PHYSICAL PROPERTIES OF CELL WATER IN PARTIALLY DRIED *SACCHAROMYCES CEREVISIAE*

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ABSTRACT The equilibrium vapor pressure, the heat of vaporization, the dielectric increment, and the NMR spectra of partially dried cells were studied in *Saccharomyces cerevisiae* with water contents varying in the range from 25 to 0.8%. The comparative study of those physical properties suggests that physical states of the microbe can be classified into four regions in accordance with the states of the cell water: the solution region, the gel region, the mobile adsorption region, and the localized water region. Much difference in the physiological properties is found between the cells in the solution region and those in the gel region, whereas the pattern changes in physical properties take place when the cells in the gel region are dried to a further extent into the mobile or the localized region. The various modes in the molecular motion of the cell water reflected in those physical properties of the cell seem to give some insight into the biological functions of the molecule in the native as well as the dried states of the cell.

INTRODUCTION

Studies of microbial cells dried to varied extents are expected to give information of interest about the properties and functions of water in native cells. However, no systematic studies of physical properties of a dried live microbe are found in the literature, although some of the properties have been intensively studied by several authors with different microbes, e.g., the heat of vaporization of freeze dried yeasts by Takano and Terui (1), the dielectric increment of freeze-dried yeasts by Sakaguchi et al. (2), and NMR spectra of *Norcardia* by Cerbon (3). The present investigation consists of biophysical and biological experiments, of which the former part is described in this paper. Data on the equilibrium vapor pressure, the heat of vaporization, the dielectric increment, and the NMR spectra are discussed with special regard to the function of the cell water.

In this respect most of freeze-dried cell preparations have rarely shown a viability

level high enough to be used for comparative studies of their physical and biological properties. On the contrary, as dried baker's yeast prepared at a relatively high temperature is known to have a remarkably high level of viability, dried cells of *Saccharomyces cerevisiae* obtained commercially as "active dry yeast" are used throughout the present experiment. By use of this microbe, samples with any water content in the range of 0.8 to 25% can easily be prepared and the viability level of the series thus prepared is the same as that of the dry yeast used as the starting sample.

MATERIALS AND METHODS

Dried yeast commercially available as ADY (active dry yeast) from the Oriental Kobo Co., Ltd., Tokyo, Japan, was used as the starting material throughout the present experiment. The ADY was manufactured by hot air drying at a relatively high temperature (presumably at about 30°C) and had a spherical pellet structure of diameters ranging from 0.5 to 1.3 mm. The pellet fractions of medium size (0.7 to 1.0 mm) were used after sieving. It had a water content of 7 to 8% and its viability was found to be invariably above 90%.

The cell water was measured as weight decrease after heating from 5 hr at 105°C in air and will be expressed in weight percentage on the wet base. Series of samples with various water contents between 4 and 25% were prepared by placing the ADY in the dessicator hygrostats which contain a large amount of sulfuric acid solution at proper concentrations. In preliminary experiments it was found that, after being left for more than 24 hr at 30°C in the atmosphere of relative humidity in the range 3 to 95%, the yeast cells approximately attain equilibrium states in regard to their water contents. Since the samples showed no hysteresis in their physical and biological properties, they could be reversibly humidified and dehumidified except in the range of high water content (>20%), where the cells became slightly sticky and slow decrease of cellular activity was observed during a prolonged standing. To obtain samples with a water content less than 4%, we dried the ADY in vacuum at room temperature.

Samples prepared as mentioned above showed little difference in their physical properties under consideration as compared with those of powdered samples obtained by use of a hydraulic press machine. This seems to be owing to the fact that only thermodynamical properties, slow rate processes, and responses to forcing with a small amplitude were concerned in the present study. Since the samples showed slight but definite decrease in their biological activities after being pressed into the powdered form, showing that some yeast cells were mechanically damaged by this hydraulic treatment, the present study was made solely with samples in the pellet form.

The dielectric measurements were made with a commercial Q-meter by the use of the parallel compensation method. A constant weight of each sample was filled into the gap of a parallel plate condenser. The sample condenser had an air capacity of 5 pF, the electrode being fixed at 2.0 mm apart.

The broadline NMR spectra were observed with Model JNM-W-30 (Japan Electron Optics Laboratory Co., Ltd., Tokyo, Japan), the high frequency used being 30 Mc modulated at a width of 1 gauss, while the high resolution spectra were obtained with Model JNM-C-60 operated at 60 Mc.

The viability of the sample cells was examined with the plating-out method in a

routine way. The colony counts thus found were invariably in fair good agreement with the values observed by use of the methylene blue staining method in the present experiment.

EXPERIMENTAL RESULTS

Equilibrium Vapor Pressure. The ADY of 7% water content was humidified up to 20% and dehumidified again to the original level by using successively several dessicators incrementally hygrostated at different humidities in the way mentioned in Materials and Methods. After a prolonged experiment for two weeks, an isotherm for the vapor pressure at 30°C was obtained as shown in Fig. 1. The

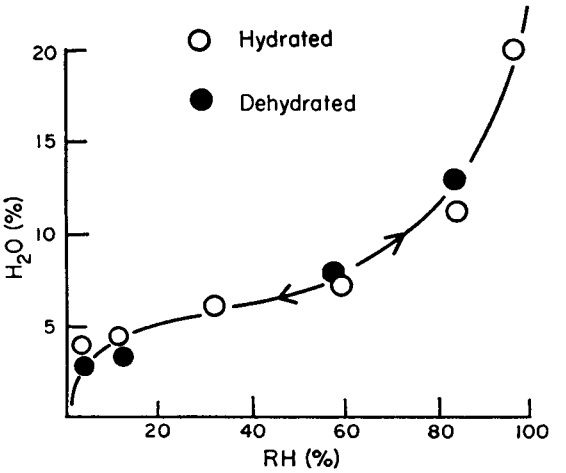

FIGURE 1 Sorption isotherm of dried yeast cells at 30°C. Water contents in percentage on the wet base are plotted against the relative humidity of the vapor phase. Open circles represent values found when the original samples (4%) was incrementally humidified, while closed circles are those observed when the wet samples (21%) thus prepared was dehumidified back to its original water level.

ordinate represents the water contents of the yeast in each dessicator and the abscissa denotes the relative humidity of the corresponding atmosphere. The curve shows no hysteresis loop, indicating that this isotherm approximately represents the equilibrium properties of the cells. The shape of the curve is similar to that usually obtained for nonbiological materials with the adsorbed water. It shows an inflection point at about 10% and indicates the existence of a strong adsorbing site corresponding to the water range below 4%.

Heat of Vaporization. By using many sets of the dessicator hygrostats set at 4°, 18.5°, 28.5° and 38.5°C, respectively, a group of sorption isotherms were obtained for samples in the water range of 4 to 23%. The curves indicated that, when the water content is held constant, the samples gave higher vapor pressures at

higher temperatures. The temperature variation of the equilibrium vapor pressure thus obtained allows the calculation of the apparent heat of water vaporization (ΔH) by use of Fig. 2 in accordance with Clausius-Clapayron's relation. Since the calculation was made under the condition of constant water content, the values thus obtained may correspond to the molar differential heat content discussed, for example, by Eley and others in the thermodynamical studies of pure protein-water systems (4).

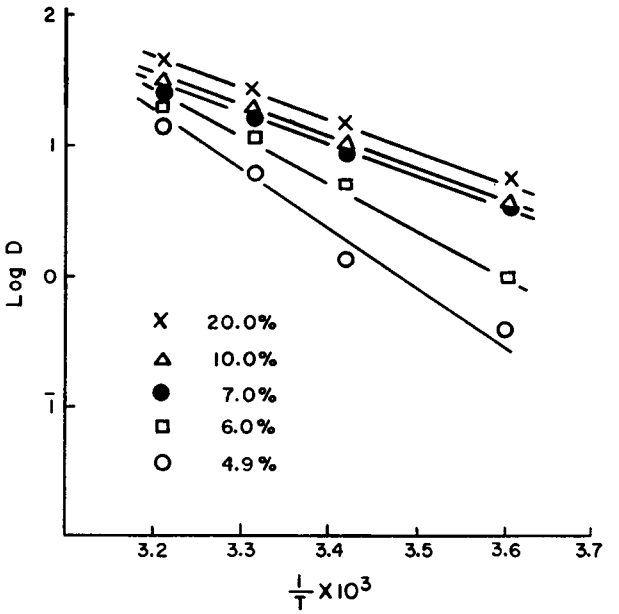

FIGURE 2 Vapor pressure of dried yeast cells as function of temperature. The water content is taken as a fixed parameter. D represents the vapor pressure in absolute humidity (gr/m^3) as known from the concentrations of H_2SO_4 solutions used in the hygrostats and T the temperature of each hygrostat.

As shown in Fig. 3 which represents ΔH as function of the cell water contents, the heat of vaporization is almost equal to that of pure water (10 kcal/mol) for the cells with a water content of 10% and above, while it increases with decreasing water contents in the range below 10% until it amounts to 20 kcal/mol at 5%. The general pattern of the curve is in agreement with that obtained by Takano and Terui using freeze-dried yeast (1).

Dielectric Increment. The apparent dielectric constant of the partially dried cells was measured by use of the resonance method. The sample condenser had an air capacity (C_o) of 5 pF. The measured sample capacitance (C) showed a broad frequency dispersion over the frequency range between 50 kc and 50 Mc, whereby the dispersion intensity was large for wet cells and almost zero for cells

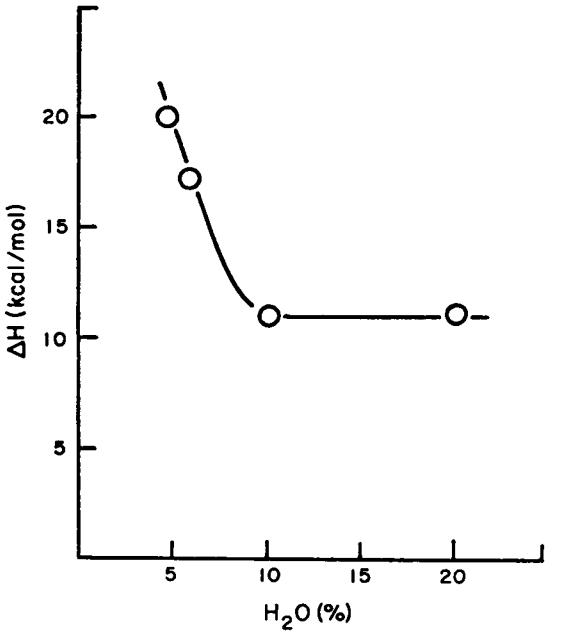

FIGURE 3 Heat of vaporization (or sorption) of dried yeast cells. The values were calculated from the tangents of the lines shown in Fig. 2.

with water less than 5%. The values of apparent dielectric constants (ϵ') obtained as the ratio of C to C_0 at 50 kc, 1 Mc and 50 Mc were plotted against the cell water contents in Fig. 4. Curve (C) shows a break point at 11%, while the remaining curves (A and B) show two break points at 7 and 11%, respectively, along the abscissa representing the sample water contents.

The dielectric polarization observed as ϵ' of those heterogeneous samples is considered to consist of molecular and structural terms, the latter being due to the existence of the pellet structure. However, it can be shown that the contribution from the mixed structure of yeast pellets and air can be discarded on account of the low conductivity and low dielectric constant of the air phase. Accordingly, since the orientation polarization of solid high polymers cannot take place at 50 Mc, curve (A) in Fig. 4 seems to represent only the contribution from the cell water, while curve (C) corresponds to the molecular polarization as a whole. If so, the difference between the two curves gives roughly a relative estimate for the magnitude of the orientation polarization of organic molecules in the cell, thus showing their varied degree of mobility in the yeast cells with various water contents.

As shown in Fig. 4, the states of the dried cells can be classified into three regions (a), (b), and (c) in accordance with the observed dielectric properties. The combined region of (a) and (b) may be the bound water region, as called by biologists, and (c) the free water region. As will be discussed later, however, the discrimina-

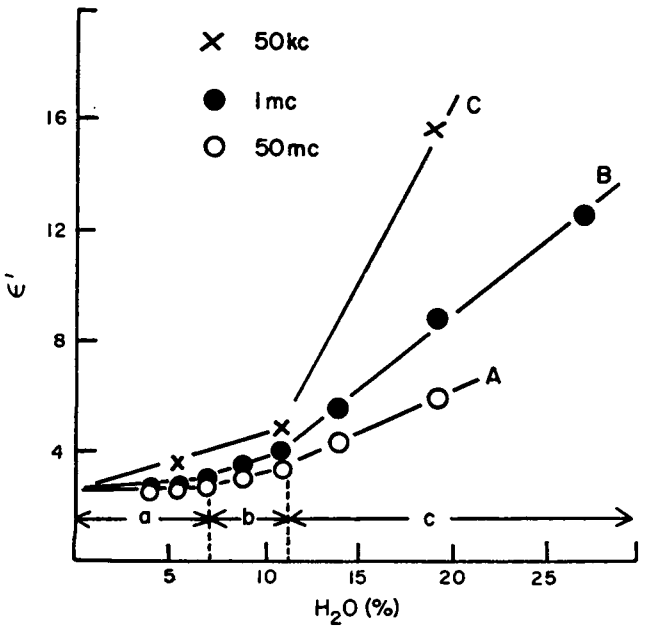

FIGURE 4 Dielectric increment at room temperature of dried yeast cells. Apparent dielectric constants of the pellet samples are plotted against the water contents.

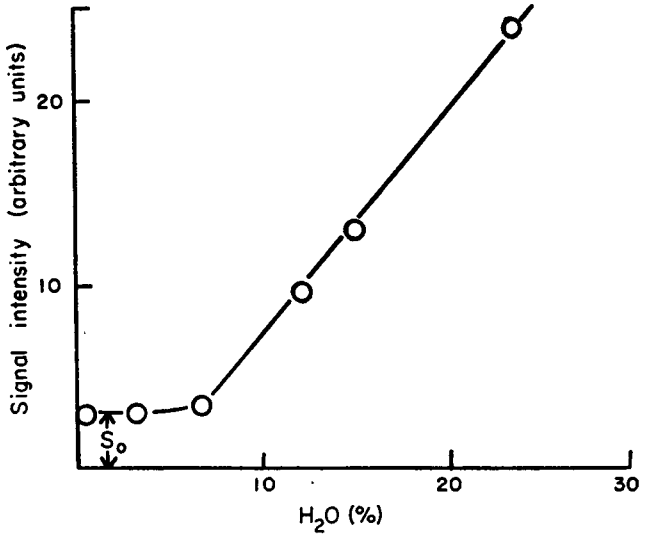

FIGURE 5 Broadline NMR of dried cells of yeast. Peak-to-peak values of signal intensities of the narrow peak are plotted against the water contents, S_o representing the residual signal at apparently zero water content.

tion reflects merely the difference in several modes of the molecular motion of the water.

NMR Spectra. The broadline spectra found with the series of dry yeast prepared as above showed a two component absorption. Their shape is similar to that of other organic substances with adsorbed water, given a narrow proton peak, due to the water molecules, accompanied by a broad background, due to the proton on organic cell constituents.

The signal intensities of the narrow peak was plotted against the water contents in Fig. 5. The ordinates correspond to the first derivatives of absorption signal and the abscissas correspond to the water contents of the dried cells.

Since the contribution from the background proton was small as compared with that of the water proton, uncorrected peak-to-peak values are shown. The curve shows a break point around 7% and remains at constant level down to 0.8%.

In order to obtain more detailed information about this peculiar pattern, we observed higher resolution spectra of the narrow peak mentioned above by using 60 Mc. Typical examples of the spectral structure shown by the narrow peak are given in Fig. 6. The position of the main component (P_1) coincides with that of the

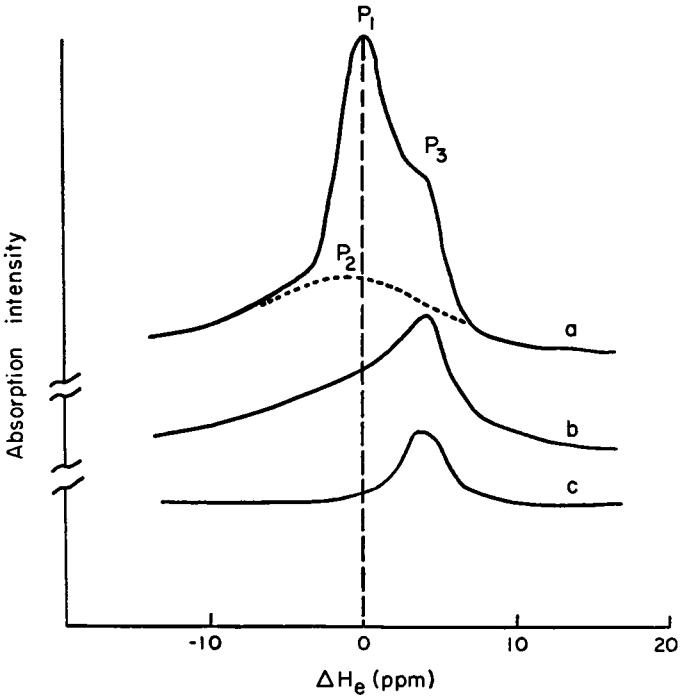

FIGURE 6 High resolution NMR spectra of dried yeast cells, representing the three component structure of the narrow peak in Fig. 5. The abscissas denote resonance shifts (ppm) with respect to pure water used as the external reference. The water contents are (*a*) 20.6%, (*b*) 12.6%, and (*c*) 2.9%.

absorption of pure water used as the external reference. The main peak decreases in height until it disappears with samples below 13%, while the broader component (P_2) remains unchanged with samples above 13%, being diminished to zero with highly dried samples (5%) as shown in curve (*c*). The side component (P_3) remains unaltered with all samples used, thus giving rise to the residual signal (S_0) in Fig. 5.

Physiological Properties. For the sake of comparison, the respiration rates of the yeast cells are shown in Fig. 7, where the rate of oxygen uptake was de-

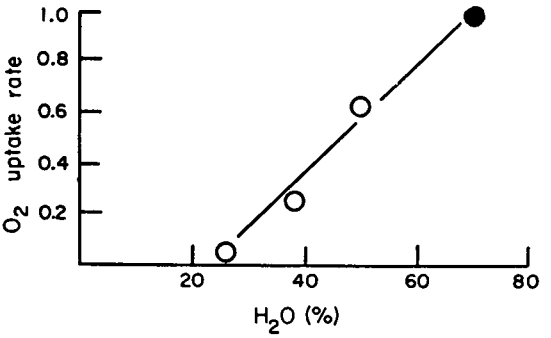

FIGURE 7 Respiration rate of partially dried yeast cells. Oxygen uptake rates at 30°C measured with the Warburg apparatus are plotted in relative units. The closed circle represents the internal respiration rate of the native cells.

termined in a routine manner with the Warburg apparatus. The rate decreased linearly with the decreasing water content to 20%, where the oxygen uptake apparently stopped, suggesting that no marked physiological activity was observed with yeast cells having a water content less than 20%.

DISCUSSION

The results of physical and physiological experiments described above can be rearranged in a diagram as shown in Fig. 8. Open circles in each row show where the curves had break points when respective physical properties were plotted against the water contents. Break points in the physical rows seem to scatter around 5 and 10%, while the break point in the physiological row is found around 20%. Thus the states of dried yeast cells can be classified into four regions *A, B, C,* and *D* as denoted in the top row of the diagram.

The properties and functions of the cell water vary as the yeast cells are transferred from a state in a region to a different state in another region. In region *D,* which may be called a "solution domain," the cell water functions as a continuous medium so that biochemical reactions can proceed at somewhat retarded rates. In region *C,* which may be called a "gel domain," water molecules cannot serve any longer as a continuous solvent for biochemical reactions involving conformation

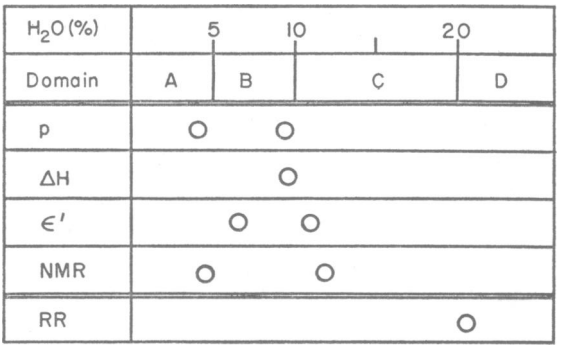

FIGURE 8 Domain diagram for the physical states of dried yeast. Circles denote the water contents where the pattern changes are observed for each physical property: *P*, equilibrium vapor pressure; ΔH, heat of vaporization; ϵ', apparent dielectric constant; NMR, proton nuclear magnetic resonance. Respiration Rate (RR) is shown as a physiological property for the sake of comparison.

changes of high polymer constituents. Hence at the water content of 20% a remarkable change was observed with the physiological property, while no drastic change was found around 20% with physical properties such as vapor pressure, heat of vaporization, dielectric polarization and NMR absorption. These are all concerned with either almost reversible processes with a small amplitude or rate processes at an extremely slow time rate. In regions *A* and *B*, which may be called a "localized water region" and a "mobile adsorbed region," respectively, the water looses its various modes of molecular motion in an incremental manner, showing pattern changes each time in related physical properties.

Since, as shown in Fig. 6, the water molecules in region *A* do not contribute to the narrow peak of the NMR absorption spectra, they seem to be highly localized by a strong interaction with organic cell constituents, whereby the heat of adsorption is estimated not less than 20 kcal/mol (Fig. 3) and the equilibrium vapor pressure at room temperature is less than 15% in relative humidity (Fig. 1). The water in this region may be irrotationally bound to some strongly ionizable sites on the constituent molecules, showing no dielectric polarization as observed in range (*a*) in Fig. 4.

The water in region *B* seems to be adsorbed to less ionizable polar sites in different manner. Its heat of adsorption varies from 20 to 10 kcal/mol as found in Fig. 3 and its line width of NMR absorption is 10^{-2} to 10^{-1} gauss, indicating that molecular exchange might possibly take place in a limited way between adjacent sites of adsorption.

Since the water molecules in region *C* show a fairly narrow line width in NMR spectra and a large dielectric polarization, it seems probable that the molecules in this region are highly mobile. Since the appearance of the P_1 peak does not affect, as mentioned before, the shape of the P_2 peak, the water clustering in region *C* is

likely to occur mostly around nonionizable polar sites different from those previously occupied by the water in region *B*.

The side peak (P_s) has so far not been identified. As it is found 3 to 4 ppm upfield in reference to the main peak, it might be assigned to unassociated water molecules which have no hydrogen bonds between them as in bulk water. If so, presumably, the water could not be measured by the weight loss method described above, probably because it is contained within the hydrophobic molecular cages of some polymer substances. Alternatively, the peak might be caused by the proton in the methyl groups on some organic compound which is in a liquid state at room temperature.

It seems appropriate at this stage to add a short discussion about a possible cause of the high viability found with the yeast in the present experiments. The drying and rehumidifying processes in the present work were invariably taken in a slow and isothermal way. Thus, although the state of the highly dried yeast cells is quite different from that of the native yeast, the former is connected with the latter by way of the quasi-reversible process which can proceed in either direction along the same pathway specified approximately by a minimum free energy contour at each step of drying or humidifying. For instance, when the yeast cells pass region *C* during a drying process, many drastic conformation changes must take place inside the cell, e.g. ion pairing or clustering and neutralization of zwitter-ions, because under such dried environments with small dielectric constants, separately charged conformation of each molecule is energetically less stable than in the wet state. If the process is reversed and the dried cell is rehumidified, all the conformation changes will be reversed and the ions will dissociate by the slow addition of water molecules.

The dried cells obtained by a drying process of this type are, therefore, in a stable state under the water-free condition, being less sensitive to the ambient temperature, whereas rapidly freeze-dried cells are not in a stable state under dried conditions so that it must be kept at low temperature to prevent the dissipation of the information content necessary to regain, when rehydrated, the original state. Moreover, during a rehydration process, there seems to be much probability for those cells to recover unsuccessfully the native state, thus loosing to a varying extent the viability or biological activities observed with the microbe before drying.

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