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Comparison of fermentation properties and specific enzyme activities of free and calcium-alginate-entrapped *Saccharomyces cerevisiae*

Bärbel Hilge-Rotmann and Hans-Jürgen Rehm

Institut für Mikrobiologie, Universität Münster, Corrensstrasse 3, D-4400 Münster, Federal Republic of Germany

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Summary. Physiological properties have been determined for calcium-alginate-entrapped *Saccharomyces cerevisiae* in comparison to cells in suspension under identical culture conditions. Cells grown in the form of microcolonies in the alginate beads showed faster glucose uptake and ethanol productivity with simultaneously decreased product and cell yields. Increased specific hexokinase and phosphofructokinase activities could be determined in these cells. Immobilized single cells showed only slightly enhanced glucose turnover and no higher specific hexokinase activity. The significant alterations in physiology are apparently connected with growth of the cells in aggregates.

Introduction

Immobilized cells show various modifications in physiology and biochemical composition when compared to suspended cells. Altered morphological forms as well as higher or lower metabolic activity, growth rates and product yields have been noticed. Since cells react sensitively to unphysiological conditions, many observed changes are a result of the immobilization technique used or toxic material components of the carrier (Mozes and Rouxhet 1985; Navarro and Durand 1977). Altered metabolism after entrapment in a natural polymer or after adsorption to an inert carrier (Holcberg and Margalith 1981; Marcipar et al. 1979) is more difficult to explain. Often the environment of the immobilized cells, which is totally different from that of the suspended cells (e.g. reduced water activity, a_w), is held to be responsible (Hahn-Hägerdal 1986). Diffusional limitations can also affect the physiological activity particularly of cells entrapped in a gel matrix (Gosmann and Rehm 1986, 1988; Hannoun and Stephanopoulos 1986). However, the mechanisms underlying these alterations are not completely understood at present. Information is necessary on the biochemical composition, metabolism, and metabolic regulation of immobilized cells in comparison to suspended cells.

At present only a few researchers have described the biochemical composition of immobilized cells. These include recent comparisons of the content of macromolecular components and intracellular metabolites with those of free cells (Doran and Bailey 1986, 1987; Galazzo and Bailey 1989; Keweloh et al. 1989, 1990). The intention of the present work was to examine the influence of immobilization on the physiology of microorganisms thereby contributing to the knowledge on immobilized cell metabolism.

Cells of *Saccharomyces cerevisiae* were entrapped in calcium-alginate beads and their physiological properties were compared with those of free cells. The nontoxic gel matrix can be dissolved, releasing the entrapped cells for physiological investigations. This study focuses on determining the glycolysis-regulating enzymes hexokinase and phosphofructokinase of free and immobilized cells in the course of ethanolic fermentations.

Materials and methods

Microorganism. All investigations were carried out with *S. cerevisiae* IMM 30.

Medium. The yeasts were propagated under aerobic conditions in a medium containing (per litre): 10 g glucose, 1.5 g yeast extract, 2.5 g NH_4Cl, 5.5 g K_2HPO_4, 0.25 g $MgSO_4$, 0.01 g $CaCl_2$, 1 g NaCl, 3 g citric acid, pH 5.0. This medium was also used in fermentations under anaerobic conditions with 100 $g \cdot l^{-1}$ glucose.

Experimental procedure. In order to obtain a real comparison between free and immobilized cells, all cells were derived from the same inoculation culture and treated the same way during all investigations. Because alginate beads diluted the medium, this effect was reached in suspended cell cultures by adding an equal volume of saline. In accordance with the precultures of suspended cells the immobilized cells were grown in alginate beads for the same time. The fermentations were carried out in several parallel batch cultures at 30° C using stirred 1-l fermentor. At 2-h intervals one of these parallel cultures was harvested for analyses.

Offprint requests to: H.-J. Rehm

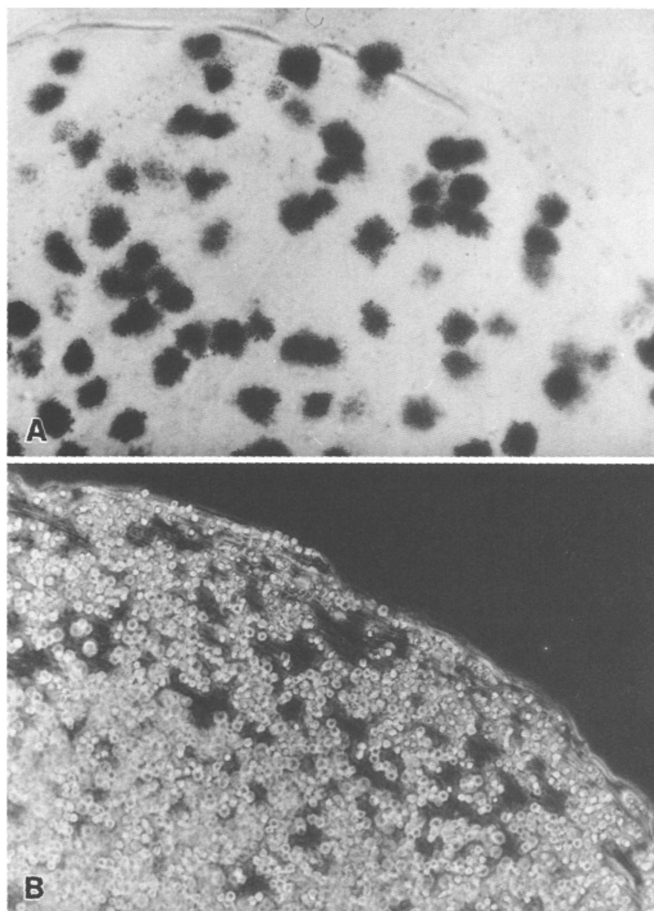

Fig. 1. A Section of an alginate bead. Cells of *Saccharomyces cerevisiae* were grown in the gel in the form of microcolonies during a 12-h preculture. **B** Section of an alginate bead. Cells of *S. cerevisiae* were immobilized just before fermentation and are regularly distributed as single cells in the gel

Immobilization. Alginate (Manugel DJX) was obtained from Alginate Industries, Hamburg, FRG. The entrapment of cells was done according to Eikmeier and Rehm (1987). Cells of an inoculation culture were suspended in sodium alginate by stirring for 10 min to obtain a 3% alginate solution. The mixture was pressed through a thin cannula (30 x 0.65 mm) into a 2% $CaCl_2$ solution, and left to harden for 1 h. The gel beads had a diameter of about 3 mm. The free Ca^{2+} was then washed out twice with saline, followed by transferring the gel beads into the medium.

Release of immobilized cells. The gel beads were dissolved by stirring in 0.05 *M* sodium hexametaphosphate, which liberated the cells within 10 min without loss of viability. After washing twice with saline the released cells were available for analyses.

Analyses. Glucose uptake, ethanol and glycerol production were monitored by HPLC using an Aminex HPX 87H column (BioRad, Richmond, Calif, USA). For measuring enzyme activities crude cell-free extracts were obtained by ultrasonic disintegration and centrifugation. The protein content of the crude cell-free extract was measured according to Bradford (1976). After sonication it was microscopically controlled such that at least 80% of the cells was broken. No differences between free and immobilized cells could be observed during disruption. The protein content of the crude cell-free extracts ranged between 3 and 10 mg/ml depending on the cell mass sonicated. The crude extracts compared with one another had a protein content within the same scale.

Determination of hexokinase and aldolase activity specific activity was carried out according to Bergmeyer (1974), and the phosphofructokinase activity was determined according to Stellwagen and Wilgus (1973). Spezific enzyme activities were calculated as nanokatal/mg protein (nkat/mg protein). Numbers of cells were determined by counting in a Thoma chamber.

Morphological observations were made with sectioned alginate beads under a phase-contrast microscope. For more detailed information about the methods used in this study, see Hilge (1987).

Results

Two types of entrapped cells were compared with the suspended cells. The first type was precultured in the

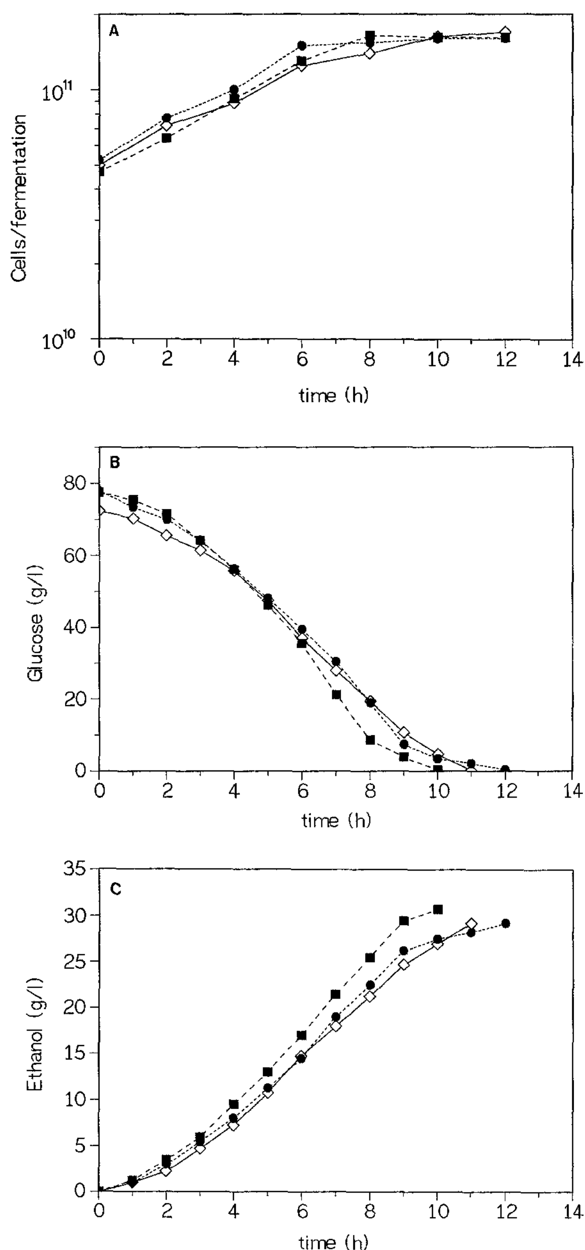

Fig. 2. A Total cell amount in the fermentor during fermentation. **B** Glucose consumption during fermentation. **C** Ethanol production during fermentation. ◇, suspended cells; ■, immobilized microcolonies; ●, immobilized single cells

Table 1. Comparison of fermentation data of suspended and immobilized cells of *Saccharomyces cerevisiae*

	Suspended cells	Immobilized microcolonies	Immobilized single cells
Maximum glucose consumption (g/l·h)	9.18	12.48	9.75
Maximum ethanol productivity (g/l·h)	3.5	4.12	3.65
Ethanol yield from glucose (mol/mol)	1.49	1.29	1.46
Cell yield from glucose (cells/g)	$1.79 \cdot 10^9$	$1.58 \cdot 10^9$	$1.38 \cdot 10^9$

Glucose consumption and ethanol productivity are the maximum values the cells reached during fermentation. Ethanol yields are calculated on the basis of these maxima. The values of cell yields present the total amount of cells produced during the fermentation

gel beads in the form of microcolonies (Fig. 1 A). In addition, a preculture of free cells was immobilized just before being transferred for fermentation: these entrapped cells were regularly distributed as single cells in the alginate beads at the start of fermentation (Fig. 1 B). The fermentations to be compared were started with nearly the same amount of cells (Fig. 2 A). The course of glucose consumption and ethanol production are given in Fig. 2 B and 2 C. Under identical culture conditions the entrapped cells grown as microcolonies showed clearly faster glucose consumption and ethanol formation, especially in the second half of the fermentation, in comparison to suspended cells or cells singly distributed in the alginate.

The results of these batch fermentations are summarized in Table 1, which presents the maximum values of glucose consumption and ethanol productivity reached by the cells in the course of fermentation. Although showing a significantly enhanced metabolism the ethanol yield from glucose for the entrapped microcolonies was lower than for free or entrapped single cells. The cells immobilized just before fermentation showed a slightly enhanced glucose turnover in comparison to free cells and, like the other immobilized cells, a decreased cell yield from glucose.

Concomitant with these fermentation data a stimulation of the metabolism-regulating enzymes hexokinase and phosphofructokinase could be determined in the entrapped cells grown in colonies. Compared with suspended cells, cells of the aggregates showed significantly higher kinase activities as well as a different development during the course of fermentation. While the specific hexokinase activity of the free cells was constant during fermentation, the entrapped microcolonies showed about twice the activity at the start of fermentation. After decreasing, the level increased again in the second half of the fermentation. The specific hexokinase activity of regularly distributed cells was similar to that of suspended cells (Fig. 3).

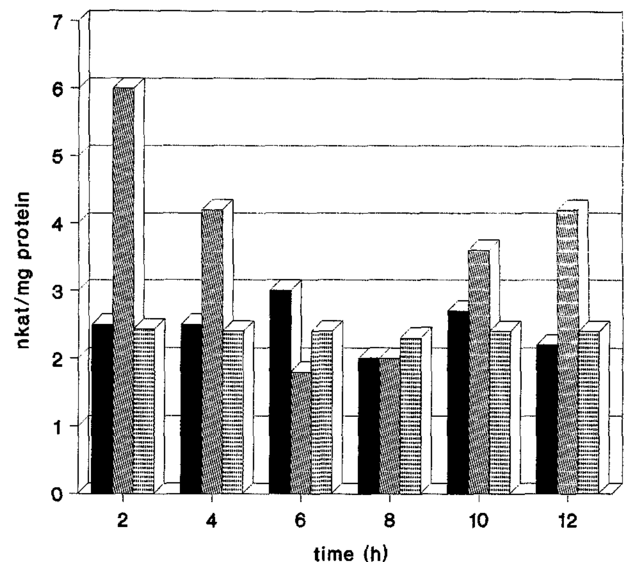

Fig. 3. Specific hexokinase activity of suspended cells (■) immobilized microcolonies (▩) and immobilized single cells (▤) of *S. cerevisiae* during fermentation

Even in their specific phosphofructokinase activity the immobilized cell colonies showed a clear increase at the end of fermentation and about 11-fold higher activity in comparison to free cells, in which the specific phosphofructokinase activity decreased over the course of fermentation. The cells singly distributed in the alginate beads produced a specific phosphofructokinase activity on the scale of the immobilized microcolonies during fermentation (Fig. 4).

The specific activity of aldolase was also determined as an example of an enzyme uninvolved in the

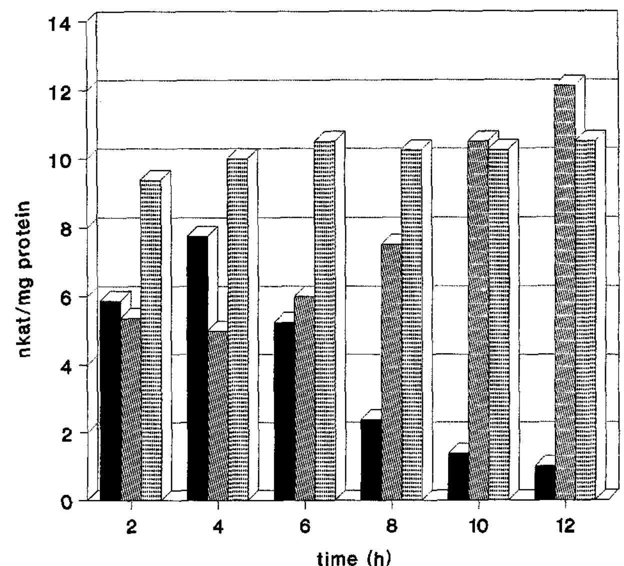

Fig. 4. Specific phosphofructokinase activity of suspended cells (■), immobilized microcolonies (▩), and immobilized single cells (▤) of *S. cerevisiae* during fermentation

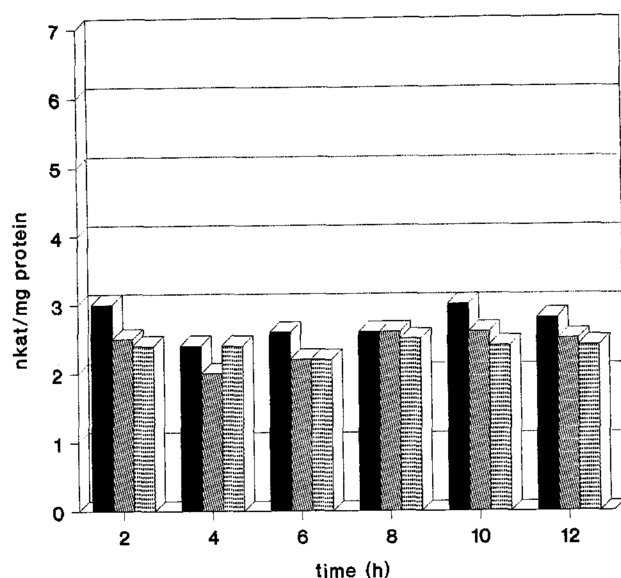

Fig. 5. Specific aldolase activity of suspended cells (■), immobilized microcolonies (▨) and immobilized single cells (▤) of *S. cerevisiae* during fermentation

regulation of glucose turnover. There was no clear difference between suspended cells and the two types of immobilized cells (Fig. 5).

Discussion

This study shows that faster substrate uptake rates and ethanol productivity with simultaneously decreased product and/or cell yields characterize the metabolism of *S. cerevisiae* when entrapped in calcium-alginate beads in comparison to free cells. The enhanced metabolism of the immobilized cells may be caused by a stimulated reaction step within glycolysis. This was suspected by Doràn and Bailey (1986), when they obtained similar fermentation data with *S. cerevisiae* immobilized in gelatine coats.

This study shows with exact data that immobilization directly affects the initial and pacemaker reactions of the glycolytic pathway. Specific activities of hexokinase and phosphofructokinase determined during the course of batch fermentations were significantly higher in immobilized cell colonies when compared to identical cells in suspension. Cells immobilized just before fermentation also showed enhanced specific phosphofructokinase activity. No difference between free and immobilized cells could be measured with respect to their specific aldolase activity, which is not involved in the regulation of the metabolism. Substrate turnover, which was increased in a different range in both types of immobilized cells, can be explained by this enhanced specific phosphofructokinase activity. The mechanisms underlying this kinase stimulation are still open to speculation.

One well-known regulating factor of phosphofructokinase activity is oxygen. As determined by several authors (Beunink et al. 1989; Gosmann and Rehm 1986, 1988; Hiemstra et al. 1983) the O_2 supply can be diminished up to 25% in a gel matrix. The more than tenfold higher specific phosphofructokinase activities in entrapped cells in comparison to free cells are apparently not caused by their better protection against oxygen by the alginate, because all fermentations were carried out under anaerobic conditions. The fermentations were started with nearly the same amount of cells but their distribution in the fermentor was completely different: the free cells were spread over the whole fermentation broth whereas the immobilized cells reached a ca. tenfold higher concentration in the carrier brought into the medium.

The dense cell-to-cell or cell-to-carrier contact may play a role in the change in cell behaviour when immobilized by directly affecting the cell envelope. Ellwood et al. (1982) postulated an altered metabolism of microorganisms growing at surfaces with changes in their membrane permeability for protons. Alterations in the physico-chemical properties and dynamic behaviour of membranes of immobilized cells (Keweloh et al. 1990) seem to be a more likely trigger of the observed stimulated reactions.

Another stimulating factor of phosphofructokinase is fructose-2,6-P_2. On formation of this effector in a side reaction of glycolysis, participation of cAMP cannot be excluded (Avigad 1981; Bartrons et al. 1982; Kren and Rehacek 1983). Because the formation of cAMP is catalysed by a membrane-bound enzyme, one could bring the effector fructose-2,6-P_2 into the discussion against the background that immobilization may affect the cell envelope by close cell-to-cell or cell-to-carrier contact. Altered concentration of an effector of a regulation step is an assumption also made by Galazzo and Bailey (1989), when they found differences in intermediate metabolite levels between free an Ca-alginate-entrapped *S. cerevisiae* by nuclear magnetic resonance analysis.

Galazzo et al. (1987) determined a lower intracellular pH in Ca-alginate-entrapped *S. cerevisiae* and suspected that this could be the reason for enhanced glucose turnover, because hexokinase and phosphofructokinase can be stimulated by a lower pH in vitro. As this study shows, the significant differences in kinase activities between free and immobilized cells were determined under identical test (pH) conditions. Obviously a lower intracellular pH or altered concentration of an effector in immobilized cells cannot be the only reasons for enhanced kinase activities. The results of measurements with crude cell-free extracts suggest that the immobilized cells may also have greater amounts of kinases in proportion to their total protein content.

From an enhanced specific phosphofructokinase activity an increase in the specific hexokinase activity could be measured in immobilized cell colonies. The immobilized single cells showed only a slightly enhanced substrate turnover, apparently caused by increased specific phosphofructokinase activity, but no higher specific hexokinase activity. The increase in specific hexokinase activity in the second half of the fermentation may be due to the fact that the immobilized

cell aggregates shifted their metabolism towards the production of storage materials. All intracellular processes such as biosyntheses that are supplied with constituents of glycolysis are reflected in an altered glycolytic activity (Käppeli et al. 1986).

The product of the hexokinase reaction, glucose-6-P, is the branch-point between anabolic and catabolic pathways. The hexokinase reaction provides C-constituents for the macromolecular cell components. As the fermentation data show (see Table 1), significantly lower product and cell yield from glucose could be calculated for immobilized cells than for suspended cells. Further results by quantitative measurements (Hilge and Rehm 1989) and other authors (Doran and Bailey 1986) confirm that immobilized cells contain higher amounts of storage and structural polysaccharides, DNA and RNA in comparison to free cells under identical culture conditions. The trigger factor for this effect may lie once more in the very dense cell packing within the gel whereby the cells lack sufficient space to bud. A shift in their metabolism towards production of storage polysaccharides seems to be possible at the expense of cell and product yields.

The high initial specific hexokinase activity of immobilized microcolonies (see Fig. 3) may derive from the preculture, where the gel beads were harvested at the beginning of the stationary phase. Reaching the stationary phase in the fermentation resulted in an increase in specific hexokinase activity again. This interesting course of specific hexokinase activity in immobilized microcolonies could also be observed with sucrose as carbon source (Hilge 1987). It is not clear why immobilized single cells did not show an increase in their specific hexokinase activity in the second half of fermentation although they showed the initial stages of a shift in metabolism with the lower cell yield from glucose. Apparently activation of hexokinase is connected with cell growth in form of microcolonies. Keweloh et al. (1989) also describe a correlation between increasingly altered metabolism and the increasing size of microcolonies of *Escherichia coli*.

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