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Characterization of the formulated cream and powder during the spray drying process of hebernem-s product

Caracterización de la crema formulada y del polvo durante el proceso de secado por aspersión del bionematicida hebernem-s

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ABSTRACT

Spray drying is increasingly investigated and widely applied in different industrial fields due to the diversity of the dried products that are obtained. In the present work, we determined the average diameter and area of *Tsukamurella paurometabola* C-924 cells contained in the formulated cream before the drying stage; the viability of the cream at different temperatures (10, 20 and 30 °C) and times (6, 15 and 24 hours); the diameter of HeberNem-S powder particles after spray drying; the stability of the HeberNem-S product at near ambient temperatures without applying vacuum conditions; and the adsorption isotherms of the HeberNem-S product. The area of the *T. paurometabola* cells had an average value of 3.154 µm^2, while the average diameter was 3.208 µm. The average diameter of the dehydrated powder particles of the HeberNem-S product was 39.203 µm. In the temperature range from 10 to 30 °C, storage time has a greater influence than temperature on survival, while the survival is less than 0.9 after 10 hours of storage. In order for the HeberNem-S product to meet the quality specifications, it must be packaged in an environment with relative moisture less than 60%.

Keywords: Scale up; HeberNem-S; Adsorption isotherms; Spray drying; *Tsukamurella paurometabola* C-924.

RESUMEN

El secado por aspersión es siendo progresivamente investigado y ampliamente aplicado en diferentes campos industriales debido a la diversidad de los productos secos que son obtenidos. En el presente trabajo determinamos el diámetro y el área promedio de células de *Tsukamurella paurometabola* C-924 contenidas en la crema formulada antes de la etapa de secado; la viabilidad de la crema a diferentes temperaturas (10, 20 y 30 ºC) y tiempos (6, 15 y 24 horas); el diámetro de las partículas de polvo de HeberNem-S después del secado por aspersión; la estabilidad del producto HeberNem-S a temperaturas cercanas al ambiente sin aplicar condiciones de vacío; y las isotermas de adsorción del producto HeberNem-S. El área de las células de *T. paurometabola* tuvieron un valor promedio de 3,154 µm^2,

mientras que el diámetro promedio fue de 3,208 µm. El diámetro promedio de las partículas de polvo deshidratadas del producto HeberNem-S fue de 39,203 µm. En el intervalo de temperatura desde 10 hasta 30 ºC, el tiempo de almacenamiento tiene una mayor influencia que la temperatura sobre la supervivencia, mientras que la supervivencia es menor de 0,9 después de las 10 horas de almacenamiento. Con el fin de que el producto HeberNem-S cumpla con las especificaciones de calidad, éste tiene que ser empacado en un ambiente con una humedad relativa menor de 60%.

Palabras claves: Escalado; HeberNem-S; Isotermas de adsorción; Secado por aspersión; *Tsukamurella paurometabola* C-924.

1. INTRODUCTION

Spray drying (or atomization) is a unit operation widely used in the production of high value-added products in the food, fertilizer, chemical, pharmaceutical and biotechnological industries (Schuck *et al*., 2016; Shishir & Chen, 2017; Cotabarren *et al*., 2018).

Spray drying allows the transformation of a feed from the fluid state to the dry particulate form by spraying the feed into a hot drying medium (Swati & Wagh, 2014). It constitutes a continuous particle drying operation, and provides a high flexibility with respect to the feed mixture because the liquid feed can be supplied in various forms, that is, as solutions, suspensions or emulsions (Bhonsale *et al*., 2019). The dried product can be in the form of powders, granules or agglomerates depending on the physical-chemical properties of the feed, the dryer design and the desired final properties of the powder (Swati & Wagh, 2014). The nature of the particulate product ranges from pure substances, solid mixtures, composite materials to viable biological components (Bhonsale *et al*., 2019).

Since it is a single-step continuous particle production process, it can be described as a robust technique that avoids a significant amount of manual handling of the dried product (Langrish *et al*., 2020). It can produce a final product with high quality and low water activity, and reduce its weight, thus facilitating its transportation and storage (Phisut, 2012).

Its field of application ranges from milk powder to very complex formulations of composite materials used in the area of medicine, as well as biological products for which only a few drying technologies are feasible (Bhonsale *et al*., 2019). In this way, spray drying is a cost-effective, continuous and relatively easy-to-operate process (Sosnik & Seremeta, 2015).

Spray drying consists of three stages: feed atomization, drying of the liquid droplets, and powder recovery. During atomization, the liquid feed is pumped through an atomizer into the drying chamber and distributed into tiny liquid particles in a large volume. Atomization maximizes the volumetric surface area of the liquid feed to increase the efficiency and effectiveness of drying (Shishir & Chen, 2017). Atomized droplets and hot air interact in a drying chamber, that is, small droplets are dispersed in a hot air medium offering large surface areas for mass and energy transfer, while ensuring short exposure of the product to high temperatures (Cheow *et al*., 2010; Fatnassi *et al*., 2013). The hot air increases the temperature of the drop, which leads to an increase in the evaporation of the water contained in the droplets. A dry layer develops on the surface of the drop as the moisture content of the drop reaches the critical point. The resulting powder particles can have oval or spherical shapes with a smooth or rough crust, depending on the product characteristics and the drying conditions (Tonon *et al*., 2008; Phisut, 2012; Caparino *et al*., 2012).

The hot air blown into the drying chamber can flow in various directions, such as parallel, countercurrent, and mixed flow. The parallel direction is preferable for heat sensitive compounds, in which the feed is passed through the atomization followed by the drying airflow path (150 - 220 ºC) and the final powder is

exposed to moderate temperatures (50 - 80 ºC), which limits its thermal degradation. Once the drying has finished, the dry particles are separated from the humid air by means of a cyclone, and recovered at the bottom of the cyclone through a collection container (Shishir & Chen, 2017).

The most commonly used commercial atomizers in industrial practice are rotary atomizers, pressure nozzles, and two-fluid nozzles. Pressure and two-fluid nozzles create tiny droplets with a larger volume distribution than rotary atomizers. In this way, they are preferred in applications where it is desired to obtain small particle sizes (Walters *et al*., 2014).

With proper calibration, spray drying provides relatively narrow particle size distributions in the nano- to micro-particle range, and good control over particle properties such as residual solvent content, morphology, and density. The main variables to be controlled during this operation include flow and temperature of the feed and drying air; concentration, surface tension, viscosity and density of the liquid feed; flow configuration and geometry of the atomizer. Any variation of these process conditions or in the properties of the liquid feed will therefore affect the properties of the powder obtained (Bhonsale *et al*., 2019).

Spray drying has been used to dehydrate a large number of feeds, ranging from sugarcane juice (Guzmán & Castaño, 2002; Díaz *et al*., 2007); concentrated juice of tamarillo (Yanza, 2003); green barley juice (García *et al*., 2004); aqueous extract of *Boerhaavia erecta* (López *et al*., 2008); pomegranate extract (Miravet, 2009); milk-juice blends and whole milk (Bahnasawy *et al*., 2010); calcium and magnesium citrate (Rodríguez *et al*., 2015); buffalo milk (Rodríguez, 2017); sodium hydroxide solution (Olufemi & Ayomoh, 2019); mixtures of blueberry juice-maltodextrin mixtures (Leyva *et al*., 2019) and fruit pulps (Salazar & Yunga, 2019).

Because of its reproducibility, mode of operation, high production capacity, and short product exposure to elevated processing temperatures, this process has become very popular in the biotech and pharmaceutical industries (Poozesh & Bilgili, 2019).

Specifically in the case of biological compounds and microorganisms, spray drying has been used in various applications, including proteases produced by submerged cultivation of the bacterium *Bacillus* sp. in presence of adjuvants (Pires *et al*., 2022), protein precipitates (Schmid, 2011), six biocontrol yeast strains (Asenjo, 2015), microencapsulated *Lactobacillus pentosus* (Hernández *et al*., 2018); the model probiotic *Lactobacillus rhamnosus* GG (Broeckx *et al*., 2019); *Lactobacillus kefir* CIDCA 8348, *Lactobacillus plantarum* CIDCA 83114 and *Saccharomyces lipolytica* CIDCA 812 isolated from kefir (Golowczyc *et al*., 2010); *Candida sake* CPA-1 yeast (Abadias *et al*., 2005); *Lactobacillus acidophilus* (Behboudi-Jobbehdar *et al*., 2013); *Lactobacillus reuteri* KUB-AC5 (Hamsupo *et al*., 2005; Jantzen *et al*., 2013) and *Bacillus clausii* (Vázquez *et al*., 2020).

According to (González, 2000), scale up is the process by which the successful startup and economic operation of a commercial-scale unit is achieved based, at least in part, on results of research carried out on a smaller scale. In order to apply the concept of scale up, it is essential that the design be implemented on the basis of investigations that have to be carried out on a scale smaller than the industrial one, but it is not required to go through all the conventional stages into which research and development processes are divided. The entire process, from laboratory to commercial scale, through bench, pilot and semi-industrial scale, is long and expensive and must be reduced as much as possible, in order to shorten the time between the conception of a product and its introduction into the market.

One of the biggest barriers to insert a spray-dried product in the market is scale up. Often, the biggest challenge is to obtain the same particle size and structure as attained at laboratory scale, due to the complex interactions between process conditions, product structure and properties (Ferreira *et al*., 2020).

Scale up in spray drying becomes difficult with respect to the high equipment costs at industrial scale; the need for new optimizations to calibrate the desired particle size distribution, as well as its morphology; to maintain reproducibility from laboratory to the industrial scale during the scale up process, while the yield/recovery of the particle could be limited by the type of collection container used (Ameri & Maa, 2006; Vehring, 2008). This leads to the development and use of design methods based on computer modeling to minimize trial and error procedures, as well as to save time and money, helping to improve the control over product properties (Grasmeijer *et al*., 2013; Keshani *et al*., 2015; Al-Khattawi *et al*., 2017). The practical experience and knowledge of the operator with respect to the scale up process, the product properties from pilot scale experiments and the influence of variables such as heat and mass transfer and moisture, are also very important factors for the successful scale up of spray dryers to the industrial scale (Al-Khattawi *et al*., 2017).

In the Center of Genetic Engineering and Biotechnology (CGEB) of Camagüey, an ecological product with bionematicidal action is produced in two commercial presentations: liquid (HeberNem-L) and solid (HeberNem-S), whose effectiveness in the control of nematodes was evaluated with good results, especially in protected cultivation houses (Mena *et al*., 2006). HeberNem is currently produced through a submerged fermentation process, where the active principle is the bacteria *Tsukamurella paurometabola* strain C-924 (Mena *et al*., 2003). However, it has the drawbacks of the large volume and weight it occupies when being stored, transported and exported, in addition to the fact that its stability in real time is limited to one year at 4 °C.

Due to the above and the need to satisfy the demand of the national and international markets, a powder formulation (HeberNem-S) (Hernández, 2009) was developed by introducing a spray dryer into the production process. HeberNem-S occupies less than 75% in volume than the liquid formulation, and its stability is extended to 2 years at 4 °C.

The drying process of HeberNem-L is the last step in the production of HeberNem-S. This product is obtained satisfactorily in compliance with international standards, but its low-scale production does not satisfy current market demand, so it is necessary to increase production levels. At present there is insufficient and limited theoretical-experimental information on the drying stage of HeberNem-L, which limits the scale up of the spray drying stage. In this way, the objective of this two-part study is to present a proposal for the scale up of the spray-drying operation of HeberNem bionematicide, supported by empirical, phenomenological and mathematical models that will contribute, in the future, to scale up this drying step to obtain higher production volumes of HeberNem-S, in order to satisfy the growing demand for this product. In this first part, the spray drying process to obtain HeberNem-S product is described; while the average diameter and area of *T. paurometabola* cells in the formulated cream before the drying step; the cream viability at different temperatures and times; the diameter of HeberNem-S powder particles after spray drying; the stability of the HeberNem-S product at near ambient temperatures without applying vacuum conditions; and finally the adsorption isotherms of the HeberNem-S product are determined.

2. MATERIALS AND METHODS

2.1. Description of the drying process of HeberNem bionematicide in its different stages

The drying process of HeberNem (*Tsukamurella paurometabola* C-924) consists of the following stages: the first stage comprises two essential operations, the cream formulation and preheating. The formulation of the cream is accomplished by mixing the biomass of *T. paurometabola*, which comes from a centrifugation step in the fermentation area, with sucrose and supernatant at 2% dry matter. Subsequently, it is slowly agitated for 12 h in a temperature range between 4 and 10 ºC in a jacketed blending tank (Hernández, 2009). This operation is performed to strengthen the organelles, the cell wall, and to reduce

damage of the microorganism during the drying process. The preheating of the formulated cream to be dried is carried out in a double tube heat exchanger, through which the formulated cream enters at 10 ºC and is preheated to 37 ºC using water at 56 ºC as the heating agent. At this stage, there are no reliable data available to characterize the cells of *T. paurometabola* C-924, such as the mean diameter and surface area, as well as the influence of temperature on its diameter and real time stability. This prevents a more rigorous analysis that might help to intensify and increase production.

Once the cream is preheated, the second stage of the process begins. The cream is transported into the drying chamber of the spray dryer by means of a screw-type pump, at a rate of 14±1 kg/h, which is specific for viscous substances and microorganisms (Potter *et al*., 2015). Inside the dryer, the formulated cream is atomized in a rotary atomizer at 25,000 rpm, forming a spray of particles in the order of 10 to 40 μm, which increases the air-droplet contact surface. The atomized droplets come into contact with a mass of hot air that is produced by an extractor, and heated by a liquefied gas burner, reaching a temperature of 130 ºC. There begins the drying of the droplet that occurs in two phases. In the first phase, all the water that is on the surface of the droplet is evaporated at a constant rate in a small period of time. In the second stage the water is extracted from the interior of the atomized particle at a decreasing mass transfer rate (Treybal, 1980; Masters, 1991).

The dried particle is dragged by the air flowrate to the cyclone where the physical separation of powder and air occurs, due to the difference in densities between them. The powder falls by gravity directly into a collection vessel while the air passes through a filter which guarantees that the powder particles that remain in it are not ejected to the atmosphere.

The collected powder is then manually packaged into bags of three-layer material with an amount of 250 g per bag. The particle diameter of the dry product is unknown, which influences its quality control. The larger the particle diameter, the smaller the contact surface, thus decreasing its effectiveness when applied to agricultural crops.

When the powder is transported from the dryer to the packaging section, it suffers practically irreversible damage. The moisture that the powder absorbs under the changing environmental conditions of the packaging room may be influencing this, directly affecting its stability and homogeneity, aspects that have not been studied in depth.

The fundamental equipment of this process is the parallel flow spray dryer (Niro, Anhydro). The product obtained in this process (HeberNem-S) meets the specific international requirements for a bionematicide (FAO, 2004), but its low-scale production does not satisfy market demand, so it is necessary to increase production levels and, with it, the capacity of the drying stage. This implies that research should be directed towards the study of the spray drying scale up.

2.2. Determination of the diameter and average surface area of *Tsukamurella paurometabola* C-924 cells in the formulated cream before the drying stage

This experiment was designed with the objective of evaluating, in the formulated cream, the size of *T. paurometabola* C-924 cells under different culture conditions. Both the average cell diameter and surface area were determined, in order to evaluate a potential pretreatment of the cells from the fermentation stage before drying.

For the development of this experiment the following methodology was carried out:

• Culture samples were taken from two production fermentation batches (52.1307 and 52.1310), covering three of the four cellular physiological stages, that is, lag, exponential and stationary phases. Samples of

the formulated cream were also taken before starting the drying process under different temperatures of the formulated cream (10, 20 and 28 ºC).

• For each sample taken, the Feret diameter (statistical diameter that depends on the orientation and shape of the particle, and represents the average distance between two parallel tangents with respect to the projected perimeter) and the area of the cells were measured using a graduated plate and an optical microscope with a digital camera (Carl ZEISS, AXIOSTAR Plus). The images were processed and analyzed with the Image J 1.36b program (Hernández, 2009).

• 10 mL of sample were taken in each fermentation batch, making dilutions (starting from 1/10 and finishing to 1/100) to guarantee isolated cells.

• 7 samples were taken for both fermentation batches according to the process stage (Tables 1 and 2), measuring 20 cells for each sample using the Image J 1.36b program, while their average value was determined using an Excel spreadsheet.

Table 1. Stages of the process in which the samples for batch 52.1307 were taken.

Sample	Description
1	4 hours after the start of fermentation, consuming yeast extract the cells.
2	At 16 hours of fermentation, after the start of sucrose consumption by the cells.
3	4 h before the end of fermentation, once the stationary phase has been reached, at hour 40 of fermentation.
4	At the end of the fermentation, approximately at the hour 44.
5	Minutes before starting the drying process, approximately 24 hours after formulating the cream at a cream temperature of 10 ºC.
6	Moments before starting the drying process, approximately 24 hours after formulating the cream at a cream temperature of 20 ºC.
7	Moments before starting the drying process, approximately 24 hours after formulating the cream at a cream temperature of 28 ºC.

Source: Own elaboration.

Table 2. Stages of the process in which the samples for batch 52.1310 were taken.

Sample	Description
1	4 hours after the start of fermentation, consuming yeast extract the cells.
2	At 28 hours of fermentation, after sucrose consumption by the cells started.
3	In the stationary phase of fermentation, at hour 56 of fermentation.
4	Before making the formulation.
5	Moments before starting the drying process, approximately 24 hours after formulating the cream at a cream temperature of 10 ºC.
6	Moments before starting the drying process, approximately 24 hours after formulating the cream at a cream temperature of 20 ºC.
7	Moments before starting the drying process, approximately 24 hours after formulating the cream at a cream temperature of 28 ºC.

Source: Own elaboration.

2.3. Determination of the viability of the cream at different temperatures and times

This experiment was carried out with the objective of identifying the influence of temperature (10, 20 and 30 °C) and time (6, 15 and 24 hours) on the formulated the cream (that is, the influence on its viability).
1.5 L of the biomass obtained at the end of the fermentation batch of *T. paurometabola* C-924 was taken, and formulated taking into account the procedure established for HeberNem-S (CIGB, 2015). The five samples to be studied were placed in five 50 mL Erlenmeyer flasks covered with aluminum foil. Three replicates of the experiment were carried out.

To adjust and control the temperature of the cream, two cryostats (Julabo F 32) were used for the temperatures of 10 ºC and 20 ºC, while a shaken temperature-controlled incubator (New Brunswick Scientific C-25) was employed for the temperature of 30 ºC.

The experiment was designed in Design Expert 6.0 software. A response surface experimental design was elaborated to determine at what temperature and time the viability values begin to decrease and therefore obtain an optimal control range for both variables in relation to the viability in the formulation stage prior to the spray drying procedure. The experimental design was carried out in duplicate.
The viability of the different samples was performed by counting on plates using the drop plate method (Herigstad *et al*., 2001). Survival was determined as the ratio between the viability of each sample and the viability at the beginning of the experiment. For values greater than 1, the condition of equaling it to unity was established.

2.4. Determination of the diameter of the powder particles of spray-dried HeberNem-S

The mean diameter of the powder particles of batch 52S.1303 was determined. This parameter is necessary for the calculation of the drying time. Two replicates were made following the protocol shown below:

- A 1 g sample of the powder was taken.
- The solid sample was spread on a slide.
- Photos were taken under an optical microscope connected to a digital camera (Carl ZEISS, AXIOSTAR Plus).

Images were analyzed using the Image J 1.36b program by means of a graduated plate and a microscope.

2.5. Determination of the stability of the HeberNem-S product at temperatures close to ambient and without considering vacuum

This experiment was carried out with the objective of evaluating the behavior of the viability parameter at temperatures of 16, 28 and 37 ºC without applying vacuum conditions, to observe if it is possible for the product to be homogeneous during the packaging stage on a scale greater than the current one.

- A study was carried out on the powder collected after drying at temperatures of 16, 28 and 37 °C with a time of 7 days between one sample and the other.
- A total of 6 samples were processed in each replicate for a study time of 42 days.
- 10 g of HeberNem-S was taken from two batches produced and packaged in bags of the same material in which the product is packaged for commercialization.
- The bags contained half of their maximum capacity.

The experiment was designed in the Statgraphics Plus 5.1 program, in which an response surface experimental design was elaborated to determine at what temperature and time the viability values begin to decrease and, consequently, obtain an optimal control range for both variables, in relation to the

viability in the packaging stage carried out without vacuum. Two replicates were made for the experimental design. The viability of the different samples was determined by the drop plate method (Herigstad *et al*., 2001). Survival was determined as the ratio between the viability of each sample and the viability at the beginning of the experiment.

2.6. Determination of HeberNem-S adsorption isotherms

This experiment was designed to determine the product moisture under different temperature (10, 20, 28, 37, 50 and 60 ºC) and relative moisture of the room values, by obtaining adsorption isotherms.

The determination of the adsorption isotherms of HeberNem-S was carried out applying the static gravimetric method (Lutovska *et al*., 2017), which is a relatively simple method that consists of placing small amounts of sample of the studied substance in a container, in the interior of which the relative moisture of the air and the temperature are kept constant. When the equilibrium between the moisture percentage in the sample and the atmosphere of the container in which it is included has been established, the weight of the sample stops varying. Thus, a point of the equilibrium isotherm is obtained in which the equilibrium moisture has been reached (Pita & Rojas, 2001). Drying agents are used to achieve constant air moisture. These can be saturated saline solutions of mineral components, molten or granular calcium chloride, phosphorus pentoxide, magnesium perchlorate, caustic soda or sulfuric acid solutions at certain concentrations.

Table 3 shows the saline solutions used in this study. These salts were dissolved until reaching a saturated saline solution. The solution was allowed to settle for 2 days to reach equilibrium. After this time, six closed glass containers containing 5 g of the powdered product (HeberNem-S) each were placed.

Table 3. Chemical components and relative moisture used to obtain the adsorption isotherms of HeberNem-S.

Chemical component	Relative moisture (%)
H_2O	100
KCl	82
$MgCl_2$	69
NaOH	42

Source: Own elaboration

After 5 days, the equilibrium was established between the moisture percentage of the sample and the atmosphere of the container. Then 1 g of the sample was taken per container and weighed on a laboratory weighing scale (Sartorius, MA35). Thus, a point of the isotherm was obtained in which equilibrium moisture was reached (Pita & Rojas, 2001).

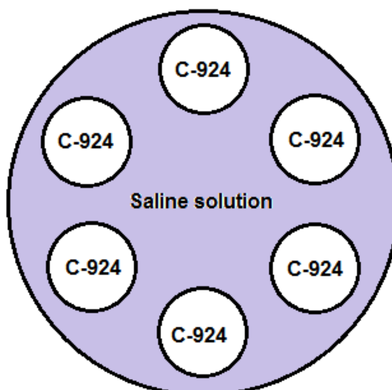

Figure 1. Container loading scheme for the determination of HeberNem-S adsorption isotherms.
Source: Own elaboration.

3. RESULTS AND DISCUSSION

3.1. Determination of the average diameter and area of *Tsukamurella paurometabola* cells in the formulated cream before the drying stage

Tables 4 and 5 show the results of the area and diameter of *T. paurometabola* cells for the two fermentation batches carried out (52.1307 and 52.1310).

Table 4. Results of area and diameter of *T. paurometabola* cells for batch 52.1307.

Sample	Area (µm^2)	Standard deviation	Diameter (µm)	Standard deviation
1	4.487	1.180	3.624	0.869
2	3.408	1.118	2.249	0.532
3	2.871	0.572	2.530	0.423
4	3.014	0.949	2.773	0.604
5	1.844	0.598	2.113	0.330
6	1.821	0.733	2.329	0.707
7	2.165	0.658	2.367	0.525

Source: Own elaboration.

According to the results shown in Table 4, the highest cell area value was obtained in sample 1, with 4.487 µm^2, while the lowest value of this parameter corresponded to sample 6 (1.821 µm^2). Regarding cell diameter, sample 1 presented the highest value of this parameter, with 3.624 µm, while the lowest value was obtained in sample 5 (2.113 µm). Both parameters decrease after 4 hours of fermentation, reaching minimum values in the formulation stage.

Table 5. Results of area and diameter of *T. paurometabola* cells for batch 52.1310.

Sample	Area (µm^2)	Standard deviation	Diameter (µm)	Standard deviation
1	4.446	0.587	4.302	0.678
2	2.496	1.00	2.318	0.532
3	4.481	0.650	3.708	0.406

4	3.321	0.702	3.125	0.606
5	2.892	0.501	2.722	0.398
6	2.781	0.598	2.651	0.448
7	3.063	0.708	2.788	0.532

Source: Own elaboration.

Table 5 shows that the highest value of the cell area corresponded again to sample 1 (4.446 µm^2), while sample 2 presented the lowest value of this parameter with 2.496 µm^2. Correspondingly, the highest value of cell diameter corresponded to sample 1 (4.302 µm), as was the case in batch 52.1307, while the lowest value of this parameter was obtained in sample 2, with 2.318 µm. As also occurred in batch 52.1307, both parameters decrease after 4 hours of fermentation to reach minimum values in the formulation stage. The authors of this study consider that in batch 52.1310 the cells experienced some type of stress during the fermentation stage corresponding to sample 2 (28 hours of fermentation, after the sucrose consumption by the microorganism began), given the low values of cell area and diameter obtained at this point.

In general, the cell area was within the range of 1.821-4.487 µm^2 (average: 3.154 µm^2), while for the diameter their values ranged between 2.113-4.302 µm (average: 3.208 µm) in the different stages considered. Thus a separation by filtration should be carried out using membranes with a pore diameter smaller than 2 µm, to ensure a high retention efficiency, membranes with a pore diameter of less than 1 µm should be used.

It can also be noted that the area of the cells is practically invariable in the formulated cream at the three temperatures evaluated, which suggests that the intracellular water content is practically constant at the end of fermentation and during formulation. These results indicate that the formulation process does not lead to a decrease in intracellular volume, whose content is mainly water, so the addition of some osmoregulatory compounds to the formulated cream should be considered to favor the drying process.

3.2. Viability of the cream at different temperatures and times

The viability of the cells in the first replicate (batch 52S.1301) is shown in Table 6, while the viability in the second replicate (batch 52S.1302) is shown in Table 7. Samples RHN-13034 and RHN-13049 were not determined due to contamination issues. Viability values greater than 1 were equalized to the unity due to system restrictions.

Table 6. Design of experiment and results of cream viability for drying batch 52S.1301 (first replicate).

Temperature (°C)	Time (h)	Sample code	Survival
10	6	RHN-13028	1.00
20	6	RHN-13029	1.00
30	6	RHN-13030	1.00
30	6	RHN-13031	1.00
10	15	RHN-13032	0.85
20	15	RHN-13033	0.88
30	15	RHN-13034	Contaminated
10	24	RHN-13035	0.83
10	24	RHN-13036	0.71
20	24	RHN-13037	0.93
30	24	RHN-13038	0.72
30	24	RHN-13039	0,73

Source: Own elaboration.

Table 7. Design of experiment and results of cream viability for drying batch 52S.1302 (second replicate).

Temperature (°C)	Time (h)	Sample code	Survival
10	6	RHN-13041	1.00
20	6	RHN-13042	0.94
30	6	RHN-13043	0.83
30	6	RHN-13044	1.00
10	15	RHN-13045	0.84
20	15	RHN-13046	0.64
30	15	RHN-13047	0.91
10	24	RHN-13048	0.93
10	24	RHN-13049	Contaminated
20	24	RHN-13050	0.76
30	24	RHN-13051	0.76
30	24	RHN-13052	0.78

Source: Own elaboration.

The compiled data was processed with software Design Expert 6.0, through which a first-order mathematical model was obtained:

$$\arcsin(sqrt(Sup)) = 1{,}42538 - 0{,}013504 \cdot Time - 9{,}32159E - 0{,}04 \cdot Temperature \quad (1)$$

In the response surface graph (Figure 2) it can be observed that viability decreases with time and that the temperature factor influences too, although to a lesser extent, than time in the interval studied. This coincides with a report by (Hernández *et al*., 2008), which indicated that the cells of *T. paurometabola* C-924 develop thermotolerance when treated at sublethal temperatures, specifically above 45 ºC.

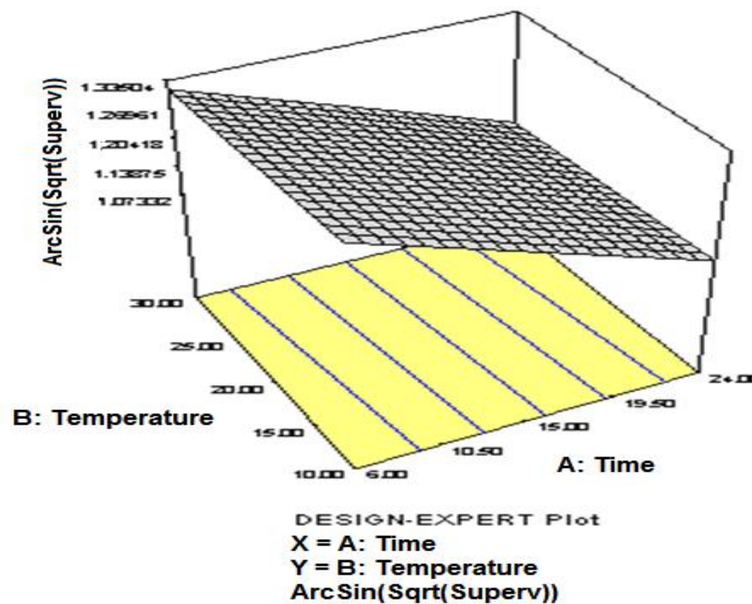

Figure 2. Response surface graph obtained by Design Expert 6.0.
Source: Own elaboration.

Taking into account that it is desired to maintain a high survival rate to reduce process losses, that is, to keep viability between 0.9 and 1, the strength must be greater than 1.25×10^{12} cfu/mL according to product specifications (CIGB, 1992). Survival was determined using the mathematical model shown above and the

analysis was performed at different conditions of temperature and time. Considering this, a graph was obtained using the software Design Expert Plot (Figure 3), in which the blue line represents the border for the survival value of 0.9, while to the right of the border survival values of less than 0.9 are found.

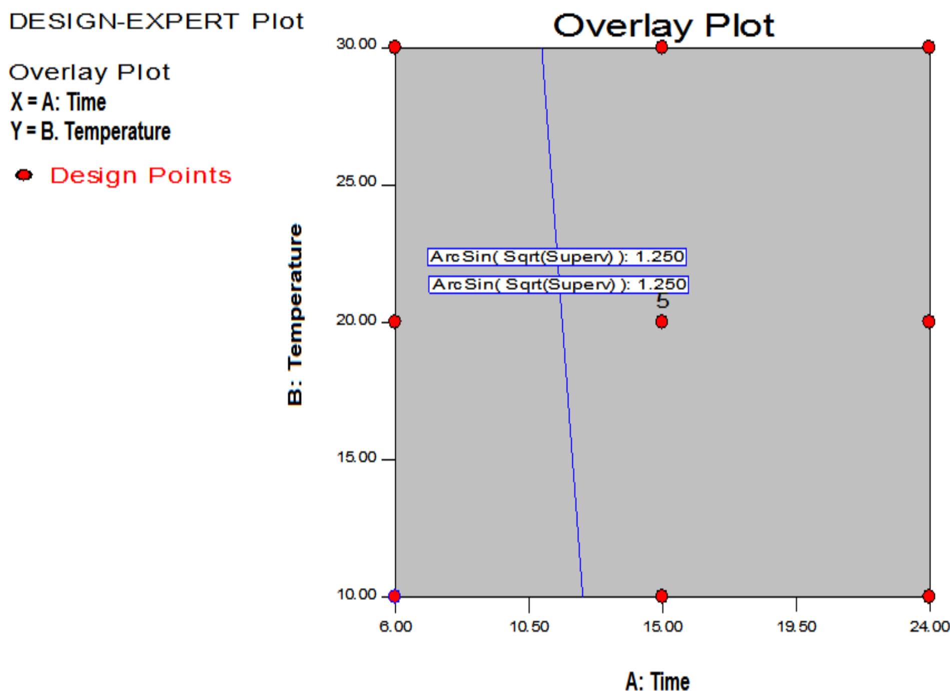

Figure 3. Survival border graph for the value of 0.9 obtained by Design Expert 6.0.
Source: Own elaboration.

It can be seen that, for the temperature range studied (10 to 30 °C), survival is less than 0.9 approximately after 10 h of storage, which implies that the microorganism begins to lose properties after this time. As such, additional studies should be carried out at temperatures below 10 °C and considering times greater than 10 h, in order to evaluate the response of the system over a wider range of these variables.

3.3. Diameter of the powder particles of the spray-dried HeberNem-S

Table 8 shows the results provided by the ImageJ software regarding the mean diameter of the HeberNem-S powder particles.

Table 8. Results of the mean diameter of HeberNem-S powder particles for drying batch 52S.1303.

Number	Diameter (µm)	Number	Diameter (µm)	Number	Diameter (µm)
1	52.27	11	44.44	21	42.22
2	33.48	12	50.00	22	33.33
3	49.17	13	40.28	23	30.56
4	30.08	14	36.11	24	37.50
5	45.92	15	31.94	25	38.89
6	33.48	16	35.56	26	35.28
7	33.08	17	34.72	27	47.22
8	41.67	18	36.11	28	38.89

9	36.11	19	40.28	29	38.33
10	40.28	20	47.22	30	41.67
Mean					39.203
Standard deviation					5.994

Source: Own elaboration.

The data obtained were processed in the Statgraphics Plus 5.1 program, resulting in the following statistical summary:

• Samples = 30.
• Mean diameter = 39.203 µm.
• Standard deviation = 5.99423 µm.
• Minimum = 30.08 µm.
• Maximum = 52.27 µm.

The average diameter of the dehydrated powder particles of HeberNem-S product was equal to 39.203 µm, with a standard deviation of 5.99423.

In (Hernández *et al*., 2018) microcapsules of *Lactobacillus pentosus* bacterium, characteristic of those produced by spray drying, were obtained, where some concavities on the surface were found as a result of the rapid evaporation of water. In this study it was estimated that the average size of the microparticles was below 20 µm.

In (Behboudi-Jobbehdar *et al*., 2013) a mean diameter of 10.96 ± 0.63 µm was obtained for microcapsules of *Lactobacillus acidophilus* bacterium, which is comparable with the values reported in the case of other probiotic formulations produced by spray drying (10-20 µm).

According to (Jantzen *et al*., 2013), the average diameter of the *Lactobacillus reuteri* bacterium was 5.5 ± 0.3 µm and 4.9 ± 0.3 µm for an outlet temperature of the drying process of 55 and 65 ºC, respectively.

3.4. HeberNem-S product stability at temperatures near ambient without applying vacuum

Storage conditions (moisture, temperature, etc.) can significantly affect the stability of dehydrated products. There are very few studies related to the stability of cells, because in most cases the useful life times are estimated by researchers working with private companies, and the results are considered sensitive information and, therefore, a trade secret. Regarding the monitoring of the state of anhydrobiosis in bacteria, there are only limited reports. Most authors describe the stability of anhydrobiotic cells using first-order models (Achour et al., 2001; Ananta *et al*., 2005; Ziadi *et al*., 2005; França *et al*., 2007). However, each microorganism has its own particularities and the patterns of thermal death can be different for dried cells and cells in liquid suspension.

Figure 4 shows the behavior of the viability of HeberNem-S for the 2 batches studied under three temperatures and for 42 days without applying vacuum.

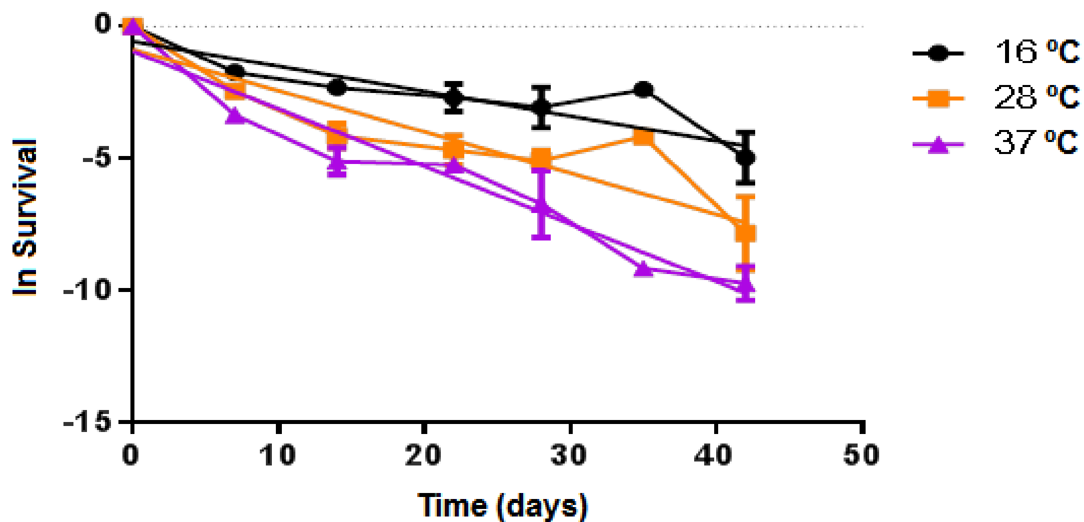

Figure 4. Behavior of the viability of two batches of HeberNem-S under three temperatures without applying a vacuum for 42 days.
Source: Own elaboration.

The first order models used to evaluate the stability of desiccated cells, as in the case under study, describe the thermal degradation and oxidation mechanisms that occur inside the cells when subjected to periods of shelf life or stability. In the kinetics described in this experiment, it is observed that, in the first 7 days at the different temperatures evaluated, a significant decrease in survival occurs, which does not coincide with that reported by (Hernández *et al*., 2008) in accelerated stability studies of the same product, which may be related to the presence of oxygen.

In the case of the curve obtained for the survival temperature of 16 ºC, it is observed that cell death tends to change its slope after the first 7 days, which may be related to the decrease in oxygen in the packaging bags used with airtight sealing. For temperatures of 28 and 37 ºC, the influencing factor is thermal degradation, so no abrupt change in the slope is observed. Another factor that could influence these results is that reported by (Morgan *et al*., 2006), which states that, as the powder moves away from the manufacturing time, a greater tendency to an oscillating increase and decrease in the viability appears, due to the presence of non-cultivable viable cells, which can be up to 50% of the total cells in this particular sample. This could justify some oscillations that existed in the survival values during this study.

According to (Behboudi-Jobbehdar *et al*., 2013) storage temperature impacts the viability of hydrobiotics through two main mechanisms: first, the increase in temperature increments the rate of metabolic activity in the cells (and other chemical or enzymatic reactions that could also occur, for example: lipid oxidation) and, secondly, it will modify the molecular mobility of the water, since the matrix will move towards a state close to rubbery as the ambient temperature approximates the transition temperature, thus increasing the molecular mobility of water.

In a study carried out by (Hernández *et al*., 2018) it was shown that the viability of the probiotic bacterium *Lactobacillus pentosus* decreased at a drying temperature of 140 ºC, resulting in a viability of 98.9% at an inlet temperature of 100 ºC. These authors reported that the loss of viability of this bacterium during drying was probably due to dehydration and thermal inactivation phenomena. Also in this study, the concentration of the bacteria remained at 9 log cfu/g until the end of the established storage period (12

weeks) at 4 ºC, while at 30 ºC the concentration of *L. pentosus* decreased to values below 6 log cfu/g from week 6.

Also, in (Golowczyc *et al*., 2010) it was shown that storage temperature was a critical parameter that affected the survival of the three microorganisms studied (*Lactobacillus kefir* CIDCA 8348, *Lactobacillus plantarum* CIDCA 83114 and *Saccharomyces lipolytica* CIDCA 812), resulting in higher survival rates when low storage temperatures were applied. The survival of cells after spray drying stored under both atmospheric and vacuum conditions were also studied, and no significant differences were observed. This may have occurred because the relative reaction rate is minimal under water activities of about 0.3 in the dried samples. In this work it was also specified that the composition of the growth medium, the addition of cryoprotectants to the growth or drying medium, the temperature and pH of the growth phase, as well as the growth phase, are variables that should be considered influential in the viability of lactic acid bacteria cells during the drying process and the subsequent storage of the biomass in the dry state.

Other authors (Abadias *et al*., 2005) evaluated spray drying as a method of dehydration of the yeast *Candida sake* CPA-1, concluding that the drying outlet temperature had a greater influence on cell death than the inlet temperature, while survival decreased with increasing temperature. It was also found that spray drying at a temperature of 150 ºC was optimal in terms of viability, powder recovery and moisture content of the dried product.

In (Behboudi-Jobbehdar *et al*., 2013) it was obtained that the total viable counts of the *Lactobacillus acidophilus* bacterium determined as 8.55 and 7.48 log cfu/g after 30 days of storage at 4 and 25 ºC, respectively, indicating good storage stability after spray drying. In this work, the inactivation rates of powders stored for 30 days at cold temperatures (4 ºC) and at room temperature (25 ºC) in airtight sealed containers were calculated by adjusting the total viable count data to a first-order reaction model. A fourfold increase in the inactivation rate of *L. acidophilus* was also observed at room temperature (25 ºC) compared to the lower temperature studied (4 ºC).

On the other hand, (Hamsupo *et al*., 2005) determined the viability of the *Lactobacillus reuteri* KUB-AC5 bacterium during its storage at 4 ºC and 30 ºC for 118 days, concluding that the viability of the samples was stable during their storage at 4 ºC for 4 months, while the total number of viable bacteria decreased rapidly when the storage temperature increased up to 30 ºC. In this work, a model for predicting the viability during storage of this strain of *L. reuteri* was obtained after spray-drying, which was based on the Arrhenius equation, thus correlating the viability of cells during storage with storage temperature.

Finally, (Jantzen *et al*., 2013) determined the viability of bacterium *Lactobacillus reuteri* bacterium at a storage temperature of 4 ºC for a period of 7 and 28 days after having been subjected to a spray-drying process, concluding that the encapsulated dry cell viability decreased by 1 log cycle after a 28-day storage period.

3.5. Adsorption isotherms of the HeberNem-S product

Figure 5 shows the results of the adsorption curves for the different values of relative moisture established in this study.

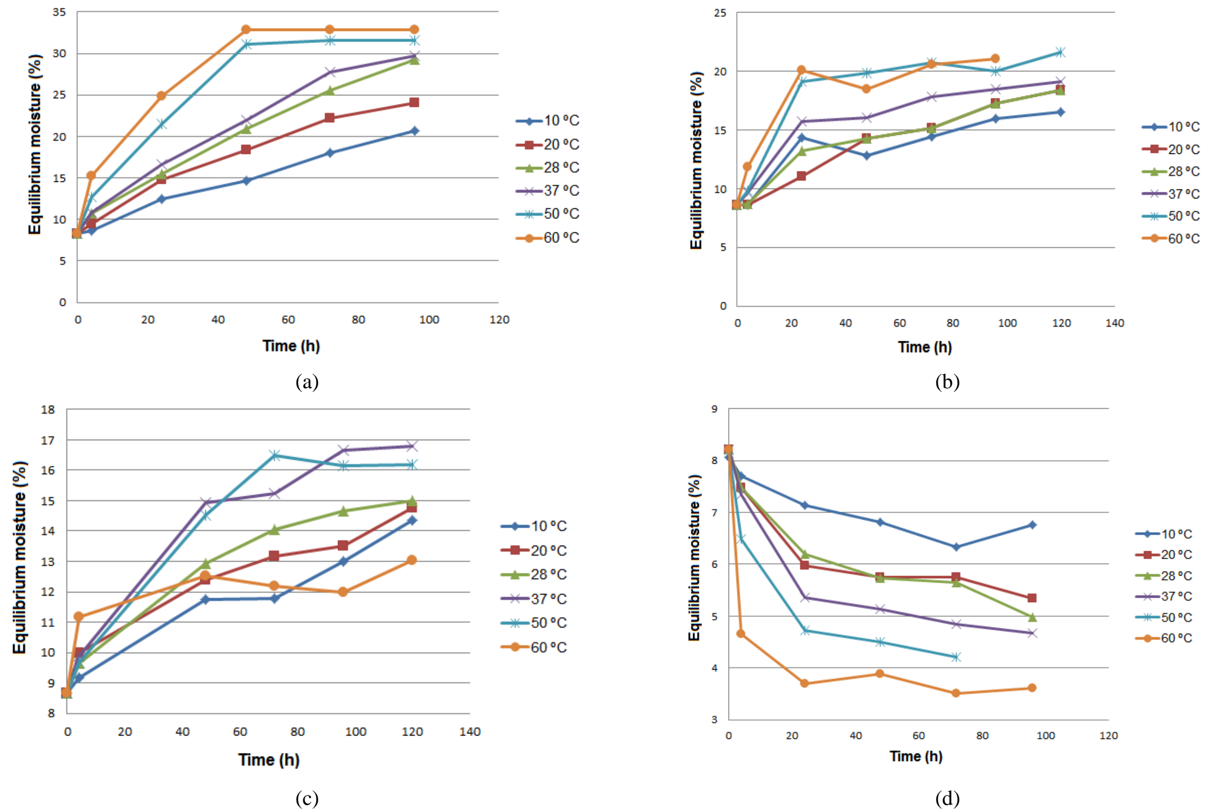

Figure 5. Results of the adsorption isotherms for a relative moisture of: (a) 100%. (b) 82%. (c) 69%. (d) 42%.
Source: Own elaboration.

For a moisture of 100%, it can be observed that, in the first four hours, and as the temperature increases, the change in equilibrium moisture is greater; reaching the equilibrium approximately 96 hours after the product is exposed. At temperatures of 50 and 60 ºC the equilibrium condition is reached faster than at lower temperatures.

In the case of an 82% relative moisture, the slope of the curves was lower and the equilibrium moisture was reached at a time of 120 h. The same was observed in the experiment carried out at 69% relative moisture.

In the case of relative moisture of 42%, the product does not adsorb water and begins to dry, reaching a greater slope at temperatures of 50 and 60 ºC. In this case, the equilibrium condition is reached faster in the other samples, that is, at 96 hours.

With the data of equilibrium moisture depending on the evaluated temperatures (10, 20, 28, 37, 50 and 60 ºC) and the relative moisture studied (42, 69, 82 and 100%) (Figure 6), a regression analysis was performed to adjust these data to a quadratic polynomial, resulting in the following correlation:

$$H_{eq} = 1{,}15 - 0{,}22 \cdot T + 0{,}10 \cdot RH - 0{,}002 \cdot T^2 + 0{,}0005 \cdot RH^2 + 0{,}006 \cdot T \cdot RH \quad (2)$$

Where:
H_{eq} – Equilibrium moisture of powder (%).
T – Powder temperature (ºC).

RH – Relative moisture of powder (%).

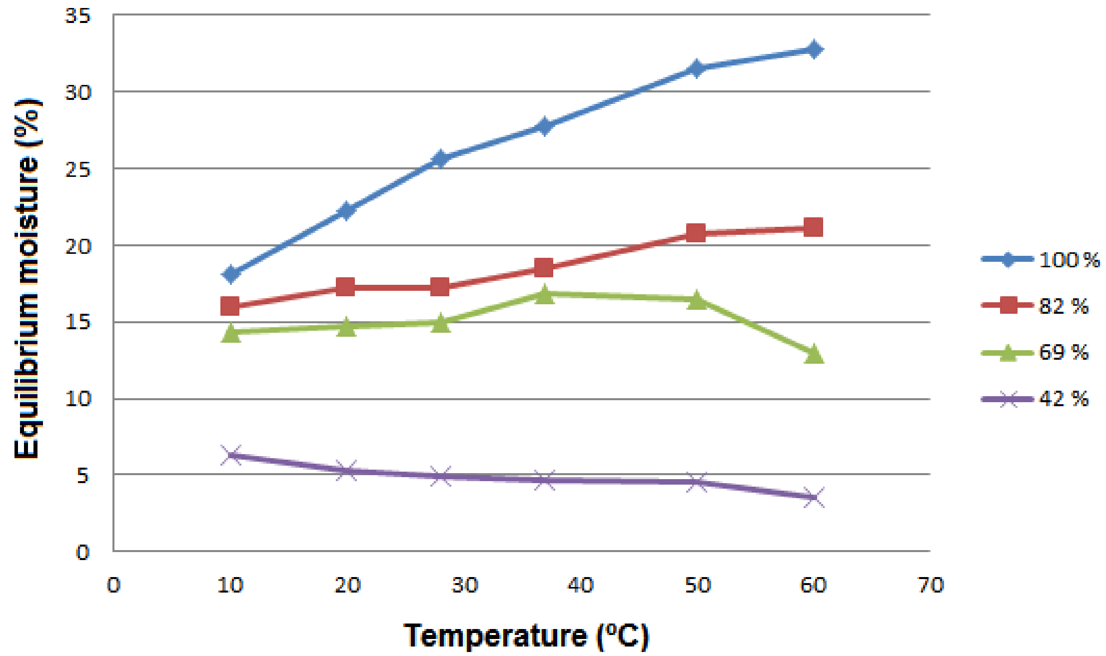

Figure 6. Equilibrium moisture of the powders obtained for different combinations of relative moisture and temperature.
Source: Own elaboration.

Figures 7 and 8 show the graphs obtained when the mathematical regression was carried out.

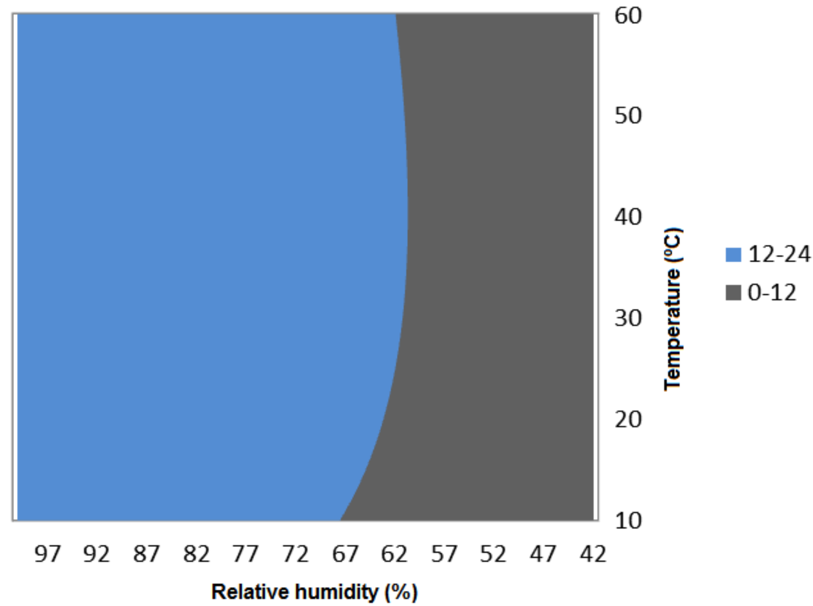

Figure 7. Dependence of temperature and relative moisture on equilibrium moisture.
Source: Own elaboration.

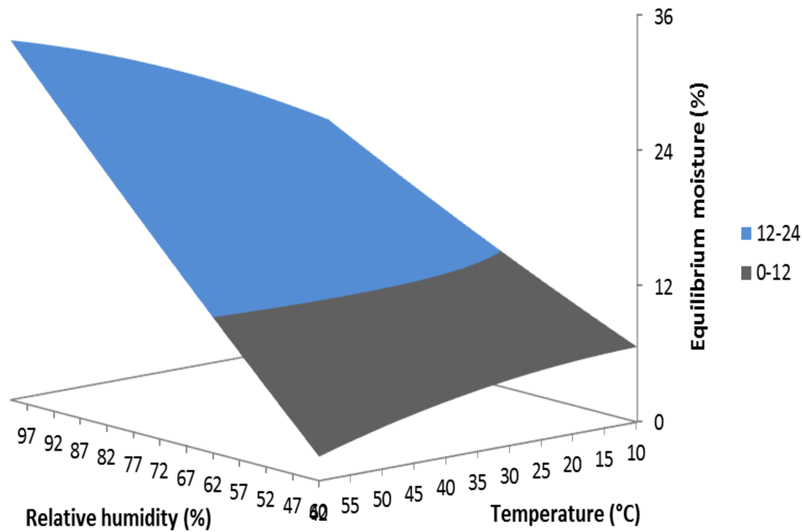

Figure 8. Dependence of temperature and relative moisture on equilibrium moisture.
Source: Own elaboration.

At a relative moisture below 50% the powder loses moisture over time, thus increasing the drying rate. At high ambient temperature values (above 28 ºC), the powder tends to stabilize the residual moisture faster, which may be caused by the increased metabolic activity of the microorganism. As the time progresses, the moisture balances and cell death caused by high temperatures begins. At low temperatures the cell death process slows down and the cell continues to absorb water to carry out its metabolism.

It can also be seen that, for relative moisture values below 60%, the equilibrium moisture of the powder is less than the maximum permissible value (12%) established by the quality specification (CIGB, 1992), which would facilitate the packaging process.

Several authors have obtained adsorption isotherms mainly for food products such as sugarcane concentrated juice powder (Largo *et al*., 2014); date powder (Dev *et al*., 2018); passion fruit pulp powder (Marques *et al*., 2010); tomato pulp powder (Goula *et al*., 2008); yogurt powder (Koç *et al*., 2010); powders rich in soluble fiber obtained from orange pomace (Pirotto *et al*., 2022), as well as from the lactic acid bacterium *Lactobacillus bulgaricus* CFL1 (Fonseca *et al*., 2001), however the adsorption isotherms of *T. paurometabola* C-924 cells after a spray drying process are not reported yet.

4. CONCLUSIONS

The area of *T. paurometabola* C-924 cells was within the range of 1,821-4,487 μm^2 (mean 3,154 μm^2), while the diameter ranged from 2,113-4,302 μm (mean: 3,208 μm) throughout all stages of the evaluated production process.

During the fermentation culture of *T. paurometabola* C-924, the largest cell diameter and area is reached in the yeast extract growth stage.

Both the diameter and the area of the *T. paurometabola* C-924 cells at the end of fermentation do not differ considerably from the values obtained at the end of the formulation process prior to drying, so the formulation process does not decrease the intracellular water content.

In the temperature range from 10 to 30 °C, storage time has a greater influence than temperature on the survival before the drying stage. Viability is generally less than 0.9 for storage times greater than 10 hours for this temperature range.

The average diameter of the dehydrated powder particles of the HeberNem-S product had a value of 39,203 µm.

In the first 7 days there is a significant decrease in survival for the three temperatures evaluated in the stability study of the HeberNem-S product.

In order for the HeberNem-S product to meet the required quality specifications, it must be packaged in an environment with a relative moisture of less than 60%, with a relative moisture lower than 50%, the HeberNem-S product loses residual moisture.

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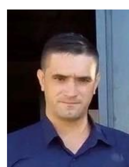

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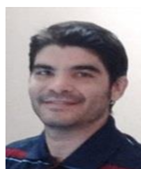

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