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Spray-drying microencapsulation of *Trichoderma harzianum* conidias in carbohydrate polymers matrices

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ABSTRACT

Microencapsulation of biological control agents in biopolymer matrices by spray-drying is a valuable alternative to produce formulations with extended shelf-life. In this work, *Trichoderma harzianum* conidia were microencapsulated in: maltodextrin DE10 (MD10), maltodextrin DE20 (MD20), gum Arabic (GA), and a 1:1 weight blend of MD10–GA. The effect of spray-drying inlet and outlet air temperatures, and the activation energy (E_a) of the biopolymers on the survival of *T. harzianum* conidia was evaluated. The highest conidia survival after spray-drying (86%) and after 8 weeks of storage at 4 °C (40%), were obtained with the MD10–GA biopolymer matrix (with the highest E_a = 31.6 kJ mol^{-1}). Such survivals were 11 and 330-fold times higher than those of non-encapsulated conidia, respectively. The evolution of reactive oxygen species (ROS), as a measure of oxidative stress, during storage at 29 °C was assessed. An inverse relationship was found between the conidia survival and ROS concentration during storage.

1. Introduction

Different authors have shown that *Trichoderma harzianum* can be used to attack pathogenic plant fungi, such as *Botrytis*, *Rhizoctonia*, *Sclerotinia*, *Pythium*, *Phytophthora* and *Fusarium* (Batta, 2004; Fravel, 2005; Wang et al., 2005). The mechanisms involved in *Trichoderma* spp. control of fungal pathogens are mycoparasitism, nutrient competition, rhizosphere competence, cell-wall degrading enzymes production, as well as induced defense responses in plants (Howell, 2003). A biological control product, able to compete commercially with chemical products, must have a minimum shelf life of 1–2 years at room temperature (Abadias, Teixidó, Usal, Viñas, & Magan, 2000; Pedreschi & Aguilera, 1997). Thus, dehydrated fungal formulations are attractive because of their long stability, easy handling and storage at room temperature (Pedreschi & Aguilera, 1997).

T. harzianum produces three kinds of propagules that can be used in formulations: hyphae, chlamydospores and conidia (Howell, 2003). The use of hyphae is not an option due to its lack of resistance to dehydration. Conidia and chlamydospores withstand adverse environmental conditions, which made them the natural choice as propagules in formulations (Jin, Harman, & Taylor, 1991; Papavizas, 1985). There are several products in the market using *T. harzianum* conidia as active ingredient, obtained either by two-phase solid fermentation or liquid fermentation. However, in both cases the biomass must be dried to obtain a stable product with prolonged shelf-life (Jin, Hayes, & Harman, 1992). Among the different drying techniques for large scale production of microorganisms containing dried powders, spray-drying is preferred due to his low cost (Morgan, Herman, White, & Vesey, 2006). The use of spray-drying can produce cellular damage as result from the elevated temperature, dehydration and oxidation of macromolecules during storage (Ananta, Volkert, & Knorr, 2005; França, Panek, & Eleutherio, 2007; Horaczek & Viernstein, 2004a, 2004b; Molina & Anchordoquy, 2008; Teixeira, Castro, & Kirby, 1996). Previous studies in our lab have shown that *T. harzianum* conidia had a low thermal inactivation energy (39.15 kcal mol^{-1}) making them susceptible to heat damage of the cellular membrane when spray-dried (Fernández-Sandoval, Ortíz-García, Galindo, & Serrano-Carreón, 2012). Also the stored dried conidia showed an inverse relationship between conidia survival and oxidative stress, evaluated as the intracellular malondialdehyde concentration.

Microencapsulation is defined as a process in which tiny particles or droplets are surrounded by a coating, or embedded in

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a homogeneous or heterogeneous matrix, to give small capsules with many useful properties (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007) and is used by several researchers to provide living cells with a physical barrier against the external environment (Riordan, Andrews, Buckle, & Conway, 2001; Ross, Desmond, Fitzgerald, & Stanton, 2005). The microcapsule may consist of a core surrounded by a wall or barrier of uniform or non-uniform thickness, which can be formed by one or more polymers (Krishnan, Kshirsagar, & Singhal, 2005). The use of microencapsulation for probiotics such as *Bifidobacterium* sp. and *Lactobacillus* sp. has been reported (Ananta et al., 2005; Corcoran, Ross, Fitzgerald, & Stanton, 2004; O'Riordan et al., 2001; Rodríguez-Huezo et al., 2007; Ross et al., 2005). For biological control agents, microencapsulation has been tested in *Beauveria brongniartii*, *Beauveria bassiana* and *Metarhizium anisopliae* conidia which are used as bioinsecticides (Horaczek & Viernstein, 2004a, 2004b; Liu & Liu, 2009a, 2009b). Recently, Jin and Custis (2011) reported that drying *T. harzianum* conidia in presence of 2% of sucrose at inlet/outlet temperature of 61/31 °C, increased their survival by a factor of three compared with non-encapsulated conidia. However, in practically all of these reports, the encapsulation matrix and drying temperatures selection were done by trial and error procedures, in which the wall materials were selected arbitrarily (Pérez-Alonso, Báez-González, Beristain, Vernon-Carter, & Vizcarra-Mendoza, 2003). This procedure is time-consuming and expensive. Although the choice of encapsulation matrix for spray-dried microorganisms has led to effective results, a systematic approach has not been taken. Matsuno and Adachi (1993) reported that materials that exhibited drying curves characterized by an early decreasing drying rate, where water evaporation was controlled by diffusion mechanisms, provided best protection oxidation. However, it is difficult to discriminate between materials showing similar drying curves. Pérez-Alonso et al. (2003), proposed a method for estimating the activation energy (E_a) of biopolymers from a quantitative analysis of the drying curves, and established that those requiring higher E_a during their drying process provided greater protection to the core material (in this case microorganism) from heat damage and offer greater resistance to oxygen diffusion through their drying matrices.

The objective of this work was to microencapsulate *T. harzianum* conidia in biopolymer matrices with different activation energies and to evaluate the effect of the spray-drying inlet and outlet air temperatures on: (a) conidia survival immediately after spray-drying; and (b) conidia survival and the evolution of reactive oxygen species (ROS) after storage at two different temperatures.

2. Materials and methods

2.1. Strain, culture medium and inoculum development

T. harzianum strain used in this study was obtained from the Centro de Investigacion en Alimentacion y Desarrollo, A.C. (Culiacan Campus, State of Sinaloa, Mexico) and preserved in potato dextrose agar (PDA) medium at 4 °C. The liquid medium used for *T. harzianum* conidia production contained: 30 g L^{-1} of glucose, 5.6 g L^{-1} of $(NH_4)_2SO_4$, 7 g L^{-1} of KH_2PO_4, 2 g L^{-1} of Na_2HPO_4, 1.5 g L^{-1} of $MgSO_4{\cdot}7H_2O$, 0.0671 g L^{-1} of $CaCl_2{\cdot}2H_2O$, 0.008 g L^{-1} of $FeCl_3{\cdot}6H_2O$, 0.0001 g L^{-1} of $ZnSO_4{\cdot}7H_2O$ and 0.5 g L^{-1} of yeast extract. The inoculum was obtained by plating the *T. harzianum* strain on PDA at 29 °C and cultured 6 days with 20 min of light exposition to induce conidation. The conidia were re-suspended in a saline solution (0.05% Tween 40, 9 g L^{-1} NaCl). This suspension was used to inoculate a fermenter to a final concentration of 10^5 conidia mL^{-1}.

2.2. Culture conditions and spore recovery

The conidia were cultured aerobically in a bioreactor with a capacity of 500 L and a working volume of 270 L. The culture was keep at a constant temperature of 29 °C, the pH was controlled at 5.6 by the addition of sodium hydroxide (50%, w/v). The bioreactor was stirred at 360 rpm with 2 Rushton turbines with a turbine diameter/tank diameter ratio of 0.5 and an aeration rate of 0.5 vvm. After 60 h of culture a wet paste of conidia was recovered by passing the culture broth through a 0.2 mm sieve (in order to separate mycelia from conidia) and centrifuged at 12,000 × *g*.

2.3. Spray-drying of Trichoderma harzianum formulations

Aqueous solutions (20%, w/v) of MD10, MD20; GA, and MD10–GA biopolymers and 1% (w/v) suspension of conidia, both in phosphate buffer 50 mM (pH 5.6), were mixed (500 mL) and spray-dried in a Bowen Engineering BE-1448 spray-dryer (Indianapolis, IN, USA) with an atomizer diameter of 0.19 mm operated with an air flow of 8.82 m^3 min^{-1} and a pressure differential of 58.9 kPa was used. Each suspension (500 mL) was fed to the dryer adjusting the inlet air temperature (T_i) to 120 °C, 135 °C or 150 °C, and the outlet air temperature (T_o) to 70 °C, 80 °C or 90 °C. The number of colony forming units (CFU) before and after spray draying was determinate by flow cytometry (Section 2.8). The percent survival of spray-dried (PS_{sd}) microencapsulated conidia, in the different biopolymer matrices, was obtained as:

$$PS_{sd} = \left(\frac{\text{CFU after spray-drying}}{\text{CFU before spray-drying}}\right) \times 100 \quad (1)$$

Also, the survival increase (SI) of each of the conidia microencapsulated in the different biopolymer matrices with respect to a non-encapsulated control treatment was obtained as follows:

$$SI = \frac{PS_{sd} \text{ microencapsulated}}{PS_{sd} \text{ non-encapsulated control}} \quad (2)$$

2.4. Determination of activation energy (E_a) of encapsulation matrix

The activation energy (E_a) of maltodextrin 10 DE (MD10), maltodextrin 20 DE (MD20), gum Arabic (GA) and a 1:1 (w:w) blend of MD10–GA was determined following the procedure of Pérez-Alonso et al. (2003). MD10 and MD20 were purchased from CP Ingredientes, SA de CV (Tlalnepantla, State of México, México) and GA from Quimica France, SA de CV (Morelia, State of Michoacán, México). The E_a values obtained were of 29.0 kJ mol^{-1} for MD10, 24.0 kJ mol^{-1} for MD20, 19.0 kJ mol^{-1} for GA, and 31.6 kJ mol^{-1} for MD10–GA.

2.5. Effect of activation energy and process temperatures on conidia survival after spray drying

A factorial design 3^3 was used to evaluate the effect of the encapsulation matrices E_a and process temperatures on the SI (Table 1). ANOVA analysis was made using a DESIGN-EXPERT software version 5.0.7 (Stat-Ease Inc., Minneapolis, MN). The main effects for each of the factors evaluated on the response were determined using the following equation:

$$\beta_i = (\gamma_i^+) - (\gamma_i^-) \quad (3)$$

where β_i is the effect of the *i*th factor on the response, and γ_i^+ and γ_i^- are the mean responses for the upper (+) and the lower (−) levels

Table 1
Experimental 3^3 factorial design used to evaluate the effect of activation energy (E_a), inlet (T_i) and outlet temperature (T_o) on the survival of *T. harzianum* conidia after spray drying.

Run	Coded variables			Process variables			Responses		
	E_a	T_i	T_o	E_a (kJ mol^{-1})	T_i (°C)	T_o (°C)	PS_{sd} (%)	SI[a]	SI[b]
1	1	1	−1	29	150	70	62.6	4.0	4.5
2	1	1	0	29	150	80	71.4	7.9	7.7
3	1	1	1	29	150	90	84.0	10.1	10.9
4	1	0	−1	29	135	70	55.3	3.6	2.7
5	1	0	0	29	135	80	62.0	4.2	4.6
6	1	0	1	29	135	90	75.4	5.4	6.5
7	1	−1	−1	29	120	70	52.6	2.0	2.1
8	1	−1	0	29	120	80	62.6	2.6	2.7
9	1	−1	1	29	120	90	69.4	4.2	3.4
10	0	1	−1	24	150	70	56.6	4.2	4.4
11	0	1	0	24	150	80	64.0	6.2	7.1
12	0	1	1	24	150	90	75.4	9.4	9.8
13	0	0	−1	24	135	70	48.6	3.2	2.9
14	0	0	0	24	135	80	53.3	4.3	4.3
15	0	0	1	24	135	90	62.7	5.3	5.8
16	0	−1	−1	24	120	70	44.6	1.7	2.6
17	0	−1	0	24	120	80	42.0	2.9	2.7
18	0	−1	1	24	120	90	46.2	2.0	2.9
19	−1	1	−1	19	150	70	50.6	3.2	3.2
20	−1	1	0	19	150	80	53.2	5.1	5.5
21	−1	1	1	19	150	90	59.9	7.8	7.7
22	−1	0	−1	19	135	70	43.2	2.9	2.0
23	−1	0	0	19	135	80	43.9	2.6	3.0
24	−1	0	1	19	135	90	45.2	2.9	4.0
25	−1	−1	−1	19	120	70	35.9	1.4	2.0
26	−1	−1	0	19	120	80	35.2	2.2	1.8
27	−1	−1	1	19	120	90	39.2	2.3	1.5
28	0	0	0	24	135	80	59.8	4.8	4.3
29	0	0	0	24	135	80	60.0	4.6	4.3
30	0	0	0	24	135	80	56.3	4.3	4.3
31	0	0	0	24	135	80	52.6	4.9	4.3
32	0	0	0	24	135	80	51.9	4.0	4.3

[a] Experimental data.
[b] Predicted data.

of the *i*th factor. Interactions of two factors were also calculated by this equation. The general equation is a second degree polynomial:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i^2 + \sum \beta_{ij} X_{ij} + \varepsilon \quad (4)$$

where Y is the estimated response, β_0 is the general mean, $\Sigma\beta_i X_i$ is the sum of main effects of the factors, $\Sigma\beta_{ij}X_{ij}$ is the sum of two-factor interaction effects, $\sum \beta_{ij}X_i^2$ is the sum of quadratic effect of the factors and ε is the lack of fit of the model (error).

2.6. Effect of storage temperature on conidia survival

The microcapsules showing best PS_{sd} immediately after spray-drying for each of the different biopolymers matrices were stored at 4 °C and 29 °C. Percent survival of the microencapsulated conidia and of the non-encapsulated control (PS_s) was calculated as follows:

$$PS_s = \left(\frac{\text{CFU at storage time } t = t_i}{\text{CFU at storage time } t = 0}\right) \times 100 \quad (5)$$

The water activity (a_w) of the microcapsules stored at 4 °C and 29 °C was also determined at the same storage times as PS_s. A Sprint TH-500 Novasina a_w meter (Pfäffikon, Switzerland) was used for this purpose (calibration range a_w = 0.11–0.97).

2.7. Scanning electron microscopy (SEM)

Dried powders were attached to SEM stubs using a two-sided adhesive carbon tape and then were coated with gold-palladium using a Polaron SC7620 Sputter Coater (Sussex, UK). Microcapsules were observed with a high vacuum scanning electron microscope LEO Model 1450 (Helsingborg, Sweden) at 3 kV.

2.8. Quantification of CFU and total conidia

Quantification of total conidia was done by using a Neubauer chamber. The number of CFUs was determined by Flow Cytometry. Dried powders were rehydrated in glucose solution 20% (w/v) and centrifuged at 6000 × *g* for 5 min. The pellet was washed and re-suspended in phosphate buffer 50 mM (pH 5.6) and propidium iodide (Invitrogen, Carlsbad, CA) was added at a final concentration of 30 μM. After 10 min of staining, red fluorescence was measured in a Becton Dickinson FACSCalibur flow cytometer (NJ, USA). The survival percentage was obtained by an inverse correlation, previously obtained in our lab (unpublished data), between PI red fluorescence (damaged cell wall) and conidia viability determined by colonies count in PDA plates.

2.9. Quantification of reactive oxygen species (ROS)

The method reported by LeBel, Ischiropoulos, and Bondys (1992) was used to quantify the specific reactive oxygen species (ROS) concentration. This method uses 2′,7′-dichlorofluorescein diacetate (DCFH-DA), a non fluorescent molecule that in presence of ROS is oxidized to fluorescent dichlorofluorescein (DCFH), which is monitored spectrofluorometrically. DCFH was prepared by mixing 0.5 mL of 1.0 mM DCFH-DA (Sigma–Aldrich, Co. St. Louis, MO, USA) in methanol with 2.0 mL of 0.01 N NaOH. The deesterification of

DCFH-DA proceeded at room temperature for 30 min, and the mixture was then neutralized with 10 mL of 25 mM NaH_2PO_4, pH 7.4. This solution was kept on ice in the dark until use. A standard curve was generated with hydrogen peroxide (JT Baker, Phillipsburg, NJ, USA) in a range of 10–100 μM. Green fluorescence was monitored using a Perkin Elmer spectrofluorometer Model LS-55 (MA, USA), with an excitation wavelength of 504 nm (bandwidth 2.5 nm) and emission wavelength of 520 nm (bandwidth 3.7 nm).

Quantification of the specific ROS concentration for the microencapsulated conidias and non-microencapsulated control stored at 29 °C was performed with 5×10^8 cells suspended in 50 mM Tris buffer, pH 5.6. DCFH was added at a final concentration of 10 μM. Samples were incubated for 15 min at room temperature. The cells were disrupted with glass pearls, and the supernatant was used to monitor green fluorescence in order to quantify the specific ROS concentration.

3. Results and discussion

3.1. Effect of activation energy of the encapsulating matrices and of process temperatures on conidia survival immediately after spray-drying

This work evaluated the effect E_a of encapsulating matrix and spray-drying temperatures (inlet and outlet) upon the survival of *T. harzianum* conidias, a competent biological control agent, using a 3^3 experimental factorial design. Three biopolymers where chosen: maltodextrins (Dextrose Equivalents 10 and 20) and gum Arabic. These biopolymers were chosen as they are commonly used for microorganism encapsulation and because their values of E_a were suitable to perform the experimental factorial design as a quantitative variable.

After validation of the empiric second-order model, we can say that, the process temperatures and E_a had a positive significant effect on SI (F test, $p < 0.001$) as can be observed in Eq. (6) ($R^2 = 0.92$):

$$SI = 4.31 + 2.18T_i + 1.44T_o + 0.80E_a + 0.58(T_i)^2 + 0.51(E_a)^2 + 1.26T_iT_o + 0.32T_iE_a + 0.47T_oE_a \quad (6)$$

Pérez-Alonso et al. (2003) proposed that the E_a of carbohydrate polymers can be used as the selection criteria for their use as encapsulating agents. Activation energy (E_a) of the encapsulating matrix wall material provides a measure of the energy required for evaporating certain mass of water from the material to be dried. These authors suggested that materials with high E_a formed a dense and structured matrix around the core (bioactive ingredient or microorganism) that works as a barrier for thermal stress as well as limiting oxygen diffusion. However, under our experimental conditions, inlet and outlet temperature individual effects and their interaction had a higher influence than E_a (as shown by the empirical Eq. (6)) upon SI. The high positive effect of T_i and T_o on SI could be explained by the fact that at the highest process temperatures the dryer's feed flow increased (data not shown), which decreased the time that the conidia are exposed to heat, and reducing thermal stress. Nevertheless, the effect of E_a is statistically significant, which is reflected in the higher survival values obtained by using the polymer with higher E_a.

Fig. 1(a–c) shows the response surface of the SI as a function of E_a, inlet and outlet temperatures. From Fig. 1 and Table 1, it is clear that the encapsulation matrix that allowed the highest SI, as well as the highest PS_{sd} was MD10, which exhibited the highest E_a. MD20, which had the intermediate E_a followed, and GA which showed the lowest E_a, exhibited also the lowest survival increase. From Fig. 1 it is also evident that, regardless of the encapsulation matrix (E_a), the combination of the highest inlet and outlet drying

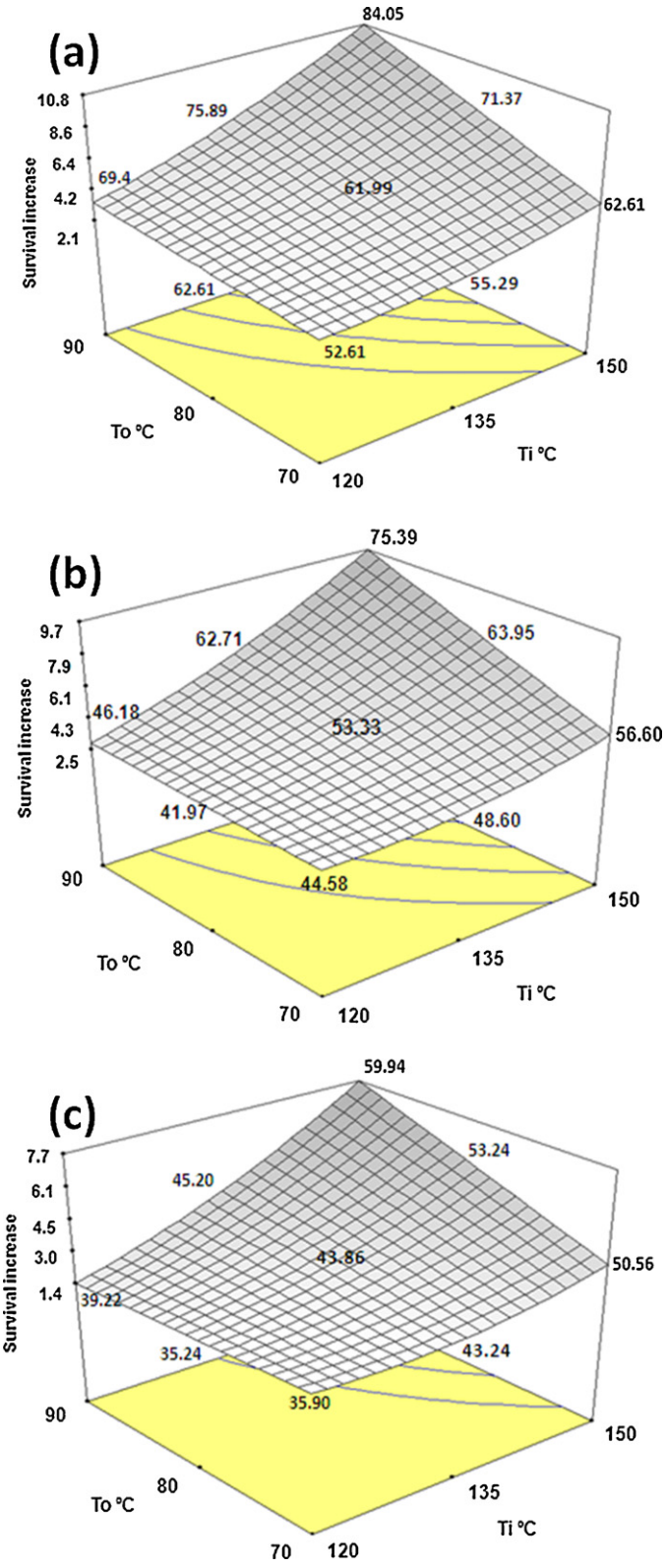

Fig. 1. Response surface graphs representing survival increase (SI) of *T. harzianum* conidia microencapsulated as a function of the inlet and outlet spray-drying temperatures using: (a) MD10 (E_a = 29.0 kJ mol^{-1}); (b) MD20 (E_a = 24.0 kJ mol^{-1}); (c) GA (E_a = 19.0 kJ mol^{-1}) as encapsulating matrices. Experimental SI values are shown for each point in the experimental design.

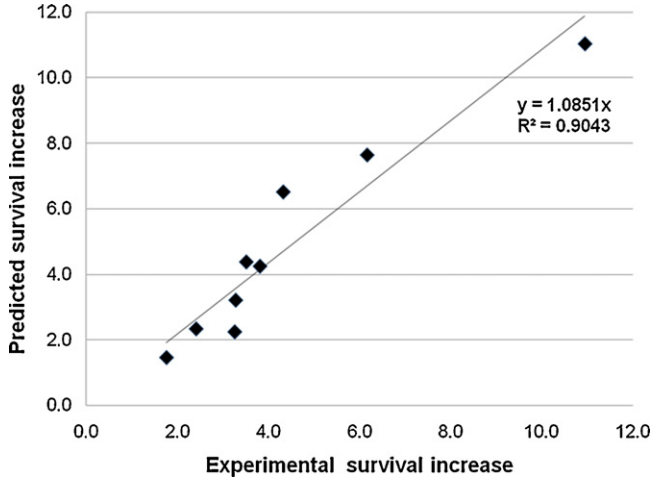

Fig. 2. Model validation through experimental vs. estimated values of the survival increase (SI) of a conidia formulation with 1:1 blend of MD10–GA (E_a = 31.6 kJ mol^{-1}).

temperatures was the condition leading to maximum *T. harzianum* PS_{sd}. These results suggest that at the lowest inlet drying temperature (120 °C), and independently of the E_a of the biopolymer used, the energy applied to the samples was not enough to produce a properly consolidated matrix.

In order to provide an additional validation of the model obtained by the factorial design, we used the experiments of the conidia formulated with the 1:1 (w/w) MD10–GA blend which exhibited the highest E_a (31.6 kJ mol^{-1}). The higher E_a displayed by this biopolymer blend was probably due to a synergistic effect occurring between both biopolymers, giving place to a continuous network structure more tightly interwoven than that resulting from either biopolymer on its own. As a result, water diffusion through the interfacial membrane is hindered to greater extent and a higher E_a is required. Fig. 2 shows the ability of the model to be extrapolated (predicted vs. experimental values) which resulted in a R^2 = 0.90 which is high enough to validate the model. Nevertheless, it is clear that under the best process temperature conditions, the use of MD10–GA blend (E_a = 31.6 kJ mol^{-1}) yielded a PS_{sd} of 86% (~11-fold survival increase). On the other hand, conidia formulated with GA (E_a = 19 kJ mol^{-1}) exhibited only a PS_{sd} of 60% (~7.7-fold survival increase).

Previous results of our group (Fernández-Sandoval et al., 2012) have shown that thermal stress (during spray-drying) and oxidative stress (during storage), are the main factors affecting *T. harzianum* conidia survival. Several reports (Guijarro, Larena, Melgarejo, & De Cal, 2006; Horaczek & Viernstein, 2004a, 2004b; Jin & Custis, 2011; Larena, De Cal, Liñán, & Melgarejo, 2003a; Larena, Melgarejo, & De Cal, 2003b) dealing with microencapsulation of fungal conidia show that this technique is very promising for its protection during spray-drying. The goal of encapsulation is to provide a microenvironment to the microorganisms, within a capsule, allowing them to survive during processing and storage (Weinbreck, Bodnár, & Marco, 2010). However, selection of encapsulating biopolymers is frequently based in trial and error experimentation. Our results are in line with those reported by Rodríguez-Huezo et al. (2007), as the biopolymers with the highest E_a provided a higher protection against cellular thermal damage, due to the formation of a dense structured matrix. It appears that higher T_i and T_o are necessary for a rapid formation of the microcapsules. Horaczek and Viernstein (2004a, 2004b) studied the microencapsulation of *B. brongniartii* and *M. anisopliae* conidia using skim milk and polyvinylpyrrolidone. They reported a 2-fold increase in the survival of spray dried conidia; however, after 6 weeks of storage at 30 °C the germination of the conidia ceased. Liu and Liu (2009a) reported that the encapsulation of *M. anisopliae* in an alginate–clay matrix, with dextrin and hydoxypropyl methyl cellulose as additives, yielded in 80% of germination after six months of storage. Larena, Melgarejo, et al. (2003b) evaluated the effect of spray-drying on conidia viability of *Penicillium oxalicum* when a blend of non-fat skim milk and sucrose were used as protective agents: the survival after drying was only 28%. Microencapsulation by spray drying of *Penicillium frequentans* using non fat skim milk as the encapsulating matrix at T_i = 150 °C, resulted in a survival of 28% (Guijarro et al., 2006). The same strategy was used to evaluate microencapsulation of *Epicoccum nigrum*, and the same trend was observed: survival after drying reached only 10% (Larena, De Cal, et al., 2003a). Only Jin and Custis (2011) have reported an experience of spray-drying microencapsulation of *T. harzianum* conidia. These authors reported a 3-fold relative increase of spore survival when compared with untreated samples using sucrose as the encapsulating matrix. We report a 11-fold relative conidia survival increase. The higher PS_{sd} can be explained by the use of biopolymers instead of a disaccharide allowing the formation of a more dense matrix.

Scanning electron microscopy (SEM) was used to evaluate the microcapsule formation. Fig. 3a shows the morphology of *T. harzianum* conidia dried without protective matrix. The average conidia size was 2–5 μm. GA, MD10 and MD10–GA formulations are presented in Fig. 3(b–d), respectively. These micrographs indicate that microcapsules with an average size of 15–20 μm were formed. MD10–GA formulation had a more dense and robust structure than that formed by GA alone which showed an irregular matrix, and probably causing as a result, that the formulations made with this biopolymer to display the lowest survival rates.

SEM micrographs of microencapsulated conidia showed differences in the encapsulating matrix morphology. It is evident that matrices possessing higher E_a formed smoother and bigger microcapsules (Fig. 3).

3.2. Influence of the encapsulating matrix E_a and storage temperatures on conidia survival

The values of a_w obtained for the different formulations after spray-drying ranged from 0.31 to 0.40, which was in the range previously found in our laboratory (Fernández-Sandoval et al., 2012) to be suitable for increasing the shelf life of dehydrated *T. harzianum* conidia.

The PS_s of non-encapsulated *T. harzianum* conidia decreased significantly after 4 weeks of storage (Figs. 4 and 5), regardless of the storage temperature (4 °C and 29 °C) used. However, when a protective matrix was used the conidia PS_s decreased only after 8 weeks of storage. Also a direct relationship between the PS_s and E_a of the biopolymer matrix was observed at both storage temperatures. The formulations with the MD10–GA matrix showed the highest PS_s. Regarding the effect of temperature, it is clear that samples stored at 4 °C retained higher viability that those stored at 29 °C for the same length of time. Nevertheless, in samples stored at 4 °C, the use of MD10–GA blend (E_a = 31.6 kJ mol^{-1}) yielded a PS_s of 40%, achieving ~330-fold on PS_s with respect to non-encapsulated conidia. On the other hand, conidia encapsulated with the same matrix and stored at 29 °C exhibited only a PS_s of 23%, increasing ~150-fold on PS_s.

PS_s decreased after 8 weeks for all the microencapsulated conidia, in a similar way to the decrease in PS_s after 4 weeks for the non-encapsulated control. These results seems to indicate that the biopolymer protective matrices retarded the diffusion of oxygen (at least for 8 weeks) into the microcapsules, limiting the amount of available oxygen for participating in the oxidation of macromolecules and causing oxidative stress.

Conidia viability during storage was higher in samples at 4 °C than at 29 °C, probably due to the higher reactivity and diffusivity

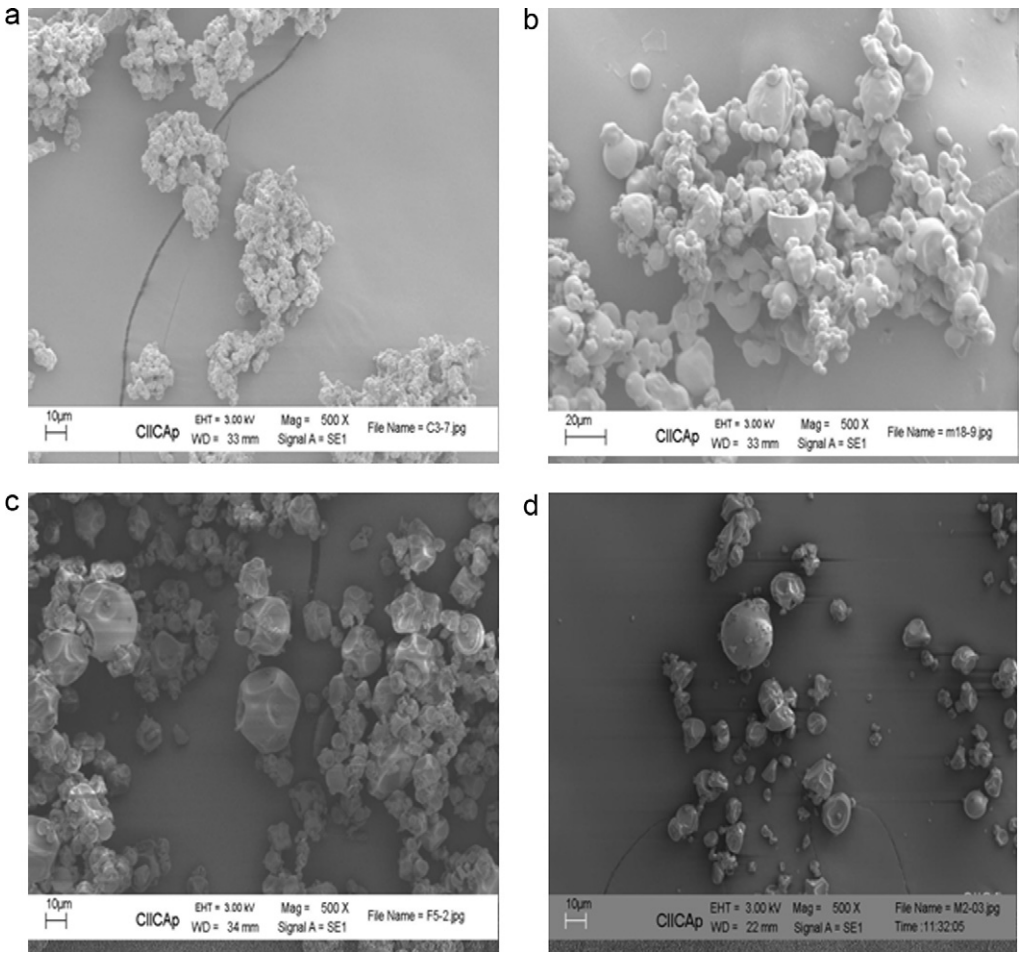

Fig. 3. Morphology of the particles of the *T. harzianum* conidia obtained by spray-drying with or without protective agent at T_i = 150 °C and T_o = 90 °C. (a) Free conidia, (b) GA microcapsules, (c) MD10 microcapsules and (d) MD10–GA microcapsules.

of intracellular ROS at the latter temperature. At 4 °C loss of cell viability after 8 weeks of storage was probably due to an increase in their metabolic activity, as the a_w increased from an initial value of 0.3–0.4 at storage time t = 0 to a value of 0.8–0.9 at t = 8 weeks. An increase in metabolic activity may be accompanied by nutrient limitation and/or oxidation of macromolecules. These results indicate control of the water activity at low storage temperatures should be considered from achieving higher survival rates.

3.3. Conidia survival as a function of intracellular ROS concentration

Table 2 and Fig. 6 show the relationship between conidia PS_s, stored for twelve weeks at 29 °C, and intracellular ROS concentration. Results are shown for the conidia microencapsulated in the MD10–GA matrix and for the non-encapsulated control. As expected, initial intracellular ROS concentration were

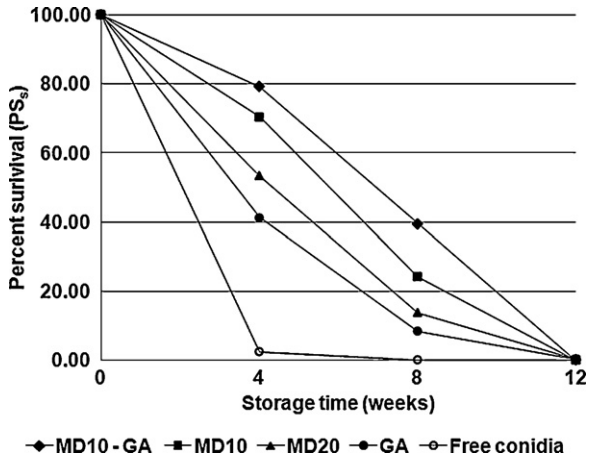

Fig. 4. Effect of activation energy on *T. harzianum* conidia percent survival (PS_s) during storage at 4 °C.

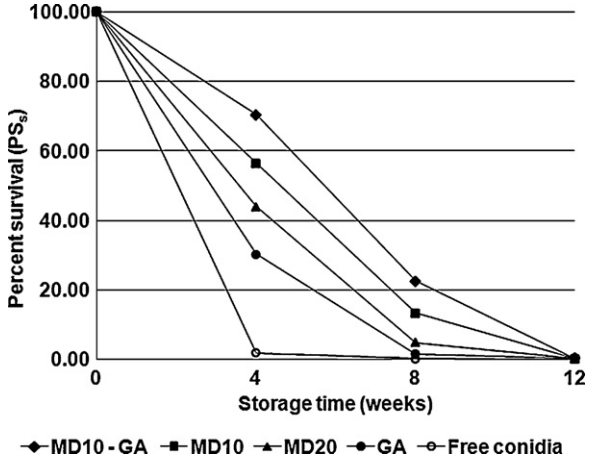

Fig. 5. Effect of activation energy on *T. harzianum* conidia percent survival (PS_s) during storage at 29 °C.

Table 2
Survival and ROS concentration of encapsulated (MD10–GA) and non encapsulated *T. harzianum spores* during storage al 29 °C.

t (week)	PS_s	nM ROS/10^6 cell
MD10–GA		
0	100	5.07 ± 1.28
4	69.8 ± 4.6	6.09 ± 2.30
8	22.5 ± 4.2	7.01 ± 1.64
12	1.32 ± 0.5	33.94 ± 2.53
Non encapsulated		
0	100	4.31 ± 1.21
4	6.1 ± 3.2	11.95 ± 1.76
8	3.8 ± 1.4	29.32 ± 1.69
12	1.8 ± 0.6	31.64 ± 2.19

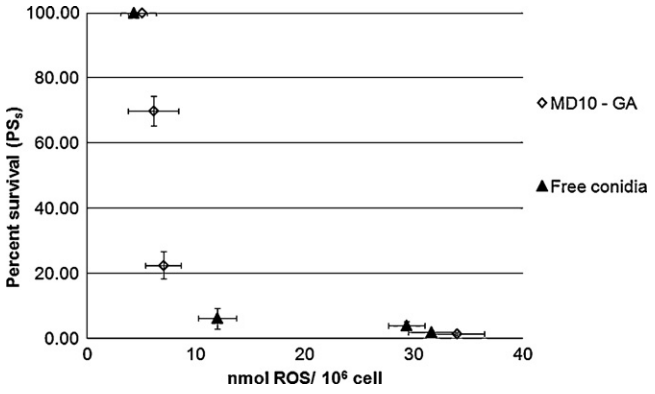

Fig. 6. *T. harzianum* conidia survival, stored at 29 °C, as a function of intracellular ROS concentration. (▲) Conidia microencapsulated on MD10–GA; (◇) Non encapsulated conidia.

similar for both samples. However, the ROS increase in non-encapsulated conidia was much faster than in microencapsulated conidia (Table 2). Nevertheless, when ROS concentration exceeded 7 nM ROS/10^6 conidia the survival of all the treatments was severely reduced (Fig. 6). Our results suggest that microencapsulation provides a protective barrier against oxygen diffusion towards the conidia reducing oxidative stress and increasing shelf life of *T. harzianum* conidia.

The evolution of conidia survival and intracellular ROS concentration seems to support the hypothesis that oxidative stress is the main cause of conidia death. Non-encapsulated conidia accumulated ROS at a faster rate than the encapsulated ones (Table 2). However, after 8 weeks, a critical intracellular ROS concentration (>6 nM ROS/10^6 cell) was arrived and survival of encapsulated conidia decrease significantly.

4. Conclusions

Activation energy of the encapsulating matrix and the drying temperatures are factors that should be taken in to consideration for enhancing the survival of *T. harzianum* conidia in spray-dried powders. In fact, the best survival (86%) was obtained for the 1:1 blend of maltodextrin–gum Arabic, which is a polymeric matrix with the highest E_a among those evaluated. During storage, a high E_a of encapsulating matrix and a low storage temperature yielded high spore survival up to 8 weeks of storage. Loss of viability after this period was probably due to oxidative stress at 29 °C, and to a combination of oxidative stress and increased metabolic activity at 4 °C. We demonstrated that the use of polymer carbohydrates with high E_a as encapsulation matrix prevent thermal stress and reduce oxidative stress during storage.

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