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Sorbitol and Gluconic Acid Production Using Permeabilized *Zymomonas mobilis* Cells Confined by Hollow-Fiber Membranes

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Abstract

Immobilization of *Zymomonas mobilis* by different methods was investigated. Experiments were performed in order to choose the most appropriate support for the immobilization of the cells. The most advantageous option was to use permeabilized cells in the bore of microporous hollow fibers. Whereas the reaction rate was about 33 g of gluconate/(g of protein·h) using hollow fibers, which is comparable to that observed by using free cells, the calcium alginate immobilized cells presented a reaction rate of 4 g of gluconate/(g of protein·h). These results can be explained by the mass transfer resistance effect, which, indeed, was much lower in the case of hollow-fiber membranes than in the alginate gel beads. A loss of enzymatic activity during the reaction was observed in all experiments, which was attributed to the lactone produced as an intermediate of the reaction.

Index Entries: Hollow fibers; *Zymomonas mobilis*; sorbitol; gluconic acid; GFOR.

Introduction

The study of enzymatic processes, as well as investigation about their potential application, has grown considerably, mainly because of relatively mild operation conditions and the enzyme specificity, which minimizes byproduct formation. An example of industrial-scale application of enzymatic processes is the production of organic acids and their derivatives *(1)*. Since the discovery of the enzyme glucose–fructose oxidoreductase *(2)*,

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which converts the sugars glucose and fructose to gluconic acid and sorbitol, respectively, efforts also have been made to develop an industrial process *(3)*. These efforts have resulted in a number of patents dealing with the production of gluconic acid and sorbitol *(4–7)*. Since the 1986 study by Zachariou and Scopes *(2)*, many investigations have been conducted in an attempt to understand the mechanisms of enzymatic reaction *(8–11)* and to increase the activity and stability of enzymes *(12–15)*.

Cells of the anaerobic bacterium *Zymomonas mobilis (2)* are capable of promoting simultaneous glucose oxidation to glucono-δ-lactone and fructose reduction to sorbitol catalyzed by GFOR. In a subsequent reaction, glucono-δ-lactone is hydrolyzed to gluconic acid both spontaneously and under the action of the enzyme glucono-δ-lactonase. To enhance selectivity, the cells can be permeabilized, allowing small compounds such as metallic ions and cofactors to diffuse out of the cells while keeping macromolecules inside. Because the NADP(H) cofactor of GFOR remains bound to the enzyme, even after the permeabilization process, the activity of the enzyme is maintained. This permeabilization procedure is quite important to avoid ethanol formation, which reduces acid production.

To achieve better operational stability and to allow easier continuous operation, permeabilized cells have been immobilized. Many immobilization methods have been applied *(16–18)*, and the most widely employed are supports based on alginate or κ-carrageenan. However, it was reported that the efficacy of the process is reduced to a lower reaction rate than that for free cells *(12,13)*. Alternatively, another promising immobilization technique consists of confining cells in the bore of hollow-fiber membranes *(19–21)*. In this case, the membrane pores should be smaller than the cells and offer a low transport resistance to the substrates and products. Furthermore, the membrane reactor can easily be operated and regenerated.

The main objective of the present study was to investigate the production of sorbitol and gluconic acid using permeabilized cells of *Z. mobilis* confined in the bore of hollow fibers or in the shell side of a hollow-fiber module. To verify the performance of the process, the initial reaction rate was compared with that obtained with free cells and cells immobilized in alginate. The system stability was evaluated by comparing the initial reaction rate after substrate replacement.

Materials and Methods

Microorganism and Culture Conditions

All experiments were performed with *Z. mobilis* CP4 (ATCC no. 31821) cultivated in a medium containing 100 g of glucose/L as the carbon source and 5 g of yeast extract/L under a controlled temperature of 30°C.

Preparation of Permeabilized Cells

Broth was cultured until the late exponential phase after about 20 h of growth. After this period, cetyltrimethylammonium bromide (CTAB) was

added directly to the broth, to give a final proportion of 0.04 g of CTAB/g of cells *(13)*. After gently stirring for 30 min, the cells were separated by centrifugation at 3000*g* and washed twice with distilled water.

Immobilization Method

Confinement in Hollow Fibers

Permeabilized cell suspension was confined in the bore of microporous polycarbonate hollow fibers or in the shell side of the module. These fibers, about 20 cm long, were assembled in a longitudinal module of polyvinyl chloride, as shown in Fig. 1A. Figure 1B is a photomicrograph of the fiber cross-section, which has outer and inner diameters of 0.68 and 0.33 mm, respectively. The setup of the membrane reactor was done by filling either the bore side of the fibers or the shell side of the module with a cell suspension.

The pore size in the outer surface of the fiber is smaller than 0.1 μm, and in the inner surface it is smaller than 1 μm. Considering the cell dimensions, the range of pore sizes in the fiber is small enough to avoid any loss of cells during the experiment. The water permeability of these fibers was determined as 912 L/(h·m^{2}bar).

Entrapment in Alginate

For the immobilization in calcium alginate, the concentrated suspension of cells was mixed with a solution of sodium alginate (8% [w/v]) *(22)*. Spherical beads were then produced by dropping the mixture into a 20 g/L calcium chloride solution through a syringe. The beads were suspended in a 0.5% (v/v) glutaraldehyde solution with stirring for 30 min. After washing with water, the beads were stored at 4°C until further use. This procedure ensures that a soft gel is produced.

Assays and Analytical Methods

Cell concentration was determined by optical density at a wavelength of 600 nm. Protein assays were done according to the binding method of Bradford *(23)*, slightly modified *(24)*, using bovine serum albumin as the protein standard. Glucose, fructose, sorbitol, and gluconic acid concentrations were analyzed by using a high-performance liquid chromatograph (Waters, model 510) with a refractive index detector and a Polyspher CH CA column. The column temperature was kept at 80°C, and water was used as the eluent at a flow rate of 0.5 mL/min.

Kinetic Studies

Kinetic studies were carried out in the experimental setup schematically shown in Fig. 2A,B. The experiments were operated in batch by adding the free cells or calcium alginate immobilized cells to the reaction medium or by circulating an equimolar fructose/glucose solution through the hollow-fiber reactor. The system temperature was maintained constant at 39°C. The pH was also kept constant at 6.2 by automatic titration using

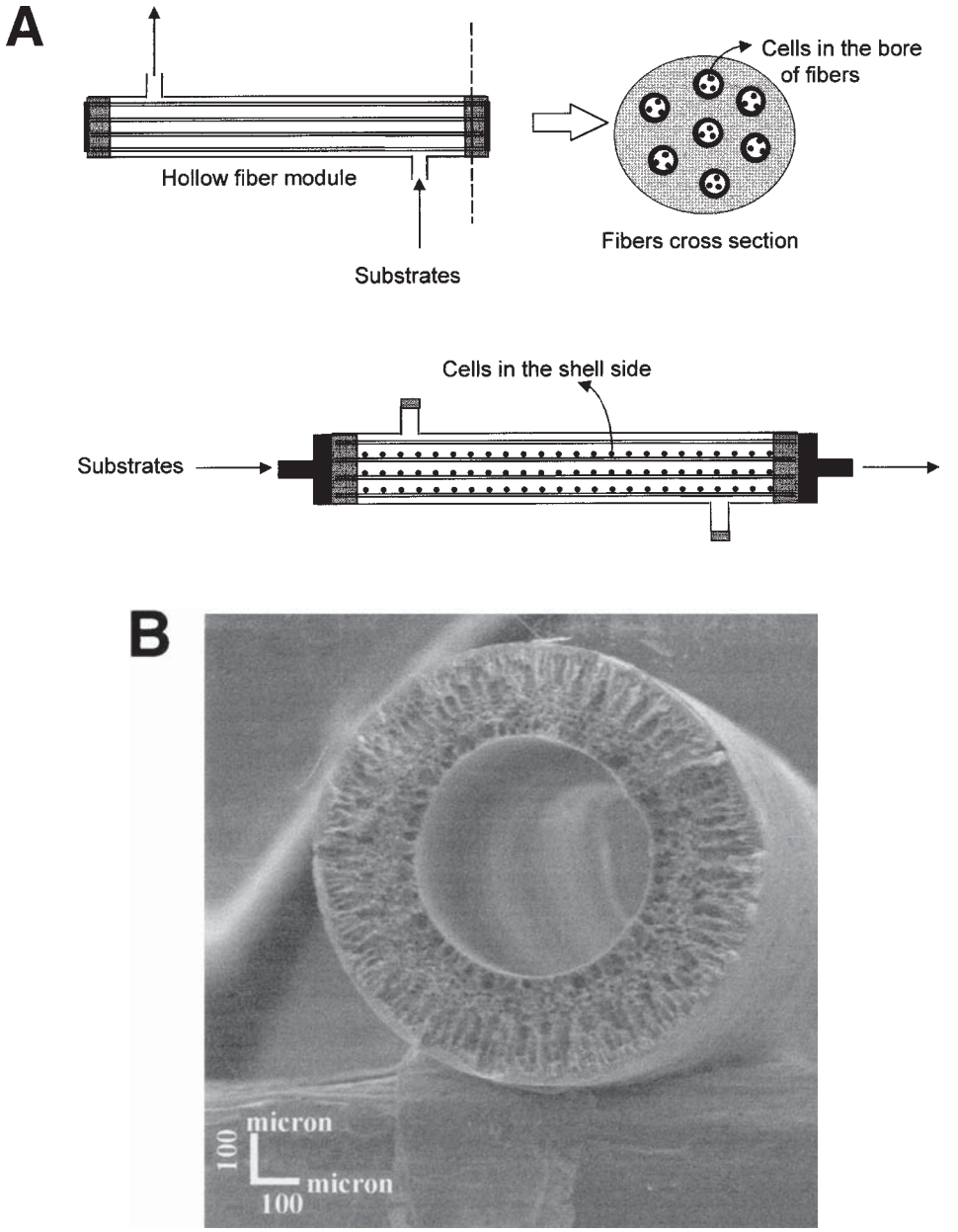

Fig. 1. Hollow-fiber module used to confine the permeabilized cells. **(A)** Scheme of the hollow-fiber bundle inside a module; **(B)** photomicrograph of the cross-section of a hollow fiber.

1 *M* NaOH as the base. This pH value was previously found to be the best for enzymatic activity *(2)*. The mass of the NaOH solution added to the system was measured during the reaction and used to monitor the evolution of the gluconic acid production.

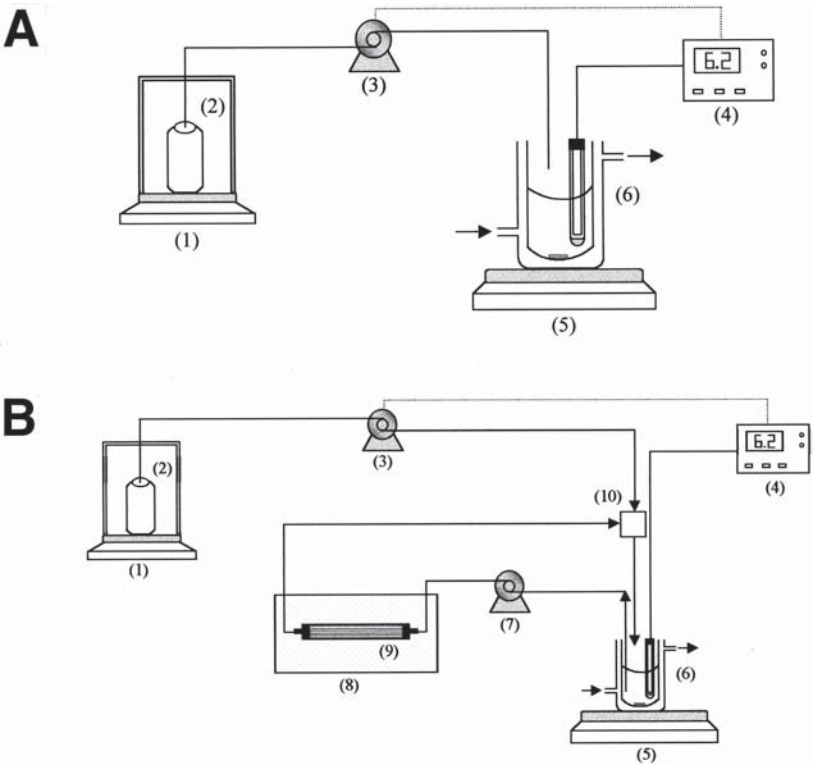

Fig. 2. Experimental setup. **(A)** Experiments with free cells and calcium alginate immobilized cells; **(B)** experiments with confined cells. 1, Digital balance; 2, NaOH tank; 3, pump; 4, pH controller; 5, reactor/substrates tank; 6, magnetic stirrer; 7, pump; 8, thermostatic bath; 9, hollow-fiber module; 10, mixture vessel.

Results and Discussion

Calcium Alginate Immobilized Cells

Before use, calcium alginate beads were observed by scanning electron microscopy (SEM). Figure 3 is a photomicrograph of an alginate particle containing permeabilized cells. Bead sizes were about 2 mm in diameter and presented an outer surface with no visible pores by SEM. The cells are dispersed in its inner part, which is much more porous.

To evaluate the GFOR stability in cells entrapped in calcium alginate beads stored at 4°C, initial specific reaction rates were obtained as a function of storage time under conditions previously described. The results are presented in Fig. 4 and show a decrease in GFOR activity with storage time. In this way, only freshly prepared beads were used in the reactions.

The reduction in specific activity was investigated by filtration of the particle storage medium through a Millipore microporous membrane of 0.45-µm maximum pore size. After filtration, the membrane was dried and a photomicrograph of its surface was taken using SEM. As seen in Fig. 5, the

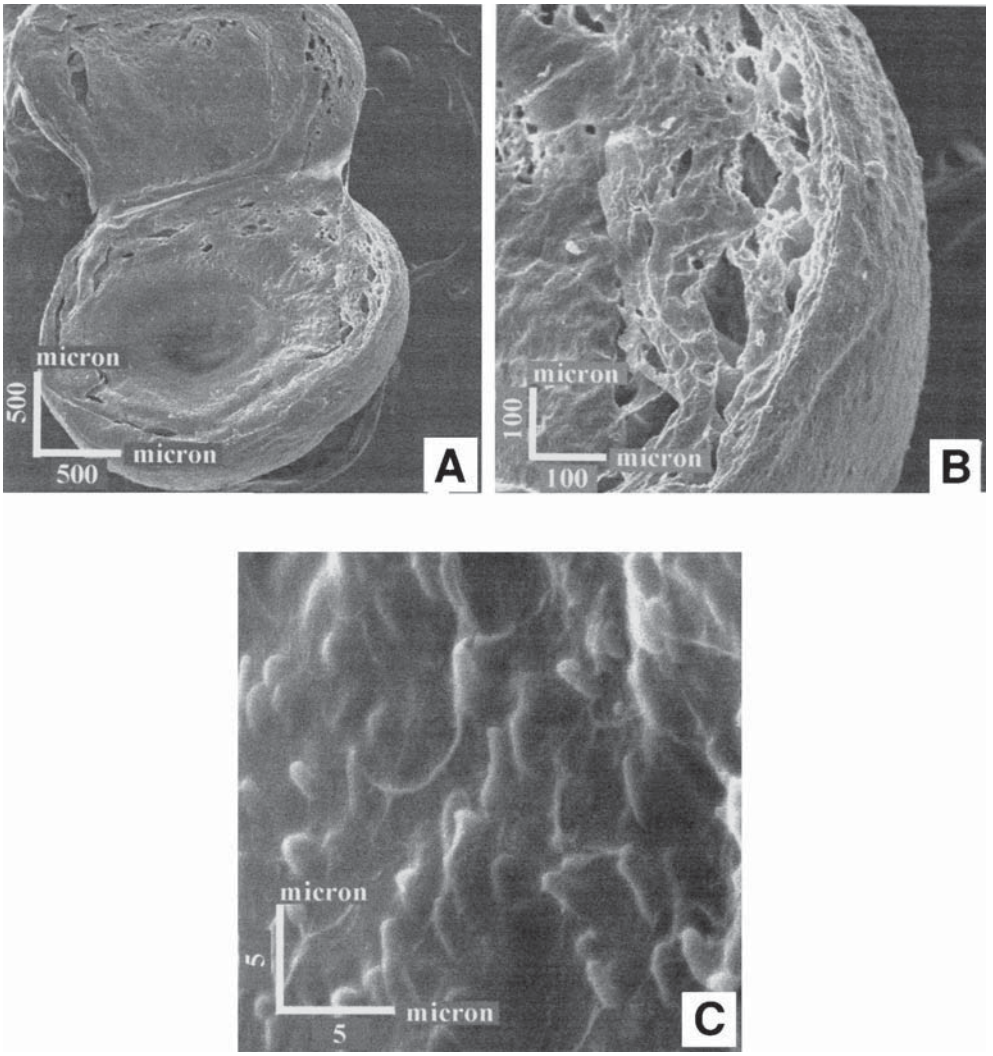

Fig. 3. SEM photomicrograph of an alginate particle. **(A)** Cross-section of the particle; **(B)** particle cross-section detail near the external surface; **(C)** cells entrapped in the inner part of the particle.

membrane surface is covered by cells, denoting release from the support to the storage medium, which explains, at least in part, the observed behavior.

Besides the leakage of cell from immobilized particles during the storage, the beads also presented low mechanical stability at reaction conditions, once it was observed that many particles were broken, liberating cells to the medium. In order to confirm this, the medium was filtered in 0.45 μm nominal pore size membrane and a photomicrograph of the membrane surface was taken, showing cells retained in it. The mechanical crack of the particle may be caused by the presence of gluconate in the reaction medium, which can act as a Ca^{2+} chelating agent *(25)*.

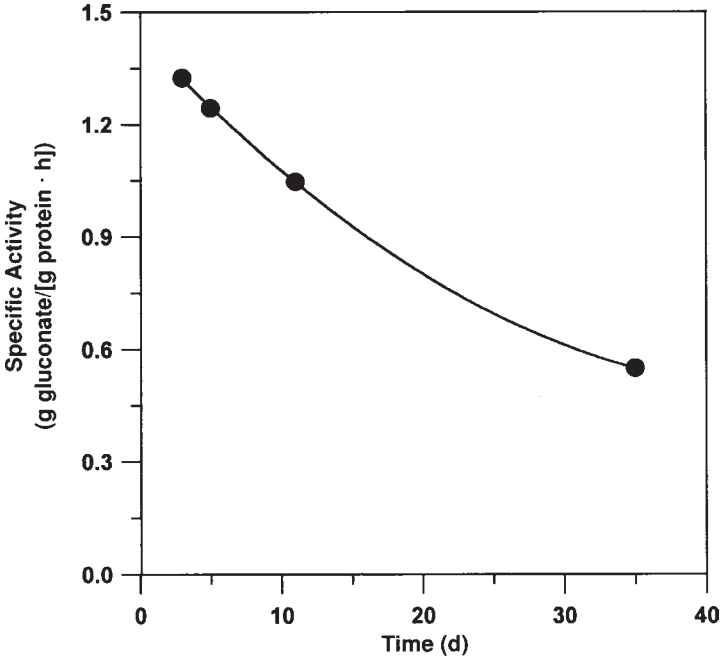

Fig. 4. Effect of the storage time on the initial specific reaction rate. Initial substrate concentration: glucose = 100 g/L and fructose = 100 g/L.

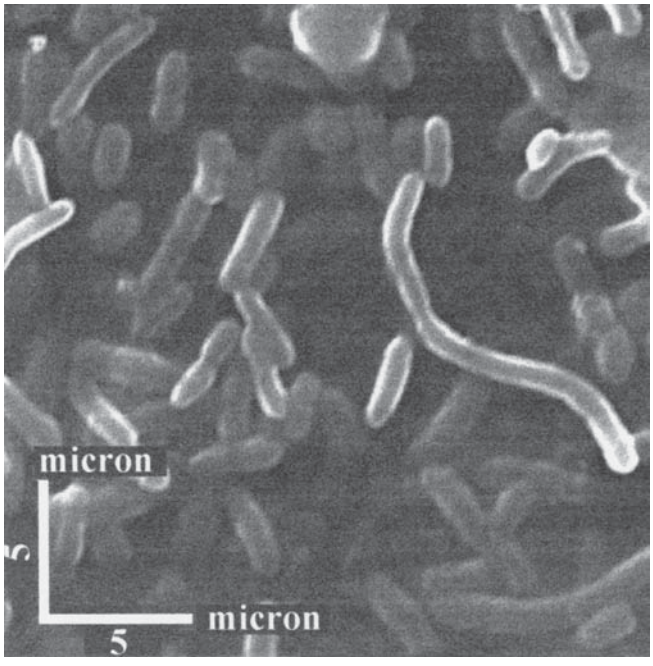

Fig. 5. SEM photomicrograph showing cells at the membrane surface after microfiltration of the storage medium.

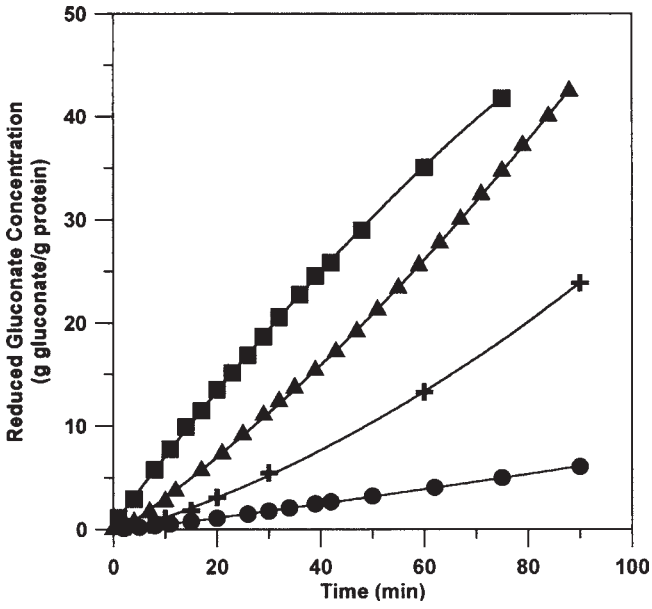

Fig. 6. Sodium gluconate production in experiments using free and immobilized cells. (■) Free cells; (▲) cells confined in the bore of hollow fibers; (✚) cells confined in the shell side of the hollow-fiber module; (●) cells immobilized in calcium alginate. Initial substrate concentration: glucose = 100 g/L and fructose = 100 g/L.

Following these results, the beads obtained did not exhibit good mechanical properties and could be considered as a soft gel, allowing cell losses. On the other hand, one may expect that such beads will provide a low mass transfer resistance to products and reactants and, consequently, a higher reaction rate than cells immobilized in more stable and rigid gels. Therefore, the immobilization procedure is appropriate in order to obtain favorable conditions to compare its performance with cells confined by microporous hollow fiber.

Batch Reaction with Immobilized Cells

The initial specific reaction rate was determined in experiments conducted with cells immobilized in calcium alginate or confined in hollow fibers. Reactions using free cells were also conducted to analyze the mass transfer effect originated by the immobilized system.

Figure 6 shows the reduced concentration of gluconate (mass of gluconate/mass of protein) as a function of reaction time for experiments using free and immobilized cells. Table 1 shows the initial specific rate of gluconate production and protein mass of each experiment. As can be seen, the system utilizing cells immobilized in calcium alginate presented the lowest initial specific reaction rate. In fact, in most gel-entrapment systems, diffusion through the gel can be the rate-limiting step reducing the reaction rate *(26)*. On the other hand, for the hollow-fiber-confined cells, the initial spe-

Table 1
Performance of the Different Cell Immobilization Methods

Immobilization method	Initial specific reaction rate (g gluconate/[g protein·h])	Protein mass (mg)
Free cells	39.0	64.5
Calcium alginate	4.2	145.7
Bore of hollow fibers	33.2	8.6
Shell side of hollow-fiber module	19.6	17.4

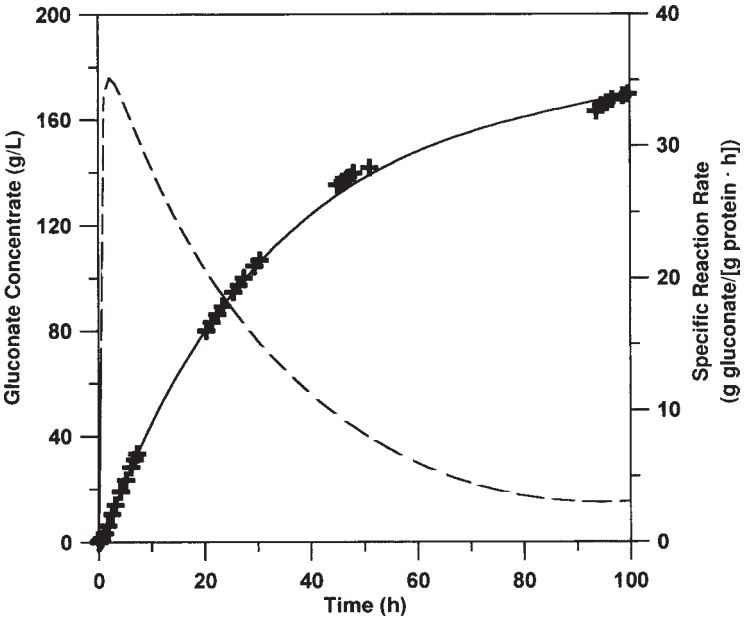

Fig. 7. Gluconate concentration (✚) and specific reaction rate (– – –) as a function of reaction time. Cells are confined in the bore of hollow fibers. Initial concentration: glucose = 200 g/L, fructose = 200 g/L, and protein = 143 mg/L.

cific rate of product formation was comparable to those using free cells. In this case, because diffusion occurs in the liquid phase through the membrane pores, the mass transfer resistance is much lower than in the gel, therefore the reaction was the rate-limiting step of the process.

The specific initial rate for the cells confined inside the fibers was 75% higher than that for cells in the shell side of the module. However, cell deposition was observed in the latter system. The main reason for these results was related to less efficient pH control because cell deposition occurred in the shell of the module.

GFOR stability of cells confined in the bore of hollow fibers was evaluated by means of reactions carried out for a longer time. Figure 7 shows the evolution of gluconate concentration and the specific reaction rate. As can be observed, there was a decrease in the enzyme activity in the early stages

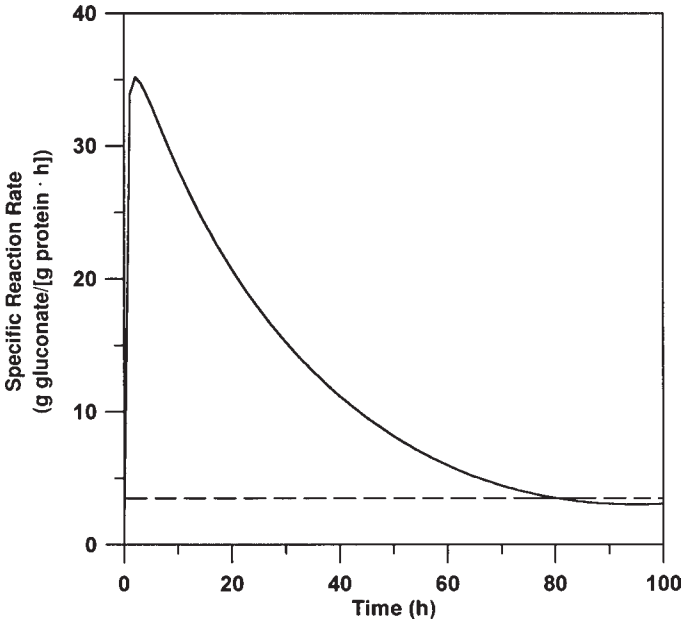

Fig. 8. Specific reaction rate for the first experiment (——) and after substrate solution replacement (– – –). Initial concentration: glucose = 200 g/L and fructose = 200 g/L.

of the reaction that should not be attributed only to the reduction in substrate concentration in the reaction medium. Under the conditions employed in the experiments, conversions higher than 70% were not attained owing to loss of enzyme activity.

To confirm the hypothesis of enzyme inactivation, after about 100 h of reaction, the medium was replaced with a fresh sugar solution. As shown in Fig. 8, the specific reaction rate for the new substrate solution was almost identical to that observed in the final stages of the previous reaction, confirming the enzyme inactivation. This inactivation has been attributed to the enzyme catalytic action itself *(11,15)*. Such an effect was not reported in other studies dealing with the same system *(3,12,13)*, most likely because of utilization of higher enzyme concentrations than in this work.

Conclusion

The use of permeabilized cells in the bore of hollow fibers showed the best performance in terms of specific reaction rate. The mass transfer resistance in this case was much reduced when compared with that of cells immobilized in alginate beads. However, if cells were confined in the shell side of the hollow fiber module, there was a lower enzyme activity, which was attributed to a local reduction in pH owing to cell deposition. This fact leads to the conclusion that the balance between cell concentration confined by hollow fibers and local pH is a very important factor for the design of this kind of reactor.

In all experiments a loss of enzymatic activity was also observed during the reaction. This inactivation has been attributed to the glucono-δ-lactone, which may inhibit the enzyme action. More experiments are being conducted in an attempt to bypass this problem and to make the enzymatic synthesis an even more attractive alternative for sorbitol and gluconic acid production.

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