REPORT

Anhydrobiotic cells of the nematocidic agent Tsukamurella paurometabola *C-924*

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ABSTRACT

Biological control of nematodes is a valuable alternative to the use of chemical nematicides in agriculture, because of the high negative impact generated by such chemicals in agroecosystems. From the Gram positive bacterium *Tsukamurella paurometabola* C-924, the bionematicide HeberNem-L™ has been developed, which is presented as a liquid formulation; nevertheless its stability is still low at 4 °C, with a shelf-life time of 6 months. One way to improve the stability of this bioproduct could be the desiccation of the cells, keeping the viability upon rehydration. Considering this aspect, the aim of our work was to obtain anhydrobiotic cells of the strain C-924 using freeze-drying and spray-drying technologies. One of the main results was the obtaining of anhydrobiotic cells of *T. paurometabola* C-924, with survival rates higher than 60%. In addition, it was shown that anhydrobiotic cells are more stable vacuum-stored at 4 °C, the rehydrated cells having nematicidal activity in field trials; therefore the powder formulation constitutes a good bionematocide candidate for agricultural use. Furthermore, a new methodology and also a mathematical model were developed to evaluate and predict the stability of desiccated bacterial cells.

Keywords: anhydrobiotic cells, nematicidal, anhydrobiosis, desiccation, spray-drying, freeze-drying

*I*ntroduction

Modern agriculture has been urged to increase the use of biocontrol agents to minimize the environmental damage of chemicals. Particularly, there are few reports on using bionematicides [1], but they mainly comprise liquid formulations of low stability and troublesome transportation due to the high volumes employed. To increase their stability, the induced anhydrobiosis process could be useful, because the cells of the biocontrol microorganism remain at a dormant state while the drying water activity decreases [2]. Hence, obtaining anhydrobiotic cells is an advantageous alternative to increase the stability of such bioproducts [3].

Anhydrobiotic cells can be obtained by using technologies such as freeze-drying and spray-drying [4]. Then, it is important to evaluate the desiccation tolerance of the bionematicidal agent and its stability at desiccation. The aim of this work was to evaluate the tolerance of the nematicidal agent *Tsukamurella paurometabola* C-924 to desiccation by freeze-drying and spray-drying procedures respectively, in order to obtain a stable formulation for biocontrolling phytopathogenic nematodes. In addition, the mathematical modelling of stability was performed in order to evaluate and predict the desiccated state in cells of *T. paurometabola* C-924.

*S*cientific novelty of results

For the first time in Cuban Science, anhydrobiotic cells were obtained from the nematicidic agent *T. paurometabola* C-924, with high survival rates (higher than 60%) when cells were desiccated using sucrose as vitrification agent by freeze-drying and spray-drying procedures, respectively. Additionally, a new general model was introduced to evaluate and predict bacterial stability at desiccation state.

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*R*esults and discussion

Obtaining anhydrobiotic cells from *T. paurometabola* C-924 by freeze-drying and spray-drying technologies

Survival rates of anhydrobiotic cells previously desiccated by freeze-drying are shown in figure 1. The statistical analysis after rehydration showed the following order in relevance for lyoprotectors: sucrose (10% (w/w) > mannitol (5% (w/w) > microcrystalline cellulose (5% (w/w); with significant differences compared to the control ($P < 0.05$). Interestingly, control cells lyophilized without mannitol or microcrystalline cellulose increased survival rate with either additive at 10% (w/w). This finding could derive from both

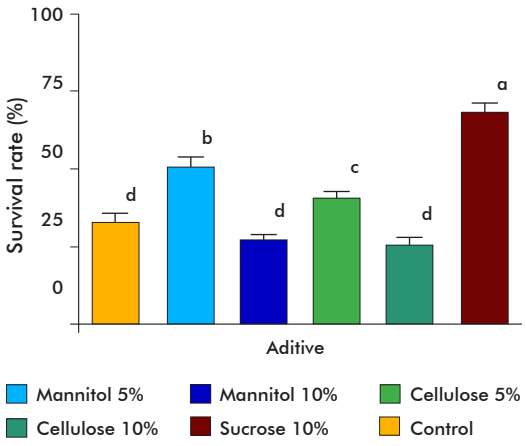

Figure 1. Survival rate percent after lyophilization of Tsukamurella paurometabola C-924 using several protectors. Survival rate data were transformed to $y = arcen\sqrt{\frac{S_p}{100}}$. The statistical analysis was performed according to Student-Newman-Keuls (n = 7).

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substances being crystalline, enhancing the crystallization process at higher concentrations during freeze-drying [5]. This likely accelerates protein denaturing [6, 7] and, consequently, cell death of C-924 cells.

Furthermore, the effect of desiccation by spray-drying on cells of *T. paurometabola* C-924 was evaluated. Figure 2 shows the response surface obtained by evaluating the effect of the output temperature and sucrose concentration on survival rates. From the trend analysis, it was observed that an increase in sucrose protected the cells better from thermal stress, which has been reported for other bacterial genera [8]. Otherwise, an increase in the output temperature negatively influenced cell survival. The mathematical model associated was:

Survival rate (%) = 57.19 - 3.12* (Sucrose %)^2 - 0.74* (T_{outlet}) (r^2 = 0.9992; F = 2118.82; P < 0.0001)

From this result, the selected experimental conditions were: T_{output} 62 °C, and sucrose concentration 10% (w/w). Using these conditions, survival rates higher than 80% were obtained. The cell membranes remained undamaged after desiccation, as evidenced by Transmission Electron Microscopy (Figure 3), contributing to cell survival after spray-drying.

Stability of anhydrobiotic cells from nematocidal agent *T. paurometabola* C-924

The evaluation of stability in desiccated cells is necessary to develop a commercially affordable formulation [3]. Moreover, prediction of such stability is relevant to estimate the shelf-life time at several temperatures in a time-consuming real-time assay.

In our experiments, the freeze-dried cells showed a linear behavior during thermal inactivation (data not shown). Otherwise, cells desiccated by spray-drying showed deviations away from the linear behavior, what led us to introduce a general model of thermal death: $S = ((m - 1)\ kt + 1)^{1/(1 - m)}$. This is a novelty in the field of Predictive Microbiology to analyze desiccated bacterial cells. In this model the parameter m is the death order and k is the rate constant (inactivation constant). In general, vaccum-stored cells were more stable than those stored without vacuum (Table 1). This may be explained based on the damage caused by reactive oxygen species in cells stored at atmospheric pressure [4, 9].

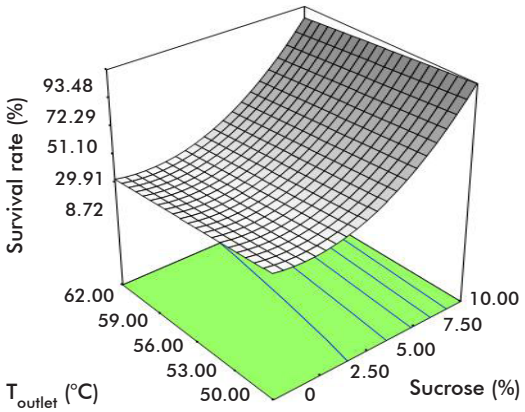

Figure 2. Response surface for the survival rate during the spray-drying of *Tsukamurella paurometabola* C-924. A 3-level factorial design was performed using sucrose as additive. Sucrose concentration was set between 0% and 10% (w/w). Outlet temperature was set between 50 °C and 62 °C. Data were analyzed with Design Expert 6.0.1.

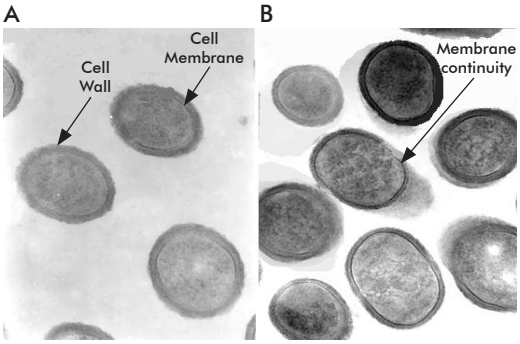

Figure 3. Electron micrographs of *Tsukamurella paurometabola* C-924 cells. A) Cells before the spray-drying process. B) Cells after desiccation by spray-drying. Bar = 100 nm.

When the real-time stability assay was performed (data not shown), no significant differences were found between the predicted inactivation constant at 4 °C $k^{est}_{4°C} = 4.5 \times 10^{-4}$ days^{-1} and the real-time constant $k^{real}_{4\ °C} = (4.6 \pm 0.35) \times 10^{-4}$ days^{-1} (P > 0.05). This finding makes robust the predictive methodology developed, by using the accelerated stability data to predict the shelf-life time of anhydrobiotic cells. In addition, the shelf-life time evaluated in real time was two years at 4 °C, making a stable formulation available, fulfilling international standards for biopesticides used to control phytopathogenic nematodes [3].

Biological activity of anhydrobiotic cells from *T. paurometabola* C-924

According to previous results, nematicidal activity of strain C-924 has been associated to the combined effect of chitinase and desulfurase activities [10]. Thus, 70% of the nematicidal activity corresponds to hydrogen sulfide production [10]. Taking this evidence into consideration, a new fast method was developed for indirect evaluation of biological activity, by measuring the desulfurase activity of anhydrobiotic cells [11]. When this method was applied to several drying experiments (n = 9), the mean desulfurase activity was 47.3 U·g^{-1}, similar to the value determined before drying.

Table 1. Kinetic parameters for the powders containing sucrose at 10% w/w. An accelerated test was performed. The normalized Survival was plotted (Survival (%)/100). All the data were fitted to the proposed model $S = ((m - 1)\ kt + 1)^{1/(1 - m)}$.

Condition of ASLT	Goodness of fit to the proposed model (R^2)	m (Order of reaction)	K (days^{-1})	Half life time (days)	Shelf life time (days)
4 °C under vacuum	0.9275	3.00 ± 1.4	(3.1 ± 1) x 10^{-2}	49	244
25 °C under vacuum	0.9666	1.63 ± 0.4	(7.4 ± 1.7) x 10^{-2}	12	29.3
32 °C under vacuum	0.9993	0.96 ± 0.06	(1.5 ± 0.6) x 10^{-1}	4.9	9
4 °C air	0.9834	2.42 ± 0.4	(1.6 ± 0.5) x 10^{-1}	7.3	27
25 °C air	0.9954	1.87 ± 0.2	(1.7 ± 0.3) x 10^{-1}	6.1	15
32 °C air	0.9955	1.24 ± 0.7	(5.1 ± 1.2) x 10^{-1}	1.5	3

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On the other hand, the nematicidal activity of C-924 anhydrobiotic cells was evaluated on banana plantations. The biocontrolling effect of the formulation on soil nematodes was evidenced, the nematicidic activity was increased due to the increment in the application dose from 1 to 2 kg·ha^{-1} (Figure 4). In this sense, the population of nematodes increased in 75% for the control plot within the first 5 days. Otherwise, the nematode population was reduced by 97% when 2 kg·ha^{-1} were applied. Moreover, the number of specimens was reduced by 80% during the same interval of time when 1 kg·ha^{-1} was applied.

This result indicates the nematicidal activity of C-924 anhydrobiotic cells, and provides enough evidence to recommend the use of such formulation as bionematicide in agronomical practices.

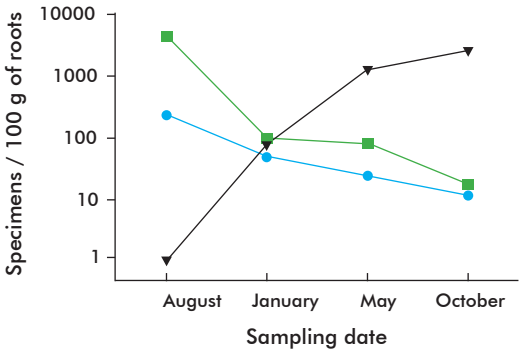

Figure 4. Nematicidic activity of *Tsukamurella paurometabola* C-924 desiccated by spray-drying. Plots of banana plantations were used to make the field trials. The formulation was applied in triplicate using doses of 1 kg·ha^{-1} (—●—); 2 kg·ha^{-1} (—■—) or control (—▼—).

Conclusions

T. paurometabola C-924 is tolerant to desiccation by freeze-drying and spray-drying procedures and using sucrose as protector, showing high survival rates (> 60%). Consequently, C-924 cells could be used as a model of an anhydrobiote. Concerning stability, C-924 anhydrobiotic cells are stable for two years at 4 °C, being biologically active after desiccation-rehydration. Consequently, this formulation could be used as a bioproduct in the control of phytopathogenic nematodes. Finally, it was possible to model the stability of the desiccated state in bacteria, using non-linear models; which allowed predicting viability in a bacterial system under desiccation stress.

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