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Anhydrobiosis in Yeasts: Changes in Mitochondrial Membranes Improve the Resistance of *Saccharomyces cerevisiae* Cells to Dehydration–Rehydration

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Abstract: Anhydrobiosis is a unique state of live organisms in which their metabolism is temporary reversibly suspended as the result of strong dehydration of their cells. This state is widely used currently during large-capacity production of active dry baker's yeast. Other strains of the yeast *Saccharomyces cerevisiae*, as well as other yeast species that could potentially find use in modern biotechnology, are not resistant to dehydration–rehydration treatments. To improve their resistance, the main factors that influence cell survival during such treatment need to be revealed. This study showed the importance of mitochondria for yeast cell survival during transfer into anhydrobiosis, a factor that was strongly underestimated until this study. It was revealed that the external introduction inside yeast cells of 50 μM of lithocholic acid (LCA), an agent that induces changes in glycerophospholipids in mitochondrial membranes, in combination with 1% DMSO, may improve the survival rate of dehydrated cells. The influence of LCA upon yeast cell resistance to dehydration–rehydration was not linked with changes in the state of the cells' plasma membrane.

Keywords: *Saccharomyces cerevisiae*; anhydrobiosis; dehydration–rehydration; mitochondria; lithocholic acid

1. Introduction

Anhydrobiosis is a unique state of live organisms in which their metabolism is temporarily reversibly suspended as a result of strong dehydration of their cells. This phenomenon was discovered by Anthony van Leeuwenhoek in 1701 and has been studied intensively for 300 years. However, only because of essential progress in the methods of biological research, we have started to understand the mechanisms behind anhydrobiosis during the last 50 years [1–4]. One large group of organisms that are able to enter the state of anhydrobiosis are yeasts, and it is generally supposed that yeasts are more resistant to the dehydration procedure in comparison to bacterial cells. There are large differences between yeast strains survival during dehydration–rehydration treatments, as well as during their storage in the state of anhydrobiosis [5–8]. It has been shown that halotolerant, thermoresistant, and psychrophilic yeasts are more resistant to dehydration than the conventional mesophilic baker's yeast *Saccharomyces cerevisiae* [9–11]. It was also revealed that some special procedures may increase the resistance of cells to desiccation stress. For example, the incubation of cells in solutions with increased osmotic pressure is very effective in increasing their resistance to desiccation stress [12–16]. The state of anhydrobiosis is now used widely in the successful long-term maintenance of yeast viability and the large-capacity production of active dry baker's yeast. In recent decades, the state of anhydrobiosis in yeasts has attracted great interest, along with other nonconventional yeasts species which may be important for new biotechnological tasks [17]. Primarily, these nonconventional strains may be used in the production of active dry wine yeasts and active dry yeast preparations for the biotechnological

production of various valuable compounds. It is now clear that for these goals it is necessary to understand the main mechanisms by which cells transfer to the state of anhydrobiosis without losing their viability during dehydration, storage in the dry state, and subsequent rehydration/reactivation.

One efficient approach for such research is an attempt to reveal the intracellular protective reactions activated by the organism during their dehydration, as well as tracking the changes in intracellular organelles that take place during cell dehydration in viable and nonviable post-dehydration yeast organisms. Many studies performed in this area have revealed a number of earlier unknown protective reactions and various protective compounds that help cells to survive in such extreme conditions. These protective compounds include trehalose, polyols, proline, glutathione, heat shock proteins, and others [18–33]. It was also shown that all intracellular organelles are subject to definite changes during dehydration and rehydration procedures [8]. It appeared that the most resistant organelles are mitochondria, which restore their activities at the early stages of the cell's reactivation process [34]. Counterintuitively, this information led to insufficient attention being paid to mitochondria during study of the mechanisms of anhydrobiosis. As a result, the information we have at the moment about the mitochondrial changes and their role in the maintenance of cells viability during dehydration–rehydration stress is still rather unclear. At the same time, studies performed during the last decade with the use of a deletion mutant collection clearly showed that the state of mitochondria is important for desiccation tolerance in yeast cells [35,36]. In another study, synthetic interspecific hybrids of two yeast species, *S. cerevisiae* and *S. uvarum*, were tested for their resistance to dehydration–rehydration stress. The results showed that hybrid strain clones containing mtDNA of *S. cerevisiae* were more resistant to drying procedures than the clones containing *S. uvarum* mtDNA. The authors of this research concluded that the resistance of mtDNA to water loss is a very important factor in the survival of dehydrated yeast cells [37]. This study showed the importance of mitochondria and their role in the maintenance of cell viability during yeast dehydration, storage in the dry state, and rehydration/reactivation.

Recent studies have revealed the existence of an additional, previously unknown protective compound for mitochondria, lithocholic acid (LCA). It was revealed that exogenously added LCA, a bile acid, attenuates mitochondrial fragmentation, alters oxidation–reduction processes in mitochondria, enhances resistance to oxidative and thermal stresses, suppresses mitochondria-controlled apoptosis, and enhances the stability of nuclear and mitochondrial DNA [38]. Further detailed studies have shown that exogenously added LCA enters yeast cells, is sorted to the mitochondria, resides mainly in the inner mitochondrial membrane, and also associates with the outer mitochondrial membrane. LCA elicits remodeling of glycerophospholipid synthesis and movement within both mitochondrial membranes, causing substantial changes in the mitochondrial membrane lipidome and triggering major changes in mitochondrial size, number, and morphology. These changes in mitochondrial morphology and the membrane lipidome alter the organelle's respiration, membrane potential, level of ATP synthesis, and reactive oxygen species homeostasis. The conclusion was made that LCA accumulates in both mitochondrial membranes and alters their glycerophospholipid compositions [39]. It also appeared that changes in the mitochondrial lipidome alter the mitochondrial proteome and its functionality. This enables the mitochondria to operate as signaling organelles that orchestrate an establishment of an anti-aging transcriptional program for many longevity-defining nuclear genes. LCA also alters numerous proteins in cellular locations outside of the mitochondria [40,41]. On the basis of these studies, we attempted to determine if the external addition of LCA to yeast cells would be essential for the stability of cells during the dehydration–rehydration process.

2. Materials and Methods

2.1. Chemicals, Yeast Strains, and Growth Conditions

The diploid yeast strains *Saccharomyces cerevisiae* 14 and *S. cerevisiae* 77 used in this study were obtained from the Microbial Strain Collection of Latvia (http://mikro.daba.lv). The strain *S. cerevisiae*

W303 was obtained from Dr. Chuang-Rung Chang (Department of Medical Sciences and the Institute of Biotechnology, National Tsing Hua University, Taiwan). The strains were maintained on solid YPG medium (1% yeast extract, 2% peptone, 2% glucose, 2% agar, *w*/*v*) at 4 °C. Yeast inocula were cultivated in 20 mL of the same liquid YPG medium (without agar) in 100 mL Erlenmeyer flasks at 30 °C for 24 h in a shaker (Environmental shaker-incubator ES-20/60, Biosan) at 180 rpm. Main cultivation of yeast was performed in ˆ250 mL Erlenmeyer flasks with 50 mL of YPG medium and 1% *v*/*v* of inoculum at 30 °C and 180 rpm for 44 h.

0.1 mM LCA (lithocholic acid, Sigma L6250) stock solution in DMSO was added to 50 mL of medium immediately after the inoculum in the amounts necessary to achieve the final concentrations of 50 or 100 µM. The amount of DMSO was calculated with the goal to reach its final concentrations in the media corresponding to 0.05% or 1%.

2.2. Yeast Dehydration–Rehydration

Yeast cells were harvested by centrifugation at 2000 rpm (Eppendorf 5810R, Germany) for 10 min. The supernatant was discarded and the cell pellet was blotted using filter paper to remove any residual liquid, and then pressed through a metal sieve (pore size 1 mm). The sieved biomass was then placed in Petri plates with a slightly opened cover for dehydration at 30 °C for up to 12 h (depending on amount of biomass) until the water content of cells was reduced to 8–10% *w*/*w*.

The water content was determined by drying approximately 200 mg of dry cells and weighing them with an accuracy of 0.1 mg to a constant weight at 105 °C.

For the standard fast rehydration, 5 mL of sterile distilled water was added to the tube with 50 mg (with an accuracy of 0.1 mg) of dry cells. The suspension was then mixed gently during 10 min at room temperature [42].

For the slow gradual rehydration, 50 mg (with an accuracy of 0.1 mg) of dry cells were placed on parchment paper in a chamber with water vapor at 37 °C for 1.5 h. This procedure was followed by fast rehydration as described above.

2.3. Yeast Viability Determination

Determination of survival rate of dry cells after fast and gradual rehydration was performed by the use of fluorescence microscopy and the fluorochrome primuline [10,43]. The yeast cell suspension was mixed with an equal volume of primuline solution (1 mg mL^{-1}), stained for 3 min, and then examined using fluorescence microscopy. In live cells, only the cell wall appears fluorescent while dead cells display a bright yellow-green fluorescence.

2.4. Determination of Plasma Membranes Permeability

The changes of cell membrane permeability during dehydration–rehydration stress were quantified by determination of the total losses by the cells of dry substances during their rehydration in water. For this analysis, measurements of dry weight changes of the samples before and after rehydration were performed.

2.5. Statistical Methods

At least 5 independent experiments (each in triplicate) were performed. Results were expressed as the mean ± standard deviation. The results were analyzed through a one-way analysis of variance (ANOVA test). By this way, significant differences among sample groups were calculated. p-value < 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. DMSO Influence on The Viability of Dry Yeast Cells

The goal of our study was to reveal if the changes of mitochondrial membranes lipids reached by addition of LCA may influence the resistance of yeast cells to dehydration–rehydration. The method of LCA introduction inside the cells is connected with the simultaneous use of DMSO. It is well-known that dimethyl sulfoxide (DMSO) is an aprotic solvent. DMSO's physiologic properties and effects are linked with its own rapid penetration and enhanced penetration of other substances across biological membranes, as well as its ability to scavenge free radicals [44]. DMSO may influence the state of the plasma membrane, including increasing its permeability. As damage to the plasma membrane is one of the main factors resulting in cell death during dehydration–rehydration, it was necessary at the beginning of this study to check the influence of DMSO addition upon the viability of yeast cells during their subsequent drying. In these first-stage experiments, we used the yeast strain *S. cerevisiae* 77, which is resistant to dehydration, and two concentrations of DMSO (0.05% and 1%). The obtained results are shown in Figure 1.

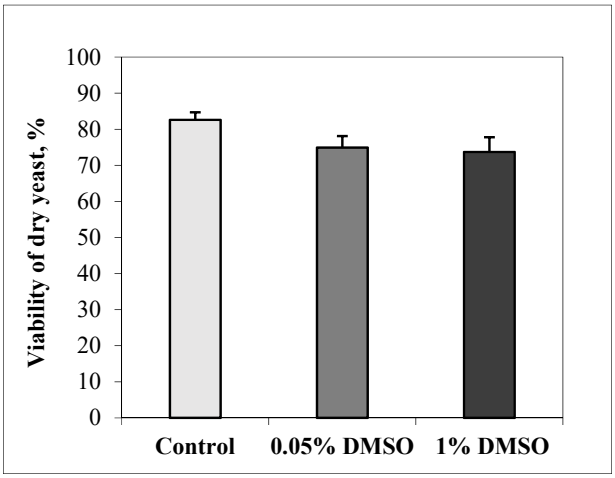

Figure 1. Influence of various concentrations of DMSO on the viability of yeast *Saccharomyces cerevisiae* 77 at dehydration–rehydration treatment.

It can be concluded that DMSO decreases the resistance of the cells to dehydration–rehydration, but that this decrease is small and has little effect. The decrease in the viability of dry yeast cells in comparison with reference conditions (without addition of DMSO) was approximately 8% and 9% for DMSO concentrations of 0.05% and 1%, respectively (no statistically significant differences were found between samples with and without DMSO, $p = 0.17$). On the basis of these results, we conclude that the use of DMSO in our experiments also at a high concentration (1%) is acceptable.

3.2. Determination of The Optimal Concentration of LCA

The second stage of the study was directed to finding which concentrations of DMSO and LCA give the best results. In this series of experiments, we again used the dehydration resistant yeast *S. cerevisiae* 77, two concentrations of DMSO (0.05% and 1%), and two concentrations of LCA (50 μM and 100 μM). LCA concentrations in these experiments were selected according to the results of Dr. V.I.Titorenko group (38–41). It is necessary to mention here that in this and in the next series of experiments we artificially decreased the viability of dehydrated cells of *S. cerevisiae* 77 comparing it with usual high viability of dehydrated cells of this strain which was obtained also in the studies described above (Figure 1). Such effect was reached by some increase of dehydration process rate. These changes were necessary in order to facilitate detection of the positive effect of LCA if it would occur. The results of these experiments (Table 1) showed that the external introduction of LCA inside

resistant to dehydration–rehydration yeast cells may additionally increase their level of viability in a dry state with statistically significant differences ($p < 0.05$). The best results were found when 50 μM of LCA was added, combined with the use of 1 M DMSO. Increase of LCA concentration to 100 μM did not give any additional positive effect (Table 1). It is important to mention that the increase in cell viability was obtained in spite of the results from the first experiments, which showed some detrimental effects of DMSO. This shows that LCA is highly important for cell viability during dehydration–rehydration, negating even the deleterious effects of DMSO. The addition of LCA produces a protective effect or influence on cells via some internal metabolic process.

Table 1. Viability of dry cells of yeast *Saccharomyces cerevisiae* 77 grown with or without various concentrations of lithocholic acid (LCA) and DMSO.

Sample	Viability, %
S. cerevisiae 77	61.0 ± 3.8
S. cerevisiae 77 + LCA 50 μM (0.05% DMSO)	54.0 ± 4.7
S. cerevisiae 77 + LCA 50 μM (1% DMSO)	68.6 ± 1.6
S. cerevisiae 77 + LCA 100 μM (0.05% DMSO)	50.9 ± 3.1
S. cerevisiae 77 + LCA 100 μM (1% DMSO)	69.5 ± 2.8

3.3. LCA Effects upon Resistance of Yeast to Dehydration–Rehydration Treatments

During the next series of the experiments, we decided to check if the previously described positive effects of LCA introduction would be maintained in another strain of yeast. These experiments were carried out using *S. cerevisiae* 14, which is only moderately resistant to dehydration–rehydration treatments. In these experiments, we attempted to determine if LCA stabilizes the plasma membrane, along with making the previously described changes to lipids in the mitochondrial membranes [38–41]. To do this we measured changes in the permeability of the plasma membrane. This was accomplished by determining losses of intracellular dry substances during rapid and gradual dry cell rehydration. The results obtained in these experiments are presented in Figure 2.

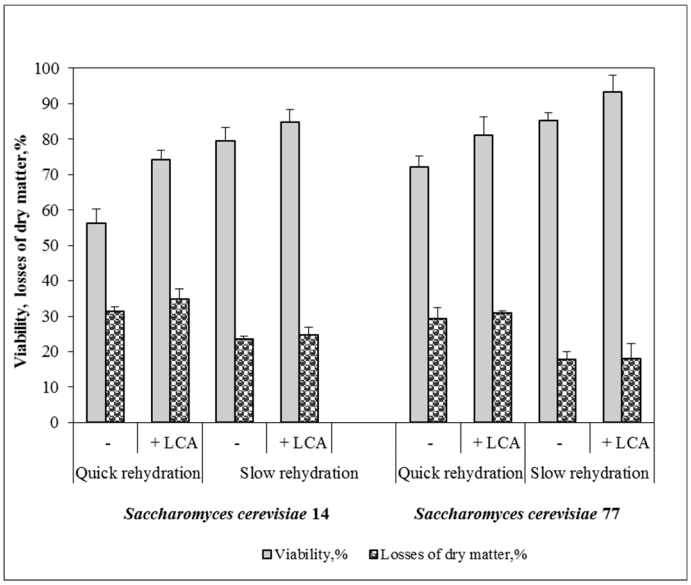

Figure 2. Influence of lithocholic acid (LCA) introduction in the cells of yeast *Saccharomyces cerevisiae* 14 and *Saccharomyces cerevisiae* 77 on their viability and permeability of plasma membrane after dehydration–rehydration treatment.

These results showed that the introduction of LCA leads to an increase in dry cell viability, from 56% to 74%, in *S. cerevisiae* 14. In the case of dehydration resistant strain *S. cerevisiae* 77, this increase

was from 72% to 81%. Gradual rehydration of yeast in water vapor before their immersion in liquid water additionally improved the viability of rehydrated cells from 74% to 85% and from 81% to 93% for *S. cerevisiae* 14 and *S. cerevisiae* 77, respectively (statistically significant differences for these groups were found, p-value < 0.05). As the additional control, we checked if the addition of the same amounts of LCA may influence the resistance to dehydration–rehydration of another, more used in laboratories yeast strain *S. cerevisiae* W303. The experiments showed that also in this case there was reached the increase of cells viability from 54% to 64%.

It is clear that the external introduction of LCA into yeast cells has a positive effect on their survival during dehydration–rehydration treatments. In previous studies, this effect was achieved mainly by improvement of the state of the plasma membrane. In some cases, it was reached by a direct decrease in the phase transition temperature of the plasma membrane lipids. This was conducted by using rehydration liquids at high temperatures (above 38–43 °C) or by gradual rehydration of the dry cells as we have carried out here, first using water vapor and then in liquid [7,8,42,45]. Previous studies have also used trehalose, sugars, and polyols as protective compounds that decrease the phase transition temperatures of membrane lipids [13,22,24].

To determine if LCA similarly improved the state of the plasma membrane, we analyzed the changes in the permeability of treated plasma membranes. The permeability is the main integral characteristic that indicates the health of the membrane. The results (Figure 2) show that LCA introduction leads to an increase in cell plasma membrane permeability in yeast strains *S. cerevisiae* 14 and *S. cerevisiae* 77. This increase was not high, being approximately 3–4% for moderately resistant yeast and 1–2% for the highly resistant strain (no statistically significant differences for these groups, p-value > 0.05). We cannot conclude from our data that this deterioration in plasma membrane state is caused by the action of LCA, as it was shown above that the addition of only DMSO led to a small decrease in cell viability (Figure 1). It is, therefore, possible that the increase of permeability after introduction of LCA is linked, not with effect of LCA, but of DMSO. Regardless, these experiments did not show any positive effect of LCA on the state of plasma membrane. It is necessary to mention here that the gradual rehydration of dry yeast cells with both strains showed a better state of the plasma membrane in reference cells without the addition of LCA and DMSO. From the point of view of our work, despite the better state of the plasma membrane as determined by its smaller permeability, the viability of dry reference cells of both yeast strains was lower by approximately 5–8% than that of those with the introduction of LCA.

Two major conclusions can be made from these results. Firstly, the external introduction of LCA, an agent that induces changes in the glycerophospholipids in mitochondrial membranes inside yeast cells influences the resistance of cells to dehydration–rehydration treatments. We once more confirmed the notion that the role of mitochondria in the processes linked with cell transition into anhydrobiosis is underestimated and that further research determining the importance of these organelles during this process is necessary. Secondly, the obtained results confirm our previous ideas on the existence of additional factors that may essentially influence the survival of cells during their transfer into anhydrobiosis, besides the state of plasma membrane. Such proposals were made earlier on the basis of the results obtained during studies on the role of various mannoproteins in yeast cell walls [46], and stability during dehydration–rehydration of psychrotolerant yeast species [11]. These ideas are supported further by our work, where the external addition of LCA changes the lipid content in mitochondrial membranes. Studies on the additional factors that may influence yeast cell survival during their transfer into anhydrobiosis should continue in future research.

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