Spray-drying encapsulation of the nematicidal agent Tsukamurella paurometabola *C-924*

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RESEARCH

ABSTRACT

Tsukamurella paurometabola C-924 is a microorganism with proven nematicidal activity which has been used for the successful development of liquid and powdered formulations to be employed under greenhouse conditions. However, the current powdered formulation, although stable at 4 °C, needs to be improved in order to extend its shelf life at room temperature. This can be attained by methodologies such as the encapsulation of the microorganism into agents with high glass transition temperatures (e.g. starch, with a Tg of 50-60 °C at residual humidity values up to 15%). In this work we report for the first time the encapsulation of *T. paurometabola* C-924 into starch by spray-drying, as well as the results of accelerated stability studies on the resulting formulation. The procedure described here yielded desiccated C-924 cells with a high survival rate (over 60%). Although increasing the concentration of starch up to 10% (w/w) did not significantly influence the survival rate during the procedure, the evaluation of the stability of the resulting formulations at 26 °C revealed a 1.56-fold enhancement as compared to unencapsulated controls.

Keywords: encapsulation, nematicide, spray-drying, anhydrobiotic cells

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RESUMEN

Encapsulación del agente nematicida *Tsukamurella paurometabola* C-924, mediante secado por atomización. *Tsukamurella paurometabola* C-924 es un microorganismo cuya actividad nematicida ha sido demostrada. A partir de este agente nematicida, se han obtenido formulaciones líquidas y en polvo para su aplicación en casas de cultivo protegidas. La formulación en polvo, pese a ser estable a 4 °C, requiere un aumento de estabilidad a temperatura ambiente, lo cual podría lograrse mediante la adición de un agente encapsulante de alta temperatura de transición vítrea (Tg). Dentro de los agentes encapsulantes más utilizados, el almidón posee un alto valor de Tg (entre 50 y 60 °C, y para valores de humedad residual de hasta un 15%). En este artículo, se informa por vez primera la encapsulación de *T. paurometabola* C-924 en almidón, mediante la tecnología de secado por atomización, así como el estudio de estabilidad de dicho formulado, mediante el método de estabilidad acelerada (MEA). Como resultado, se obtuvieron células desecadas de C-924 con una alta tasa de supervivencia (mayor del 60%). El incremento en la concentración de almidón de hasta 10% (peso/peso), no influyó significativamente en la supervivencia durante el proceso de secado. En cambio, al evaluar la estabilidad de los formulados obtenidos, el almidón proporcionó un aumento significativo en los tiempos de vida útil de las células encapsuladas, las cuales resultaron 1.56 veces más estables a 26 °C que sus homólogas no encapsuladas.

Palabras clave: encapsulación, nematicida, secado por atomización, células anhidrobióticas

*I*ntroduction

Pests are one of the major causes of losses in agricultural crops and therefore in agricultural productivity in general. The use of chemical pesticides has currently declined, due mainly to their negative ecological impact and the inherent toxicological risks associated with the human consumption of foodstuffs which have come in contact, directly or indirectly, with these agents [1]. This situation has spawned a renewed interest in the use of biological products for pest control. Such bioproducts are based on the use of organisms or microorganisms that constitute natural enemies of the pest to be controlled (or metabolites or molecules thereof) as the active ingredients for the formulation [1]. This "biological approach" lends a more natural and healthy character to agricultural production and decreases its environmental impact and toxicity.

Nematodes constitute one of the most represented taxa within the number of organisms currently regarded as agricultural pests. The microorganism *Tsukamurella paurometabola*, strain C-924, is a bacterium with proven nematicidal activity [2, 3] which has been used as the basis for the development of biological nematicide formulations by researchers from the Center for Genetic Engineering and Biotechnology (CIGB). One such formulation, in powdered form for agricultural use, has a shelf life at 4 °C of 105 days and a half life of 330 days at the same temperature [4]; however, its shelf life at room temperature (26 °C) is only 5 days, which constitutes an operational limitation for using the product. One of the potential solutions for this problem is the use of encapsulating agents with high glass transition temperatures [5, 6], which decrease the rate of the degradation and oxidation reactions that take place inside anhydrobiotic cells [7].

Starch, with a high Tg (50 to 60 °C at residual humidity values up to 15%) is one of the most commonly used encapsulating agent. Therefore, the objective of this study was the obtention of anhydrobiotic C-

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924 cells with an improved shelf life at room temperature by encapsulation into starch by spray-drying.

Materials and methods

Propagation at scales up to 500 mL

In every case, the seed train was started from stocks of *T. paurometabola* C-294 conserved in 20% glycerol at -70 °C, which were streaked into LB-agar plates and incubated for 72 h at 37 °C. An isolated colony from a grown plate was selected and used for the subsequent propagation of the cellular culture.

The propagation medium SD was prepared according to Betancourt [8] using sucrose, $(NH4)_2SO_4$, NaCl, KCl, $MnCl_2$ x 4 H_2O, $MgSO_4$ x 7 H_2O and yeast extract. The prepared culture medium was dispensed into 50 mL-and 500 mL-Erlenmeyer flasks and sterilized.

Isolated colonies obtained as described above were picked and used for inoculating six 50 mL-Erlenmeyer flasks with SD medium (prepared as previously described), which were then incubated for 20 h at 37 °C in a shaker at 250 r.p.m. (New Brunswick Scientific Edison, New Jersey, USA). These starter cultures were then used to inoculate, respectively, six 500 mL Erlenmeyer flasks with SD medium, which were incubated for further 10 h at 37 °C.

Fermentation at 24 L scale

The fermentation was performed in SD medium according to Betancourt [8], at a controlled pH of 6.6. A 35 L fermentor with an effective volume of 24 L (B.E. Marubishi, Tokyo, Japan) was inoculated by means of a 5 L siphon. The operational parameters were 37 °C and 950 r.p.m., with a volumetric flow of air of 36 L/min (1.5 v.v.m.). The fermentation was performed for 24 h.

Dry weight measurement for the harvested biomass

The obtained biomass was centrifuged and the dry weight contents were measured by drying 1 g of wet biomass (in triplicate) to constant weight in a dry weight balance (the biomass was spread evenly on the plate of the balance until a monolayer was obtained).

Measurement of suspended solids in the culture supernatant

The concentration of solids suspended in the culture supernatant was determined by weighing (in triplicate) 1 mL of the supernatant and drying to constant weight on a dry weight balance.

Estimation of the volume of supernatant to be used for the creams

The volume of supernatant to be used for the resuspension of the biomass to a level of total suspended solids of 20% was estimated according to a previously determined equation [4]:

$$V = \frac{M_p}{(200 - C_s)} - \frac{3M_p}{1.07}$$

Where:

V: Volume of supernatant to be used for resuspending the biomass to be formulated (L).

Mp: Total mass of dry biomass (g) (Determined by multiplying the dry weight percentage by the weight of the wet biomass).

C_s: Total concentration of solids in the culture supernatant (g/L).

Preparation of the biomass for the spray-drying experiments

A 2^2 factorial design was implemented for the spray-drying experiments, using the concentration of starch and the outlet temperature as independent variables, and evaluating the effect on both variables in survival.

The two variants of cell cream used were prepared as follows:

Control cream: The biomass was resuspended into culture supernatant (previously diluted 1/10) to a dry weight percentage of 20%, using a blade impeller until the obtention of a homogeneous suspension. Sucrose was added to this suspension to a concentration of 20% (w/w of dry biomass).

Starch cream: The starch cream was prepared as described above for the control cream, but adding previously sterilized soluble starch to 10% (w/w of dry biomass).

Spray-drying of the biomass used for the formulation of Hebernem-S

The formulated cream was spray-dried on a Mobile Minor™ (Niro Atomizer, Denmark) atomizer. A double stream drying system was used, with rotational atomizing speed of 30000 rpm.

The inlet temperature was set at 130 °C, and the outlet temperature was set either at 55 or 65 °C, with feeding flows of 1.9 or 1.7 L/h, respectively. The formulated cream was divided into 1 L batches, and the process was stopped after each batch, collecting the remaining powder from the walls of the drying cone.

The obtained powder was manually homogenized and dispensed into three-layered polyester-aluminum-polyethylene bags (0.013 cm thick, with oxygen permeability of 0.001 cm^3/645 cm^2/24 h and permeability to steam of 0.0010 g/645 cm^2/24 h). The bags were sealed under vacuum on a TECNOVAC sealer (TECNOVAC, Italy).

Measurement of residual humidity on the powders

One gram of powder was weighed per triplicate and the residual humidity was determined by drying to constant weight on a dry weight balance, spreading the powder uniformly on the weighing plate.

Accelerated stability testing of the powders

In order to estimate the stability of the formulated powders, an accelerated stability test was performed. Bags containing samples of 5 g of powder (5 replicates) were incubated at different temperatures (26, 37 and 50 °C). The samples were prepared by the method of the fourths [4]; 10 g of the powder were placed on a Petri dish and split in 4 portions, then mixing and

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homogenizing the opposing portions in order to achieve a good representation of the obtained powder.

Random samplings were performed at different time intervals with the aim of determining the viability during the incubation in order to estimate the rate constants for cell death (k) at each temperature. The viability was measured by taking triplicate 0.1 g samples from each experimental condition and resuspending them to homogeneity in 10 mL of NaCl (9 g/L). These suspensions were then serially diluted down to a dilution factor of 10^{-8}, and 100 µL from each dilution were spread on the surface of LB agar plates, followed by a 72 h incubation at 28 °C. At the end of the incubation period, the colonies were counted, and the number of viable cells per mL of suspension was determined as the average of the colony-forming units (c.f.u.) for each plate multiplied by the dilution. In order to express the result as the number of viables per gram of powder, the obtained result was multiplied by 100 [multiplication by 10 taking into account the volume of solution used for the suspension, and division by 0.1 taking into account the weight of the sample (0.1 g)].

Measurement of suspensibility for the powders (with or without encapsulating agent)

Triplicate 1.5 g samples were taken and resuspended to homogeneity into 50 mL of distilled water. A 1 mL sample was immediately taken from the upper layer of the suspension and its dry weight contents were measured on a dry weight balance at 105 °C. The suspension was allowed to sit still for 1 h, and the sampling and subsequent analyses were repeated identically. Suspensiveness was defined as:

$$\% \text{ suspensiveness} = \frac{PSt_{1h}}{PSt_0} \times 100$$

Where:

PSt_0: Dry weight contents of the suspension at time zero

PSt_{1h}: Dry weight contents of the suspension after 1 hour

Statistical treatment of the experimental data

The analysis of the design was performed with the software application *Design-Expert*, version 6.0.1. The data were processed, obtaining the response surface and the equation that modeled the experimental behavior of the system. Goodness-of-fit to the model was estimated with Fisher's F test at alpha = 0.05. The influence of each variable was analyzed with a bifactorial ANOVA at alpha = 0.05.

The data from the accelerated stability studies were analyzed with the statistical software application *GraphPad Prism* 4.0. The survival rate for each time point was determined, defining Xv_0 as the concentration of viables at the start of the experiment and Xv as the concentration of viables at time t (both expressed as c.f.u. per gram), and the obtained values were transformed to their natural logarithms [ln(*S*)], calculating the mean for each point of the stability curve and its standard error (A minimum of 3 replicates per point were used in every case). The cell death constants were determined by plotting the means of the ln(*S*) values against the incubation time, and were then compared with Student's t-test at alpha = 0.05.

The stability of the powders was estimated by fitting the data to an Arrhenius model for bacterial cell death, plotting the natural logarithm of the cell death constants [ln(*k*)] against the inverse of absolute temperature (1/T). The cell death constants for 4 °C (277 K) were estimated by extrapolation to this value (*k*); and, assuming that cell death follows first-order kinetics, the shelf life of the product was calculated with the following equation:

$$T_{7/10} = -\frac{\ln 0.7}{k}$$

The suspensiveness values for the control and the sample encapsulated with 10% starch were compared by the Student's t test with an alpha of 0.05, transforming the data with the equation, and determining they followed a normal distribution by the Kolmogorov-Smirnov's test with an alpha of 0.05 (Statgraphics Plus 5.0).

$$y = \arcsin \sqrt{\frac{\% \text{ suspensibility}}{100}}$$

Results and discussion

Effect of the encapsulating agent and outlet temperature on the survival of *T. paurometabola* C-924

The results presented here constitute the first report in the international literature on the encapsulation of *T. paurometabola* strains. In order to evaluate the effect of the encapsulation and of the outlet temperature on the survival of spray-dried *T. paurometabola* C-924, a 2^2 factorial design was implemented. Figure 1 shows the response surface obtained after 12 experimental runs (4 experimental points per triplicate). There is a tendency towards a decrease in survival with increasing outlet temperatures. Upon statistical analysis, the following mathematical model for this tendency was obtained:

$$S = 186.16333 + 4.11200 * C(\text{starch}) - 1.66733 * Ts - 0.076933 * C(\text{starch}) * Ts$$

Where:

S: Survival (%).

C (starch): Starch concentration (% w/w of biomass)

Ts: Outlet temperature at the spray-drier

These results corroborate the existence of a significant effect for outlet temperature on the cellular survival of C-924 (P = 0.0013). On the other hand, although the coefficient for the concentration of starch was positive, it did not have a statistically significant effect on the response variable (P = 0.2719), and neither did the interaction of both factors (P = 0.3941).

The decrease in survival rates with increasing outlet temperatures is a result that has been previously reported for other bacterial species [6, 8, 9], and is

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related to the thermal denaturation of proteins and other biomolecules that play a relevant role in survival of the bacterial cell [10]. However, the absence of an effect for a starch concentration of 10% (w/w of dry biomass) does not coincide with previous results by To *et al.* [9], who found that an increase in the concentration of total solids tended to improve the survival of *Brevibacterium linens*, a microorganism which is phylogenetically close to *T. paurometabola*. On the other hand, Lian *et al.* [5] obtained decreased survival rates upon encapsulation by spray-drying of two *Bifidobacterium* strains when the concentration of soluble starch was raised to 10-30% (w/w of dry biomass).

This study used sucrose as a vitrifying agent [7, 11] both for the unencapsulated control cells and the encapsulated preparations; and these experimental evidences suggest that in *T. paurometabola* C-924 the protective effect of sucrose against rehydration is more relevant than the protection theoretically afforded by starch against high temperatures during desiccation. In other words, this strain is equally resistant to desiccation in the absence or presence of starch during spray-drying, at least in the 0-10% (w/w of dry biomass) range.

Additionally, it is important to notice that the *T. paurometabola* C-924 survival rates are higher than 60% [4], underlining the high tolerance of this strain towards the drying process. These survival rates are larger than those reported for other bacterial genera [5, 6, 12].

Physical properties of the obtained powders

Figure 2 shows the data from the residual moisture and suspensiveness assays. The residual moisture figures both for the control and encapsulated powders were relatively high (lower than 18%) in comparison with the values previously reported for this strain [4]; however, in all cases the obtained formulation was a fine powder, with better suspensiveness values (lower than 93%) than those reported for *Bacillus subtilis* strains [12]. While a residual moisture content higher than 10% [13] has a negative effect on the stability of the formulation, the fact that more than 93% of the powder does not sediment 1 hour after its resuspension in water is an operational advantage during application of the product in the field, since its homogeneity during aspersion into the ground is guaranteed.

The statistical analysis revealed no significant differences in suspensiveness among the formulations with or without starch ($P > 0.05$). The practical consequences of this finding are that a powder formulated with 10% (w/w of dry biomass) starch would not demand the introduction of new operational guidelines during the resuspension and application of the product in the field.

Accelerated stability of the powders

Once the survival rates were determined for the obtained formulations, they were subjected to an accelerated stability study. The study used powders stored in three-layered bags to block the diffusion of

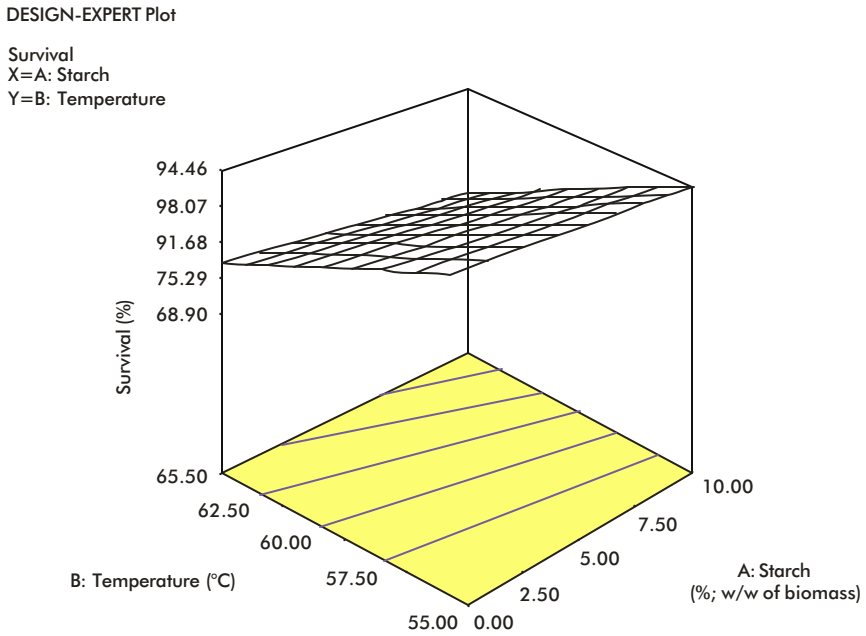

Figure 1. Response surface obtained from the evaluation of the effect of the starch concentration and outlet temperature on the survival of spray-dried Tsukamurella paurometabola C-924.

oxygen and water into the sample. In general, these tests are very useful from the point of view of Predictive Microbiology [14, 15], since they allow the prediction of the kinetic behavior of a system in terms of the rate of bacterial death at a specified temperature [16].

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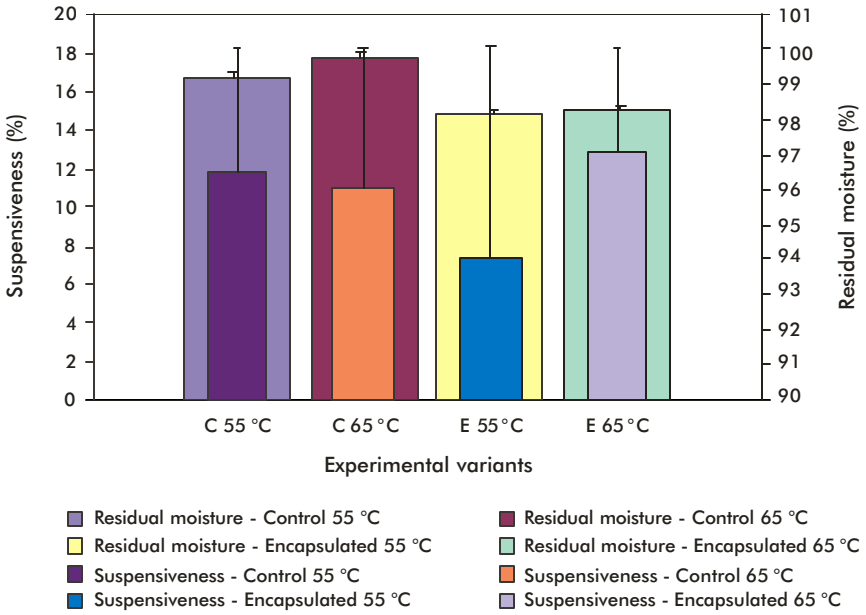

Figure 2. Residual moisture and suspensiveness values for the powders obtained from spray-dried *Tsukamurella paurometabola* C-924, with or without starch as encapsulating agent. The symbols used to represent the experimental variants are: C 55 °C - control biomass spray-dried at an outlet temperature of 55 °C; C 65 °C - control biomass spray-dried at an outlet temperature of 65 °C; E 55 °C - encapsulated biomass, spray-dried at an outlet temperature of 55 °C, and E 65 °C - encapsulated biomass, spray-dried at an outlet temperature of 65 °C.

Figure 3 shows the thermal death curves for spray-dried C-924. Cell death followed first-order kinetics in every case (the thermal death constants for each temperature are presented in Table 1). The presence of starch in the formulation resulted in a statistically significant ($P < 0.05$) decrease in the slopes of the curves, indicative of a slower rate for degradative reactions in the starch formulation relative to the control. Although this finding apparently suggests that the results obtained during drying and those from the stability study are mutually contradictory, they in fact reflect different phenomena. It all seems to indicate that the presence of a polymer, such as starch, during fast desiccation does not compete with the vitrifying effects of sucrose. However, the data show a long-term protective effect of starch on the already desiccated cells. According to some authors, the polymeric structure of starch very likely decreases the rate of some reactions that reduce the stability of anhydrobiotic cells [6], and this would explain our experimental results.

The kinetic behavior observed here is analogous to that obtained by Lian *et al.* [5], who obtained better protection for encapsulated *versus* non-encapsulated cells upon incubation in simulated gastric juice of *Bifidobacterium* encapsulated in soluble starch.

The *Arrhenius* diagram for the death constants determined experimentally is shown in Figure 4. The transformed values of the constants [ln(*k*)] plotted against the inverse of absolute temperature fitted well a straight line model, with high regression coefficients for both the control and encapsulated formulations. This implies that the degradation reactions only depend on the temperature (at least in the range studied in this work) [7], since a dependency on other factors such as molecular diffusion would result in a non-linear behavior, better described by William-Landen-Ferry kinetics [7].

The activation energies for the thermal death process were calculated for the control and the encapsulated formulations. The activation energy (E_a) estimated for the control was 26.4 kcal/mol and that estimated for the encapsulated formulation was 27 kcal/mol, indicating that the processes associated with thermal death are similar in both cases [15]. These E_a figures are similar to those published by Ziadi *et al.* [15] during the analysis of accelerated stability studies of two *Lactococcus* strains desiccated by lyophilization (an average of 20 kcal/mol for both).

Table 1 summarizes the kinetic parameters calculated from the data obtained during the accelerated stability study. The most important parameters for our purposes are the shelf life (which indicates the period of time, after manufacturing, during which the formulation maintains its biological activity and is fully functional) and the half life ($T_{1/2}$), defined as the time it takes for the biological activity of the preparation to decay to 50% of its starting level. Based on previous practical experience acquired during the use of liquid C-924 formulations in the field, the half life was defined as the period of time after manufacturing during which the biological activity of the cellular suspension (5×10^{11} cfu/mL) decays to no more than 70% of its starting value, that is, the shelf life was defined as the $T_{7/10}$.

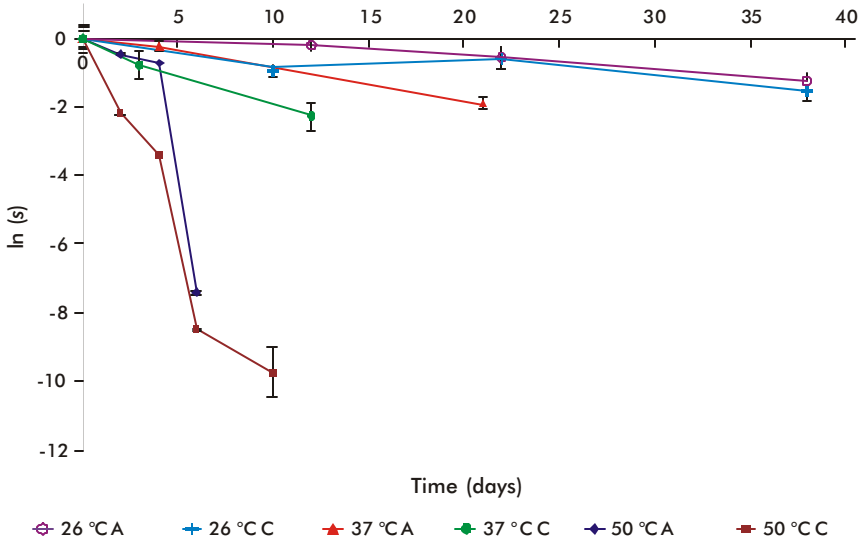

Figure 3. Thermal death of spray-dried Tsukamurella paurometabola C-924. The data shown correspond to the variability in survival at 3 temperatures (26, 37 and 50 °C). The survival values were transformed to their natural logarithms and used to fit a first-order model (ln(*s*) = -kt). A represents the formulation encapsulated in starch; C represents the unencapsulated control.

Table 1. Thermal death kinetic parameters for spray-dried *Tsukamurella paurometabola* C-924. Both formulations used 10% (w/w) sucrose as vitrifying agent. The table shows the half-life ($T_{1/2}$) and shelf life ($T_{7/10}$) respectively, as well as the thermal death constants

Temperature (°C)	Control (unencapsulated formulation)			Starch-encapsulated formulation		
	$T_{1/2}$ (days)	$T_{7/10}$ (days)	K (days-1)	$T_{1/2}$ (days)	$T_{7/10}$ (days)	K ($days^{-1}$)
4	573.9*	295.3*	$^{a}1.21 \times 10^{-3}$*	986.2*	507.5*	$^{e}7.03 \times 10^{-4}$*
26	16.8	8.7	$^{b}4.12 \times 10^{-2}$	26.4	13.6	$^{f}2.63 \times 10^{-2}$
37	3.5	1.8	$^{c}0.20$	5.2	2.7	$^{g}0.13$
50	0.6	0.3	$^{d}1.12$	0.9	0.5	$^{h}0.77$

* The parameters for 4 °C were estimated using the *Arrhenius* model derived from the thermal death constants.
The presence of differing letters to the left of the death constants denotes statistically significant differences ($P < 0.05$).

Upon examination of the values of the parameters defined above (estimated with the thermal death models for each temperature, as shown in Table 1), it is evident that $T_{7/10}$ and $T_{1/2}$ are superior for the encapsulated formulation, including, importantly, the $T_{7/10}$ at 26 °C (used as reference for room temperature). In other words, the encapsulated formulation is 1.57-fold more stable at room temperature than the control, thus confirming our working hypothesis.

A global analysis of the results indicates that the strategy of encapsulation is useful for improving the stability at room temperature, but further work should be aimed at decreasing the residual moisture contents of the obtained formulations, which are still too elevated in comparison to previous studies [4, 17]. The latter goal could, in turn, be achieved by careful set up of the inlet temperature during spray-drying, which is expected to decrease residual moisture. However, setting up this parameter requires additional and careful optimization in order to balance the beneficial effect of a diminished residual moisture with the potential decrease in cell survival to be expected from higher inlet temperatures [12].

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On the other hand, a comparison of the $T_{7/10}$ figures at 4 °C reveals a 1.7-fold higher value for the encapsulated formulation, which therefore has a shelf-life longer than 1 year at this temperature (1.39 years). This compares favorably with the stability of similar solid biopesticide formulations available in the international market, which have shelf lives no longer than 6 months at 4 °C [18].

Additionally, it should be added that in these experiments the stability of the control formulation at 4 and 26 °C was higher than that previously reported [4]. This phenomenon may be associated to changes in the fermentation process which, apparently, have improved the thermotolerance of strain C-924.

Conclusions

The obtention of anhydrobiotic *T. paurometabola* C-924 cells encapsulated in starch by spray-drying increases the survival rates (higher than 60%) independently of the concentration of the encapsulating agent (in the 0-10% range). However, the encapsulation in starch confers higher stability to desiccated C-924 cells during storage at room temperature.

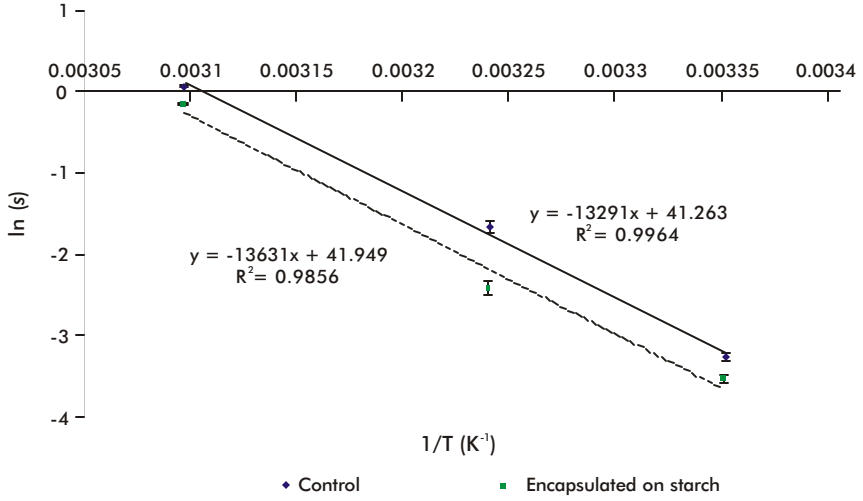

Figure 4. *Arrhenius* model for spray-dried *Tsukamurella paurometabola* C-924. The data shown correspond to the natural logarithms of the death constants vs. the reciprocal of the incubation temperature (26, 37 and 50 °C), both for the encapsulated and the control formulations.

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