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Cell surface damage and morphological changes in *Oenococcus oeni* after freeze-drying and incubation in synthetic wine

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ABSTRACT

The aim of the present study was to evaluate the effects of freeze-drying in the presence of trehalose as a cryoprotectant, followed by incubation in synthetic wine, on surface damage, viability and L-malic acid consumption of the oenological strain *Oenococcus oeni* UNQOe 73.2. After freeze-drying, no significant differences were observed in the number of viable cells (for both acclimated and non-acclimated cultures) respect to the fresh culture. In contrast, loss of viability was observed after wine incubation for 24 h, being acclimated freeze-dried cells the best conditions for this. After the preservation process, small changes in cell morphology were observed by Atomic Force Microscopy (AFM). The Zeta potential and AFM showed that 24 h of wine incubation was enough to induce several cell surface modifications. Plate count data allowed us to establish that surface damage is an important factor for loss of viability, regardless of the acclimation treatment. Although the number of surviving *O. oeni* cells decreased dramatically after incubation in synthetic wine for 15 days, the consumption of L-malic acid was higher than 70%, with freeze-dried cells showing a better performance than fresh cultures. These results demonstrate that *O. oeni* freeze-dried cultures could be applied to direct wine inoculation, to conduct malolactic fermentation, maintaining its technological properties and reducing the time and costs of the winemaking process.

1. Introduction

Oenococcus oeni is one of the main lactic acid bacteria (LAB) responsible for malolactic fermentation (MLF) during the winemaking process. MLF occurs after alcoholic fermentation and may be either spontaneous, i.e. carried out by indigenous LAB, or induced by commercial MLF starter cultures. MLF consists in the conversion of L-malic acid into L-lactic acid, reducing the acidity of wine and improving its microbiological stability and organoleptic characteristics [27,35]. In red wines produced in North Patagonia, Argentina, MLF usually occurs spontaneously and randomly, guided by the natural microbiota present in the grapes and cellar. Nevertheless, any delay in the starting of this process can lead to alterations in the wine quality. To control this fermentation process, some authors have suggested the use of MLF starter cultures [25–28]. However, in order to maintain the specific and distinctive features of Patagonian wine, the use of autochthonous strains able to adapt to the conditions of that specific wine-producing area has been recommended [9,28]. The *microbial terroir* of typical fermented food and beverage production represents a dynamic sector of applied research in food microbiology [18]. In this context, several studies have been performed on the characterization of *O. oeni* biodiversity with the aim to select putative autochthonous starter cultures [9,10,21,24,32].

In previous works, different *O. oeni* strains were isolated from Patagonian red wines and their phenotypic, genotypic and technological properties have been reported [7,8,36]. However, few strains were able to survive in wine medium because bacteria undergo rapid death due to the harsh environment (low pH values of 3.0–3.6, high ethanol and molecular SO_2 concentrations). For this reason, only the strains better adapted to these conditions could be considered as candidates for MLF starter cultures [17]. Also, in order to be considered as MLF starter candidates, strain cultures must withstand preservation processes such as freezing and freeze-drying [4,6,40]. Freeze-dried cultures should ideally have the same technological properties before and after being preserved. However, the loss of water during this process might cause cell membrane damage, protein and DNA denaturation, thus significantly decreasing cell survival [29,30,39]. Taking this into

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consideration, the optimization of the preservation processes is an important step for the development of dried starter cultures that retain their technological properties.

The high ethanol concentration in wines (10–14% v/v) is the main cause of the decrease in LAB viability, being cell membranes the first target of damage. To overcome this harmful condition, LAB could be pre-adapted to grow in these environments by acclimation treatments. Acclimation consists in the previous incubation of bacteria in a medium containing low ethanol concentrations (4–10% v/v) [3,12,25,34]. This process improves the viability and L-malic acid consumption of *Lactobacillus plantarum* and *O. oeni* cultures inoculated in a synthetic wine [3,7]. In this regard, different authors have reported that changes in lipid composition during the acclimation treatment counteract the toxic effect of ethanol [22,31]. This adaptation mechanism has also been reported for other enological LAB strains such as *L. plantarum* [5,37]. In addition, we have previously assessed that previous acclimation of freeze-dried *L. plantarum* cultures increases their ethanol resistance after incubation in wine containing 13 or 14% v/v ethanol [4,6]. In the same strains, acclimation treatment previous to freeze-drying induces damage on the cell surface, with negative consequences after wine incubation, being Atomic Force Microscopy (AFM) and Zeta potential the most appropriate methodologies for this kind of studies [6].

Several authors have studied the preservation of *O. oeni* strains, and trehalose and glutamate have been shown to be the best protecting agents [40,41]. However, the effects of freeze-drying on the surface properties of *O. oeni* strains and their impact after inoculation of dried cells under wine conditions have not been reported yet.

In this context, the aim of the present work was to study the effect of freeze-drying of acclimated or non-acclimated *O. oeni* on surface properties and on the resistance to wine-like medium before and after the dehydration process. In order to fulfill this goal, the surface damage of *O. oeni* was evaluated by AFM and Zeta potential measurements, and the impact of these surface changes on survival and consumption of L-malic acid after incubation in synthetic wine was assessed.

2. Material and methods

2.1. Strain, medium and growth conditions

O. oeni UNQOe 73.2 (GenBank Accession Number *16S rRNA* KC562903) was isolated from Patagonian Pinot noir wine and selected as a potential MLF starter culture due to its oenological properties [7]. Cells were grown in 10 mL of MLO broth (glucose, 10 g/L; fructose, 5 g/L; yeast extract, 5 g/L; tryptone, 10 g/L; $MgSO_4$ 7 H_2O, 0.2 g/L; $MnSO_4$ 4 H_2O, 0.05 g/L; cysteine/HCl, 0.5 g/L; diammonium citrate, 3.5 g/L; Tween-80, 1 mL/L, and tomato juice, 100 mL/L, pH 4.8) [11], at 28 °C for 7 days.

2.2. Acclimation conditions

Cells in the early stationary phase (approximately 5×10^9 CFU/mL) were harvested by centrifugation at 5000 × *g* for 10 min and suspended in the same volume of a modified acclimation medium containing 6% (v/v) ethanol. Its composition was defined according to Bravo-Ferrada et al. [7]. After incubation at 21 °C for 48 h, the acclimated cells were harvested by centrifugation and prepared for freeze-drying.

2.3. Sample preparation for freeze drying

To prepare samples for freeze-drying, 1 mL of the culture, previously acclimated or non-acclimated, was harvested by centrifugation at 5000 × *g* for 10 min. Pellets were washed twice with NaCl 0.85% (w/v), and resuspended in 1 mL of 20% (w/w) aqueous solution of trehalose, previously sterilized using 0.2-μm pore sterile filters [33]. The protective effects of a suspension of sodium glutamate, trehalose and sodium glutamate/trehalose 50:50 were previously studied [40], but because better results were obtained for samples resuspended with trehalose, in this study, all experiments were carried out with this protectant.

2.4. Freeze-drying procedure

Aliquots of 1 mL containing cell suspension in trehalose were transferred into 5-mL glass vials under aseptic conditions and frozen for 24 h at −80 °C. Then, a freeze-dryer Labconco Freeze Dry System/Freezone 4.4 was used for this process, which lasted for 24 h. The process was carried out with the condenser at −50 °C and the chamber pressure was lower than 0.05 mbar.

2.5. Culturability after freeze-drying and incubation in synthetic wine

The culturability of acclimated and non-acclimated freeze-dried cultures and fresh cultures (as control) was studied before and after incubation in synthetic wine. The number of viable cells was measured by plate count in MLO agar at 28 °C for 7 days. Incubation in synthetic wine was set at 21 °C for 24 h. The composition of the synthetic wine was similar to that described in Ref. [4], but adapted to the composition of the Patagonian red wines, with lower pH and higher ethanol concentration (5 g/L tartaric acid, 4.5 g/L malic acid, 0.6 g/L acetic acid, 2 g/L glucose, 2 g/L fructose and 14.0% (v/v) ethanol, pH 3.5) [3].

2.6. Atomic force microscopy imaging

The surface and morphological changes of cells exposed to the different treatments were analyzed by AFM. Cell suspensions with 1×10^9 CFU/mL were spun down at 3000 × *g* for 10 min and washed twice with 0.85% NaCl to remove the medium. A 100 μL droplet of each sample was applied onto a glass slide and allowed to rest at 25 °C for 1 h. After deposition, the sample was rinsed 10 times with Milli-Q water and air-dried at 25 °C. On average, five individual bacterial cells were imaged at high resolution for each sample. AFM images were acquired using a JPK NanoWizard II (Berlin, Germany) mounted on a Zeiss Axiovert 200 inverted microscope (Jena, Germany). Measurements were carried out in intermittent contact mode, using ACT silicon cantilevers (AppNano, Huntingdon, UK), with a nominal tip radius of 6 nm, typical frequencies of 300 kHz and a spring constant of 40 N/m. All images were obtained with similar AFM parameters (set point, scan rate and gain). Height and error signals were collected and images were analyzed with the JPK image processing software v. 4.2.61.

2.7. Surface roughness analysis

The data generated from the AFM height images were used to calculate the surface quadratic roughness of the bacterial cell surface. Using the software Gwyddion v. 2.19 (Czech Metrology Institute, Brno, Czech Republic), the bacterial cell shape was estimated through the application of a mean filter to the raw data. Subtraction of the treated image from the original height image generated a flattened representation of the bacterial cell surface. The surface roughness of a selected area of this flattened image was then calculated from the height standard deviation, *i.e.*, the root-mean-square value (R_{rms}) of the height distribution in Equation (1),

$$R_{rms} = \sqrt{\sum_{i=1}^{n} \frac{(z_i - z_m)^2}{N - 1}} \quad (1)$$

where, N is the total number of data points, z_i is the height of the *i* point, and z_m is the mean height [1,19]. Roughness values were measured over the entire bacterial cell surface, on areas with a constant size of 75 × 75 nm^2.

2.8. Zeta potential measurements

Zeta potential studies were performed at 25 °C on a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK), equipped with a 633-nm He-Ne laser. Cell dilutions were prepared using 10 mM HEPES buffer, pH 7.4, containing 150 mM NaCl, and then filtered using a 0.22-μm pore-size filter. The bacterial suspensions were dispensed into disposable Zeta potential cells with gold electrodes and allowed to equilibrate for 15 min at 25 °C. Viscosity and refractive index were set to 0.8872 cP and 1.330, respectively. Bacterial concentrations were 2×10^9 CFU/mL, in order to acquire high enough count rates. The Zeta potential was determined from the mean of 10 measurements (100 runs each), for control and acclimated cells. The Zeta potential for each sample was calculated from the measured value of electrophoretic mobility, using the Smoluchowski equation [23]. The complete experiment was carried out twice for each sample, using independently grown cultures.

2.9. Survival and L-malic acid consumption after incubation in synthetic wine

Bacterial viability in the wine environmental conditions was studied at laboratory scale by using synthetic wine, as mentioned in Section 2.5. To this end, 10 mL of synthetic wine was inoculated with the rehydrated cells (approximately 5×10^7 CFU/mL), at 21 °C, without shaking, for 15 days. Bacterial viability was monitored by MLO plating at different times (1, 7 and 15 days), at 28 °C. The bacterial inactivation rate was determined according to Equation (2):

$$\text{Log } N/N_0 = -kt \quad (2)$$

where N is the CFU/mL at a given time of wine incubation, N_0 is the CFU/mL before wine incubation, t is the time of wine incubation expressed in days, and k is the constant of viability loss expressed in days^{-1}.

The remaining concentration of L-malic acid was determined by the enzymatic assays (L-Malic Acid MegaQuant™ Format enzymatic kit, Megazyme International, Wicklow, Ireland), on days 1, 7 and 15 of wine incubation. The percentage of L-malic acid consumed (MAC %) was calculated following Equation (3):

$$MAC\% = 100 - ([MA_t]100/[MA_0]) \quad (3)$$

where $[MA_0]$ is the initial concentration of L-malic acid in the synthetic wine and $[MA_t]$ is the concentration measured at different days of incubation.

2.10. Reproducibility of results and statistical analysis

Measurements were performed in duplicate, from three independent cultures of each group studied. Analysis of variance (ANOVA) or *t*-test was carried out using Graph Pad Prism. Data are shown as mean ± standard deviation (SD).

3. Results

3.1. Resistance to the dehydration process

Fig. 1 shows the culturability (expressed as CFU/mL) of the *O. oeni* strain UNQOe 73.2 exposed to different treatments: freeze-dried cells, previously acclimated or not, and fresh culture (as control), before and after incubation in synthetic wine for 24 h at 21 °C. In the presence of trehalose as a cryoprotectant, *O. oeni* UNQOe 73.2 showed to be highly resistant to the freeze-drying processes. No significant differences were observed between freeze-dried acclimated or non-acclimated cells and the fresh culture. However, after 24 h of incubation in synthetic wine, a decrease in culturability was observed in all the conditions assayed. Surprisingly, the fresh culture was the condition most affected by wine incubation, with a decay of 1.5 log units. In contrast, previously acclimated freeze-dried cells were the most resistant to the wine stressful environment.

3.2. Atomic force microscopy

O. oeni frequently occurs as linear chains from 2 to 8 spherical cells (Fig. 2A) or larger chains with lateral prolongations (Fig. 2B), with an average cell diameter of 0.98 μm and a relatively smooth surface. In a few cases, a small protuberance is observed in the center of the cell (Fig. 2C), probably due to the budding division structures, as previously described [38].

Cell surface changes were then assessed using AFM on freeze-dried acclimated and non-acclimated *O. oeni* cells, before and after 24 h of incubation in synthetic wine. Fresh culture in MLO medium was also assayed as a control (Fig. 3).

Images of control cells (Fig. 3A), freeze-dried cells (Fig. 3C) and acclimated freeze-dried cells (Fig. 3E), suspended in physiological solution, allow noticing that the surface and morphology of the freeze-dried cells suffered small modifications, but no lysis or membrane disruption. The non-acclimated cells appear as non-spherical cells, and the binary fission region appears more irregular than in control cells. In the case of acclimated freeze-dried cells, no differences in the chain length were observed, but the budding structure of the center of the cells appears smaller. On the other hand, clear damage on the cell surface can be observed in fresh and freeze-dried non-acclimated cells after inoculation in synthetic wine, together with an increase in their surface roughness and, in some cases, membrane disruption and extensive leakage of the intracellular content (Fig. 3B and D). Interestingly, when cells were previously acclimated in a medium with 6% ethanol prior to the freeze-drying process, no dramatic effects were observed on their surface, although some alterations were noticed (Fig. 3F). Finally, after incubation in synthetic wine, the linear chain structure was completely lost in all conditions, and cells grouped as grapes.

To quantify the damage exerted by the freeze-drying process and the subsequent incubation in synthetic wine, the roughness of the *O. oeni* cell surface was analyzed. The surface roughness of untreated (i.e. fresh culture) cells was 3.04 ± 1 nm, which is in good agreement with previously published data [2,4]. As it can be seen in Fig. 4, the values of surface roughness were not significantly affected by the freeze-drying or acclimation treatments, relative to the control (fresh culture). Furthermore, no significant changes were either observed when acclimated and subsequently freeze-dried cells were incubated for 24 h in synthetic wine, in good agreement with the AFM imaging qualitative evaluation (Fig. 3F). In contrast, the values of surface roughness of non-acclimated fresh culture and non-acclimated freeze-dried cells incubated in synthetic wine for 24 h were significantly increased (8.5 ± 2.9 and 12.6 ± 5, respectively) (Fig. 4).

3.3. Zeta potential

The variation in Zeta potential was measured in control cells before the freeze-drying process. In order to correlate the damage observed in control cells, the Zeta potential was also measured after incubation with synthetic wine. The Zeta potential of control cells, in MLO broth, was close to −14 mV (Fig. 5). These values were not significantly affected in freeze-dried cells, with or without acclimation in 6% ethanol. On the other hand, the values of Zeta potential for freeze-dried cells after incubation for 24 h in synthetic wine were significantly different, reaching more negative Zeta potential values, close to −18 mV. However, when cells were acclimated prior to freeze-drying and then incubated in synthetic wine, the Zeta potential values remained close to those of the fresh culture (control).

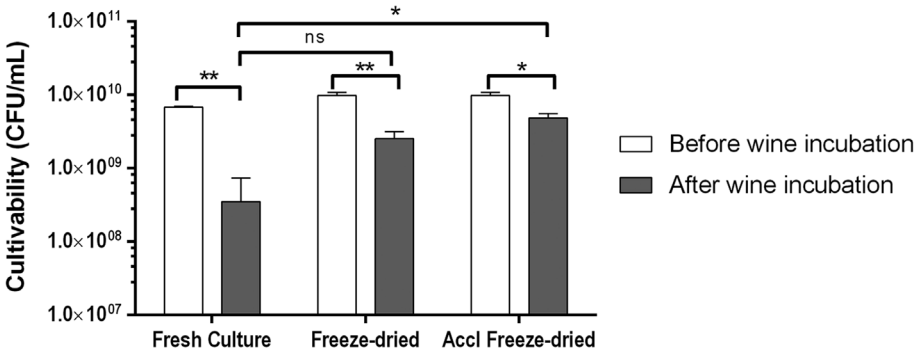

Fig. 1. Culturability of freeze-dried *Oenococcus oeni* UNQOe 73.2 strain before and after incubation in synthetic wine for 24 h (ethanol 14% v/v, pH 3.5, at 21 °C); fresh culture (growth in MLO medium) was added as a control. Freeze-dried culture was previously acclimated (Acc Freeze-drying) in ethanol, 6% (v/v), or non-acclimated (Freeze-dried). Data are presented as mean ± SD.*p < 0.05; **p < 0.005; unpaired *t*-test.

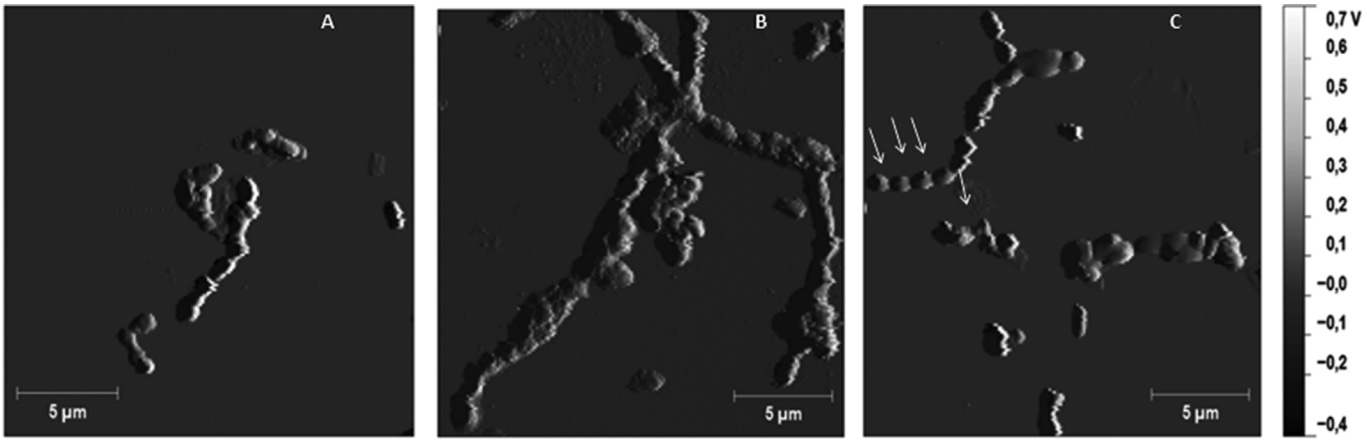

Fig. 2. Atomic Force Microscopy (AFM) error images of *O. oeni* control cells growing in MLO medium. All images are 20 × 20 µm^2. A: Chains with 2–8 spherical parts can be observed, B: Outward protrusion of the lateral surface of the cell chains or cell clusters, C: Outward protrusion in the center of the surface of spherical cells, forming small protrusions.

3.4. Survival and L-malic acid consumption under synthetic wine conditions

As expected from previous works [7], after incubation in synthetic wine, a dramatic loss of viability was observed in the three conditions assayed (Fig. 6). However, the inactivation constant (k) (Table 1) of both freeze-dried cultures (acclimated and non-acclimated) was lower than that obtained for fresh culture, and no significant difference was observed between acclimated and non-acclimated cultures ($P < 0.05$). Finally, after 15 days of wine incubation, the number of viable cells was 1.5 log unit higher for the acclimated and non-acclimated freeze-dried cultures than for the control culture (fresh culture).

The evolution of L-malic acid consumption along incubation in synthetic wine is shown in Fig. 7. Until 7 days, no significant difference in the MAC% was observed between the fresh and freeze-dried cultures, but, after 15 days, both the non-acclimated and acclimated strains were able to consume more L-malic acid than the control culture, with the MAC% being higher than 70%. No difference was observed between acclimated and non-acclimated freeze-dried cultures, in agreement with the results of Fig. 6.

4. Discussion

In this work, we studied the effects of freeze-drying of *O. oeni* and further inoculation in wine-like medium in relation to changes in the cell surface and impact on technological properties: survival and malic acid consumption under synthetic wine conditions. In addition, we evaluated the effect of acclimation treatment prior to freeze-drying.

In concordance with previous reports, *O. oeni* showed a higher resistance to the freeze-drying process in the presence of trehalose [17,40]. Although several *O. oeni* MLF starter cultures are commercially available as dried products, to our knowledge, few studies have evaluated the impact of direct inoculation of dehydrated *O. oeni* strains in wine media [26]. For this reason, it is interesting to study the correlation between surface damage and resistance to hydric (freeze-drying) and ethanol (wine incubation) stresses.

The selection of the Patagonian *O. oeni* strain UNQOe 73.2 as a potential candidate for MLF starter cultures was based on its oenological capabilities, particularly survival and tolerance to harsh wine environment [7]. As previously reported by our group, the acclimation process is a way to improve the culturability of *O. oeni* strains in a wine environment [7,12,13]. However, no studies of the effect of the acclimation treatment previous to a conservation process had been conducted yet. In this study, acclimation showed to be beneficial to protect *O. oeni* cells from the wine stress factor during the first day after inoculation. As shown in Fig. 1, the number of viable cells after 24 h of wine inoculation was significantly higher than that in fresh culture.

In order to dissect the effects of freeze-drying and wine incubation at surface level, AFM and Zeta potential studies were conducted. The results obtained showed no drastic changes in the surface properties or cell morphology after freeze-drying. In contrast, incubation in synthetic wine led to cell surface damage in fresh culture and non-acclimated freeze-dried cells, but not in acclimated cells. This could be due to the fact that acclimation in low ethanol concentration induces adaptation mechanisms on the cell envelope, such as changes in fatty acid composition and membrane protein expression, making the membrane less permeable to ethanol [6,14,15]. The lower impact on the cell surface properties of acclimated cells (Fig. 3C and E) is in concordance with the higher survival values shown in Fig. 1. In addition, this observation is in good agreement with the roughness and Zeta potential values (Figs. 4 and 5). The maintenance of the Zeta potential correlates with the preservation of structures of surface macromolecules and the physiological state of acclimated cells [16,20,23,33,34].

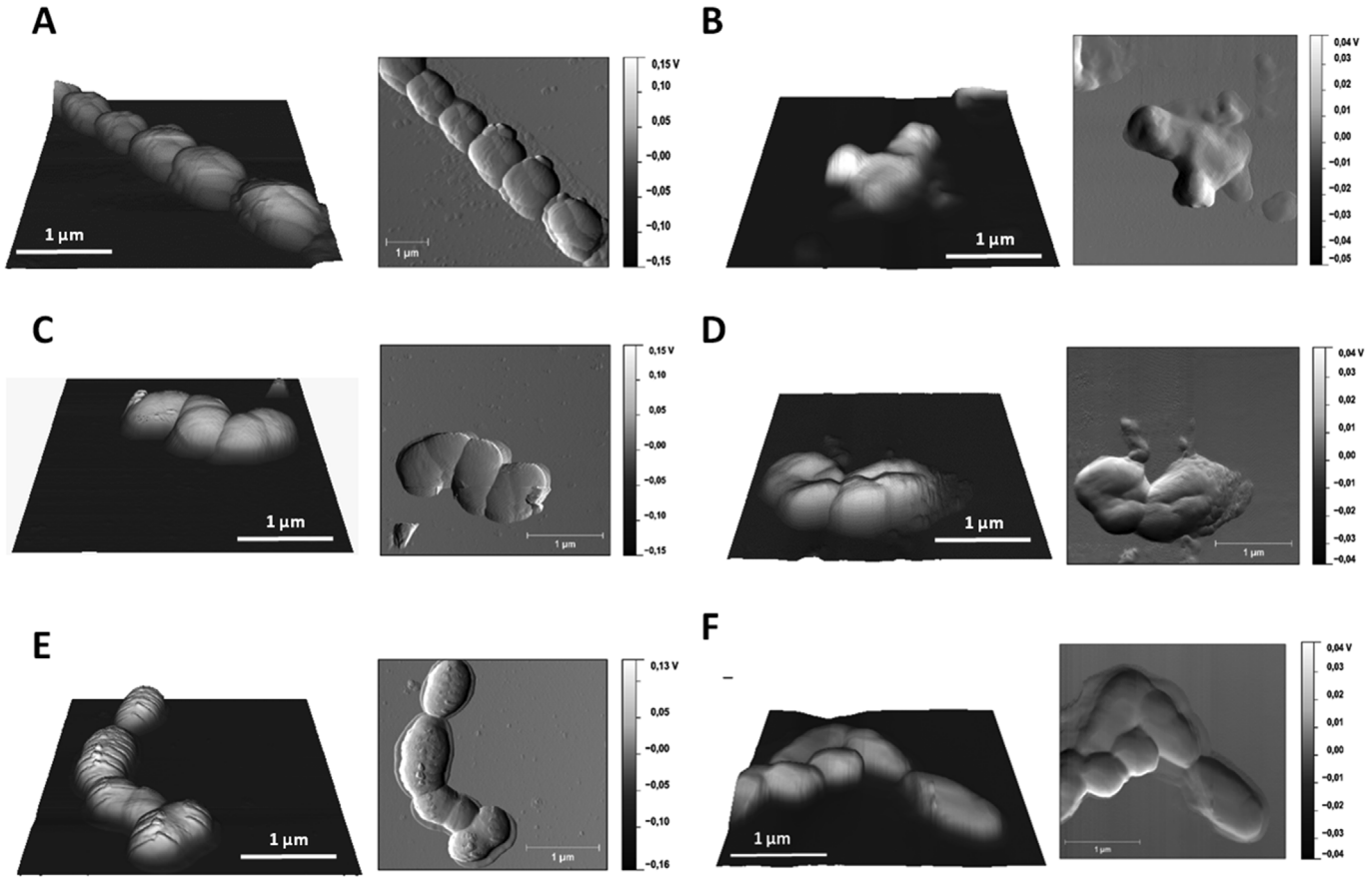

Fig. 3. AFM orthogonal projections from height images (left) and AFM error images (right) of *O. oeni* UNQOe 73.2 cells. (A) Control cells grown in MLO medium without freeze-drying. (B) Cells grown in MLO medium and incubated for 24 h in synthetic wine. (C) Cells grown in MLO medium, freeze-dried and resuspended in physiological solution. (D) Cells freeze-dried and incubated for 24 h in synthetic wine. (E) Cells acclimated in ethanol, freeze-dried and resuspended in physiological solution. (F) Cells acclimated in ethanol, freeze-dried and incubated for 24 h in synthetic wine.

AFM is useful to evaluate cell damage on oenological strains of *L. plantarum* and *L. helveticus* after drying [6,29]. Although no previous AFM images have been reported for *O. oeni*, the images obtained in the present work could clearly be correlated with the electron microscopy images published by Wang et al. [38].

Although no difference was quantified in the roughness of freeze-dried cells in AFM images (Fig. 3A, C and E), a slight change in the morphology was observed between freeze-dried cells and controls. In non-acclimated cells, no protrusion (corresponding to budding) in the center of the cells or changes at the level of sleeve membranes (in the region of the binary fission) were observed (Fig. 3C). In contrast, in acclimated freeze-dried cells, the sleeve membrane was more conserved, and a protrusion on the cell surface was observed, although smaller than in control cells, indicating higher resistant to dehydration than non-acclimated cells (Fig. 3E).

In cells exposed to wine conditions, in addition to an increase in the roughness (Fig. 4), changes were observed in their morphology in the three conditions evaluated. The linearity of the chains was lost and a multiple asymmetry budding was observed. Wang et al. [38] described this type of changes due to oxidative stress. In addition, secreted debris and a grape-like appearance were observed after inoculation in synthetic wine, possibly due to induction of a pomegranate-shape structure

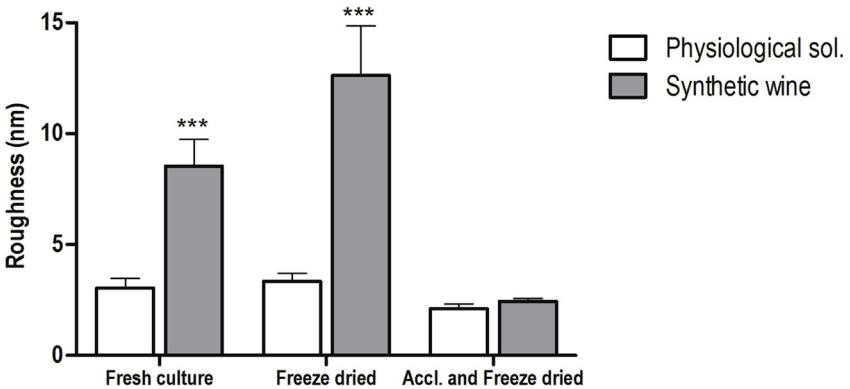

Fig. 4. Surface roughness of *O. oeni* UNQOe 73.2 cells subjected to different conditions, calculated using AFM data. Data are presented as mean ± SD. ***p < 0.001; one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs.* the control column (fresh culture in physiological solution).

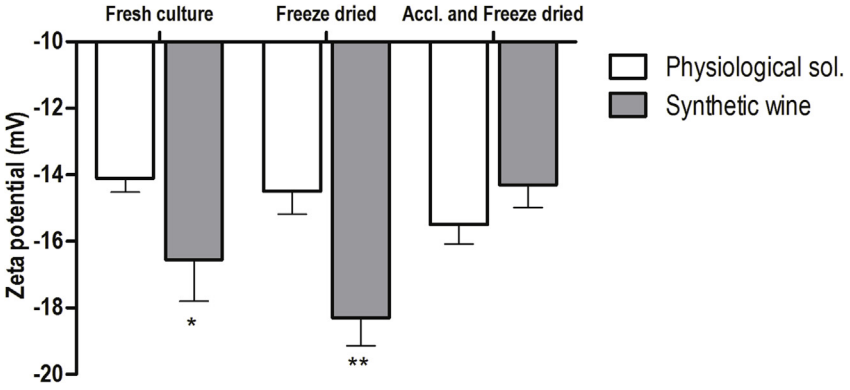

Fig. 5. Zeta potential measurements of *O. oeni* UNQOe 73.2 cells under different conditions. Data are presented as mean ± SD. *p < 0.05; **p < 0.01; one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs.* the control column (fresh culture in physiological solution).

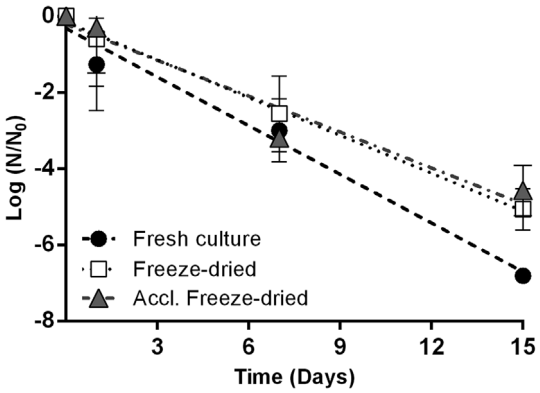

Fig. 6. Relative survival fraction (Log N/N_0) of freeze-dried *O. oeni* UNQOe 73.2 cells, acclimated (▲) or non-acclimated (□) and fresh culture as control (●) incubated in synthetic wine with ethanol 14% (v/v) and pH 3.5 at 21 °C for 15 days. N = CFU/mL after incubation in synthetic wine for different times; N_0 = CFU before wine incubation. Dashed lines indicate the linear regression for each condition.

Table 1
Constant of viability loss (k), of *Oenococcus oeni* UNQOe 73.2, during incubation in synthetic wine at 21 °C. k and R^2 were obtained by adjustment fitting of Equation (2) from Fig. 6.

Condition	k ($days^{-1}$)	R^2
Fresh Culture	−0.43 ± 0.04	0.9821
Freeze-dried	−0.33 ± 0.01**	0.9972
Accl. Freeze-dried	−0.31 ± 0.05**	0.9440

**Significantly different with respect to fresh culture ($p < 0.05$).

as a part of mechanism of resistance [38].

From a technological point of view, different results were observed after the different times of incubation in synthetic wine. The fresh culture was the most affected by the low pH and high ethanol concentration, and a fast loss of viability was observed. Surprisingly, both freeze-dried conditions showed a higher number of viable cells than the control after 15 days of incubation, and no differences between acclimated and non-acclimated cells were observed. These results indicate that, although acclimated cells appeared to be more adapted to the wine stressful environment, after longer incubation periods of time, non-acclimated cells are also able to adapt and survive in the wine environment. In addition, both *O. oeni* acclimated and non-acclimated cells were very efficient in consuming most of L-malic acid.

Finally, we analyzed the dehydration by freeze-drying of *O. oeni* UNQOe 73.2 taking into account a basic and applied perspective. Cell surface damage and morphological changes were observed, mainly

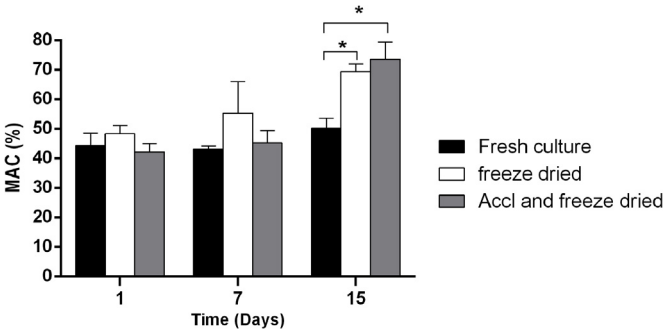

Fig. 7. Percentage of L-malic acid consumption (MAC %) of freeze-dried *O. oeni* UNQOe 73.2, acclimated (gray bars) or non-acclimated (white bars), and fresh culture as control (black bars) after 1, 7 and 15 days of incubation in synthetic wine with ethanol 14% v/v and pH 3.5 at 21 °C. Data are presented as mean ± SD. *p < 0.05 one-way ANOVA followed by a Dunnett post-test for multiple comparisons *vs.* the fresh culture.

after inoculation under wine stressful conditions, being acclimated cultures more resistant to dehydration and subsequent wine inoculation for 24 h. From a technological point of view, we demonstrated that freeze-dried *O. oeni* UNQOe 73.2 directly inoculated in wine was able to consume L-malic acid efficiently, with good perspective to be applied as a MLF starter culture in industrial winemaking processes. It should be pointed out that direct inoculation of dried cultures is an easy practice to apply in the cellar, thus reducing the costs and time of the wine-making process.

Conflicts of interest

The authors state that there are no conflicts of interest that might bias this work.

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