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Immobilization of *Lactobacillus casei* cells to ceramic material pretreated with polyethylenimine

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Summary. The cells of *Lactobacillus casei* were adsorbed to Poraver, foam glass particles pretreated with polyethylenimine (PEI). Exposure of cells for a relatively short period to Poraver beads coated with a high concentration of PEI resulted in maximal adsorption with good retention of metabolic activity. The immobilized cells were tested in packed-bed and stirred-tank reactors for lactic acid production. Stirred-tank operations were more effective in terms of productivity but the support was sensitive to attrition. The beads exhibited good mechanical stability to withstand pressure in the packed-bed reactor.

Introduction

Cell immobilization technology continues to attract attention for providing stable preparations for continuous or batch fermentation processes with the aim of making them cost-effective by increasing their volumetric productivity. Entrapment is by far the most popular method as judged from the number of research papers published. For applications on a large scale, however, immobilization by adsorption seems technically more attractive as it is easily performed without involving toxic chemicals, and it may be based on the use of inexpensive carrier materials. Adsorbed cells are exposed more directly to the reaction medium, providing better mass transfer conditions than that achieved with the entrapped cells.

A broad range of support materials have been used for cell adsorption, which include wood chips, sawdust, ion exchange resins, porous bricks, glass, foam, cotton cloth and ceramics (Mattiasson 1983; D'Souza 1989). Efforts made to increase cell loading and improving the binding strength between the support and the cell surface include pretreatment with the charged polymer polyethylenimine (PEI) (D'Souza and Kamath 1988), starvation of cells in distilled water prior to immobilization (Van Haecht et al. 1984), facilitation of cell surface binding using Al^{3+} ions (Van Haecht et al. 1985) and hydrodynamic deposition (Salter et al. 1990).

Cell adsorption using PEI is an extremely simple and straightforward procedure resulting in the cells becoming firmly attached to the matrix. The choice of the support material is important. The ideal immobilization matrix would be the one that has a rigid structure to retain the cells and favorable mechanical properties required during scaling up. The study reported in this paper was set up to investigate the possibility of using PEI-treated foam glass particles (trade name, Poraver) as a support for immobilizing cells of *Lactobacillus casei*, and to evaluate their performance both in stirred-tank and packed-bed reactors for the production of lactic acid.

Materials and methods

Microorganism and culture medium. L. casei (DSM 20021) was used for this study. The organism was maintained on MRS-agar medium (De Man et al. 1960) and subcultured fortnightly.

The culture medium used for cultivation of *L. casei* and lactic acid production, unless specifically mentioned, contained per litre: glucose, 30 g; yeast extract, 10 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; sodium citrate, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.005 g; $MnSO_4 \cdot H_2O$, 0.0031 g; $FeSO_4 \cdot 7H_2O$, 0.002 g; ascorbic acid, 0.005 g (Guoqiang et al. 1991). The medium components were sterilized individually by autoclaving at 120° C for 15 min.

Growth conditions. Cultivation of *L. casei* was performed as described earlier (Guoqiang et al. 1991). The cells were grown in a fermentor (Chemoferm FLC-B-3, Hägersten, Sweden) with a working volume of 2 l. The inoculum used was 100 ml of an overnight culture of *L. casei*. The cultivation temperature was 42° C and the pH was maintained at 6.0 by automatic titration with 3 M NaOH. Stirring was maintained at a constant speed of 200 rpm. At the end of the exponential phase, cells were harvested under sterile conditions at 10000 rpm for 10 min at 4° C in a Sorvall RC-

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5B centrifuge. The cell pellet was then washed thoroughly with sterile 0.9% NaCl solution and stored at 4° C in a solution of 2 g yeast extract/l.

Chemicals. PEI was purchased from Sigma (St. Louis, Mo., USA). 3,5-Dinitrosalicylic acid, lithium lactate and 4-phenylphenol were obtained from Aldrich (Steinheim, FRG). Other chemicals of analytical grade were obtained from standard sources.

Immobilization matrix. Poraver beads were a kind gift from Dennert Poraver (Postbauer-Heng, FRG).

Immobilization procedure. Five grams of Poraver beads (2–4 mm diameter) were washed thoroughly with tap water and dried at 120° C. These beads were then added to 50 ml PEI (2 g/l) solution, pH 7.0 (in 0.02 M KH_2PO_4) and autoclaved for 15 min at 120° C. Poraver beads autoclaved in buffer without PEI were used as a control. Both the PEI-coated and control Poraver beads were later dried at 50–70° C. The dried beads were then mixed with 50 ml of *L. casei* cell suspension in 0.02 M potassium phosphate buffer, pH 6.5, and the mixture incubated for about 13 h at room temperature in a tube (5 × 10 cm) with gentle shaking on a rocking table. Thereafter, unadsorbed cells were removed by washing the beads thoroughly with sterile distilled water. The beads were later rinsed about three times with culture medium and used for further studies.

Batch lactic acid fermentation using immobilized cells. L. casei cells immobilized to Poraver using different PEI concentrations and adsorption times were used for fermentation of glucose to lactic acid. The beads were transferred into 100-ml conical flasks containing 30 ml culture medium and incubated at 42° C. About 2 g calcium carbonate powder (particle size, 0–1 mm) was added into the flasks to neutralize the lactic acid produced. The flasks were sealed with rubber stoppers with syringe needles passing through to achieve nearly anaerobic conditions and to have an outlet for any gas produced. The flasks were shaken at intervals so as to mix the $CaCO_3$ properly with the medium. Aliquots of the medium were withdrawn at different time intervals for analyses of glucose, lactic acid and free cell density.

After completion of a batch process, the immobilized cells were reused after being washed once with 50–60 ml sterile distilled water and twice with similar volumes of culture medium. After three batch processes, the yeast extract concentration in the culture medium used for lactic acid fermentation was lowered to 2 g/l.

Continuous lactic acid production. The immobilized cells were incubated twice in the culture medium until the glucose was fully utilized each time. The beads were then transferred into a column or a stirred-tank reactor. The water-jacketed column (1.0 × 30 cm) packed with Poraver beads was maintained at 42° C. The culture medium containing glucose at a concentration of 12 g/l was continuously pumped upward into the column. In order to prevent the pH from dropping too much in the column, the medium was buffered in 0.1 M sodium phosphate, pH 6.8.

A water-jacketed stirred-tank reactor with an internal diameter of 10 cm and height of 14 cm, and fitted with a lid with five openings, was used. An overhead stirrer and a pH electrode were fitted through two of the openings. Poraver beads (200 ml sedimented volume = 65 g) and the culture medium were mixed in the reactor to a total working volume of 600 ml. Fresh medium was continuously pumped in via a tube passing through the lid and reaching the base of the reactor. Another tube starting from the medium surface was used for channelling of the processed medium out of the reactor. The pH was controlled by automatic titration with 4 M NaOH. The temperature was maintained at 42° C and stirring at 100 rpm. In both cases, samples of the eluate for analyses were taken at different time intervals.

Analyses. The concentration of glucose was measured by a modification of the dinitrosalicylic acid method (Miller 1954).

Total lactic acid content was estimated according to the method described by Lawrence (1975). The lactic acid was converted to acetaldehyde by concentrated sulphuric acid in the presence of copper sulphate, and the acetaldehyde formed was measured colorimetrically using 4-phenylphenol.

The amount of suspended cells was monitored by measuring the absorbance at 620 nm using a Shimadzu UV-120-02 spectrophotometer. The amount of absorbed cells was quantified by measuring suspended cells before and after the adsorption process.

Results

Adhesion of L. casei cells to PEI-coated Poraver beads: optimization of immobilization parameters

Native Poraver beads did not exhibit significant adsorption of *L. casei* even after a few days incubation with the cells. However, coating of the beads with PEI resulted in the cells adhering strongly to the matrix, which was observed as a significant drop in the optical density at 620 nm (OD_{620}) of the cell suspension (Table 1). Variation of the adsorption conditions showed that drying of the beads after PEI treatment was effective for maximal adsorption. Repeated experiments indicated that buffering of the solution for maintaining the optimal pH of the solution during autoclaving and adsorption was essential. The optimal pH for PEI coating was about 7.0, and during adsorption it was 6.5.

Different particle sizes of Poraver were used to investigate the efficiency of cell adsorption and the catalytic performance of the immobilized preparation. The change in OD_{620} of the cell suspensions when mixed with PEI-coated Poraver of varying diameters is shown in Fig. 1. It is obvious that in the beginning, the smaller particles were more efficient for adsorbing cells, but after some hours of exposure their capacity seemed to reach a plateau. However, it was seen that the smaller particles were more brittle and therefore broke more easily into even smaller pieces, thereby reducing their usefulness in this context. Beads with a diameter of 2–4 mm exhibited optimal adsorption characteristics and

Table 1. Adhesion of *Lactobacillus casei* cells to Poraver beads

Mixing time (h)	$OD_{620\,nm}$ of cell suspension mixed with Poraver beads			
	Native (untreated) beads	PEI-coated beads[a]		
		a	b	c
0.0	1.55	1.80	1.82	1.82
1.0	1.60	1.27	1.34	1.05
15.0	1.48	1.12	0.43	0.54

[a] Poraver beads autoclaved in polyethylenimine (PEI) solution (2 g/l) were used for adsorption of cells either (a) directly. (b) after drying at 50° C, or (c) after washing once with distilled water and then drying

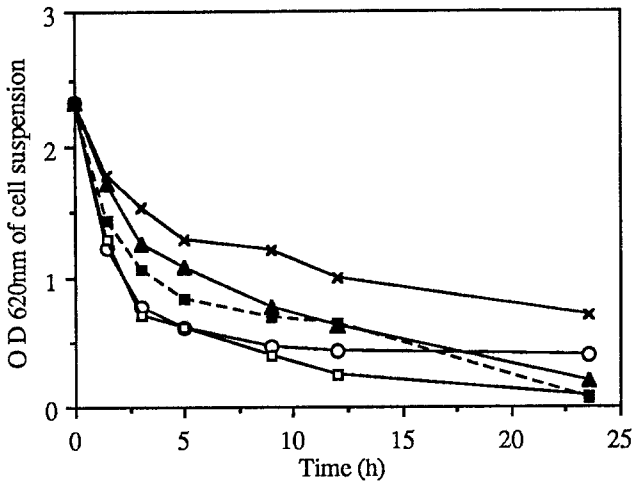

Fig. 1. Time course of cell adsorption on polyethylenimine (PEI)-coated Poraver beads of different diameters: 0.25–0.5 mm (○), 1–2 mm (□), 2–4 mm (■), 4–8 mm (▲), and 8–16 mm (×)

Table 2. Adhesion of *L. casei* cells to Poraver beads treated with different concentrations of PEI

Mixing time (h)	PEI concentration (g/l)				
	0.1	1.0	2.0	10.0	25.0
0.0	1.98	1.98	1.98	1.98	1.98
1.0	1.62	1.56	1.55	1.27	0.02
13	1.37	1.07	1.02	0.21	nd

nd, not done

suitable mechanical stability. Hence, these beads were used for further studies.

To evaluate the effect of PEI used in the pretreatment step on the adsorptive capacity of the support, Poraver beads coated with different concentrations of PEI were used for cell adsorption. Table 2 shows that the higher the PEI concentration used, the more efficiently were the cells adsorbed. The beads treated with 25 g PEI/l adsorbed the cells almost completely within just 1 h. Subsequent washing of the immobilized preparations showed no cell leakage.

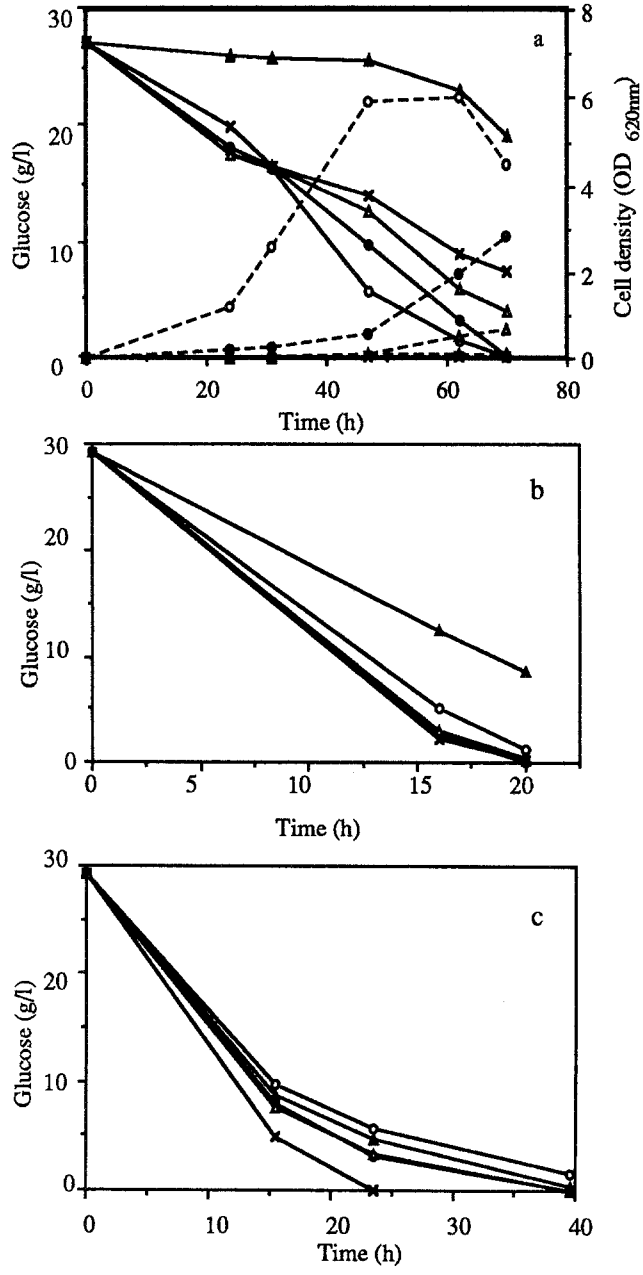

Fig. 2. Glucose utilization and free cell density (OD_{620nm}) during the first batch (**a**), glucose utilization during the third (**b**), and fourth (**c**) batches of fermentation by *Lactobacillus casei* cells adsorbed to Poraver beads coated with different PEI concentrations. The PEI concentrations used were 0.1 (○), 1.0 (●), 2.0 (△), 10.0 (▲) and 25.0 (×) g/l. The adsorption time was 1 h for the beads coated with 25 g PEI/l, and 13 h for the other preparations

Evaluation of immobilized L. casei cells in a batch process

The effect of immobilization conditons such as the PEI concentration and cell adsorption time on the viability and metabolic activity of the adsorbed *L. casei* cells was studied by means of fermentation of glucose in a batch process.

First, the cells adsorbed to Poraver coated with varying concentrations of PEI were used. The time-dependent glucose utilization and the density of free cells in the medium, the latter resulting from the cell division on the support, showed a negative correlation to the amount of PEI used (Fig. 2a). However, contacting cells with Poraver modified with 25 g PEI/l for just 1 h gave a preparation with a relatively higher glucose consumption rate than the one utilizing a lower concentration of PEI (10 g/l) but a longer adsorption time (13 h). Reusing the immobilized cells after appropriate washing showed, during the second run, the highest rate of glucose utilization by the preparation based on 1.0 g PEI/l (data not shown). However, during the third run, the preparation that was pretreated with the highest PEI concentration and had the lowest adsorption time, attained the same activity as those modified for a longer time (13 h) with

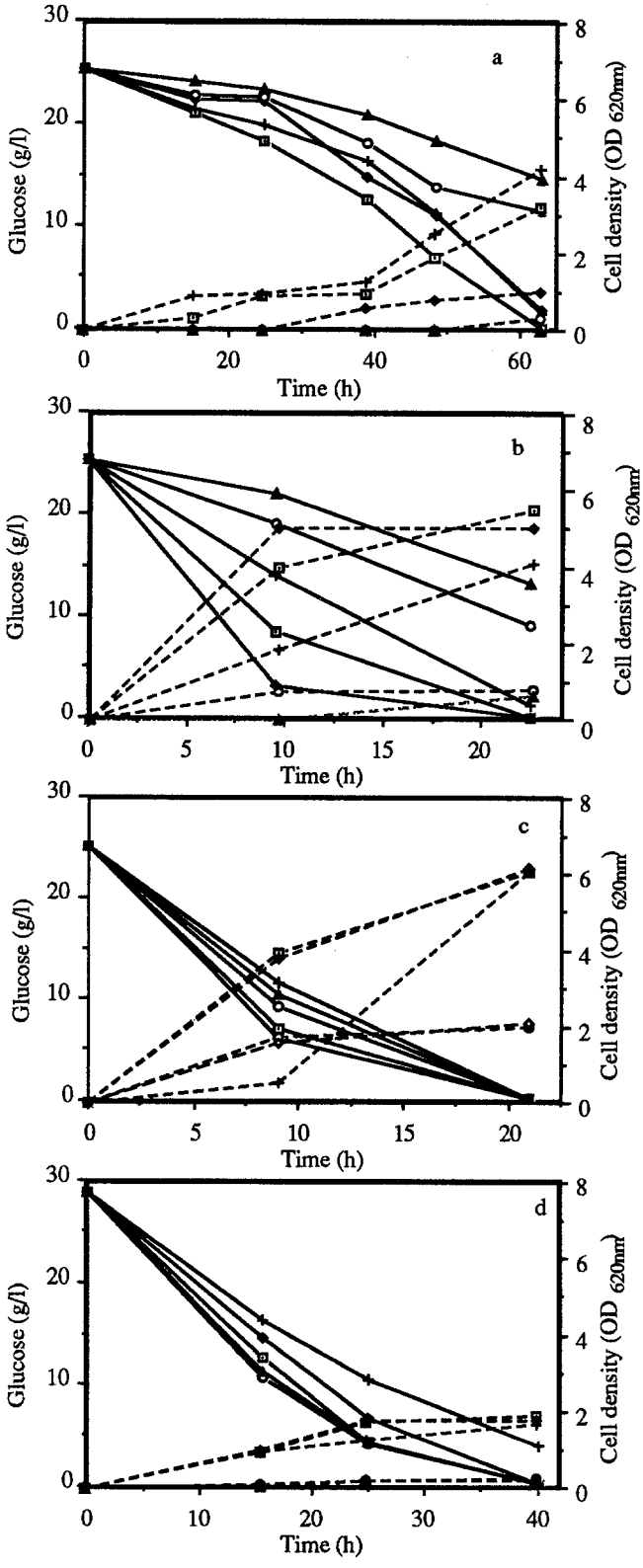

Fig. 3. Effect of PEI concentration and adsorption time on glucose utilization and amount of free cells during the first (**a**), second (**b**), third (**c**), and fourth (**d**) batches of fermentation by immobilized *L. casei* cells. The PEI concentrations and adsorption times used were 2 g/l, 1.5 h (□); 2 g/l, 8 h (◆); 10 g/l, 1.5 h (○); 10 g/l, 8 h (▲); and 0.0 g/l, 8.0 h (×)

lower concentrations (1.0 and 2.0 g/l) of PEI solution (Fig. 2b). This was particularly interesting because the use of this preparation (coated with 25 g PEI/l) showed very few free cells in the medium compared with other preparations, indicating that the kinetics of the system was mostly controlled by the adsorbed cells. Maximum leakage of cells was observed with the beads treated with 0.1 g PEI/l.

In the fourth batch, the yeast extract concentration in the medium was reduced to 2 g/l in order to reduce the production of free cells and thus the interference by free cells on the kinetics of the system. Here, the highest glucose utilization rate was observed for the preparation modified with the largest amount of PEI (Fig. 2c). It was, therefore, concluded that beads exposed to high concentrations of PEI for a short period have a large capacity for the adsorption of cells.

In another series of experiments, cells adsorbed to modified Poraver for varying periods of time were used. Poraver treated with 2 and 10 g PEI/l, and then exposed to the cells for 1.5 and 8 h, respectively, were used for lactic acid production. In the first cycle of experiments it was seen that a short time of exposure to a low-degree derivatized Poraver exhibited both a high glucose consumption rate and liberation of cells into the medium. On the other hand, the highly substituted preparations showed a reduced glucose consumption rate and also a dramatically decreased rate of cell release into the medium (Fig. 3a).

Upon repeated cycles, the difference in glucose consumption rate between all preparations was reduced (Fig. 3b, c and d). However, the cell density in the supernatant even after four cycles was markedly different between preparations with a high and low degree of substitution (Fig. 3d). These data were interpreted in terms of a process where highly substituted Poraver favoured adsorption of the newly grown cells. In these cases the kinetics of the systems was mainly controlled by the immobilized cells. However, for the low-substituted Poraver more of the newly grown cells leaked into the surrounding medium, thereby levelling out any differences in overall glucose consumption due to initial conditions.

Reactor experiments aiming at production of lactic acid

Preliminary studies on continuous lactic acid production in a stirred-tank and a packed-bed reactor were carried out using cells adsorbed for about 13 h to Poraver modified with 2 g/l solution. Prior to loading in the reactors, the immobilized cells were incubated in the culture medium to ensure that the cells were fully viable and the bead surface was well covered with cells.

In the stirred system, glucose was almost completely (99.2%) utilized with a lactic acid productivity of 5.2 g/l per hour at a dilution rate (D) of 0.18 h^{-1} (Fig. 4). Lactic acid productivity increased with increasing dilution rate and reached 9.8 g/l per hour at $D = 0.77\ h^{-1}$, however with a substantial amount of the glucose still left in

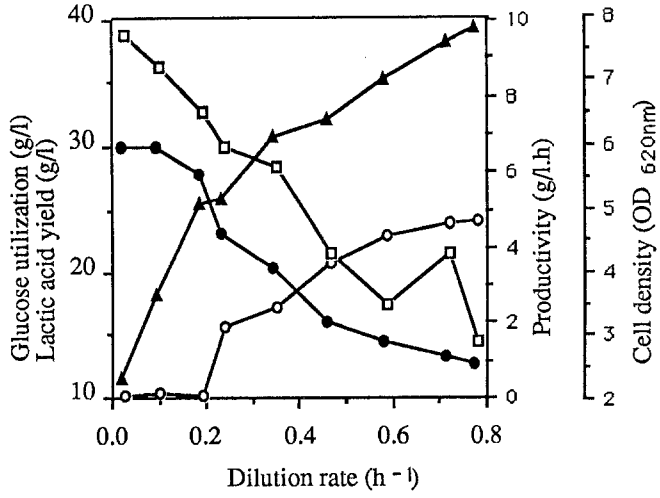

Fig. 4. Continuous culture of immobilized *L. casei* cells in a stirred-tank reactor with varying dilution rate: O, glucose utilization; ●, lactic acid yield; ▲, productivity; □, cell density

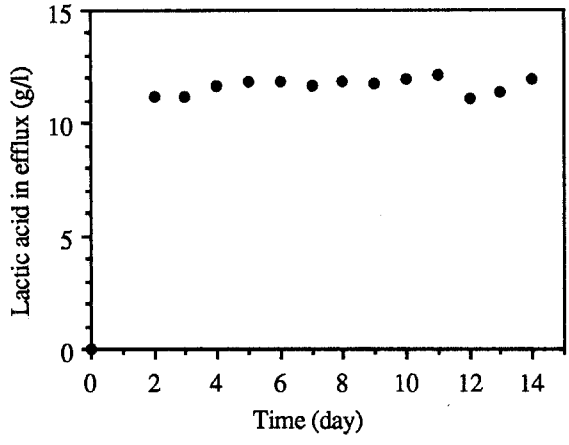

Fig. 5. Continuous production of lactic acid by *L. casei* cells adsorbed to PEI-coated Poraver beads in a packed-bed reactor at a dilution rate of 0.11 h^{-1}

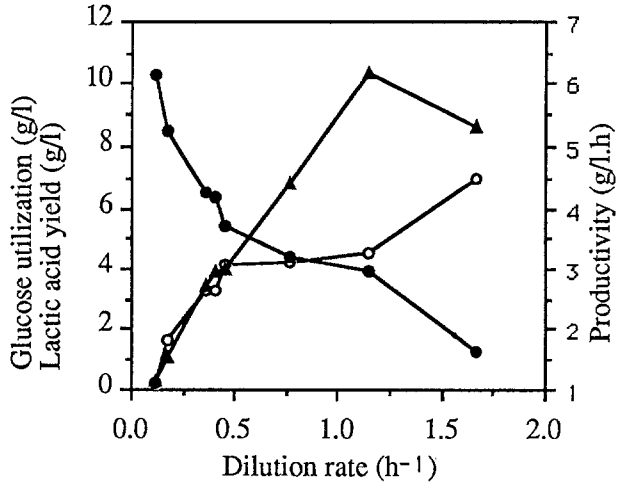

Fig. 6. Continuous culture of immobilized *L. casei* cells in a packed-bed column with varying dilution rate. Symbols as in Fig. 4

the medium. For a production situation it is more important to fully utilize the substrate than to achieve very high productivity figures, and therefore $D = 0.18\ h^{-1}$ was regarded as the optimum with the system studied here.

The packed-bed experiments were performed using a small reactor. Lactic acid production was maintained constant over a period of 14 days at $D = 0.11\ h^{-1}$ (Fig. 5). The pH of the eluate was between 4.5 and 5.0. Conversion of glucose to lactic acid was more than 90% and the productivity 1.17 g/l per hour. At $D = 1.1\ h^{-1}$, maximum productivity was achieved (6.16 g/l per hour), but with only 60% glucose utilization (Fig. 6).

Discussion

Poraver, an inexpensive sedimentary ceramic material has been found in a study to be suitable for settlement and colonization of bacteria, and hence has potential for waste-water treatment. Having a porous structure, it could provide a good support with a large surface area for the adsorption of cells. In the present study, however, native Poraver had poor adsorption characteristics for the *L. casei*, but pretreatment with the charged polymer PEI made the support a suitable adsorbent for cells.

High concentrations of PEI seemed to be helpful in the long run for maximal loading of the cells on the support. Even if these concentrations of PEI initially seemed to inhibit the metabolic function of the cells, this effect appeared to be nullified during repeated fermentations. A few viable cells on the support were enough to give rise to a large number of cells that continued to get adsorbed on the beads, ultimately giving favorable reaction kinetics per unit weight of the support.

This investigation showed that the support had excellent mechanical properties for withstanding pressure in packed-bed reactors, while being somewhat brittle and sensitive to attrition under stirred conditions. However, the lactic acid fermentation process was more efficient in the stirred-tank operation. This is probably due to a better pH control in that system compared to the packed-bed reactor. Such an observation has also been reported earlier (Boyaval and Goulet 1988). Hence, for the present application it may be more suitable to use the Poraver immobilized cells in a fluidized-bed reactor. This reactor configuration has earlier been found to be effective for obtaining high lactic acid productivity (Krischke et al. 1991).

It may be interesting to evaluate PEI-modified Poraver as a support for the adsorption of a number of microorganisms for other applications.

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