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Activity of Yeast Cells Solubilized in a Water-in-Oil Microemulsion**

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Abstract: The respiration of Baker's yeast solubilized in a water-in-oil microemulsion (water content up to 4% (v:v)) was investigated by following the consumption of oxygen after addition of glucose by a Clark electrode. The activity of cells in the concentration range 1–100 mg /mL (ca. 10^6–10^8 cells/mL) could thus be measured in the organic medium and followed as a function of time. The influence of added ethanol and of fermentable sugars has also been tested. The results show that the polarographic method is reliable for studies of cells solubilized in organic solvents and that yeast in this organic medium although displaying lower rates, has the same behavior as in water concerning the oxygen consumption and ethanol fermentation.

Bacterial cells and cell particles can be solubilized in organic solvents, such as hexadecane, isopropyl palmitate and isooctane, by means of non-ionic surfactants (e.g. Tween®, lecithins, asolectins) which build reverse micelles or water-in-oil microemulsions in such solvents[1–4]. It seems that the solubilization is mediated by a micellarlike system, as in the case of proteins in organic solvents[5,6]. However the picture of a particle, hosted in the water pool of reverse micelles, is hardly applicable for cells being too large to be compatible with the size of thermodynamically stable water droplets in classic reverse micelles. The question of the real structure of these cell-containing microemulsion systems is still open.

In fact cells, up to a concentration of 10^6–10^7 cells/mL (1–10 mg/mL) can be solubilized in a water-in-oil microemulsion yielding a transparent solution with practically no scattering above $\lambda = 300$ nm, and there is no cell sedimentation, even after weeks. Such systems are interesting not only from the scientific but also biotechnological view. On the one hand, as shown for example by *Haag* et al.[7], *Nakamura* et al.[8], and *Fadnavis* et al.[9], these systems are useful for microbial transformations in organic solvents. On the other hand one can also be interested in fundamental questions, such as whether and to what extent the metabolism, the structure, and the growth rate of the cells is changed in the new environment. Presently structural studies by microscopy and light scattering, as well as studies on the growth rate and the basic biological behavior are pursued in our group.

Measurements of cell viability in this new system are usually performed by plating out aliquots on agar-dishes containing normal aqueous growth medium. It would be much better to have a rapid and reliable test of cell viability and activity directly in organic solvents. The aim of this communication is to illustrate one of such a procedure, based on polarography. Polarographic techniques for measuring changes in oxygen concentration dissolved in aqueous solutions have been already used since the 1940's to evaluate photosynthesis or respiration[10], and later the Clark electrode became useful also to study oxidative enzymes, as shown by the example of glucose determination with immobilized glucose-oxidase[11]. Recently, the polarographic determination of cell respiration was proposed as a reliable way to determine cell viability[12]. At the best of our knowledge, polarography has not been applied yet to organic solutions, although the group of *Palmieri* attempts to use this method to follow enzymatical reactions in reverse micelles[13].

Experimental:

Materials and Methods

Commercial Baker's yeast from Eridania Z.N., Genoa, was used for the experiments. Tween 85, ethanol, isopropyl palmitate (IPP), isooctane, glucose,

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bis(2-ethylhexyl) sodium sulfosuccinate (AOT) were from Fluka, lipoxidase (lipoxygenase E.C. 1.13.11.12) and linoleic acid from Sigma. All reagents (at the highest available purity) were used without purification.

The polarographic assays were performed with a Gilson K-IC oxygraph, fitted with a Clark electrode and a water-jacketed cell. The electrode surface was coated with KCl gel (Beckman) and equipped with a Teflon membrane.

The calibration of the oxygen electrode was achieved via the lipoxidase (1.5 mg/mL) catalyzed peroxidation of 2–10 µL 100 mM linoleic acid in 1 mL of a AOT/IPP/isooctane (300 mM AOT in isooctane/IPP, 20:80 v/v) solution, equilibrated at 25°C against air for 3–4 h. Direct calibration in the Tween/IPP system was not possible because lipoxidase is active towards Tween 85. The calibration was repeated every 24 h, finding no change in the electrode response.

Preparation of the Solutions

3 g pressed baker's yeast were resuspended in 25 mL basal medium (110 mM glucose) as described by *Meikle* et al.[14] and shaken for 4 h at room temperature. The solubilization of the yeast cells was carried out by putting 400 µL of the cell suspension into 20 mL of a 100 mM Tween in IPP solution producing an initial w_0 value of 11 ($w_0 = H_2O$/Tween (mol/mol)); the rate of the oxygen uptake is very sensitive to the strain and the way of preparing the aqueous yeast suspension before the solubilization.

Measurements

The reaction was started by adding 20 µL 220 mM glucose solution into the polarographic cell containing 1 mL yeast in the organic medium. Assuming that cells had consumed all the initial glucose fed with the basal medium, the final water-pool concentration of glucose was again 110 mM, while the w_0 raised from the initial value (11) to the final value of 22. This corresponds to 2.2 M water in IPP or ca. 4% (v/v). After a short time of stirring the solution became clear and the oxygen decrease was determined during 15–30 min at 25°C.

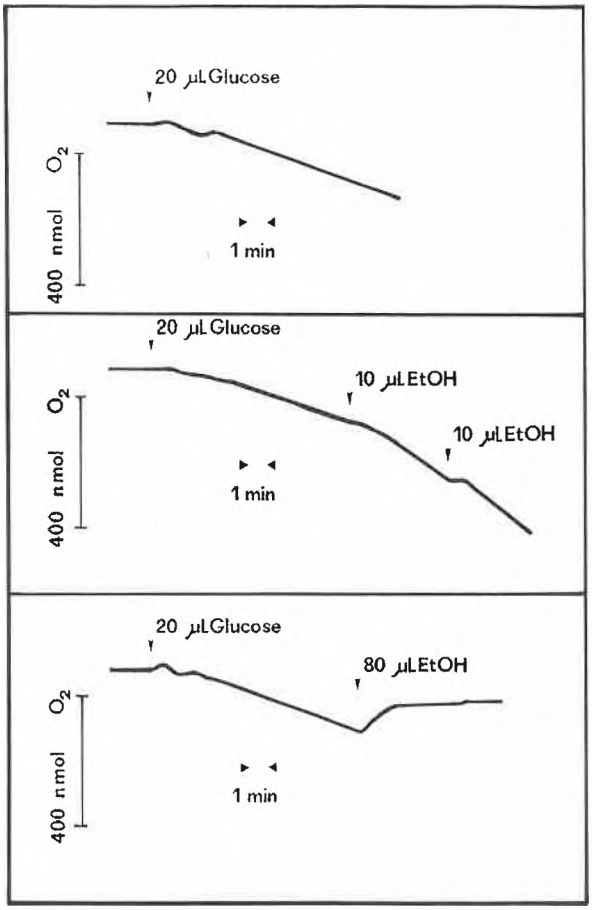

Fig. 1. The actual polarographic recording of oxygen consumption after addition of 220 mM aqueous glucose in a microemulsion system of 100 mM Tween 85/IPP containing 2.5 mg/mL baker's yeast (overall concentration). The final w_0 ($w_0 = H_2O$/Tween 85) is 22. Ethanol is added in pure form.

Fig. 1 shows typical polarographic responses in the organic medium after addition of glucose. The activity (defined as nanomoles of oxygen consumed per minute and milligram of yeast) was 8.1. Yeast is also able to metabolize other sugars under such conditions. The rates with fructose and mannose are equally good as with glucose (the latter surprisingly slightly higher); sucrose and lactose gave 85–89% of the glucose activity, maltose 79%, whereas xylose resulted inactive.

It is known that yeast also grows in media with ethanol as substrate, and that small amounts of ethanol favor the respiration of yeast cells in aqueous solutions[15]. It would be interesting to see whether the same feature was present in IPP microemulsions. Fig. 2 illustrates the influence of added ethanol. The consumption rate of oxygen increases up to a factor of three by an ethanol concentration of 2% v:v (overall concentration), and decreases to ca. 50% of the initial value at a concentration of 5%, i.e. ethanol became more and more toxic.

Fig. 3 shows the respiration rate of solubilized cells as a function of time. The initial increase of rate, perhaps a consequence of the cell growth or adaptation to the higher glucose concentration, is followed by a decrease. This is due to at least two factors: to the diminution of viability and

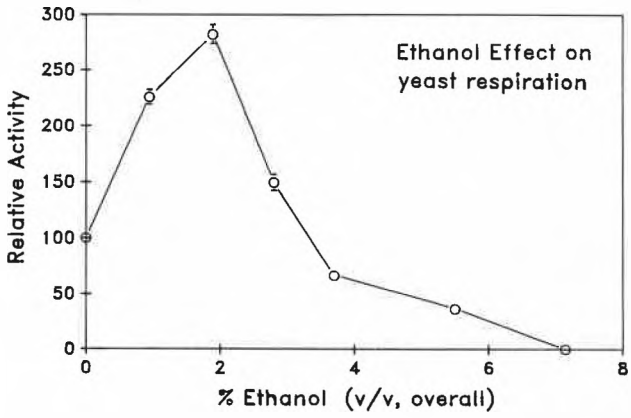

Fig. 2. The influence of concentration of ethanol on the oxygen uptake of yeast cells solubilized in the same system as in Fig. 1 (relative to an ethanol-free microemulsion, take as 100%).

to the consumption of the initial glucose in the stock micellar solution (yeast cells turn into the anaerobic state). In fact, when glucose is again added to the stock solution, the activity rises to the maximal value (even a little higher because of the meanwhile ethanol production).

Another effect which can be usefully studied with the present method is the relation between concentration of cells and activity. One particularly interesting question with this regard is if there is a linear relation between cell concentration and activity, and whether this linearity can be extended even beyond the limits of a solution, i.e. up to very concentrated cell suspensions. Fig. 4 shows some typical data: curve a) represents the absolute oxygen uptake, whereas in curve b the rate is normalized to the cell concentration. The concen-

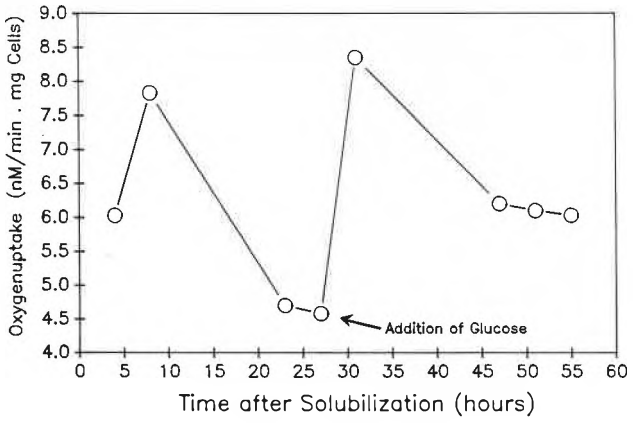

Fig. 3. Oxygen uptake in a cell-containing microemulsion system (as in Fig. 1) as a function of time after yeast solubilization.

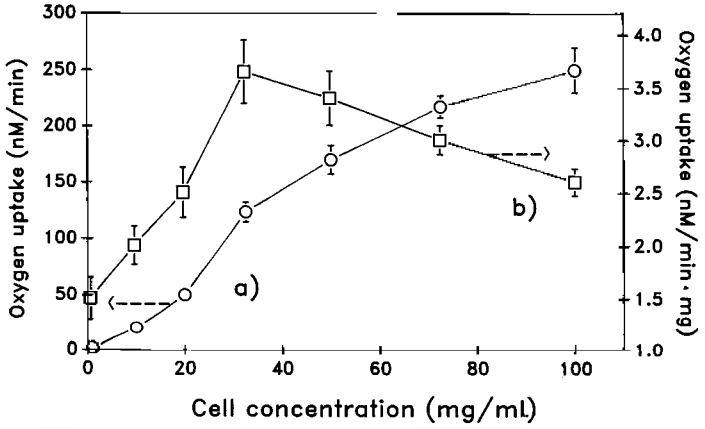

Fig. 4. Dependence of the cell concentration on the oxygen uptake in a water-in-oil microemulsion (as in Fig. 1): a) absolute O_2 uptake; b) uptake normalized to the cell concentration (per mg of fresh cells).

tration of the cells in the organic solution is varied in the range of ca. 10^6–10^8 cells/mL (1 to 100 mg/mL). The limit of a clear solution, as already mentioned, is between 10^6 and 10^7 cells/mL (1–10 mg/mL), so that the higher concentration values in Fig. 4 correspond to suspensions. It is apparent that the linearity is present only in the lower concentration range. After this point, which is near to the change from clear solutions to emulsions, the relative activity begins to decrease and the absolute values tend to reach a plateau.

This tendency also suggests that it is not always advantageous to work with highly concentrated cell systems, to have an optimal turnover, although this statement perhaps is only valid for transformations similar to the investigated reaction under our experimental conditions. It remains to be seen, whether this behavior is a general feature of water-in-oil microemulsion.

Preliminary studies were also made, to see, whether the present method can be extended to other cells. In some experiments, soybean cells were isolated[16] and solubilized in 100 mM Tween/IPP transferring 20 µL of the aqueous cell suspension into 1 mL organic solution. We were able to observe oxygen consumption (even in the presence of sodium hydrogencarbonate). Surprisingly, we observed a threefold increase of oxygen consumption by illuminating the cells (by means of a slide projector lamp). This could be ascribed to photorespiration, as it is well-known that soybean is a C_3-type plant. However, further experiments are necessary to confirm this interpretation. Until now we have been unable to observe oxygen evolution deriving from the photosyntheses of solubilized soybean cells.

This work shows that oxygen, glucose, and ethanol can be utilized as metabolites by yeast cells in microemulsion solutions – in other words, yeast is able to carry out specific reactions in organic media, containing only 4% water (v:v). The good reactivity observed here encourages applied research in this new field of «microbiology in organic solvents» (more precisely in water-in-oil microemulsions) possibly coupled with polarography as a fast and reliable technique to monitor the reactivity.

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[16] Soybean leaves were rinsed with distilled water and excess water absorbed on filter paper. The leaves were then gently peeled with a razor blade and cut into 2–3 cm long pieces, which were then vacuum-infiltrated (25 s) with a solution containing 0.4% pectinase in 0.1 M acetate buffer, pH 4.5. After 90 min of enzymatic digestion, the solution was filtered on a 100 µm filter and the cells were harvested on a bellow-standing 20 µm filter. After prolonged rinsing with Gamborg B-5 medium, the cells were collected from the filter and resuspended in Gamborg B-5 medium, pH 6.5.