

## Inert supports for lactic acid fermentation – a technological assessment

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**Abstract.** Production of lactic acid using *Lactobacillus delbrueckii* NRRL B445 recently renamed *L. rhamnosus* was studied in continuously recycled packed reactors at pH 6.3 and 42°C. Four inert adsorbent supports were used for immobilization: Raschig rings of sintered glass (Schott, FRG), beads of sintered glass (Schott), beads of porous glass (Poraver; Dennert, FRG) and irregular ceramic particles (Otto Feuerfest, FRG). The best support was found to be the beads of sintered glass, yielding the highest volumetric lactic acid productivity. Zeta potentials of *L. rhamnosus* showed the cells to be negatively charged at all pHs studied, the charge becoming less negative with increasing ionic strength. The surface charge did not control adhesion. A comparison between the immobilization carried out in batch and continuous tests with the different supports demonstrated that extrapolation from batch adsorption curves to continuous operation can introduce large errors. The effect of dilution rate was also studied: a saturation concentration of adsorbed cells was achieved at all dilution rates, i.e., the immobilized cell component was almost invariant. Different diameters of Poraver beads were tested; clear evidence for mass transfer limitation was shown. Finally, the effects of pH and substrate concentration under immobilization were evaluated. The results indicate that pHs above or below the optimum for suspended cell systems can be used in the immobilized reactor while maintaining lactic acid productivity. To simplify downstream processing by keeping the glucose concentration close to zero in the effluent, the glucose concentration in the feed has to be chosen in conjunction with the dilution rate.

### Introduction

Given the low productivity of batch processes for lactic acid production, recent research has produced numerous studies, in particular using continuous processes, aiming

at increasing the cell concentration in the reactor. Cell recycling over a filtration unit has been extensively reported (Vick Roy et al. 1982; Ohleyer et al. 1985a, b; Mehaia and Cheryan 1986, 1987b; Taniguchi et al. 1987; Major and Bull 1989; Xavier et al. 1991). Despite the high volumetric productivity thus obtained, the specific productivity is low and the system requires a cell bleed stream. During long-term runs it is difficult to prevent fouling of the filtration membranes (Crespo et al. 1992).

Immobilization of the organisms has also been used in a hollow-fibre reactor (Mehaia and Cheryan 1987a; Vick Roy et al. 1983) and in packed beds of cells entrapped in Ca-alginate (Stenroos et al. 1982; Nomura et al. 1987), in agar and polyacrylamide (Tuli et al. 1985) or in  $\kappa$ -carrageenan (Buyukgungor et al. 1984; Audet et al. 1988). The gels have great problems of stability with time (Nomura et al. 1987; Audet et al. 1988) and the hollow fibre is critically mass-transfer-limited (Hongo et al. 1986; Stieber et al. 1977). As for the production of lactic acid, the use of inert supports for immobilization has been lacking (Hartmeier et al. 1987; Krischke et al. 1991).

In the present work inert supports, mainly made up of glass, were chosen for lactic acid production by immobilized cells of *Lactobacillus rhamnosus*, as it is known that these bacteria adsorb to the glass walls in continuous stirred-tank reactor (CSTR) experiments (Gonçalves et al. 1991). This type of support material was also chosen because: (1) it is easy to handle, (2) it generally requires no further preparation for immobilization, (3) it can be reutilized, (4) it is steam sterilizable, and (5) it is generally inexpensive. This work reports technologically relevant comparisons of different inert supports as well as the interpretation and predictions that can be obtained from different types of culture-batch and continuous.

### Materials and methods

**Microorganism.** The organism used was *L. delbrueckii* NRRL B445, a homofermentative lactic acid producer obtained from the

**Table 1.** Characterization of porous supports

Support	Form	Material	Company	Mean particle diameter (mm)	Mean pore diameter ( $\mu\text{m}$ )	Porosity (%)	Density ( $\text{g}/\text{cm}^3$ )
Sikug	Beads	Sintered glass	Schott <sup>a</sup>	1.0–2.0	60–300	55–60	2.48
Rings	Raschig Rings	Sintered glass	Schott <sup>a</sup>	$7.0 \times 2.0 \times 2.0$	60–100	60	
Bi86	Irregular particles	Ceramic	Otto Feuerfest <sup>b</sup>	1.0–2.5	22	70	3.21
Poraver	Beads	Recycled glass	Shaumglass <sup>c</sup>	1.0–2.0 2.0–4.0 4.0–8.0	<200	60–70	0.225 <sup>d</sup> 0.200 <sup>d</sup> 0.175 <sup>d</sup>

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<sup>d</sup> CO<sub>2</sub> is physically entrapped inside the beads

**Table 2.** Continuous fermentation with different immobilization supports

Support	D (l/h)	LacOH (g/l)	Productivity (g lacOH/l/h)	Specific productivity (g lacOH/g cell/h)	$Y_{P/S}$ <sup>a</sup> (g/g)	Total cell conc (g/l)
P (4–8)	0.48	28.61	13.71	0.40	0.67	34.39
P (2–4)	0.31	46.79	14.69	0.70	0.62	20.79
P (1–2)	0.37	43.04	16.01	0.67	0.75	23.99
Sikug	0.39	51.40	20.05	0.78	0.76	34.36
Bi86	0.47	44.32	20.83	0.93	0.63	22.51
Rings	0.34	30.35	10.23	0.43	0.62	33.94
CSTR	0.33	23.15	7.64	1.36	0.71	5.60

D, dilution rate; lacOH, lactic acid;  $Y_{P/S}$ , product yield; P, Poraver; CSTR, continuous stirred tank reactor

<sup>a</sup> Average for all dilution rates tested (see text)

U. S. Department of Agriculture Northern Regional Research Laboratory, Peoria, USA. This has now been renamed *L. rhamnosus* (ATCC Catalogue 1985; Collins et al. 1989).

**Media.** The medium utilized during fermentation tests was made up as follows: 15 g/l of yeast extract, 0.2 g/l of KH<sub>2</sub>PO<sub>4</sub>, 0.2 g/l of K<sub>2</sub>HPO<sub>4</sub>, 0.1 g/l of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.03 g/l of MnSO<sub>4</sub>·7H<sub>2</sub>O. The glucose concentration was variable.

**Supports.** The inorganic porous materials are characterized in Table 1. The supports were used after washing with deionized water and sterilized by autoclaving during 25 min at 1 atm and 121°C. For the Poraver support only the portion that adsorbed water after autoclaving was used, as the rest floated due to physically entrapped CO<sub>2</sub>.

**Zeta potential measurement.** The procedure and the equipment utilized were the same as have been described by Krekeler et al. (1991).

**Analytical procedures.** The free cell concentration was expressed as the dry cell weight. The immobilized cell concentration was obtained as the difference between the dry weight of the total solids (cell and supports) measured after drying at 106°C for 72 h, and the weight of the supports after 6 h of drying at 400°C; prior to drying, the supernatant was decanted and the supports were rinsed three times with 50 ml of a 0.9% (w/v) NaCl solution to eliminate non-adsorbed cells. The total cell concentration in the tubular reactors was obtained as the sum of freely suspended and immobilized cells, after converting the concentration of immobilized cells (g cell dry weight/g support) into grams cell dry weight per litre reactor volume.

Lactic acid and glucose concentrations were determined by HPLC. The conditions and the equipment used were the same as previously described (Gonçalves et al. 1991).

**Batch adsorption experiments in shaking flasks.** Cells in the exponential phase were centrifuged at 8000 rpm for 20 min (MLW T52, FRG) and suspended in culture medium at two cell concentrations (0.02 and 2.0 g cell dry weight/l). These two values were chosen so that saturation levels could be guaranteed. The support material was previously sterilized and added to 50 ml culture medium with 20 g/l of glucose and grown for 24 h with agitation (100 rpm) in 100-ml erlenmeyer flasks on a rotatory shaker at 42°C (GFL, FRG). After incubation the amount of adsorbed cells was determined as described in the analytical procedures.

**Experimental tests in continuous recycled tubular reactor.** The reactor used comprises a jacketed column approximately 24 cm high with an i.d. of 2.5 cm and a total volume of 190 ml; the temperature was kept at 42°C. The amount of support used in each experiment is shown in Table 2. Reactors were sterilized by autoclaving (121°C, 1 atm, for 35 min). After cooling, they were filled with previously sterilized culture medium, inoculated with *L. rhamnosus* in the exponential phase, and grown batchwise for 24 h. From then on the reactor was fed continuously with a peristaltic pump (Watson Marlow, 502S, UK). In order to have a perfectly mixed reactor the effluent of the reactor was continuously recycled with the help of a peristaltic pump (the recycling rate was 8 l/h, that is 80 times the maximum feed rate of fermentation medium used throughout this work). In this way it was possible to keep the pH of the medium constant using one pH controller (ProMinent-Dulcometer system type PHD, FRG) adding 10% (w/v) ammonia solution. Tracer tests showed the reactor to behave as a perfectly mixed bulk liquid, although one should bear in mind that intraparticle diffusion takes place inside the supports.

## Results and discussion

### Adsorption and surface phenomena

Although dependent on many other parameters, adsorption of hydrophilic microorganisms is said to be mainly dependent on electrostatic interactions (Mozes et al. 1987). Thus the characterization of the cell surface in terms of charge can provide information about the adsorption characteristics of the cells to surfaces of inert supports. The surface of bacterium used in this work, *L. rhamnosus*, is negatively charged for all the pHs studied, as can be seen in Fig. 1. For pH 6.6, often used for lactic acid fermentation, the high ionic strength renders the cell surface less negatively charged (Fig. 1). Since the glass surface of the support is also negatively charged (Krekeler et al. 1991) the adsorption to the supports or to the glass walls of the fermentors that was observed in the tests with CSTRs at high dilution rates (Gonçalves et al. 1991) can be ascribed to one or more of the following effects:

1. High ionic strength of the culture medium, resulting in less negatively charged cell surfaces (Fig. 1).
2. Modification of the cell surface charge and/or support surface charge by some components of the fermentation medium. Light chemical modifications of the cell surface has been an important field of experimentation (van Haecht et al. 1985). Thonart et al. (1982) demonstrated that the adsorption of negatively charged cells to negatively charged supports can be significantly increased due to the presence of starch in the medium.
3. Increased cell hydrophobicity obtained, for example, during exponential growth or at high growth rates in a chemostat, without marked change in zeta potential as reported by van Loosdrecht et al. (1987).

To test the first hypothesis, the zeta potential of cells immersed in culture medium was measured as being  $-13.9$  mV, i.e., not a strong enough drop to explain, on its own, the adsorption to the glass surface. The second hypothesis can be one of the main causes if some constituents of the medium of fermentation are responsible for changes in the support surface; this hypothesis was not tested here. The influence of hydrophobicity over and above the surface charge effect has also clearly been shown to be a strong factor in explaining the adsorption of negatively charged cells to negatively charged supports (Krekeler et al. 1991). Thus, from the surface charge results presented here and the evidence that these negatively charged cells attach to negatively charged surfaces of glass, it can be concluded that one cannot predict microbial adsorption in complex media based on zeta potential alone.

### Adsorption tests

Although conclusions are often drawn from batch adsorption tests, differences in adsorption capacity between batch and continuous tests for these acidogenic bacteria are striking, as can be seen in Fig. 2. Such differences can be explained not only by the different hy-

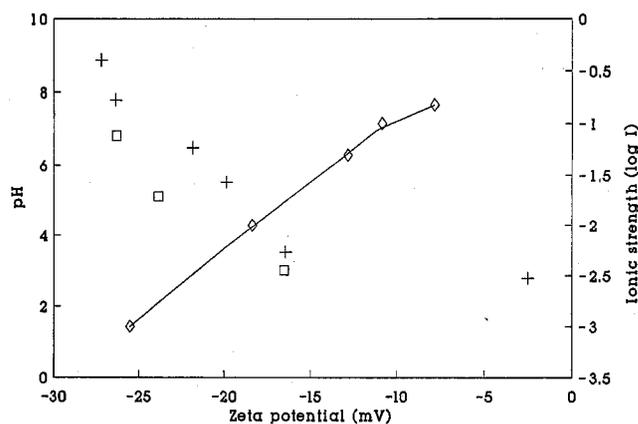


Fig. 1. Influence of pH using cells of two ages ( $\square$ , 48 h;  $+$ , 16 h) and the ionic strength ( $\diamond$ ) on the zeta potential of *Lactobacillus rhamnosus*

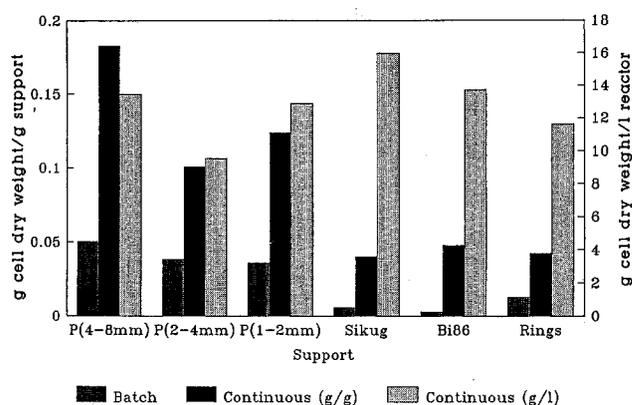


Fig. 2. Immobilized cells in batch and continuous tests with different inert supports: P, Poraver; Bi86, ceramic particles

drodynamics of the reactors but also from physiological and cell surface modifications occurring during the tests. Under microscopical observation, cells changed from isolated rod shapes into thinner, chain-linked consortia with increases in dilution rate. In effect, in the batch adsorption experiments the pH decreased from 6.6 to 3.5 while in continuous tests the pH was maintained constant in the range 6.3–6.6. Since a drop in pH is accompanied by a shift in zeta potential towards the less negative region (see Fig. 1), if the zeta potential was the main factor controlling adsorption, higher cell adsorption would be expected in the batch tests, in opposition to the experimentally obtained values (see Fig. 2).

As can be seen in Fig. 2, different supports present different adsorption capacities, but no correlation with chemical composition of the supports is apparent. The Poraver support presented the best result in terms of specific cell adsorption (g cell dry weight/g support) but when this value is converted to grams cell dry weight per litre volume of reactor, a more usual unit to compare different reactor systems, the best support is found to be Sikug (Fig. 2). The larger pore diameters of Sikug and Poraver compared to the ceramic material (Bi86) did not enhance the concentration of adsorbed bacteria (Fig. 2).

When extrapolating results obtained from batch adsorption curves to continuous operation large errors can take place. As has been shown by the results presented here, the adsorption of cells to inert supports is controlled by different parameters besides pH, ionic strength, cell surface charge and hydrophobicity. In fact the composition of the medium of fermentation and product concentration, the cell physiology and the hydrodynamics of the reactor play a large role and thus scale-up requires long-term continuous runs.

### Continuous fermentation tests

*The effect of the dilution rate.* Given the difficulties associated with aseptically removing samples of support and to keep the reactors running under comparable conditions, namely dilution rates, it was decided to perform one test run for each dilution rate. Once steady state conditions had been achieved and soluble concentrations measured, the tubular reactors were disassembled and the immobilized cell concentrations determined as described in the analytical procedures section.

As can be seen in Fig. 3, the immobilized cell concentration (cells in the support after washing with 0.9% NaCl solution) did not significantly change with the dilution rate. Thus, one can almost define a saturation concentration of adsorbed cells and see that such saturation was achieved for all dilution rates tested. Figure 3 also depicts a very striking effect concerning the non-adsorbed cell concentration; in effect, although the immobilized cell component is almost constant at any dilution rate, the values of the total cell concentration show a large variability decreasing with an increase in the dilution rate, as washing of the bed more homogeneously removes the interstitially settled cells.

*Different supports tested.* The same supports used in the batch adsorption assessment were also tested in continuous fermentation. The tubular reactors described in Materials and methods were continuously fed with culture medium with approximately 100 g/l of glucose. To permit a close comparison with the results obtained in the reactors with immobilized cells a continuous tubular reactor was operated without immobilization support. As expected and as is clearly depicted in Table 2 and Figs. 4 and 5, the systems with immobilization supports attained higher volumetric productivities and could be run at higher dilution rates than the CSTR.

From Fig. 5 for the three Poraver size ranges, there is evidence of a slight influence upon reactor performance due to particle size: this seems clear evidence of mass transfer limitation. Nevertheless, as can be seen from Fig. 5 and Table 2, the 2–4 mm Poraver had, at the end of the run, the smallest total cell concentration; this might explain the final point of the 2–4 mm curve at dilution rate 0.58 1/h, which, on diffusion limitation reasoning, should stay above the curve representing 4–8 mm Poraver, which also shows the maximum total cell concentration.

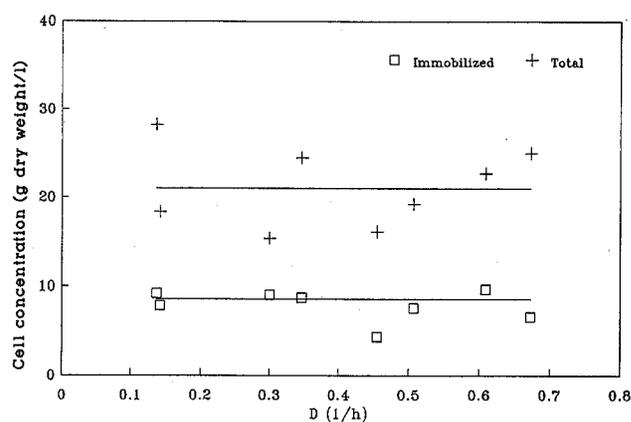


Fig. 3. Total (+) and immobilized (□) cell concentrations as a function of dilution rate (D)

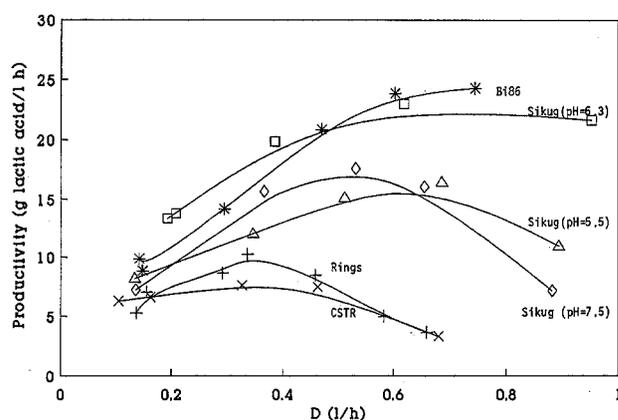


Fig. 4. Lactic acid productivities for different inert supports as a function of dilution rate and for Sikug support at different pHs. CSTR, continuously stirred tank reactor

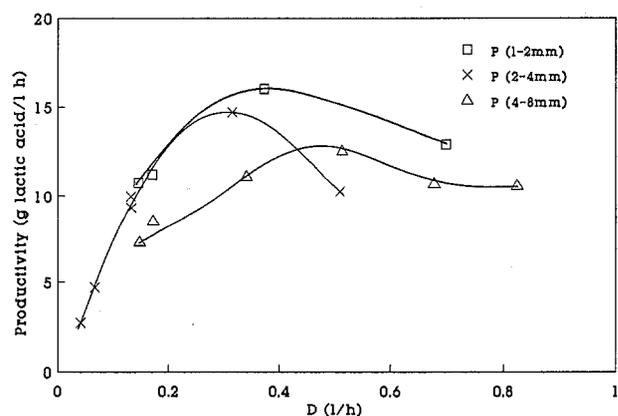


Fig. 5. Lactic acid productivities as a function of dilution rate for Poraver supports with different diameters: □, 1–2 mm; ×, 2–4 mm; △, 4–8 mm

Nevertheless, the Poraver supports did not behave well at high dilution rates: although the total cell concentrations obtained at average dilution rates were reasonable (Table 2), at large dilution rates, the productivity dropped sharply (Fig. 5) and so did the immobilized

cell concentration (data not shown). It seems possible that no strong interaction forces were present between cell and support in this case and that only weak van der Waals forces or even simple loose entrapment takes place, as cells are lost from the support at larger dilution rates. On the other hand, the Sikug and Bi86 supports presented better lactic acid productivities, even at large dilution rates, resulting from a stable high cell concentration in the fermentor. Thus it can be assumed that the forces responsible for the immobilization of the cells in these supports are strong.

For all the dilution rates tested and for all the supports tested the glucose consumed varied linearly with the lactic acid produced. The constants of proportionality were calculated by linear regression of the results and are presented in Table 2 as  $Y_{P/S}$ , the product yield. The ceramic Bi86 support showed low mechanical stability throughout the test runs, as it turned to powder. This was the main reason why it was not selected for further tests on glucose concentration and pH effects; the support selected was Sikug.

**The effect of pH.** Lactic acid fermentation is strongly inhibited by the lactic acid produced (Leudeking and Piret 1959; Gonçalves et al. 1991). Moreover, the specific growth rates are very low above pH 6.8; thus there is a short range of operationally practical pH (Gonçalves et

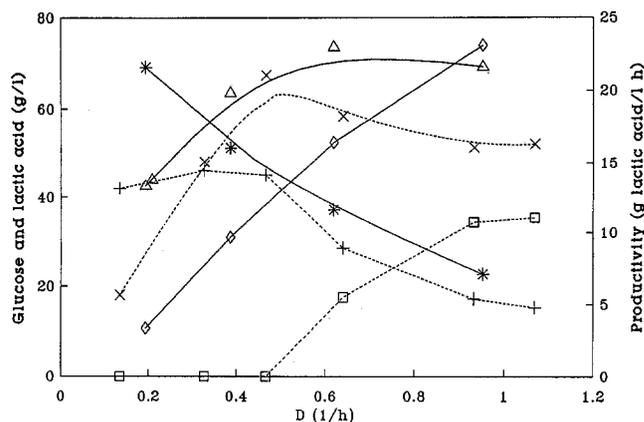


Fig. 6. Glucose, lactic acid and lactic acid productivities as a function of dilution rate at different glucose feed concentrations:  $\square$ , glucose; +, lactic acid;  $\times$ , lactic acid productivity at 50 g glucose/l; and  $\diamond$ , glucose; \*, lactic acid;  $\Delta$ , lactic acid productivity at 100 g glucose/l

al. 1991). This parameter is also important for immobilization processes.

Using the Sikug support as a matrix, three continuous fermentation tests were run at different pHs (5.5, 6.3, 7.5). Figure 4 shows the variation in volumetric productivity with the dilution rate for the pHs tested. As

Table 3. Lactic acid production by high cell density systems

Bioreactor Process	Microorganism	Substrate (g/l)	Lactic acid conc (g/l)	Cell conc (g dry weight/l)	Volumetric productivity (g lactic/l/h)	Reference
<b>Hollow fibre</b>						
Batch	<i>Lactobacillus delbrueckii</i>	Glucose	2	480	100	Vick Roy et al. (1982)
Continuous	<i>L. bulgaricus</i>	Lactose <sup>a</sup>	13	100	2.6	Mehaia and Cheryan (1987a)
<b>Membrane recycle</b>						
Continuous	<i>L. delbrueckii</i>	Glucose	35	54	76	Vick Roy et al. (1982)
Continuous	<i>L. delbrueckii</i>	Glucose	60	130	66	Ohleyer et al. (1985a)
Continuous	<i>L. delbrueckii</i>	Lactose	40	80	38	Ohleyer et al. (1995a)
Continuous	<i>L. delbrueckii</i>	Glucose	60	140	150	Ohleyer et al. (1985b)
Continuous	<i>L. bulgaricus</i>	Lactose <sup>a</sup>	14 <sup>d</sup>	43	85	Mehaia and Cheryan (1986)
Continuous	<i>L. bulgaricus</i>	Lactose <sup>a</sup>	117	63	84	Mehaia and Cheryan (1987b)
Continuous	<i>L. helveticus</i>	Lactose <sup>a</sup>	25 <sup>d</sup>	64	22	Boyaval et al. (1987)
Continuous	<i>L. casei</i>	Lactose	40 <sup>d</sup>	49	— <sup>b</sup>	Taniguchi et al. (1987)
Continuous	<i>S. cremoris</i>	Lactose	25 <sup>d</sup>	81	— <sup>b</sup>	Taniguchi et al. (1987)
Continuous	<i>L. delbrueckii</i>	Glucose	40	8	12	Major and Bull (1989)
Continuous	<i>L. delbrueckii</i>	Glucose	68	94	40	Xavier et al. (1991)
<b>Immobilized</b>						
Continuous	<i>L. bulgaricus</i>	Lactose	53.8	4.8	5.1 <sup>d</sup>	Stebier and Gerhardt (1981)
Continuous	<i>L. delbrueckii</i>	Glucose	39.8	— <sup>b</sup>	3.3 <sup>d</sup>	Stenroos et al. (1982)
Continuous	<i>L. delbrueckii</i>	Glucose	27.2	8.1	34.0	Buyukgungor et al. (1984)
Batch	<i>L. casei</i>	Lactose <sup>a</sup>	33.0	— <sup>b</sup>	0.5	Tuli et al. (1985)
Continuous	<i>L. delbrueckii</i>	Glucose	0.8 <sup>d</sup>	9.5	3.3	Hartmeier et al. (1987)
Batch	<i>L. delbrueckii</i>	Glucose	70.1	— <sup>b</sup>	5.3 <sup>e</sup>	Nomura et al. (1987)
Batch	<i>L. bulgaricus</i>	Lactose <sup>a</sup>	10.0	— <sup>b</sup>	1.7 <sup>d</sup>	Audet et al. (1988)
Batch	<i>S. lactis</i>	Lactose <sup>a</sup>	8.0	— <sup>b</sup>	1.3 <sup>d</sup>	Audet et al. (1988)
Continuous	<i>L. casei</i>	Lactose <sup>a</sup>	13.5 <sup>d</sup>	105 <sup>c</sup>	13.5	Krischke et al. (1991)
Continuous	<i>L. delbrueckii</i>	Glucose	51.4	34.4	20.1	This work

<sup>a</sup> From whey permeate

<sup>b</sup> Not presented

<sup>c</sup> Grams cell dry weight per kilogram support material

<sup>d</sup> Estimated from reported data

<sup>e</sup> g/l gel/h

expected, the lactic acid production was highest at pH 6.3. Since for pH 7.5 the lactic acid production is still large in comparison with suspended cells, it can be said that the lactic acid thus produced results mainly from non-growing immobilized cells.

*The effect of glucose concentration.* Two glucose concentrations were tested in continuous tests using Sikug as the immobilization support. This parameter is important since in industrial processes the remaining unfermented glucose can complicate the extraction and purification of lactic acid. When 100 g/l of glucose was used, only for the very low dilution rates (lower than 0.1 1/h) did the glucose concentration in the effluent stay close to zero (Fig. 6). When 50 g/l of glucose was used, all the substrate was converted to lactic acid up to dilution rates of 0.45 1/h; for higher dilution rates the two systems presented similar lactic acid productivities (Fig. 6), although these are slightly higher for the larger glucose feed concentration.

#### Comparison of different systems

Table 3 summarizes the data for high-cell-density systems reported in the literature compared with the results presented here. It is clear that the immobilized systems or the hollow-fibre reactor systems result in small volumetric productivities and/or low lactic acid concentration being obtained in the broth. The cell recycling systems present high volumetric lactic acid productivities as a direct result of the much larger cell concentrations obtained in these systems; nevertheless, most of the very high performance data shown (Table 3) have been obtained in short run experiments, often only 24 h. Longer term tests, namely Xavier et al. (1991), yielded results that are more closely comparable with the immobilized results reported here; these were obtained under bleeding stream strategies. Given the process simplicity, the immobilization of lactic acid bacteria in inert supports presented in this work seems to be a good practical alternative for producing lactic acid, since high lactic acid concentrations can be obtained with consistently high volumetric lactic acid productivities at reasonable lactic acid concentrations in the broth.

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