APPLICATION OF THE TECHNIQUE OF CELLULAR PERMEABILIZATION TO THE STUDY OF THE ENZYMATIC ACTIVITIES OF SACCHAROMYCES CEREVISIAE IN CONTINUOUS ALCOHOLIC FERMENTATION

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Summary:
A method of assaying the intracellular enzymatic activities of Saccharomyces cerevisiae with a very small amount of biomass, based on cellular permeabilization is described. An example of this method as applied to a small volume continuous fermenter running at low dilution rate is given.

INTRODUCTION
In the course of a general study of the degradation of malic acid by Saccharomyces cerevisiae during alcoholic fermentation in must, we were led to follow up enzymatic activities in cells cultivated in a continuous laboratory-scale fermenter running at low dilution rate.

In these conditions, to measure the intracellular enzymatic activities in acellular extracts is not to be thought of, as the volume of culture involved generally leads to the sacrificing of the fermenter, or at least, to the loss of its steady state which is very precarious at low dilution rate.

The technique of permeabilization while allowing the use of limited quantities of cells, in addition presents the advantage of maintaining the "in vivo" molecular associations.

This work has led us then, to investigate the optimal conditions of permeabilization for a strain of Saccharomyces cerevisiae presenting an enological interest in order to arrive at reliable determination of malate dehydrogenase and alcoholic dehydrogenase activity by taking quantities of culture sufficiently small so that the steady-state of the continuous fermenter is not disturbed.

MATERIAL AND METHODS
Growth media. The medium for batch cultures (YEPD-110) contains (g/l) yeast extract : 10 ; peptone : 20 ; glucose : 110, pH adjusted to 3.5.
The medium for continuous cultures is a synthetic medium containing in tartaric acid/tartrate buffer 5 x 10^{-2}M (pH 3.5). Nitrogen source (g/l) (NH_4)_2SO_4 : 1.88. Carbon source (g/l) L(-) malic acid : 4.5 ; glucose : 110. Vitamins (g/l) Calcium pantothenate : 15 x 10^{-5} ; inositol : 2 x 10^{-3} ; nicotinic acid : 2 x 10^{-3} ; pyridoxine=HCl : 25 x 10^{-5} ; thiamine=HCl : 25 x 10^{-5} ; biotin : 1 x 10^{-5}. Trace elements (g/l) H_3BO_3 : 5 x 10^{-3} ; CuSO_4,5H_2O : 2.7 x 10^{-5} ; KI : 1 x 10^{-3} ; MnSO_4,H_2O : 3 x 10^{-3} ; NaMoO_4, H_2O : 2 x 10^{-4} ; Fe_2(SO_4)_3 : 26 x 10^{-4} ; ZnSO_4,7H_2O : 4 x 10^{-4} ; CoCl_2, 6H_2O : 12 x 10^{-5} ; NiSO_4,6H_2O : 1 x 10^{-4}. Mineral salts (g/l) KH_2PO_4 : 1.5 ; MgSO_4,7H_2O : 0.5 ; CaCl_2,2H_2O : 0.5. Anaerobic growth factors (g/l) Tween 80 : 2.16 ; sodium oleate : 2 x 10^{-2} ; ergosterol : 6 x 10^{-2}.
Growth conditions. Batch cultures are carried out in 300 ml flasks with capillary gas outlet, containing 250 ml of medium. The cultures carried out at 28°C, are shaken by intermittent magnetic stirring. Continuous
cultures are carried out in 300 ml flasks with bubbling CO₂ outlet. The volume of medium in the fermenter is 250 ml. The inflowing medium rate is set up at 12 ml/h to assure a dilution rate of 0.048 h⁻¹. Samples of the outflow are kept at +4°C. The cultures are run at 28°C with permanent magnetic stirring.

Acellular extract preparation. Cells harvested by centrifugation are washed twice with imidazole buffer 75 x 10⁻³M (pH 3.5). The resulting pellet is resuspended into 10 ml of imidazole buffer 75 x 10⁻³M, glutathion 0.3 M (pH 7.5) so as to obtain a dry weight concentration of 60 g/l. A sample (100 µl) of this suspension is used to assay protein. 8 g of glass beads (diameter 0.45-0.50 mm) are added to the remaining part and the cells are crushed for five minutes at speed 2 in a cell homogeniser MSK Braun. The cell-free homogenate is centrifuged at 20,000 g for 20 minutes. The supernatant obtained is adjusted to a volume of 10 ml with imidazole buffer 75 x 10⁻³M, KCl : 0.1 M ; MgCl₂ : 10⁻²M (pH 7.5) and used for enzymatic and protein assays.

Enzymatic assays. Alcohol dehydrogenase (E.C : 1.1.1.1) and malate dehydrogenase (E.C : 1.1.1.37) activity have been determined respectively according to Racker (1960) and Witt et al. (1966).

The specific activities are expressed in milliunits per milligram of extractible protein. One milliunit corresponds to one nanomole of substrate (ethanol or L(-) malate) transformed per minute.

Protein determination. Protein concentration was measured by the method of Lowry et al. (1951) after extraction of protein at 100°C for a period of 45 minutes with dimethyl sulfoxide (50 % V/V).

Cell permeabilization. Unless otherwise stated in the text, the procedure is the following : 5 ml of culture are filtered through a millipore filter (RAWP type, pore size 1.2 um) and washed with 6 ml of ice cold water. The cells retained on the filter are resuspended in 1 ml of imidazole buffer 75 x 10⁻³M, KCl : 0.1 M, MgCl₂ : 10⁻²M (pH 7.5) to obtain a dry weight concentration of about 9 g/l. 50 µl of 0.3 M glutathion, 10 µl of triton X - 100 10 %, and 50 ul of the mixture toluene/ethanol (1/4, V/V) are added rapidly to the suspension which is vigorously shaken up on a stirring machine of the cyclomixer type for 5 minutes.

After stirring the suspension is filtered and the cells are washed as before. The permeabilized cells are resuspended in 3 ml of imidazole buffer 75 x 10⁻³M (pH 7.5). This suspension kept at 4°C, is used for enzymatic determinations.

RESULTS AND DISCUSSION

Adaptation of the method of permeabilization. The most common agents of permeabilization used with yeast are toluene, dimethylsulfoxide (Adams, 1972), triton X - 100 (Felix, 1987) and digitonin (Volland et al., 1973).

Serrano et al. (1973) have shown, with Saccharomyces cerevisiae grown on glucose, that a permeabilization using the mixture toluene/ethanol (1/4, V/V) permitted an "in situ" enzymatic study of a certain number of enzymes involved in the glycolysis and krebs cycle. According to their work it seems however that a systematic study of the conditions of permeabilization is necessary for each individual case.

First and foremost we were unable to detect any influence of the chemical nature of the buffer used during permeabilization (Tris-HCl, phosphate, imidazole) on the accessibility of the alcohol and malate dehydrogenase activities.

Over and above the permeabilization agents, the addition of detergents to improve the accessibility of enzymes had already been practiced (Serrano...