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EXPERIMENTAL ARTICLES

Correlation between the Cellular Content of Mobile Water and the Viability of Lyophilized Yeast Cells

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Abstract—Spin-echo NMR studies showed that lyophilized yeast cells contain isolated mobile water (IMW), whose content varied from 0.25% (of the dry weight of cells) in lyophilized exponential-phase yeast cells to 3.8% in lyophilized lag-phase and stationary-phase yeast cells. The viability rate of yeast cells varied from 20% in a lyophilized preparation of exponential-phase cells to 86% in a lyophilized preparation of early-stationary-phase cells. In a lyophilized preparation of yeast cells grown in a chemostat mode at a constant specific rate, the content of IMW depended on the growth-limiting factor, being minimal in the case of growth limitation by the carbon source. In the latter case, the viability of cells was also minimal. The data obtained show that there is a correlation between the IMW content and the viability of yeast cells in lyophilized preparations.

Key words: yeasts, lyophilization, mobile water, viability.

Some eukaryotic cells, yeasts in particular, are known to contain water in different forms—free, bound, and isolated mobile water (IMW). In the process of lyophilization, when cells lose more than 80–85% of their water, they pass to a state of anabiosis, which enhances cell survival under harsh conditions [1, 2]. Lyophilized yeast cells still contain an amount of water, which is isolated in various cellular structures [3]. Cryofractographic studies showed that the freeze-fractured replicas of yeast cells whose growth was limited by nitrogen contained five times fewer globular subparticles than did the freeze-fractured replicas of cells whose growth was limited by carbon. In these two cases, the content of IMW in the lyophilized yeast cells was 3.8 and 1.6%, respectively [4]. Pinto da Silva hypothesized that these globular subparticles are polyenzyme complexes, one of their functions being related to membrane permeability [5].

The content of IMW in the lyophilized preparations of yeast cells depends on the growth phase from which they were taken for lyophilization and varies from tenths of percent in the case of exponential-phase cells to 2.5–4% in the case of stationary-phase cells, the mobility of the isolated water being almost the same in these two cases [6]. The low content of IMW in the exponential-phase cells correlated with their low mechanical strength and high sensitivity to various physical and chemical factors.

The mechanical strength and the content of IMW in *Candida utilis* and *Saccharomyces cerevisiae* cells grown on ethanol or glucose in chemostat cultures were found to be dependent on the cultivation temperature in the range 20–40°C. The cellular content of IMW was minimal at the optimal growth temperature, increased at elevated temperatures, and dropped at supraoptimal temperatures [7]. Furthermore, the content of IMW in lyophilized preparations of yeast cells grown in chemostat cultures at constant specific rates depended on the growth-limiting factor (the carbon, nitrogen, or phosphorus source) [8].

It should be noted that the effect of growth-limiting factors on the state of lyophilized yeast cells was studied by evaluating their viability after rehydration [9]. These studies showed that the viability of yeast cells was minimal when they were taken for lyophilization from the exponential growth phase and maximal when they were taken from the stationary growth phase [9, 10]. In this case, the IMW content of cells also depended on their growth phase. These observations suggest that the content of IMW in yeast cells may be used as a characteristic of their physiological state.

This study is an attempt to reveal a correlation between the viability of lyophilized cells and the cellular content of IMW.

MATERIALS AND METHODS

Experiments were carried out with the yeasts *Candida utilis* Y-405; *Saccharomyces cerevisiae* Y-773; and

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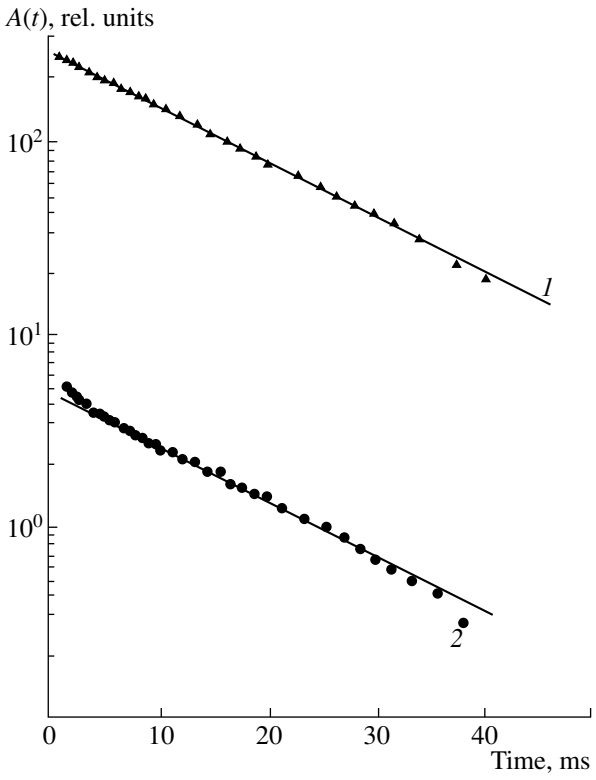

Fig. 1. The spin echo signal declines for the lyophilized preparations of *C. utilis* cells taken from (*1*) the stationary growth phase and (*2*) the phase of active growth.

Saccharomyces vini varieties Beregovskaya, Pino-14, Aligote, and Bordo-20.

The yeast *C. utilis* was grown, in batch or continuous mode, in an ANKUM-2 fermentor in a synthetic medium containing (g/l) NH_4NO_3, 1.5; KCl, 0.3; NaCl, 0.1; $MgSO_4 \cdot 7H_2O$, 0.25; $FeCl_3 \cdot 5H_2O$, 0.0025; KH_2PO_4, 3.54; Na_2HPO_4, 6.92; and glucose, 20.

In the case of chemostat cultivation, the dilution rate of the nutrient medium was 0.25 h^{-1}, the concentration of dissolved oxygen corresponded to 30% saturation, the pH of the medium was maintained at a level of 6.7–6.9 by adding 0.5 N NaOH, and the temperature was 30°C. For each growth-limiting factor (0.05 g/l glucose, 0.0007 g/l phosphate (^{32}P), and 0.016 g/l nitrogen), the yeasts were continuously cultivated over the time period corresponding to 30 cell generations [11].

The *S. vini* varieties were grown in batch mode for 36 h at 24°C on a shaker (180 rpm) in Erlenmeyer flasks with Reader medium containing 3% glucose as the carbon source.

For spin-echo NMR studies, yeast cells were grown in batch culture to the desired growth phase or in continuous culture with the particular growth-limiting factors. Then the cells were harvested by centrifugation, suspended in 10 ml of tap water, frozen, and lyophilized for 10 h in an Edwards lyophilizer. The lyophilized yeast biomass was stored in sealed ampules for 6 months. Spin-echo NMR measurements were performed using lyophilized biomass samples from 0.5 to 1 g in weight. The results were recast to the same amount of biomass (1 g).

Spin-echo NMR measurements were performed by the Hahn method [12]. To improve the accuracy of experimental results, each point of spin echo decline was recorded 10–20 times. The amplitude of spin echo signals was expressed in arbitrary units (100 units corresponded to 30 mg H_2O). The amount of mobile water was calculated from the initial amplitude of echo signals, which was obtained by extrapolating the rectilinear portion of the slow component of the spin echo decline on a logarithmic scale to zero time. It should be noted that the mobile groups of lipid molecules contribute little to the slow component of the spin echo decline due to the low content of lipids in yeast cells.

The viability rate of lyophilized yeast cells was determined by plating yeast cells onto agar medium before and immediately after lyophilization, as well as after 6 months of storage of the lyophilized biomass in sealed ampules. The results were expressed in colony-forming units (CFU).

RESULTS AND DISCUSSION

The detection of IMW in the lyophilized yeast biomass is possible due to different spin echo signals from the protons of water molecules with different degrees of mobility. The relaxation time T_2 of the slow component of the spin echo signal decline of IMW in lyophilized yeast cells is an order lower than the T_2 of water molecules with normal mobility and three orders higher than the T_2 of water molecules with low or zero mobility [1].

Figure 1 shows the spin echo signal declines of the lyophilized preparations of *C. utilis* cells taken from the phase of active growth (curve *2*) and from the stationary growth phase (curve *1*). As can be seen from this figure, the spin echo signals of the stationary-phase yeast cells are considerably higher in amplitude than those of the actively growing cells, whereas the relaxation time T_2 (and, hence, the mobility) of water molecules remaining in the lyophilized biomass does not depend on the growth phase of yeast cells.

Figure 2 shows the consumption of glucose from the cultivation medium of *C. utilis*, the specific growth rate of yeast cells, and the content of IMW in the lyophilized preparations of yeast cells taken at different cultivation stages. As is evident from a comparison of the three curves in this figure, the specific growth rate of yeast cells reached a maximum (0.54 h^{-1}) by the sixth hour of cultivation (when the concentration of glucose in the medium decreased from 20 to 8 g/l) and then rapidly fell as glucose was consumed. The content of IMW was maximum (about 3.8% of the dry weight of cells) in the lyophilized preparation of yeast cells taken from the lag phase (when the specific growth rate was close to zero) and then gradually decreased as the

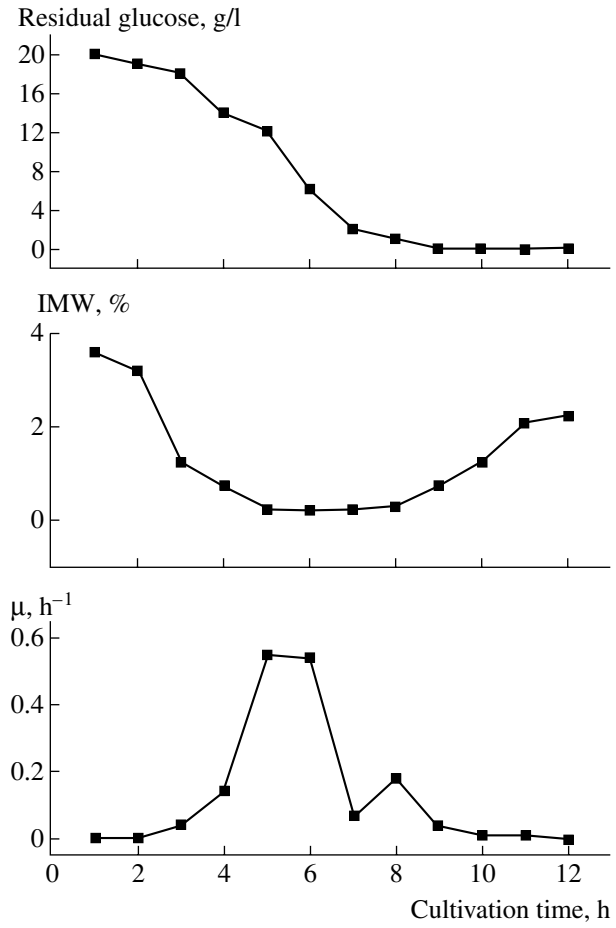

Fig. 2. The IMW content in the lyophilized preparations of *C. utilis* cells characterized by different specific growth rates.

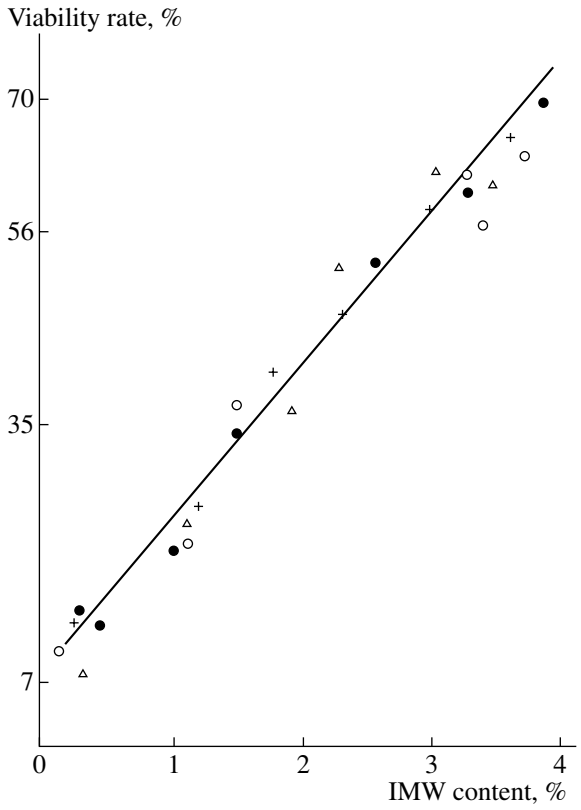

Fig. 3. Correlation between the content of IMW in lyophilized yeast cells and their viability rate (data of four experiments).

specific growth rate increased. The IMW content was minimum (0.25% of the dry weight) in the case of exponential-phase yeast cells. These data indicate that the content of IMW in yeast cells and their specific growth rate are related inversely, which can be explained by the increased sensitivity of actively growing cells to various environmental physical and chemical factors [3].

It should be noted that the content of IMW in yeast cells depends not only on their specific growth rate during batch cultivation but also on the growth-limiting factor during continuous cultivation. The table summarizes experimental data on the content of IMW in lyophilized preparations of yeast cells taken from batch cultures at different growth stages and from chemostat cultures with different growth-limiting factors.

In the case of batch cultivation, the maximum content of IMW (3.9%) was observed in the lyophilized preparation of the stationary-phase *C. utilis* cells. This preparation was also characterized by the maximum viability (86%). The lyophilized preparation of the exponential-phase yeast cells contained little IMW (0.22%) and showed minimal viability (20%).

In the case of continuous cultivation at a constant specific growth rate of 0.25 h^{-1}, the minimal content of IMW (0.15%) was observed in the lyophilized preparation of *C. utilis* cells taken from the carbon-limited chemostat culture. This preparation was also characterized by the minimal viability (12%). At the same time, *C. utilis* cells taken from the nitrogen- or phosphorus-limited chemostat cultures contained a considerable amount of IMW (3.7–3.8%) and showed a high viability (69–72%).

It should be noted that the use of propynol B-400 at a concentration of 0.1 ml/l as an antifoaming agent during cultivation led to a drastic decline in the cellular content of IMW. This could be due to an altered permeability of intracellular membranes in the presence of the surface-active propynol.

After 36 h of batch cultivation, the *S. vini* varieties Beregovskaya and Pino-14 obviously reached the stationary growth phase and were characterized by cellular contents of IMW equal to 3.8 and 3.4%, respectively, and viabilities after lyophilization and subsequent rehydration equal to 70 and 63%, respectively. Two other *S. vini* varieties (Aligote and Bordo-20) grew more slowly, so that the 36-h-old cells of these varieties were in the retardation growth phase and exhibited

The IMW content and the viability rate of different yeast cells subjected to lyophilization

Yeast strain	Growth phase or limitation factor	IMW content, %	Viability rate, %
C. utilis Y-405	Exponential phase	0.22	20
"	Stationary phase	3.90	86
"	Phosphorus-limited chemostat	3.70	69
"	Carbon-limited chemostat	0.15	12
"	Nitrogen-limited chemostat	3.80	72
S. vini var. Beregovskaya	Stationary phase	3.80	70
S. vini var. Pino-14	Stationary phase	3.40	63
S. vini var. Aligote	Retardation phase	1.50	36
S. vini var. Bordo-20	Retardation phase	1.90	43

lower values of IMW content (1.5 and 1.9%, respectively) and viability (36 and 43%, respectively).

Figure 3 summarizes experimental data on the IMW content and viability for all the yeasts and cultivation conditions investigated. As is evident from this figure, in the range of IMW content from 0.15 to 3.9%, the IMW content and the viability are directly correlated. This correlation can be used for evaluating yeast viability in lyophilized preparations from the IMW content. It should be noted that none of the lyophilized preparations investigated was characterized by 100% viability of yeast cells, which indicates that cells differ in resistance to lyophilization even in the stationary growth phase.

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