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Critical water activity for the preservation of *Lactobacillus bulgaricus* by vacuum drying

E. Elizabeth Tymczyszyn, Rosario Díaz, Andrea Pataro, Natalia Sandonato, Andrea Gómez-Zavaglia *, E. Anibal Disalvo

Laboratorio de Fisicoquímica de Membranas Lipídicas, Cátedra de Química General e Inorgánica, Facultad de Farmacia y Bioquímica (UBA), Junín 956 (1113) Buenos Aires, Argentina

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ABSTRACT

Lactobacillus delbrueckii subsp. *bulgaricus* was dried under vacuum at different temperatures and its preservation evaluated analyzing the evolution of three parameters throughout the process: *lag* time, percentage of membrane damage and ζ potential.

Microorganisms were dehydrated at 30, 45 and 70 °C in a vacuum centrifuge for different times. The a_w achieved for each time of drying was correlated with the cell recovery at all the temperatures assayed. The recovery of microorganisms was evaluated by means of: a) kinetics of growth in milk after drying, as a measure of the global damage; b) quantification of the membrane damage using the fluorescent dyes SYTO 9 and PI; c) determination of changes in the superficial charges (ζ potential) as measured of the increase in the hydrophobic residues exposed in the bacterial surface after dehydration. These changes correlate well with the bacterial damage occurred during the dehydration process.

The Page's equation allowed fitting of a_w and time of drying, thus making possible the determination of the appropriate dehydration conditions (time-temperature ratios) for which no cell damage occurs.

The evaluation of three parameters (lag time, percentage of membrane damage and ζ potential) allowed us to conclude that at the lowest temperature of dehydration, the first target of damage is the cell membrane. However, this damage is not decisive for the bacterial recovery after rehydration, as are the increase in the lag time and the changes in the ζ potential, as was observed for *L. bulgaricus* dehydrated at 45 and 70 °C for larger times.

1. Introduction

Starters of lactic acid bacteria are usually preserved by freeze thawing and lyophilization (De Antoni et al., 1989; Carvalho et al., 2002). In spite of being efficient methods, freezing and freeze drying (Roser, 1991) have high manufacturing costs and energy consumption (Santivarangkna et al., 2007a). For this reason, increasing attention has been paid on alternative drying processes such as spray drying (Teixeira et al., 1995; To and Etzel, 1997; Meng et al., 2002), fluidized bed drying (Selmer-Olsen et al., 1999) and vacuum drying (Santivarangkna et al., 2006; Santivarangkna et al., 2007a; Cardona et al., 2002; Morgan et al., 2006; King and Su, 1993). Among these alternative processes, vacuum drying has been described to be the most promissory method to preserve sensible biological material because of its acceptable cost-effectiveness balance (Santivarangkna et al., 2007a). However, the conditions of vacuum drying (time, temperature) must be optimized to allow the best bacterial recovery after dehydration-rehydration, avoiding cellular damages.

It has been proposed that bacterial death results from the inactivation of critical sites in the cells (Texeira et al., 1997). Membranes, nucleic acids and certain enzymes have been identified as cellular targets of damage caused by dehydration (Tomlins and Ordal 1976; Gould, 1989). It has been reported that after dehydration-rehydration the microorganisms can be recovered even when the cellular membrane is damaged. In addition, it has also been observed that an increase in the absolute value of the ζ potential can be associated with an increase in the lag time (Tymczyszyn et al., 2007). Changes in this parameter were correlated with a loss of the original orientation of the surface macromolecules and thus, the capacity to recover the surface properties after rehydration (Pembrey et al., 1999). This indicates that there are other bacterial structural parameters besides the membrane integrity affecting the bacterial viability after dehydration-rehydration. In this sense, data obtained by Differential Scanning Calorimetry reveal that damage produced in membrane lipids, ribosomes and DNA are reversible, whereas damages produced in proteins are not (Teixeira et al., 1997; Lepock et al., 1990).

* Corresponding author. Junín 956 2° P. (1113) Buenos Aires, Argentina. Tel.: +54 11 4964 8249x21; fax: +54 11 4508 3653.
E-mail address: angoza@qui.uc.pt (A. Gómez-Zavaglia).

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Nomenclature

Water activity	a_w
Zeta potential	ζ potential
k	drying rate constant in the Page equation.
n	drying time index in the Page equation.
a_{w0}	initial water activity ≅ 1
t	time

When applying vacuum drying, it is important to consider that a thermal stress takes place in parallel to the hydric stress, probably inducing irreversible damages. For this reason, the exposure of microorganisms to high temperatures should be as short as possible and the correct choice of times and temperatures of dehydration is crucial to achieve the best vacuum drying conditions.

The challenge of making vacuum drying a wide spread methodology for microorganisms' preservation is the difficulty of defining standardized conditions that allow the comparison of results obtained in different laboratories. The reason of this difficulty is that the times and temperatures for the dehydration processes are related with the drying conditions (*i.e.*: exposure surface, pressure of the vacuum system, weight or volume of the sample, etc.), which in general are dependent on the equipment used. Therefore, to make results comparable, it becomes necessary to refer the experimental conditions, to a parameter that is independent to these experimental conditions, for example, the water activity of the sample after dehydration in a given condition.

In consequence, considering that both time and temperatures of drying affect the final water activities of the samples, the definition of drying conditions in terms of the final water activity becomes important to define correlatable parameters with the state of dehydration of the cells. This fact would help to attain the best conditions for the preservation processes. Therefore, this work aims to investigate the kinetics of dehydration of *L. bulgaricus* in order to analyze the values of water activity below which the damage of cells is irreversible under the conditions assayed. The values obtained should represent the best *temperature/water activity* balances allowing the highest recovery with the lowest damage. To attain this goal, *L. bulgaricus* was dehydrated at three different temperatures (30, 45 and 70 °C) for different times until a constant water activity was attained. The viability, cell membrane integrity and ζ potential of *L. bulgaricus* was measured after rehydrating cells previously dried up to those previously defined a_w values. This approach gets an insight on the inactivation mechanisms of *L. bulgaricus* along with the vacuum drying process.

2. Material and methods

2.1. Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was isolated from a fermented product (Gómez-Zavaglia et al., 1999). The strain was maintained frozen at −80 °C in 120 g/l non-fat milk solids. Cultures were grown in MRS broth (De Man et al., 1960).

2.2. Dehydration procedure

Volumes of 1.2 ml cultures in the stationary phase were harvested by centrifugation at 10,000 *g* for 10 min and after that, washed in water. Pellets were dehydrated at 30 °C, 45 °C or 70 °C for different times in a vacuum centrifuge (Integrated Speed Vac System ISS 100, Savant Instrument Inc., Farmerdale, USA). Drying was carried out by applying a maximum Vacuum < 10 Torr ≅ 13.33 mbar ≅ 1.33 kPa. Taking into account that the boiling point of water at this pressure is ≅ 11.14 °C [Handbook of Chemistry and Physics. Editor in chief: David R. Lide — 72ND edition 1991–1992 — pages 6–13], the water was removed over the boiling point at all the temperatures assayed. The dehydrated pellet obtained in all cases was rehydrated in an equal volume of distilled water (1.2 ml).

2.3. Water activity

Water activity was measured after drying samples at 30 °C, 45 °C or 70 °C for different times, in all the conditions analyzed using a Novasina water activity instrument (Pfaeffikon, Switzerland). The equipment was calibrated using standard solutions of known Relative Humidities (the minimum amount of sample used was the corresponding to a pellet obtained from 10 ml of culture). The times of dehydration have been correlated with the water activity after each condition assayed.

2.4. Growth in milk after drying

After rehydration during 15 min, 1 ml of rehydrated cells were used to inoculate 10 ml of Ultra-High Temperature (UHT) milk and the growth kinetics were followed by measuring the decrease of pH during incubation at 37 °C. The lag time corresponding to each condition of dehydration was plotted against the time of drying.

2.5. Zeta potential

The ζ potential of whole cells was determined in a Zeta-Meter System 3.0 (Zeta-Meter INC.—USA) by measuring the rate of migration of the cells in the stationary layer when a constant electric field was applied (Koch, 1984). The effective electrical distance of the cell was calculated by using KCl solutions of known conductivity at 25 °C. The rate of migration was determined by microscopic observation of the displacement of individual cells in a rectilinear and uniform movement along a reticular lattice. The potential was fixed at 40 V. At least 20 determinations were made for each sample.

2.6. Membrane damage

3 µl of the dye mixture containing SYTO 9® and Propidium iodide (PI) 1:1 (Kit BacLight Molecular Probes L-7012) were added to 1 ml of cells dried under different conditions and rehydrated (as described above) and incubated for 15 min at room temperature. Both probes are nucleic acid stains: SYTO 9 is a green fluorescent probe and PI is a red-fluorescent probe. These dyes differ in their spectral characteristics and in their ability to penetrate healthy bacterial cells: SYTO 9 generally labels all bacteria in a population (those with intact and those with damaged membranes) whereas, PI penetrates only when bacterial membranes are damaged, causing a reduction in the SYTO 9 fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO 9 and PI stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red.

Cell suspensions were adjusted to 1.10^8 bacteria/ml. The fluorescence emission spectra were measured in a Perkin Elmer Luminescence spectrometer Model LS 55; the excitation wavelength was set at 470 nm and the emission wavelength from 490 to 700 nm.

The percentage of the membrane damage was calculated as described in Tymczyszyn et al., 2007:

$$\%\,\text{membrane damage} = \frac{\text{IF control} - \text{IF sample}}{\text{IF control}} \times 100$$

IF = fluorescence intensity at 500 nm.

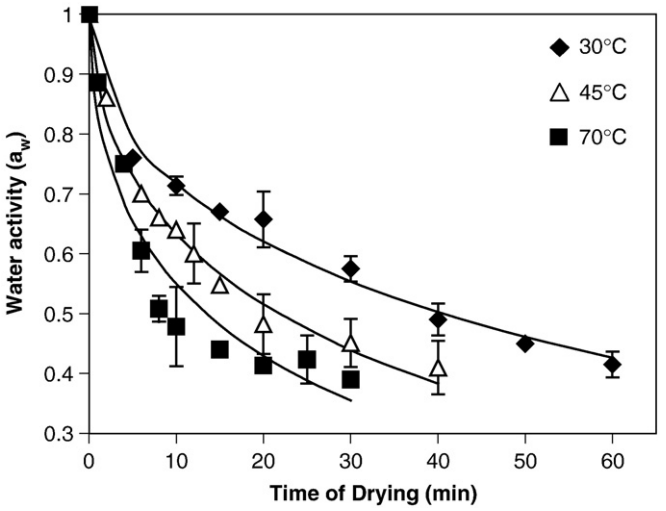

Fig. 1. Water activity (a_w) as function of the drying time for microorganisms dehydrated at 30 °C (◆), 45 °C (△) and 70 °C (■). The isotherms were fitted according to the Page's equation (Simal et al., 2005).

$$a_w = a_{w0}\ \exp[-kt^n]$$

where: a_w=water activity after drying; a_w=initial water activity≅1; k=drying rate constant; t=time; n=drying time index. The empirical constants of the models were computed using the "SOLVER" function in Excel spreadsheet using methods of least squares.

2.7. Reproducibility of the results

All experiments were done on duplicate samples using three independent cultures of bacteria. The relative differences were reproducible independently of the cultures used.

3. Results

The effect of temperature during drying was evaluated by measuring the water activity of samples dehydrated during different times. Fig. 1 depicts the decrease of water activity along with the time of drying for *L. bulgaricus* dehydrated at 30, 45 and 70 °C.

The experimental results were fitted using the Page's equation (Simal et al., 2005). According to this model:

$$a_w = a_{w0}\ \exp[-kt^n]. \quad (1)$$

This equation has been already used successfully to accurately fit the drying curves of potato slices (Akpinar et al., 2003), rough rice (Iguaz et al., 2003), green bean, potato and pea (Senadeera et al., 2003), carrot (Doymaz, 2004), among others. Among microorganisms, the Page's equation has been used to estimate the dehydration of *L. lactis* (Cardona et al., 2002). In this work, the empirical constants in Eq. (1) were determined using the SOLVER function in Excel spreadsheet using methods of least squares. The drying rate constant ***k,*** the drying time index ***n*** and R^2 value are displayed in Table 1. The drying rate constant ***k*** increased linearly with the drying temperature (R^2=0.9986). This temperature's dependence can be represented through the equation:

$$k = 0.0023\,T\,(^{\circ}C) + 0.0262 \quad (2)$$

Parameter n of the Page's equation did not exhibit temperature dependence and can be considered a constant parameter (0.51<n<0.53). Similar responses were observed by Simal et al. (2005) and Senadeera et al. (2003).

The response of the samples after rehydration was evaluated by means of kinetics growth in milk. For each condition of vacuum drying (time and temperature of dehydration), the samples were rehydrated and incubated in milk at 37 °C. The kinetics of growth in milk were followed by registering the pH of each culture as a function of the time of incubation at 37 °C (data not shown). In Fig. 2A, the lag time obtained from these kinetics was plotted against the time of drying at the three temperatures assayed.

According to this figure, the slope corresponding to dehydration at 70 °C is much higher than the ones corresponding to dehydration at

Table 1
Page's equation parameters for each temperature of dehydration.

Page's model			
T (°C)	k (min^{-1})	n	R^2
30	0.0951	0.5336	0.9869
45	0.1334	0.5350	0.9878
70	0.1890	0.5140	0.9454

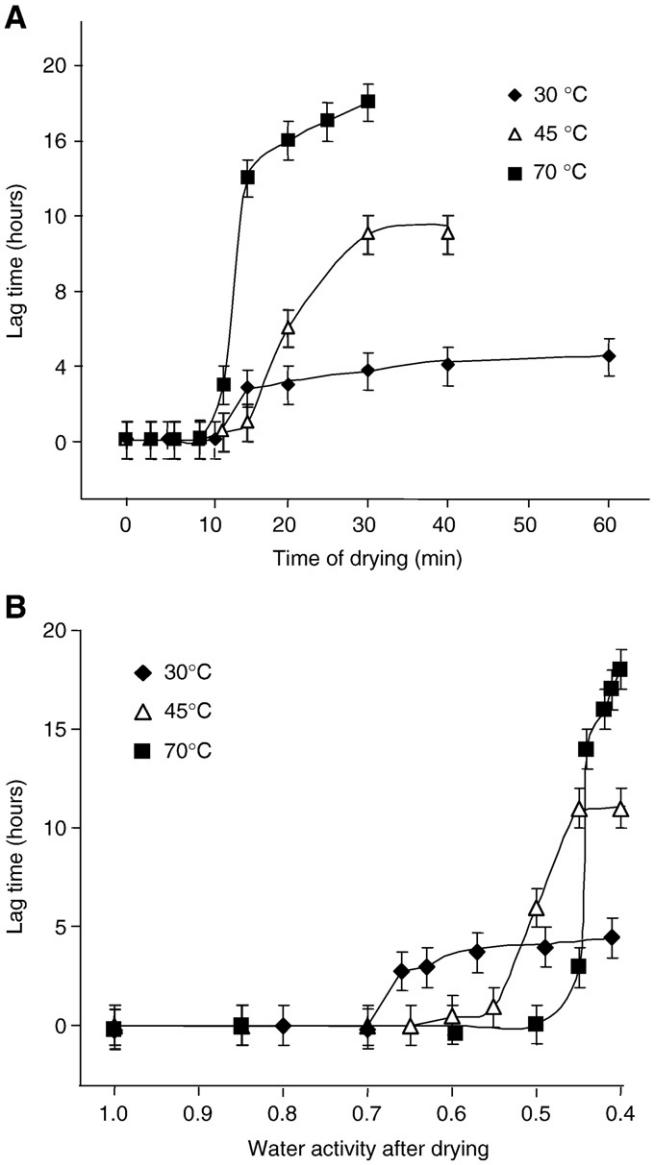

Fig. 2. A: *Lag* time of *L. bulgaricus* as a function of the drying time for microorganisms dehydrated at 30 (◆), 45 (△) and 70 (■) °C. B: *Lag* time of *L. bulgaricus* expressed as a function of the water activity (a_w) after dehydration at 30 (◆), 45 (△) and 70 (■) °C.

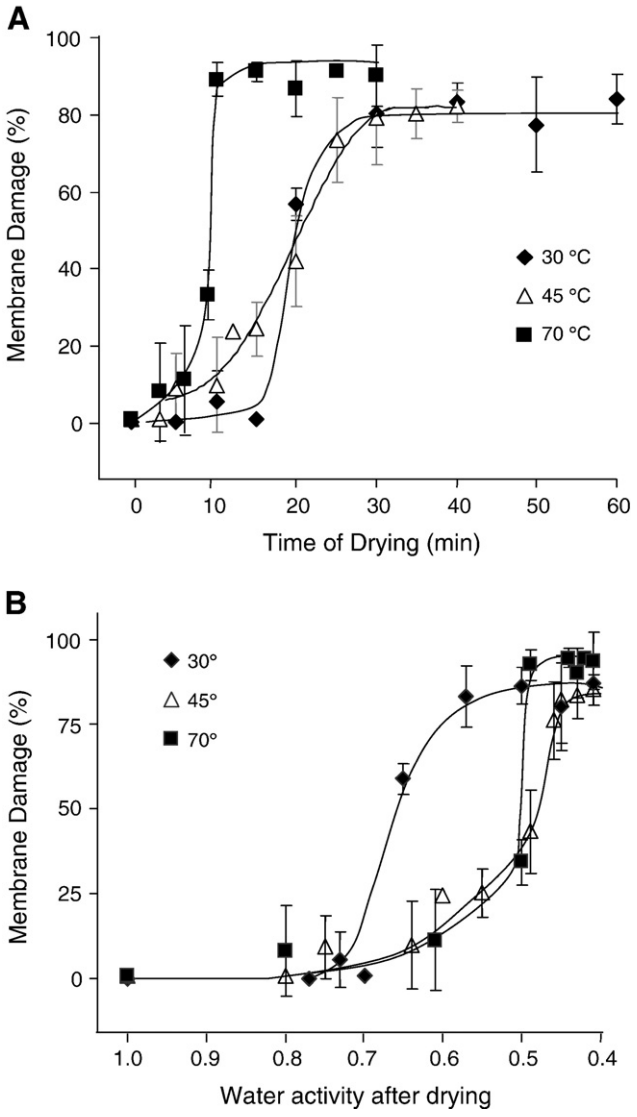

Fig. 3. A: Membrane damage of *L. bulgaricus* as a function of the drying time at 30 (◆), 45 (△) and 70 (■) °C. Membrane damage is expressed as the decay in the fluorescence emission spectra of SYTO 9 upon the times of dehydration. The % has been calculated as:

$$\%\,\text{membrane damage} = \frac{\text{IF control} - \text{IF sample}}{\text{IF control}} \times 100$$

where IF = fluorescence intensity at 500 nm. A membrane damage of 0% corresponds to the damage of the non-dehydrated microorganisms. B: The same membrane damage expressed as a function of the water activity after drying.

the other two temperatures. This indicates that, as expected, the global damage at 70 °C occurs much faster than the ones corresponding to dehydration at the other two temperatures. The behavior at 30 and at 45 °C does not show any significant difference when the slopes of both curves are compared. However, the global damage at 30 °C is lower than the one corresponding to dehydration at 45 °C (the *plateau* of the plots is *ca.* 4 h at 30 °C and *ca.* 12 h at 45 °C). It is also important to notice that for all temperatures assayed, no effect on the lag time is observed when the time of drying is shorter than 10 min.

Thus, a common state is being reached in each independent condition of time and temperature. This behavior is more clearly deduced when the lag times are plotted against the decrease of a_w (Fig. 2B). The values of a_w at each time and temperature can be obtained by interpolating the dehydration times in Fig. 1.

It is noticeable that the increase in the lag time begins at higher water activities when *L. bulgaricus* are dehydrated at lower temperatures. Thus, after dehydration at 30 °C, the increase in the *lag* time is noticeable when a_w is 0.7 or lower and cells can be further dehydrated without any noticeable increase of the lag time (a_w decreases up to 0.4 and the lag time remains constant at *ca.* 4 h). In contrast, when dehydrated at 70 °C, the increase in the lag time begins when a_w is 0.5 and progresses much faster for lower a_w. The situation at 45 °C is intermediate: the increase in the lag time begins when a_w is *ca.* 0.6 and the slope of the curve and maximum damage produced in the *L. bulgaricus* is less steep than the one observed for *L. bulgaricus* dehydrated at 70 °C.

At this point, the goal is to evaluate what kind of damage has been produced in *L. bulgaricus* at the critical points of a_w indicated in Fig. 2B.

To attain this aim, the membrane integrity was measured for each condition of dehydration. Fig. 3A depicts the membrane damage along with the time of dehydration using SYTO 9 and PI as fluorescent probes as described previously (Tymczyszyn et al., 2007). It is interesting to note that the slope of the plots at each temperature of dehydration correlates well with the values of the lag times displayed in Fig. 2A.

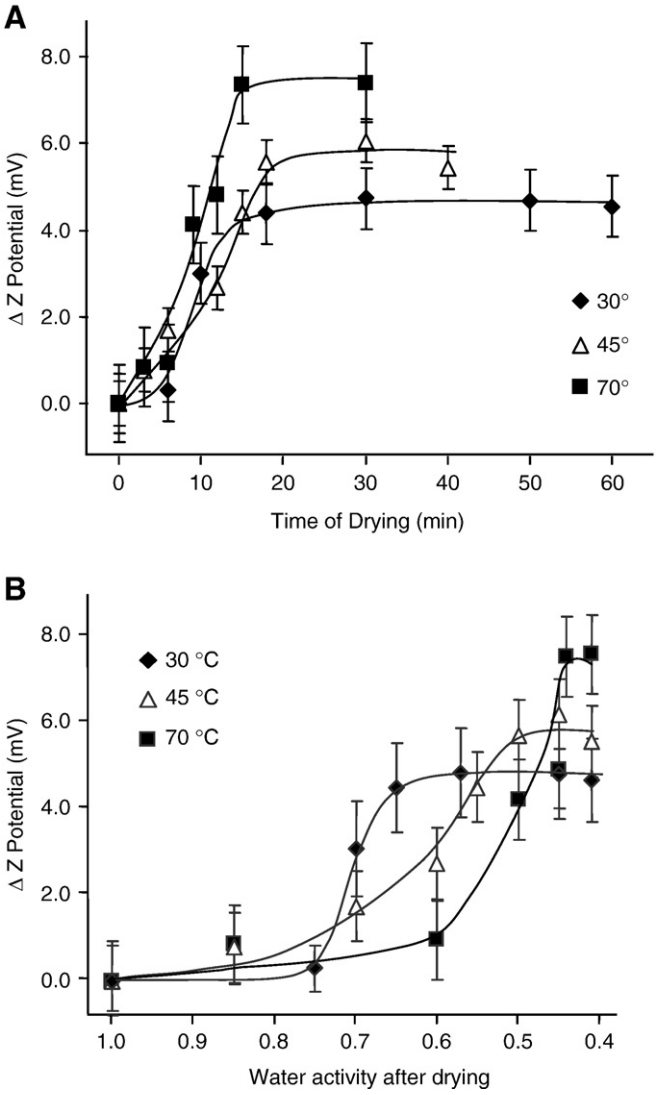

Fig. 4. A: Zeta potential (ζ) of the recovered *L. bulgaricus* as a function of the drying time. Cells were dehydrated at 30 (◆), 45 (△) and 70 (■) °C. ζ potentials are expressed as $\Delta\zeta$ potential (difference between the measured value and the value corresponding to the control -non-dried microorganisms-). B: The same $\Delta\zeta$ potentials expressed as a function of the water activity after drying.

When plotted against a_w (Fig. 3B), the membrane damage begins being noticeable when a_w is 0.7–0.75 at all the temperatures assayed. However the evolution of this damage is pretty faster at 30 °C than at 45° and 70 °C. This indicates that even when the maximum damage is similar at the three temperatures of dehydration, the lower the temperature of dehydration, the more susceptible are the cells to membrane damage. When *L. bulgaricus* are dehydrated at higher temperatures, lower a_w are required to induce noticeable membrane damage.

The ζ potential is defined as the difference in electrical potential between the surface of the microorganism and the bulk-surrounding medium. It is a measure of the net distribution of electrical charges on the bacterial surface and its sensibility to determine both damage and metabolic changes has been already reported by different authors (Fernández Murga et al., 2000; Tymczyszyn et al., 2007). In this work, the ζ potential has been evaluated as a parameter of the superficial charge of the rehydrated bacteria. Fig. 4A depicts the evolution of this parameter along with the times of dehydration at the three temperatures assayed. As soon as the time of dehydration increases, the values of ζ potential reach more positive values, as previously reported for microorganisms undergoing freezing and thawing and vacuum drying (Fernández Murga et al., 2000; Tymczyszyn et al., 2007). In consonance with the results depicted in Figs. 2 and 3, the highest slope is observed for *L. bulgaricus* dehydrated at 70 °C.

The ζ potential was also plotted against the a_w. Fig. 4B indicates that changes in ζ potential are dependent on the temperature at which the *L. bulgaricus* were dehydrated. In this sense, the changes in ζ potential are noticeable at a_w *ca*. 0.75 for 30 and at a_w 0.6 when dehydration takes place at 70 °C. In summary, the maximum change in the ζ potential observed at the lower water activities follow the order: 70>45>30 °C. It is interesting to note that the changes observed regarding the ζ potential follow the same pattern as the ones observed for the lag time at the same a_w (Fig. 2B).

4. Discussion

Vacuum drying has been used for the preservation of different microorganisms (Santivarangkna et al., 2007a) and has been described as a useful, simple and inexpensive method for microorganism preservation. However, it is difficult to compare the results obtained in different laboratories because the time and temperatures of dehydration are strongly related with the conditions of drying (exposure surface, pressure of the vacuum system, weight or volume of the sample, etc.), which in general are dependent on the equipment used. For this reason, the correct understanding of the vacuum drying conditions represents a promissory contribution for its overspread use in the future.

In this sense, the impact of this work has been to correlate the time of drying with the water activity. The application of the Page's equation allows correlating the a_w determined after dehydration in different conditions with the temperature (Eq. (2)). For the application of this equation, it is important to consider that the drying rate constant (k) is proportional to the temperature (Eq. (2)) and the drying time index (n) can be considered as constant (see Table 1 and refs. Simal et al., 2005; Senadeera et al., 2003).

The curves shown in Fig. 1 are useful for the determination of the vacuum drying conditions. The data in Fig. 1 show the curves fitting the Page's equation (Eq. (2)) when $n=0.5$. As a result, the use of these isotherms will allows one to select the optimal conditions of time and temperature of dehydration when a given a_w is required. The knowledge of these parameters before drying is very important to know which is the best condition of dehydration, thus avoiding unnecessary damages.

With this purpose, we have investigated in previous works different targets of damage upon dehydration at a given condition of time and temperature (Tymczyszyn et al., 2007). The results indicated that the cell membrane is not critical for the recovery of cells after drying. According to that paper, the maintenance of zeta potential can be correlated with the conservation of the cellular volume and the preservation of the surface properties. Taking this information as a background, we aimed to evaluate the evolution of the damage at membrane and surface potential level.

The comparison among Figs. 2B, 3B and 4B indicates that at the lowest temperature used for dehydration (30 °C), the damage observed at all the levels analyzed is noticeable at higher water activities. This observation can be explained taking into account that in a first dehydration step, extracellular water is eliminated as a consequence of a slow process of exchange with hydration water. As a result, water molecules that are bound to lipids and proteins go out because of the difference in the osmotic pressures outside and inside the cell. This process is enough to induce noticeable damage of the cell structures (membrane and surface potential) and thus, affecting the lag time.

The maximum membrane damage is similar for the three temperatures assayed and attains *ca.* 80% (Fig. 3B). In addition, the a_w value at which the membrane damage starts is 0.7 at the three temperatures assayed. Similar results were obtained by Santivarangkna et al., (2006 and 2007b). They reported that after vacuum drying *L. helveticus* at 43 °C and 100 mbar during 12 h, the cell envelope is the main damaged structure (Santivarangkna et al., 2007b) and according to another paper of the same authors, the water activity values attained using the same dehydration conditions are *ca.* 0.7–0.8 (Santivarangkna et al., 2006).

The difference is that slow decreases in a_w are enough to induce membrane damage at low temperatures of dehydration (30 °C). In other words, the bacterial membrane is more susceptible when a slow dehydration process takes place. Note that the slope of the plot corresponding to dehydration at 30 °C is the highest. According to our results, one can consider that the effect of the drying rate on the microorganisms can be somehow compared with the effect of freezing, where the cooling rate is determinant of the cellular damage (Santivarangkna et al., 2008). At very slow cooling rates, substantial osmotic contraction of the cells occurs, and this may be fatal itself. In contrast, at very high rates, there is little osmotic contraction, so that the solute concentration in the cytoplasm remains low (Santivarangkna et al., 2008). In our case, the difference between the slow and fast drying rates is the time necessary to produce the cytoplasm water diffusion outside the cell and then, the cell contraction.

The plasma membrane has a small resting tension. This process is not always reversible. When the cells are rehydrated, the bacteria are exposed to an external medium abruptly diluted, the volume increases rapidly and the area increases almost equally rapidly, with the consequent loss of semi permeability barrier. This effect is more relevant when bacteria are dried at low temperatures (low drying rate) than when the cells are dryed at high temperatures (high drying rate).

The plot corresponding to the ζ potential against the water activity after dehydration (Fig. 4B) depicts a similar behavior as the one observed for the lag time (Fig. 2B): the increase in $\Delta\zeta$ potential when curves attain the *plateau* is lower when they are dehydrated at 30 °C than at 45 °C and lower at 45 °C than at 70 °C. However, these changes occur at higher a_w for the lowest temperatures of dehydration. In addition, the a_w at which both lag time and ζ potential increases take place, are similar for both parameters. This behavior could be determined by the drying rates. The difference between high and low drying rates (high and low temperatures of dehydration, respectively) is that the changes in the ζ potential progresses more rapidly after the lowest water activity due to the high temperatures. The increase of the ζ potential to more positive values most likely indicates a decrease of the exposed charge on the bacterial surface. This decrease can be assigned to the exposure of hydrophobic groups on the bacterial surface with the consequent denaturation of the superficial macromolecules. This kind of damage was already reported by Santivarangkna et al. (2007b) in *L. helveticus*. Indeed, they observed that the cell envelope is the main target of damage during vacuum drying using Atomic Force Microscopy.

According to these results, the critical water activity (a_w at which the lag time starts increasing) is highly dependent on the dehydration rates. During cryopreservation, the usual goal is to achieve intracellular vitrification avoiding intracellular ice formation and membrane and protein damage. On the other hand, cryopreservation is similar to desiccation (Wolfe and Bryant 1999). In this work, at high drying rates, the fast diffusion of water out of the cells could help the formation of the glassy state. The most important parameter describing the glassy state is the glass transition temperature (*Tg*), which is strongly dependent on the temperature and water content, and these parameters are responsible for the stability of the starter cultures during the storage (Santivarangkna et al., 2008). However, at low temperatures (low drying rates), a thermodynamic equilibrium is created and the crystallization of the citoplasmic components occurs. As a consequence, the denaturation of cells structures takes place.

5. Conclusions

In this work, we have dealt with two relevant aspects of vacuum drying. From one side, the fitting of the Page's equation to the dehydration process at different temperatures will be very important in the future because it allows to determine the conditions of dehydration, avoiding unnecessary damages.

On the other side, it was determined that low temperatures of dehydration induce damages mainly at a membrane level. Since these injuries are not crucial for the *L. bulgaricus* recovery, the global damage of cells dehydrated at low temperatures (measured as lag time) do not experience strong increases when microorganisms are dehydrated at low temperatures.

Finally, the relevance of the drying rate must be underlined because it is determinant of the cellular damage after rehydration.

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