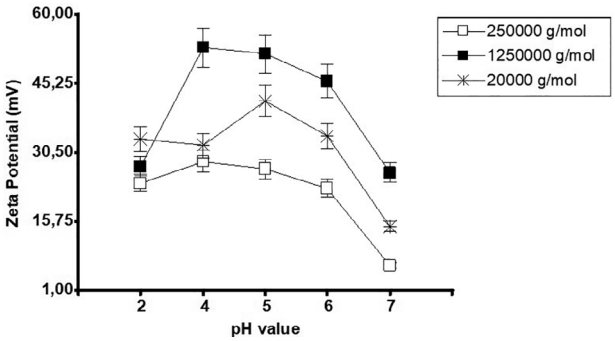

Figure 2. ζ potential *vs* pH value of chitosan at different molecular weights.

From ζ potential measurement and Eqn (6), the parameters ζ_{max} and pH($1/2\zeta_{max}$) were obtained. Then, through Eqn (5), the *P. fluorescens* M1C pK_a was found to be 3.30 $\pm$ 0.18. Similar values have been reported for other *P. fluorescens* strains.[37]

3.2 Determination of chitosan ζ potential

Figure 2 shows the chitosan (0.05% w/v) ζ potential variation as a function of pH value for different chitosan MWs. As can be seen, when the pH becomes more acidic, a higher ζ potential was obtained due to more protonated $-NH_3^+$ residues becoming available. Chitosan exhibited good solubility in acidic conditions.

At pH 7.0, the deprotonation began, and chitosan molecules were aggregated (observed visually), probably due to inter/intramolecular hydrogen bond formation and hydrophobic interactions. Therefore, the solubility and ζ potential value of these chitosan molecules decreased.

The ζ potential variation with pH is similar for all chitosan MWs evaluated. This is in accordance with the results obtained by Wang *et al.*[10] where the protonation constants (pK_a) of chitosan with different MWs were determined by potentiometric titrations and it was concluded that the pK_a values for different chitosan MWs had no obvious differences. The protonation constants of chitosan showed a slight decrease from 6.51 to 6.39 when the MWs changed from 1 370 000 to 60 000 g mol^{-1}. However, it is important to highlight that the highest ζ potential value is achieved with chitosan MW 1 250 000 g mol^{-1}.

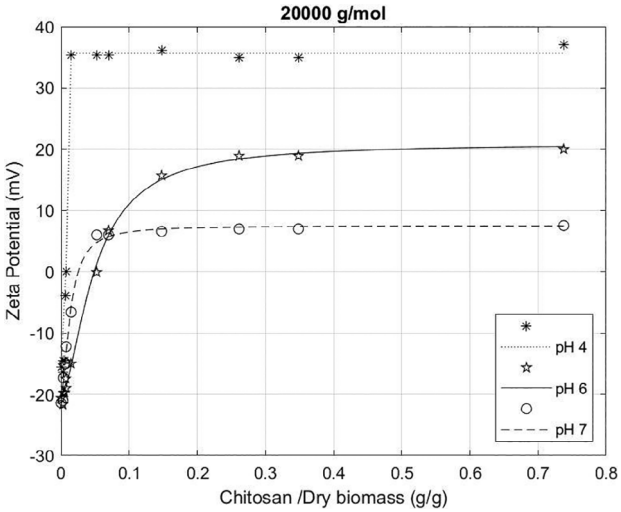

Figure 3. ζ potential of bacteria–chitosan complex with chitosan (20 000 g mol^{-1}) at different pH values. The points represent the experimental data and the lines represent the prediction values.

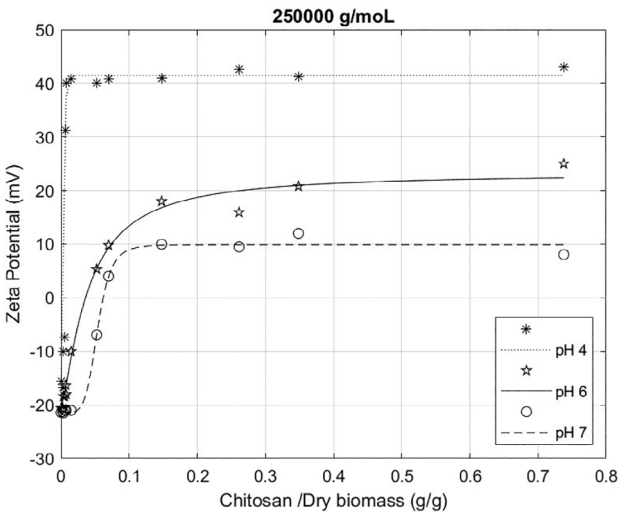

Figure 4. ζ potential of bacteria–chitosan complex with chitosan (250 000 g mol^{-1}) at different pH values. The points represent the experimental data and the lines represent the prediction values.

3.3 ζ potential analysis of bacteria–chitosan complex

Figures 3–5 show the ζ potential variation with σ (g chitosan/g bacteria) for the different chitosan MWs, 20 000, 250 000, and 1 250 000 g mol^{-1}, respectively. In general, at pH 4, an abrupt increase in ζ potential with σ can be observed, and the saturation point was achieved more rapidly, whereas at pH 6 and 7 more chitosan per gram of bacteria was needed to achieve this point.

From the experimental results, the parameters K, α, and ζ_{max} were determined through Eqn (7). The calculated parameters at different chitosan MWs and pH values are shown in Table S1.

The k parameter is directly associated with the CNP. An increase in this parameter indicates an increase in the chitosan quantity per gram of bacteria that is needed to achieve the CNP, specifically an increase in the chitosan quantity necessary to turn the bacteria–chitosan complex charge from negative to positive. On the other hand, ζ_{max} and α are indirectly associated with the

CNP, thus an increment in these parameters indicates a decrease in the necessary chitosan amount.

Figure 6 shows the parameter variations with pH values at different chitosan MWs. When the pH value was increased from 4 to 6, k increased significantly (Fig. 6(a)). α decreased significantly when the pH increased from 4 to 6 (Fig. 6(b)). Contrary to the other parameters, ζ_{max} decreased homogenously with pH for all the cases evaluated. The ζ_{max} parameter of the complex with chitosan (1 250 000 g mol^{-1}) was significantly higher than for the other chitosan MWs evaluated (Fig. 6(c)).

3.4 Effect of molecular weight and pH on the CNP

Table 1 summarizes the intersection of the prediction curves with the x axis (CNP) for different MWs at pH 4, 6, and 7. The ζ potential of the bacteria chitosan complex became positive at pH 4 when its chitosan:bacteria ratio was around 0.007 g chitosan per gram of bacteria. Whereas, at the others pH values evaluated (pH 6 and pH 7), the ζ potential became positive when its chitosan:bacteria ratio was around 0.05 g chitosan per gram of bacteria. If 0.003 g mL^{-1} of bacteria represents 1 $\times$ 10^{9} UFC mL^{-1} for *P. fluorescens* (Supporting Information Eqns (S1) and (S2)), the chitosan solution should be 0.002% and 0.015% w/v, respectively.

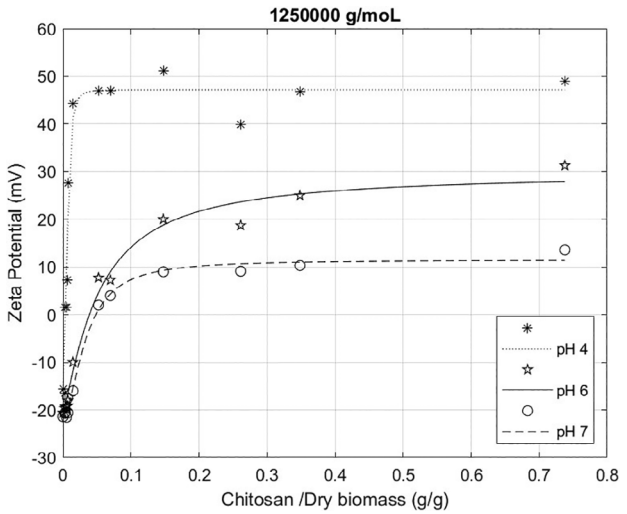

Figure 5. ζ potential of bacteria–chitosan complex with chitosan (1 250 000 g mol^{-1}) at different pH values. The points represent the experimental data and lines represent the prediction values.

The chitosan concentration at pH 6 and 7 is lower than the CS-MIC reported by many researchers.[31,33–36] This last indicates that a positively charged bacteria–chitosan complex can be developed working between pH 6 and 7 for the three chitosan MWs evaluated.

3.5 Effect of molecular weight and pH on the SP

The other point that requires special analysis is the SP, the ζ potential value at the top of the curves (ζ_{sat}). It is directly associated with the ζ_{max} parameter.

At pH 4, 0.05 g chitosan/g bacteria was needed to achieve the SP with chitosan of MW 1 250 000 g mol^{-1}. The ζ potential value was more than 45 mV. However, when the other chitosan MWs were used (250 000 and 20 000 g mol^{-1}), the polymer quantity per gram of bacteria required decreased to 0.015 g and ζ potentials greater than 40 and 36 mV, respectively, were achieved.

At pH 7, 0.35 g chitosan/g bacteria was needed to achieve the SP with chitosan of MW 1 250 000 g mol^{-1}, while the formation of the complex with chitosan MW 250 000 g mol^{-1} needed 0.15 g chitosan/g bacteria to achieve this point. Finally, the lowest chitosan MW evaluated needed 0.05 g chitosan/g bacteria. Independently of MW, the ζ potential value was around 10 mV at the SP.

The largest ζ potential that was achieved with chitosan (1 250 000 g mol^{-1}) was 30 mV at pH 6. The ζ potential for the other chitosans (20 000 and 250 000 g mol^{-1}) was 20 mV at pH 6.

In general, more chitosan per gram of bacteria could be adsorbed with high chitosan MW and a higher ζ potential was achieved at the SP. This is inconsistent with results obtained from the study of chitosan–bacteria adsorption in other research.[46] Higher MW needs more time to achieve the equilibrium configuration, thus more polymer chains per particle can be adsorbed at the surface. This is in accordance with results for other polyelectrolytes and the pK_a variation with chitosan MW reported by Wang.[10,47–49]

These last results suggest that higher chitosan MWs are preferable to develop a positively charged chitosan–bacteria complex.

On the other hand, the flocculation phenomenon increases with high MW.[50] This increases the size of the bacteria–chitosan complex and bacterial antifungal activity could be harmed. Within the ζ potential range (−30 to 30 mV), flocculation is produced by charge neutralization. The bridging flocculation appears when the bacteria surfaces are not fully saturated with chitosan and, this phenomenon increases with chitosan MW.[51] At the saturation point, the flocculation phenomenon cannot occur. At acidic conditions (pH 4), the ζ potential of the bacteria–chitosan complex

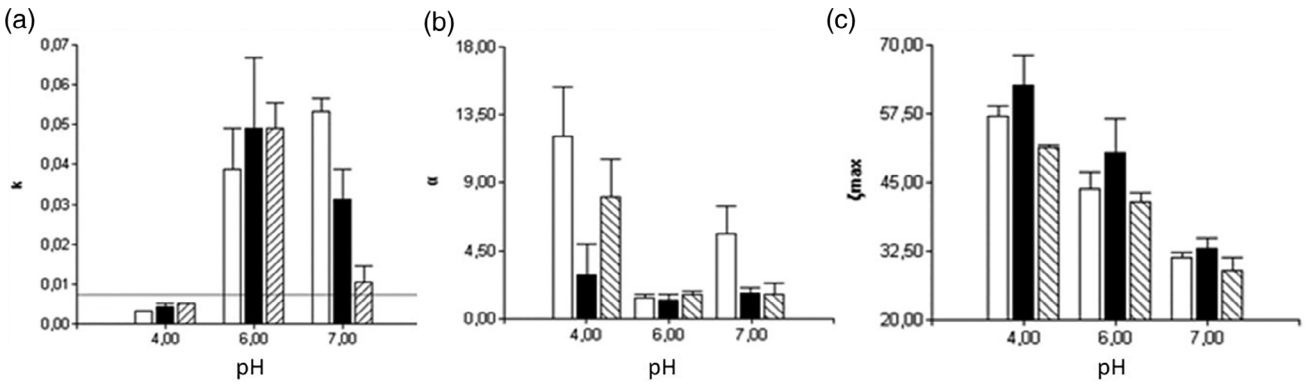

Figure 6. (A) k, (B) α, and (C) ζ_{max} *vs* pH value of chitosan at different molecular weights: 250 000, 1 250 000, and 20 000 g mol^{-1}. Bars represent standard deviation.

Table 1. *X* axis intersection for the prediction curves for different molecular weights at pH 4, 6, and 7

σ (g chitosan/ g bacteria)	pH 4	pH 6	pH 7
1 250 000 g mol^{-1}	0.007 ± 0.000	0.050 ± 0.004	0.025 ± 0.001
250 000 g mol^{-1}	0.006 ± 0.000	0.037 ± 0.007	0.062 ± 0.000
20 000 g mol^{-1}	0.005 ± 0.002	0.037 ± 0.010	0.047 ± 0.003

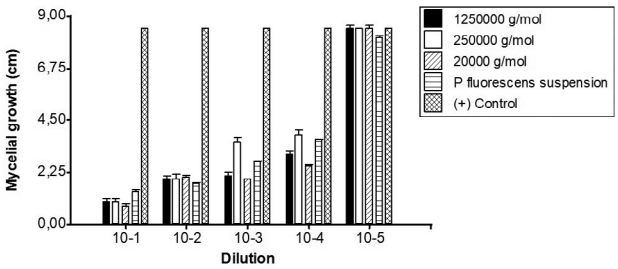

Figure 7. Mycelial growth (cm) of *Sclerotium oryzae* on agar Petri plates *vs* serial dilution of microcarrier formulation with different chitosan molecular weights, *Pseudomonas fluorescens* suspension without microcarrier formulation and (+) control inoculated with sterile water. Bars represent standard deviation.

is greater than 30 mV. In this last condition, the charge neutralization phenomenon disappears. However, the acidic conditions could be harmful to the bacteria viability. At pH 6, 0.8 g chitosan/g bacteria was needed to obtain a ζ potential higher than 30 mV with chitosan of MW 1 250 000 g mol−1. While the other chitosan MWs evaluated cannot achieve this ζ potential value at the different chitosan: bacteria ratio evaluated. More than 0.25% w/v chitosan is required for 1×10^9 UFC mL^{-1} *P. fluorescens* (see Supporting information Eqns (S1) and (S2)). This final concentration is above the CS-MIC reported.[31,33–36] In summary, a positively charged bacteria-chitosan complex was successfully obtained. Nevertheless, the flocculation phenomenon could not be prevented. If a stable formulation is desirable, a detailed analysis of CS-MIC against bacteria at pH lower than 6 is recommended to select the optimal chitosan–bacteria ratio.

3.6 Antifungal analysis

To determine if the flocculation phenomenon affects antifungal activity, one chitosan: bacteria ratio (0.16 g chitosan/g bacteria), in the ζ potential range −30 to 30 mV, was prepared. The pH value was adjusted to 6.00. The chitosan concentration selected (0.05%) is enough to turn the bacteria ζ potential from negative to positive. At this concentration, the chitosan is not harmful to the bacteria viability.[31,33–36]

Figure 7 shows the mycelial growth (cm) of Sclerotium oryzae on agar Petri plates for different serial dilutions of microcarrier formulation with different chitosan molecular weights. As can be seen, all the treatments resulted in positive antifungal activity against *S. oryzae* until dilution of 10^{-4} was reached. No differences were founded between different MWs and between the formulation with microcarrier and without it.

4 CONCLUSIONS

This work aimed to evaluate the effects of chitosan MW on the formation of positively charged *P. fluorescens*–chitosan complex and its impact on bacteria attachment to a anionic microcarrier used in biocontrol against *S. oryzae* in flooded rice fields.

The set of fitted equations (Eqns (5)–(7)) allows the parameters to be calculated and predictions to be made for the analyzed system.

The pK_a value of *P. fluorescens* is 3.30. The *P. fluorescens* ζ potential surface is a net negative for pH value greater than 2. On the other hand, the chitosan ζ potential is positive and its value tends to zero at pH near to 7.

The bacteria–chitosan complex formed by the highest chitosan MW evaluated needs more chitosan per gram of bacteria to achieve this saturation point in comparison with the other MWs evaluated. It also achieved a higher ζ potential value for all pH values evaluated.

Based on the experimental results and background information, the formation of a bacteria–chitosan complex with a net positive charge at pH and chitosan concentration that does not affect bacteria viability is possible. For this, the highest chitosan MW, which allows a higher ζ potential to be achieved, is preferable. The ζ potential of the bacteria chitosan complex is higher in acidic conditions. However, at strong acidic conditions (pH 4), the bacteria viability could be damaged. Therefore, working at pH 6 is recommendable against the other pH evaluated (pH 4 and pH 7).

At the recommended pH value (pH 6), the chitosan amount necessary to avoid flocculation problems (ζ potential > 30 mV), could be harmful to the bacteria viability. Then, the chitosan concentration should be less than this value. Despite the flocculation phenomenon appears, it demonstrated that the bacteria antifungal activity does not be affected.

Our findings provide relevant information about bacteria–chitosan interaction and may be useful in biocontrol programs that involved these two components and where bacteria adsorption to an anionic carrier or anionic surfaces is desirable.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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