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Polymers selection for a liquid inoculant of *Azospirillum brasilense* based on the Arrhenius thermodynamic model

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Plant growth promoting bacteria (PGPB) enhances the growth of their hosts and can protect them from biotic and abiotic stresses. Bacterial inoculants contain one or more of these beneficial strains in a carrier material, which must be able to maintain the viability of the cells during the time of storage, and also guarantee the biological activity of the strains once applied in the soil. These inoculants can be solid, liquid, gel or oil-based, depending on the characteristics of the strains and the shelf life expected by the producers. In this study, we used a method of accelerated degradation to select a polymer and a concentration to maintain cell stability of a liquid inoculant based on the strain C16 *Azospirillum brasilense.* A screening at 45°C was made to compare the protectant effect of five polymers on the viability of the strain (p/v): carrageenan (1.5%), sodium alginate (1%), trehalose (10 mM), polyvinylpyrrolidone (2%), glycerol (10 mM) and phosphate saline buffer as control. Carrageenan and sodium alginate showed significant differences in cell viability over the use of other polymers ($P < 0.05$). We evaluated cell viability with these two polymers at three concentrations and three different temperatures (4, 28 and 45°C) for 60 days and determined the bacterial degradation rates. Based on the Arrhenius thermodynamic model, we calculated the time required to reduce cell concentration in three log units, and observed that the protectant activity of each polymer and each concentration depends on the temperature of storage. Cell viability was best preserved in all treatments at 4°C. In general, alginate prolonged cell viability at 28°C, and carrageenan at 45°C. Alginate at 1% and carrageenan at 0.75% showed a stable behavior (superior to the control) in the three evaluated temperatures, so we conclude that they can be used for a formulation of a liquid inoculant based on the strain C16 of *A. brasilense.*

Key words: Energy of activation, degradation, cell death, kinetics, formulation.

INTRODUCTION

Plant growth promoting bacteria (PGPB) are able to enhance the growth of their hosts by increasing the supply or availability of primary nutrients, producing beneficial compounds and protecting them from biotic

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Abbreviations: PGPB, Plant growth promoting bacteria.

and abiotic stresses (Vessey, 2003). Bacterial inoculants contain one or more of these beneficial strains in an easy-to-use and economical carrier material (Bashan, 1998), which must be able to maintain the viability of the cells and protect them from the stresses they face once applied into the soil or plant surfaces. There is no universal carrier, and choosing the proper formulation is a strain-specific process as important as the selection of the bacteria itself (Rivera et al., 2014). Solid, liquid, oil and gel based formulations have been developed, each with their own advantages and limitations. Liquid inoculants are an economical technology, usually consisting in the addition of one or more polymers to the broth culture. These polymers are used to protect bacterial cells from stresses and help their establishment into the host (Albareda et al., 2008; Mugilan et al., 2011; Amalraj et al., 2013*)*. Their major disadvantage is that they cannot be stored at room temperature for long periods without compromising the viability of the bacteria and their efficacy. Here, the physical and chemical properties of polymers protect cells against desiccation and sedimentation, which is directly related to cell death (Sivasakthivelan and Saranraj, 2013; Rouissi et al., 2011).

Stability tests are used to determine the viability and biological activity of the bacteria inside these formulations, helping to establish the point where they are no longer viable and will not be of any use to the farmers. In the first steps of formulating a bacterial inoculant, methods of accelerated thermal degradation are used to select -in a short period of time, the components which will best preserve the viability of the strains. These models rely on the effect of temperature over cell viability, and with first-order kinetics, determine the experimental values of cell degradation rates. Prediction models like Arrhenius are used to calculate the energy of activation and determine the time required for cell death in the bacterial inoculant (Krumnow et al., 2009).

In this study, we aimed to select a polymer and concentration for a liquid inoculant based on the strain C16 of the PGPB *Azospirillum brasilense,* evaluating the effect of five polymers on cell viability and using the Arrhenius thermodynamic model to predict the shelf-life of the selected formulations. *A. brasilense*, a member of the alpha-proteobacteria, is one of the most studied PGPR (Steenhoudt and Vanderleyden, 2000; Okon and Labandera-Gonzalez, 1994; Baudoin et al., 2009) and its beneficial effects have been widely known in more than a hundred plant species (Bashan and Bashan, 2010), and are attributed to various mechanisms, especially nitrogen fixation and hormone production, such as auxins, cytokinins and gibberellins (Cassaan et al., 2014; Fibach-Paldi et al., 2011). Determining the best conditions to maintain the viability of the bacteria in the formulation can lead to a steady behavior in field, which will help to promote plant growth in crops of socio-economical interest.

MATERIALS AND METHODS

Bacterial strain and culture conditions

The strain C16 was isolated from *Panicum maximum*, identified as *A. brasilense,* and cryopreserved on glycerol (50%) (Cardenas et al., 2010). For its activation, the strain was incubated at 30°C for three days on Potato Agar Medium (Components g/L: potatoes peeled and sliced, 200; DL-malic acid, 2.5; KOH, 2.0; sucrose, 2.5; vitamin solution, 1.0 ml; micronutrient solution, 2 ml; bromothymol blue, 2 drops; agar, 15.0). Cellular suspensions were made in 10 ml of ABRA medium (Moreno et al., 2012), a strain-specific medium designed in our laboratory for biomass production. After 27 h, cell concentration was adjusted (OD 600 nm = 0.5).

Evaluation of the polymeric protectant effect under stress conditions

Five polymers were selected based on their use as additives in other liquid inoculant formulations (Table 1). Treatments were (w/v): alginate 1%; carragenaan 1.5%; polyvinylpyrrolidone 2%; trehalose 10 mM, glycerol 10 mM, and phosphate saline buffer (components g/l: NaCl 8.0, KCl 0.2, Na_2HPO_4 x $7H_2O$ 1.15, KH_2PO_4 0.2; pH 7.3) as a control. Flasks with ABRA medium were inoculated at 1% with the cellular suspensions and incubated for 27 h and 120 rpm. Each polymer was added at 10% of the total volume of the flasks -each flask consisting in one replicate. Three replicates per treatment were made, and the content of the flasks was dispensed in microtubes (1.5 ml) with a total of five microtubes per flask. Finally, the microtubes were incubated at 45°C for 15 days. During this time, cell concentration was determined by serial dilutions, plating 20 µL on BMS medium at 0, 7 and 15 days. At the end of the evaluation time, two polymers were selected based on their protectant effect on the preservation of cell viability.

Cell viability prediction with the Arrhenius thermodynamic model

For the two polymers chosen, three different concentrations were selected. The same procedure described above was used to mix the polymers within the cell suspensions. Three temperatures were selected to evaluate their effect: 4, 28 and 45°C, each one representing refrigeration, environmental and stress conditions, respectively. Treatments were carrageenan at 0.75, 1.5 and 3%; alginate at 0.5, 1 and 2%, and phosphate saline buffer as a control. All of them were incubated at the three temperatures mentioned above, with a total of 21 treatments. Cell concentration was measured at 0, 15, 30, 45 and 60 days. Knowing that bacterial degradation rate at constant temperature can be described by first-order kinetics, cell concentration versus time was plotted to determine the experimental values of cell death rate (k) based on Equation 1:

$$\mathrm{Log}\frac{N}{No} = -2.303 \times kt \quad (1)$$

First-order kinetics equation, where N_o is the initial concentration of cells, N is the cells concentration at a specific time, k represents the first-order rate constant and t, the time of the thermal degradation.

The first-order cell death rate (k) was replaced in the Arrhenius Equation 2, where A is the pre-exponential Arrhenius factor (1/day), Ea is the apparent activation energy (cal/mol), R is the

Table 1. Previous reports of the selected polymers for the screening with strain C16 *Azospirillum brasilense*

Polymer	Concentration	Microorganisms	References
Sodium alginate	1%	*Azospirillum brasilense, Pseudomonasfluorescens Rhizobium* sp.	Bashan and Gonzales (1999), Rivera (2014), Yabur et al. (2007)
Carragenaan	1.5%	*Azotobacter chroococcum*	Rojas-Tapias et al. (2013)
Polyvinylpirrolydone	2%	*Bradyrhizobium, japonicum Azorhizobium, caulinodans Mesorhizobium cicero Azospirillum brasilense*	Tittabutr et al. (2007), Kumaresan and Reetha (2011)
Trehalose	10 mM	*Pseudomonasfluorescens, Azospirillum brasilense*	Manikandan et al. (2010)
Glycerol	10 mM	*Pseudomonasfluorescens*	Manikandan et al. (2010)

universal gas constant (1.985 cal/ mol K), and T is temperature (K):

$$k = Ae^{-\frac{Ea}{RT}} \quad (2)$$

Taking the logarithm of the Arrhenius equation and plotting log k versus the reciprocal of temperature (1/T), the experimental value for the energy of activation was obtained (Equation 3):

$$\text{Log } k = -\frac{Ea}{2.303R} \times \frac{1}{T} + \text{Log } A \quad (3)$$

Finally, the time required to reduce cell concentration in three log units was estimated using the first-order kinetics equation.

Statistical analysis

The experimental design consisted in a complete randomized design with 21 treatments where polymers, concentrations and temperature were the factors of evaluation. The data of the screening was analyzed using the ANOVA and HSD Tukey test. To calculate the rate of cell death, the Arrhenius equation was used as reported by Sorokulova et al. (2008). Figures were made using Sigmaplot 13.0.

RESULTS

Screening under stress conditions

The first seven days of evaluation, cell viability showed no significant differences, but that tendency changed at day fifteenth, where cell concentrations differed significantly among treatments (Figure 1). Carrageenan and alginate showed the higher cell counts compared with the use of other polymers. Cells concentrations were maintained at least one log unit above, meaning that these two polymers protected cells against heat and desiccation and reduced the rate of cell death. Trehalose and glycerol also showed a protectant effect superior to polyvinylpyrrolidone, which was only similar to the control. All polymers proved to significantly protect cells against high temperature (45°C) with the exception of polyvinylpyrrolidone, but in this study, the strain C16 was more compatible with carrageenan and alginate. The observed behavior of polyvinylpyrrolidone contrasts with previous reports, where its good rheological properties, high water activities, and ability to limit heat transfer, helped to preserve cell viability of *Azospirillum, Azotobacter* and *Bacillus* strains (Leo Daniel et al., 2013; Kumaresan and Reetha, 2011). Here, the concentration of the polymer and its interaction with the liquid medium may have affected its performance (Albareda et al., 2008; Velineni and Brahmaprakash, 2011) which was also seen in glycerol and trehalose. Carrageenan and alginate demonstrated that, as seen by other authors, they have desirable characteristics as additives in liquid formulations. The viscosity and water holding capacity of these hydrophilic polymers reduced the drying rate and helped to protect cells from environmental stresses. Similar results were obtained with carrageenan on *Azotobacter chroococcum* (Rojas-Tapias et al., 2015), and alginate with *Rhizobium* and *Bradyrhizobium* strains (Tittabutr et al., 2007; Rivera et al., 2014). It is important to establish that these two polymers are used in other type of formulations like microencapsulation, which have been proved to maintain cell viability of nitrogen fixing bacteria for over fourteen years (Bashan and Gonzalez, 1999).

Cell viability prediction using the Arrhenius thermodynamic model

Figure 2 shows the dependence of cell viability as a function of time at the evaluated temperatures, where

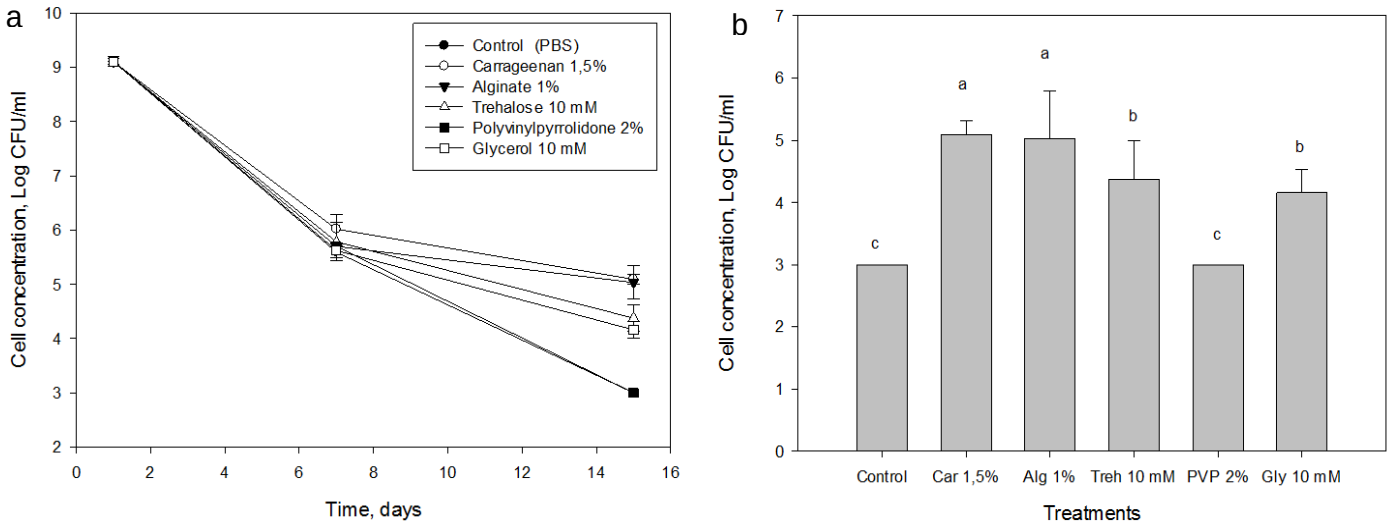

Figure 1. Effect of high temperature on cell concentration. a) Cell concentration during the assay. b) Final cell concentration at fifteenth day. Letters indicate sub homogeneous groups obtained using the HSD Tukey test.

degradation rates followed a first-order kinetics across all temperatures. At 4 and 28°C, cell viability was similar in all treatments, with a reduction of only one log unit regarding the polymer used and its concentration. At 45°C, cell viability decreased about four log units for the treatments with polymers and five log units for the control. At this temperature, we observed that carrageenan at 1.5 and 3% allowed a major viability of the strain. The apparent first-order degradation rate constants increased with temperature, with the highest values at 45°C for all treatments, as shown in Figure 3. At 4°C, alginate at 2% and control showed the highest degradation rates, as well as carrageenan at 0.75% at 28°C. On the highest temperature (45°C), carrageenan at 1.5 and 3% showed the lowest rates. Once the apparent rates of constant degradation was obtained, we used the Arrhenius equation to calculate the experimental values of the energy of activation (Table 2) and observed that, at the three concentrations used for carrageenan, the energy of activation was very similar. Alginate at 0.5%, had the highest value for energy of activation, and the control (PBS) the lowest, followed by alginate at 2%. Data of bacterial death during time helped to determine the apparent rate constants of degradation for each polymer, and the energy of activation was determined in a plot where the log of the rate is linearly related to the inverse of absolute temperature. In terms of viability, the energy of activation is the energy barrier needed to overcome cell degradation (Rojas-Tapias et al., 2015). The protectant effect of polymers was observed because the energy of activation increased when compared with the control (PBS), meaning that it takes more energy and time for cells to start degrading. The experimental values of the activation energy in our study (with a maximum value of 13.2 kcal/mol) were inferior to those described by other authors for *Azotobacter* (22.07 kcal/mol) and *Bacillus* spores (19.7 kcal/mol) when they were evaluating the effect of carrageenan, alginate and acacia gum over cell viability (Rojas-Tapias et al., 2015; Sorokulova et al. 2008), but is necessary to highlight that, *Azospirillum* is much more sensitive to temperature than those microorganisms, and *Bacillus* spores are more resistant to any kind of stress. According to Dell et al. (2007) and Huey and Kingsolver (2011), the use of the Arrhenius model is well accepted for biological processes, fitting in about 80 to 90% the behavior of thermal sensitivity among plants, microbes and animals. Although, some deviations of the model have been developed (Aquilanti et al., 2010), the results of this study show clearly that it can be useful in the selection of the components of bacterial inoculants.

The data obtained was fitted to the first-order kinetics equation to predict the necessary time to reduce cell concentration of the strain in three log units (Table 3). The time needed for this reduction at 4°C was more than a year in all treatments with the exception of alginate at 2% and the control treatment. Alginate at 1% was the only treatment predicted to prolong cell viability more than two days than the control treatment at 28°C. Interestingly, carrageenan at 1.5 and 3% are predicted to preserve cell viability at 4 and 45°C, but at 28°C the time of reduction is similar to the control. Even though the control treatment has a predicted life time lower than the others, the time needed for the reduction shows that cells can survive long periods only in the culture medium at room temperature.

The addition of polymers had a strong effect in the rate of cell death in all temperatures, and according to the

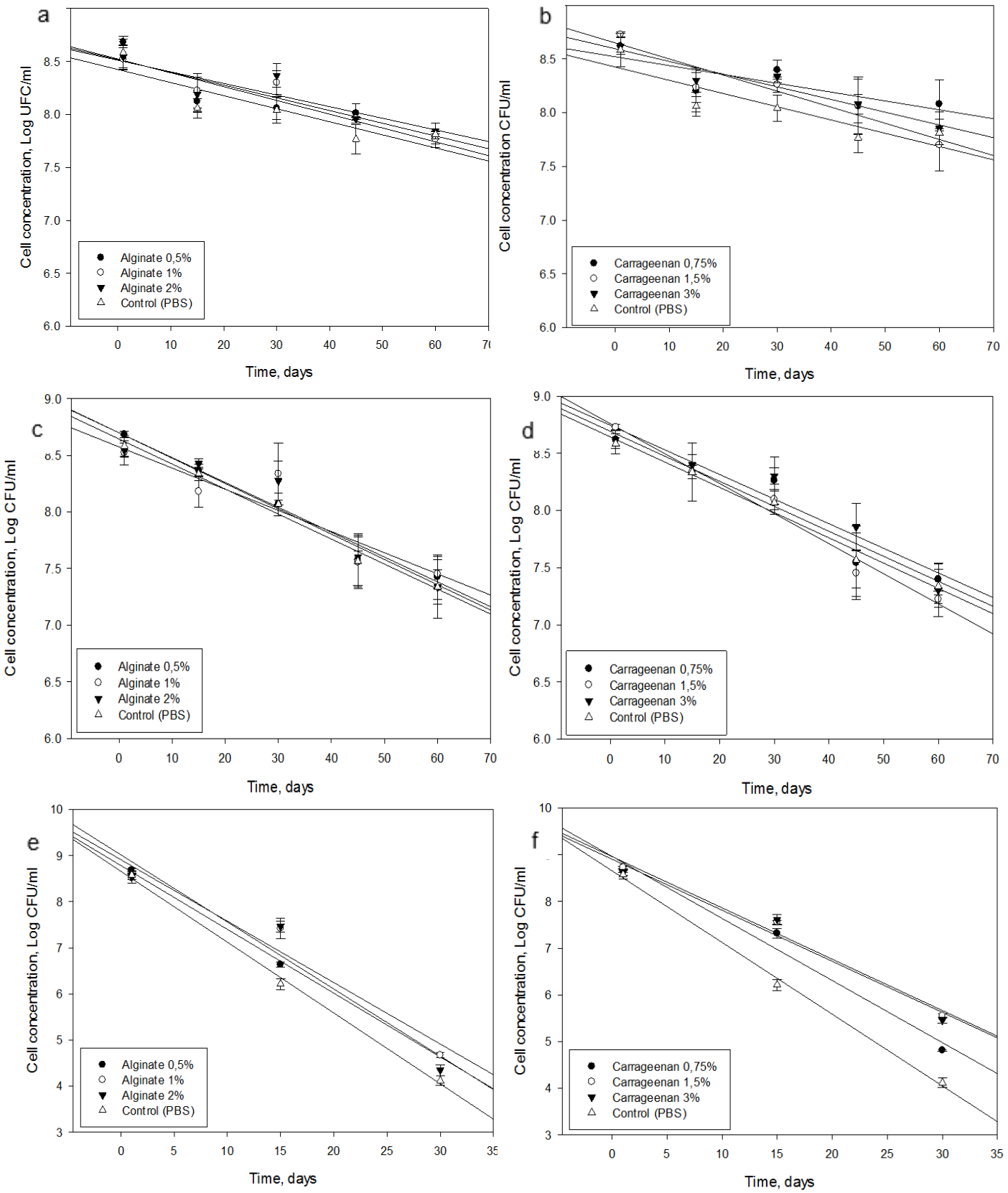

Figure 2. Apparent first order-kinetics of degradation for strain C16 *Azospirillum brasilense* with different alginate and carrageenan concentrations during storage. Experimental data of cell degradation rate at 4ºC (a, b); 28ºC (c, d) and 45ºC (e, f).

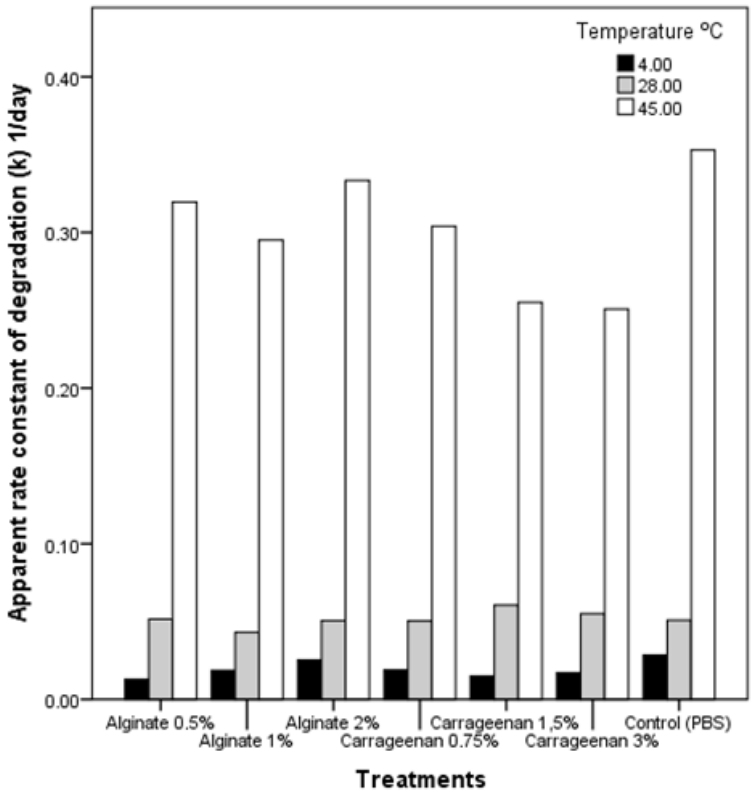

Figure 3. Apparent first-order constant of degradation of strain C16 with carrageenan and alginate at different temperatures and concentrations.

Table 2. Experimental values for energy of activation of strain C16 in all treatments. R^2 corresponds to the determination coefficient. Values of slope are the result of plotting Log (k) vs. 1/K. Ea meaning energy of activation.

Treatment (%)	R²	Slope	Ea (Kcal/mol)
Alginate 0.5	R² = 0.9538	2.90	13.233
Alginate 1	R² = 0.8763	2.46	11.197
Alginate 2	R² = 0.8507	2.28	10.423
Carrageenan 0.75	R² = 0.9104	2.49	11.384
Carrageenan 1.5	R² = 0.9794	2.59	11.873
Carrageenan 3	R² = 0.956	2.43	11.134
Control (PBS)	R² = 0.8213	2.21	10.122

Table 3. Predicted time to reduce cell concentration in three log units based on the Arrhenius equation.

Treatment (%)	4ºC	28ºC	45ºC
Alginate 0.5	568	143	23
Alginate 1	397	172	25
Alginate 2	292	146	22
Carrageenan 0.75	391	147	24
Carrageenan 1.5	492	122	29
Carrageenan 3	429	134	29
Control (PBS)	260	145	21

predicted time they can prolong shelf-life of liquid inoculants in more than 200 days at 4°C, 30 days at 28°C and 8 days at 45°C. These results are similar to those obtained by Kumaresan and Reetha (2011) and Manikandan et al. (2010), where they found that the polymers like glycerol, trehalose, and gum arabica doubled the shelf life of liquid inoculants of *A. brasilense,* and *Pseudomonas fluorescens.* Leo Daniel et al. (2013) also found that the addition of polymers as additives and adjuvants maintain cell concentration in liquid inoculants of *A. brasilsense, Bacillus megaterium* and *Azotobacter chroococcum,* reducing just two or three log units in 480 days at room temperature.

The Arrhenius prediction model allowed us to observe that, liquid inoculants can have a long shelf-life when refrigerated, but they can also survive enough time at room temperature (28°C), taking on average five months to reduce cell viability in three log units. Shelf-life prediction showed that, in general, at any concentration, carrageenan and alginate can prolong cell viability, but alginate at 1% and carrageenan at 0.75% preserved cell

viability and increased the shelf life of the formulations at the three temperatures evaluated. So, we conclude that they can be used in the formulation of a liquid inoculant based on the strain C16 *A. brasilense*.

Conflict of interests

The authors did not declare any conflict of interest.

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