Lactic Acid Fermentation in a Recycle Batch Reactor Using Immobilized *Lactobacillus casei*

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Abstract: Lactic acid production by recycle batch fermentation using immobilized cells of *Lactobacillus casei* subsp. *rhamnosus* was studied. The culture medium was composed of whey treated with an endoprotease, and supplemented with 2.5 g/L of yeast extract and 0.18 m*M* Mn^{2+} ions. The fermentation set-up comprised of a column packed with polyethyleneimine-coated foam glass particles, Pora-bact A, and connected with recirculation to a stirred tank reactor vessel for pH control. The immobilization of *L.* casei was performed simply by circulating the culture medium inoculated with the organism over the beads. At this stage, a long lag period preceded the cell growth and lactic acid production. Subsequently, for recycle batch fermentations using the immobilized cells, the reducing sugar concentration of the medium was increased to 100 g/L by addition of glucose. The lactic acid production started immediately after onset of fermentation and the average reactor productivity during repeated cycles was about 4.3 to 4.6 g/L·h, with complete substrate utilization and more than 90% product yield. Sugar consumption and lactate yield were maintained at the same level with increase in medium volume up to at least 10 times that of the immobilized biocatalyst. The liberation of significant amounts of cells into the medium limited the number of fermentation cycles possible in a recycle batch mode. Use of lower yeast extract concentration reduced the amount of suspended biomass without significant change in productivity, thereby also increasing the number of fermentation cycles, and even maintained the D-lactate amount at low levels. The product was recovered from the clarified and decolorized broth by ion-exchange adsorption. *Biotechnol Bioeng* **55:** 841–853, 1997.

Keywords: immobilized cells; lactic acid bacteria; adhesive fermentation; poly(ethyleneimine); recycle batch reactor

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INTRODUCTION

Lactic acid is a product with a wide variety of industrial applications, among the most important being its use as a preservative and acidulant in foods (Vick Roy, 1985), and as a precursor for polymers like poly(lactic acid) (Dunn et al., 1988). Whey, generated by the dairy industry, sugarcane molasses, and starch hydrolysates from a variety of agricultural products worldwide constitute the most useful raw materials for lactic acid production by microbial fermentation (Keller and Gerhardt, 1975; Mehaia and Cheryan, 1986; Shamala and Sreekantiah, 1988; Silva and Yang, 1995; Vick Roy, 1985; Zhang and Cheryan, 1994). The process of production includes two key stages: fermentation and product recovery. Lactic acid fermentation is characterized by product inhibition, which affects cell growth and metabolism, thus limiting the amount of product formed and increasing the downstream processing costs. The strategies needed to achieve high product output are to have a means for retention of high concentration of active biomass that can tolerate high product concentrations, and/or in situ product removal so as to overcome the problem of inhibition.

A successful approach has been that of continuous fermentation in a cell recycle reactor where the cells are separated by a filtration unit and recycled, while the product is removed in the permeate (Mehaia and Cheryan, 1986, 1987; Ohleyer et al., 1985a; Vick Roy et al., 1983; Xavier et al., 1991). The long-term performance of these units at high cell densities is, however, affected by fouling of the filtration membranes (Hjorleifsdottir et al., 1990; Tejayadi and Cheryan, 1988), requiring extensive cleaning protocols. Hjorleifsdottir et al. (1991) have further reported a change from homofermentative to heterofermentative metabolism of the bacteria during continuous lactic acid fermentation with cell recycling.

Immobilization of cells to a solid matrix has been an alternative means of high biomass retention. When provided with the nutrients, the cells divide within and

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on the core of the matrix, also releasing some of the progeny in the medium. Entrapment in gels has been a popular technique for immobilization of lactic acid bacteria (Boyaval and Goulet, 1988; Buyukgungor et al., 1984; Guoqiang et al., 1991; Norton et al., 1994a, 1994b; Roy et al., 1987; Stenroos et al., 1982; Tuli et al., 1985). The potential limitations of these systems are disruption of the gel beads as a result of the pressure built up by the dividing cells, and accumulation of the inhibitory product in the immediate vicinity of the cells due to the diffusional restrictions within and outside the gel layer. Adsorption on the surface of supports is a more convenient and practical approach in this respect. A few reports are known of fermentation processes based on lactic acid bacteria adsorbed to porous glass/ceramic beads (Goncalves et al., 1992; Guoqiang et al., 1992; Krischke et al., 1991), cotton fibers (Silva and Yang, 1995), and polypropylene–agricultural material composites (Demirci et al., 1993; Demirci and Pometto, 1995).

Even the type of reactor is an important consideration for lactic acid fermentation because of the necessity to control pH. The use of a stirred tank reactor allows efficient control of pH, but often leads to attrition of the support (Guoqiang et al., 1992), whereas in packed bed reactors large pH gradients are generated and a substantial fraction of the immobilized cells do not experience optimal pH for fermentation (Boyaval and Goulet, 1988). Improvement in control of pH has been achieved by the use of a packed bed reactor with recirculation (Goncalves et al., 1992), or using a fluidized bed reactor (Krischke et al., 1991). Operation of an immobilized cell reactor in a continuous mode has the potential of achieving simultaneous product removal. So far, even though high productivities have been reported in some systems by this means, this has been achieved at either low sugar concentrations or with incomplete substrate utilization.

The traditional means of obtaining pure lactic acid from the fermentation broth involves its recovery as the calcium salt, which is reacidified with H_2SO_4 yielding sulfates, adding substantially to process chemical costs and waste streams (Kaufman et al., 1995). At present, different separation procedures, including extraction (Hano et al., 1993; Honda et al., 1995; Katzbauer et al., 1995; Kwon et al., 1996; Scholler et al., 1993; Silva and Yang, 1995; Yabannavar and Wang, 1991a, 1991b), adsorption (Evangelista et al., 1994; Kaufman et al., 1995; Srivastava et al., 1992; Vaccari et al., 1993; Zihao and Kefeng, 1995), and electrodialysis (Boyaval et al., 1987; Cytko et al., 1987; Hongo et al., 1986; Nomura et al., 1987; Siebold et al., 1995), are being investigated to facilitate the recovery of lactic acid, and even to integrate these techniques with the fermentation step to get an added effect of improvement in reactor productivity by *in situ* product removal.

The present article presents studies performed on lactic acid fermentation using immobilized cells in a recycle batch reactor (Yang et al., 1995), in which the medium is continuously circulated between a column packed with immobilized cells and a reactor for pH control (Fig. 1). Foam glass, an inexpensive, porous material coated with polyethyleneimine, was used as the matrix to which *Lactobacillus casei*, an organism that produces predominantly L-lactic acid, was adsorbed during the course of fermentation. The sequential batch fermentations were aimed at complete utilization of sugar used at high concentration, with high product yield. A whey-based medium was used that was further enriched in sugar content by supplementation with glucose. Ion-exchange adsorption was used for the recovery of lactic acid with high yields.

MATERIALS AND METHODS

Materials

Whey was obtained from Skåne Mjölk (Hörby, Sweden). Pora-bact A® beads (prepared from recycled glass, and derivatized further with hydroxyl groups, having 2- to 4 mm mean diameter, 60% porosity, and 0.2-g/cm^3 density) were a kind gift from Dennert Poraver (Postbauer-Heng, Germany). Neutrase®, 0.5 L (0.5 AU/g), a metallo-protease from *Bacillus subtilis*, was bought from Novo Nordisk (Bagsværd, Denmark). Poly(ethyleneimine) (PEI; average MW 50,000 to 60,000, 50% w/v aqueous solution), lithium lactate, 3,5-dinitrosalicylic acid, bicinchoninic acid solution, and the enzymatic kit for L-lactate measurement (Cat. No. 735-10) were obtained from Sigma (St. Louis, MO). The enzymatic kit for determination of D-lactic acid was from Boehringer Mannheim GmbH (Mannheim, Germany). Amberlite IRA-400 (Rohm & Haas) was procured from ICN (Costa Mesa, CA). The remaining chemicals were obtained from standard sources.

Microorganism and Culture Medium

Lactobacillus casei subsp. *rhamnosus* (DSM 20021) was maintained on MRS-agar (Merck) medium and subcultured fortnightly.

The culture medium used for lactic acid fermentation was based on cheese whey. The pH of the whey was adjusted to 6.5, followed by addition of Neutrase (10 mL/L), an enzyme from *Bacillus subtilis* with endoprotease activity. After proteolysis at 45°C for 5.5 h, the whey was boiled for about 10 min and centrifuged to remove the precipitated, unhydrolyzed proteins. The supernatant, containing 30 g/L lactose and 5.3 g/L hydrolyzed protein, was used for lactate fermentations after supplementation with more sugar; that is, 70 g of glucose, and also 2.5 g/L of yeast extract and 0.03 g/L

of $MnSO_4 \cdot H_2O$. Inoculum preparation and immobilization of *L. casei* cells were done in the same medium, but the sugar comprised only of 40 g/L of lactose. The medium was sterilized by autoclaving at 120°C for 20 min.

Batch Fermentation with Free *L. casei* Cells

L. casei cells were cultivated in a fermentor (Multigen) with a working volume of 1 L. The inoculum used was 50 mL of an overnight culture of the cells. The medium was stirred continuously at 200 rpm. The temperature during fermentation was maintained at 42°C, pH 6, by automatic titration with 3 *M* NH_4OH with the help of a pH controller (Radiometer, Copenhagen).

Preparation of PEI-Coated Pora-Bact A Beads

Pora-bact A beads were washed thoroughly with tap water and dried at 105°C. The beads were then suspended in a 1% to 2% (w/v) aqueous solution of PEI, pH 7 (1.5 L for 1 L of beads) and autoclaved for 20 min at 120°C. Subsequently, the beads were washed and dried at 50°C for about 12 h before use. The beads autoclaved in distilled water instead of the PEI solution were used as control.

Immobilization of *L. casei* and Lactic Acid Fermentation in the Recycle Batch Reactor

The fermentation set-up comprised of a water-jacketed column (8 cm i.d.; total volume 1800 mL), packed with the treated Pora-bact A beads, and connected through both ends via silicon tubings to a water-jacketed reactor vessel (14.8 cm i.d.; total volume 3000 mL). The latter was equipped with the following: a stirring device, a pH electrode connected to a controller unit, a thermometer, a sampling device, and a port for pumping in fresh medium and withdrawing the spent broth respectively (Fig. 1). The reactor system was autoclaved after only partially filling with 2200 mL of culture medium, in order to avoid overflow; of this volume, 1000 mL occupied the void space in the column, 70 mL was in the tubings, and the remainder in the stirred vessel. One liter of medium was sterilized separately and added to the reactor at the start of the fermentation.

The reactor set-up was maintained at 42°C for fermentations, and the medium was inoculated with 5% (v/v) of an overnight-grown culture of *L. casei.* The medium was stirred continuously at 250 rpm and recirculated between the vessel and column at a rate of 50 mL/min with the help of a peristaltic pump. The flow of the medium was from the bottom to the top of the column. The pH of the medium in the vessel was maintained at 6.0 throughout the fermentation using 6 *M* NH_4OH. Samples were taken at regular time intervals for analyses of cell density, lactic acid, and reducing sugar. Once

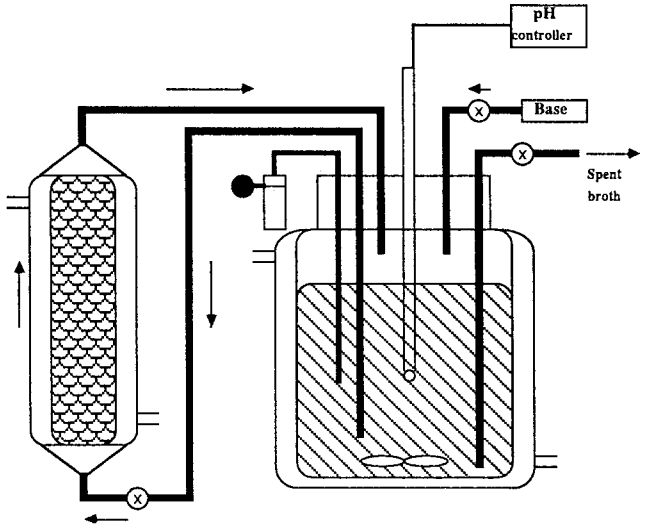


Figure 1. Schematic diagram of the experimental set-up used for lactic acid fermentation by immobilized *Lactobacillus casei* cells. The details are provided in the text. The arrows indicate the circulation of culture medium in the reactor system.

the sugar was completely utilized, the spent medium was withdrawn from the reactor. Thereafter, 0.9% (w/v) NaCl solution was circulated through the column several times to remove the cells loosely attached to the beads. The reactor was then filled with fresh medium to start a new fermentation batch.

Purification of Lactic Acid

The spent broth was centrifuged at 16,300 *g* for 15 min to remove the cells. The supernatant was mixed with 2% (w/v) activated charcoal with constant stirring for about 15 min, and then filtered through sintered glass funnel by applying low pressure. Lactic acid was then recovered from the clear, decolorized filtrate by ion-exchange chromatography. The ion exchange resin (Amberlite IRA-400) was pretreated according to the manufacturer's recommendations. One kilogram of the resin suspended in deionized water was packed in a column (9.0 i.d. × 35 cm) and backflushed with water at a flow rate of 25 mL/min. About 4 bed volumes of 4% (w/v) NaOH were then passed from the top of the column to replace —Cl groups on the resin with —OH groups, and finally the column was rinsed with water until the pH of the eluate was 7. The filtrate (1200 mL) was passed over the column at 25 mL/min. Subsequently, the column was washed with water and the bound acid was eluted with 2N HCl. The eluate was collected in 100-mL fractions. The resin was subsequently regenerated for further use.

Analytical Methods

The concentration of reducing sugar was measured by a slight modification of the procedure using dinitrosalicylic acid reagent (Miller, 1954).

Total lactic acid content was determined by HPLC (Beckman, CA) using a Rezex organic acid analysis column (300 × 7.8 mm; Phenomenex, CA) and a UV detector. The injection volume of the sample was 50 μL. The column was eluted with 0.014 *M* H_2SO_4 at a flow rate of 0.5 mL/min for 30 min. The temperature of the column was maintained at 45°C. The retention time of lactic acid under these conditions was 20.53 min.

The concentration of L-lactate was estimated using an enzyme kit (Sigma), wherein H_2O_2 liberated by the oxidation of lactate by lactate oxidase is used by peroxidase for oxidative condensation of chromogen precursors to produce a colored dye with an absorption maximum at 540 nm. D-Lactic acid was estimated using the enzymatic kit according to the procedure described by Boehringer Mannheim (1995). Here, the enzyme, D-lactate dehydrogenase, catalyzes the oxidation of D-lactate in the presence of nicotinamide adenine dinucleotide (NAD^+). The product, pyruvate is trapped by glutamic-pyruvic transaminase in the presence of D-glutamate, while the NADH formed is quantitated by absorbance at 340 nm.

The density of free cells in the medium was monitored by measuring the absorbance at 620 nm using a Shimadzu UV-120-02 spectrophotometer. For measurement of dry weight, cells from a definite volume of broth were collected on a 0.45-μm cellulose acetate filter (Sartorius, Germany), washed with 0.9% saline and dried at 105°C until a constant weight was reached (12 to 14 h). For routine purposes, the cell density (OD_{620nm}) was converted to dry weight (g/L) using a calibration curve.

The amount of cells immobilized on the support was estimated by the difference between the dry weight of the immobilized biocatalyst, that is, the beads with the cells, and that of the beads prior to use. The dry weight was obtained after heating at 105°C for 72 h. Prior to drying, Pora-bact A beads with immobilized cells were rinsed three times with sufficient 0.9% (w/v) NaCl solution to remove the loosely bound cells.

The reactor productivity was obtained by dividing the final lactate concentration (g/L) by the total fermentation time (h).

The protein content of the whey was estimated by the bicinchoninic acid method (Smith et al., 1985).

RESULTS AND DISCUSSION

Batch Fermentation in Whey Hydrolysate Medium with Free Cells of *L. casei*

Lactic acid bacteria are very fastidious with respect to their nutrition requirements (Peters and Snell, 1953). Besides yeast extract, hydrolyzed whey protein has also been reported to provide the short chain peptides favored by the bacteria to meet their demand for assimilable nitrogen (Berg et al., 1981; Krischke et al., 1991; Leh and Charles, 1985). Hence, in agreement with earlier reports (Krischke et al., 1991; Leh and Charles, 1985), enzymatic hydrolysis of the whey protein prior to fermentation stimulated the growth of *L. casei* and production of lactic acid from whey lactose. The highest fermentation rate was obtained for whey treated with 10 mL/L of Neutrase, an endoprotease from *Bacillus subtilis*, at 45°C for 5 to 6 h (not shown). However, as reported earlier by Krischke et al. (1991), further supplementation of the whey hydrolysate with yeast extract and Mn^{2+} ions was needed to improve reactor productivity (Fig. 2). An optimal fermentation rate with a biomass yield of 5.1 g/L (OD_{620nm} = 12.2) and productivity of 3 g/L · h was achieved with the addition of 2.5 g/L of yeast extract and 0.18 m*M* Mn^{2+} ions. Under these conditions, sugar was completely utilized with a product yield of 92.6%. Similar values of productivity, cell yield, and product yield were also obtained if the fermentation was performed in whey permeate (ultrafiltrate) supplemented with the retentate pretreated with Neutrase, and yeast extract and Mn^{2+} ions as above.

The fermentations performed with sugar levels present in native whey result in relatively low lactate concentrations. To increase the product concentration and reduce downstream processing costs, it is desirable to process as high concentrations of substrate as possible. Fermentation in concentrated whey hydrolysate medium containing 70 g/L of lactose while maintaining other additions same as above, yielded even higher amounts of biomass (OD_{620nm} = 15.0) and productivity (4.0 g/L · h). Alternatively, the whey hydrolysate medium was supplemented with lactose or glucose to make the total reducing sugar concentration to 100 g/L. Irrespective of the sugar added, the reactor productivity was 3 g/L · h and biomass yield was about 6.1 g/L (OD_{620nm} =

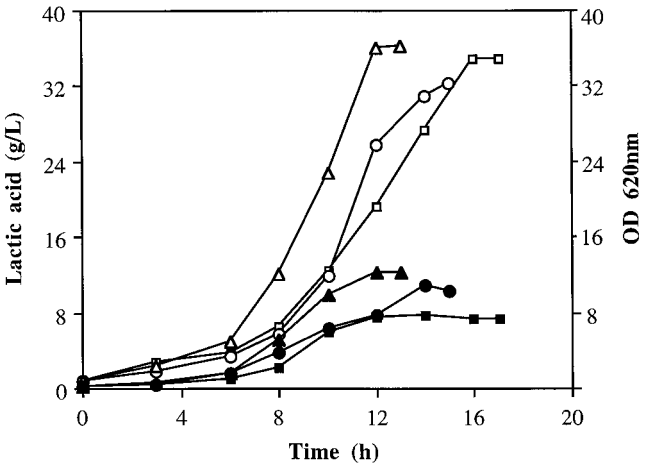


Figure 2. Lactic acid production (open symbols) and cell densities (closed symbols) during batch fermentation using free cells of *L. casei* in a culture medium of whey hydrolysate supplemented with (a) 2.5 g/L of yeast extract (○, ●), (b) 0.18 m*M* Mn^{2+} (□, ■), and (c) 2.5 g/L of yeast extract and 0.18 m*M* Mn^{2+} (Δ, ▲).

14.5), but in the medium with added glucose, the sugar could not be completely utilized during fermentation (Fig. 3). Our recent studies have further confirmed the dependence of the organism on the presence of lactose for bringing about efficient fermentation (unpublished observations). These observations are in contrast to the report of Ohleyer et al. (1985b) in which glucose was the preferred carbon source used by *L. delbreuckii.*

Immobilization of *L. casei Cells* to Pora-bact A Beads

L. casei cells were adsorbed to the foam glass Pora-bact A beads, directly in the reactor system to be used for lactate fermentations (described in Materials and Methods). The reactor filled with the whey hydrolysate medium containing 40 g/L of lactose, 2.5 g/L of yeast extract, and 0.18 m*M* Mn^{2+} ions was inoculated with the cells. The medium was continuously recirculated through the bead-packed column and pH was controlled in the stirred vessel. Figure 4 shows the profiles of free cell density and corresponding lactic acid production in the reactors with native Pora-bact A beads and the beads coated with PEI, respectively. In the case of native (uncoated) beads, the free cells in the medium increased in number with time up to 14 h when maximum cell density (OD = 10.8, equivalent to 4.5 g/L cells dry weight) was reached. Complete substrate consumption was also achieved at the same time. The fermentation profile was more or less similar to that obtained with free cells under batch conditions.

When beads coated with 1% PEI were inoculated with *L. casei*, the absorbance of the culture medium at 620 nm dropped to zero at 6 h and remained so for 24 h. Subsequently, the free cells started to appear in the medium and gradually increased in number, reaching a maximum absorbance of 6.8 (2.8 g/L cells, dry weight) at 48 h. A similar pattern was observed with the beads coated with 2% PEI, but it took a much longer time (58 h) before the free cells appeared in the medium. Maximum cell density obtained after 74 h of fermentation was relatively lower (OD = 5.8, equivalent to 2.4 g/L cells dry weight). The entire substrate was utilized in each case.

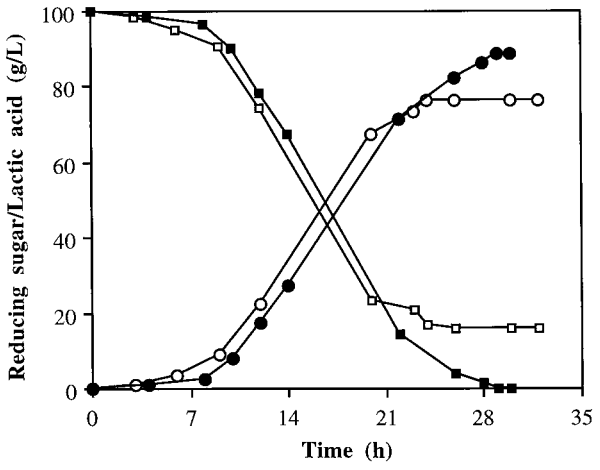


Figure 3. Batch lactic acid fermentation with free *L. casei* cells in whey hydrolysate medium supplemented with 2.5 g/L of yeast extract, 0.18 m*M* $MnSO_4$, and lactose (closed symbols) or glucose (open symbols) making the total reducing sugar concentration 100 g/L. The sugar consumption is shown as square symbols, and lactic acid production as circles.

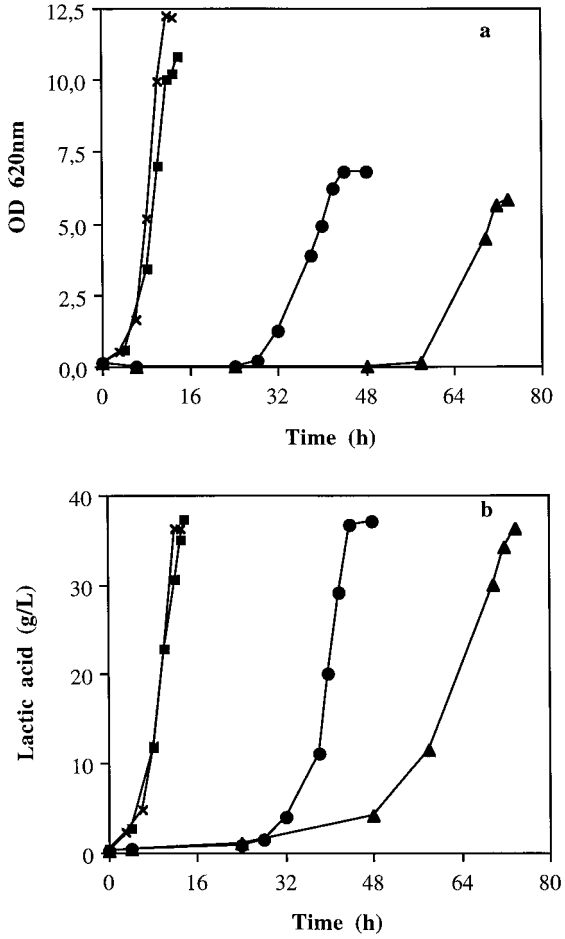


Figure 4. Profiles of (a) free cell density, and (b) lactic acid formation in the whey hydrolysate medium during the first stage of cell adsorption to Pora-bact A beads in a recycle batch reactor. The poly(ethyleneimine) concentration used for coating the beads was 0 (■), 1% (●), and 2% (w/v) (▲), respectively. Comparison is also made with batch fermentation (x) with free cells. The medium was supplemented with 2.5 g/L of yeast extract and 0.18 m*M* $MnSO_4$. The initial concentration of lactose was 40 g/L.

An earlier report by Goncalves et al. (1992) showed that spontaneous adsorption of cells to foam glass particles, Poraver® (Dennert-Poraver, Germany), during continuous fermentations, was much higher than under batch conditions. It was also shown, however, that there was no strong interaction between the cell surface and the beads as the cells were lost from the support at high dilution rates. Our observations reported previously confirm the lack of interaction of *L. casei* cells with

the native Pora-bact A beads in batch, while the cell adsorption could be facilitated by polycation coating of the matrix. This effect has also been reported earlier from our studies with Poraver beads (Guogiang et al., 1992). Growth of the adsorbed cells is delayed perhaps because of the inhibitory effect of the polycation through its interaction with the bacterial cell wall. The higher the PEI concentration, the longer the lag period. Once the cells start to divide, the progeny continues to adsorb to the support surface, while some cells are also released into the medium. The amount of free cells is lower with higher concentration of PEI used for coating, suggesting higher capacity of the support for cell adsorption.

Repeated Recycle Batch Fermentations with Immobilized *L. casei* Cells

The fermentation broth was pumped out from the reactor after the cell adsorption stage, and the cells loosely adsorbed to the beads were washed away by circulating 0.9% saline through the column. The cells remaining bound to the beads were then used to initiate the subsequent fermentation. This procedure was repeated after each fermentation cycle. We emphasize here that rigorous washing of the column between batches, as done here for the purpose of demonstrating the initiation of fermentation by immobilized cells, is not essential, but intermittent cleaning could be useful to remove the loosely attached cells.

The culture medium for the fermentations was supplemented with glucose to bring the total sugar concentration to 100 g/L. In the system with native Pora-bact A beads, the time taken for complete fermentation increased with the number of cycles (Fig. 5a), even though very high densities of suspended cells (average of 5.5 g/L) were consistently obtained. Finally, after five batches, the substrate could not be utilized completely. As noted previously, the cells were not adsorbed strongly to the foam glass beads; the cells which formed the inoculum for subsequent fermentation were those simply entrapped in the pores of the matrix and those bound to the bead surface by weak forces. The fermentation was thus dependent on the free cells, which were, in general, sensitive to inhibition by high concentrations of the product present in the reactor. It is additionally possible that the nutrient levels in the reactor became limiting for the bacteria whose need for nutrition increased under these inhibitory conditions (Ohleyer et al., 1985b). During repeated fermentations the stress factor was accumulated, which was seen in lowered lactic acid production.

Fermentations by the cells immobilized to PEI-coated beads started without undergoing any lag period, in contrast to the initial cell adsorption stage. Repeated recycle fermentations showed a stable performance (Fig. 5), and sugar was utilized completely in all the

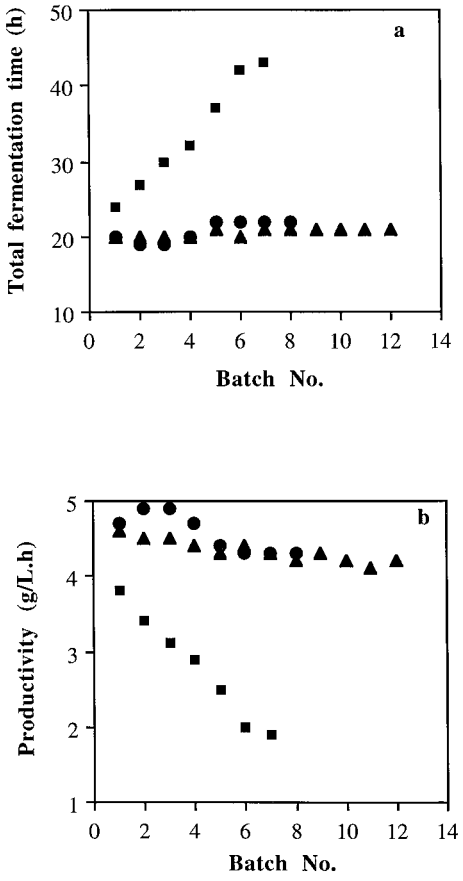


Figure 5. Time taken for fermentation (A) and reactor productivities (B) during repeated recycle batch fermentations in whey hydrolysate medium with *L. casei* adsorbed to Pora-bact A beads pretreated with 0% (■), 1% (●), and 2% (w/v) (▲) polyethyleneimine solution. To the medium were added 2.5 g/L yeast extract, 0.18 m*M* $MnSO_4$, and glucose to a total reducing sugar concentration of 100 g/L.

cycles with about 93% being converted to lactic acid. A higher productivity was achieved: an average of 4.6 g/L · h and 4.3 g/L · h with 1% and 2% PEI-coated beads, respectively, compared to 2.9 g/L · h with uncoated beads (Fig. 5b). The amount of suspended biomass during repeated fermentations was lower than that obtained with uncoated beads, around 4.4 g/L and 3.3 g/L for beads coated with 1% and 2% PEI, respectively.

The amount of cells immobilized to the beads coated with 2% PEI was determined after 12 cycles of fermentation and was found to be about 0.49 g/g; that is, about 176 g per 1800 mL (360 g) of beads present in the reactor. Although we do not know how many of these cells were viable, it is clear that the immobilized cells formed the bulk of the catalyst in the reactor determining the fermentation. However, a rather small increase in reactor productivity obtained with higher cell density implied that only a fraction of the immobilized cells were participating actively in the fermentation. The cells were seen to form a film around the beads and it may seem that only the cells in the outer layer of the film were in contact with the substrate.

In general, immobilized cell systems do present lower specific productivities as compared with the free cell fermentations (Goncalves et al., 1992; Krischke et al., 1991; Norton et al., 1994a). In our system, accumulation of high product concentration under batch conditions could additionally contribute toward limiting productivity, a problem which can be reduced by running the fermentation in a continuous mode. The advantage gained, however, in this system, in comparison with the free cell system, was the sturdy nature of the adsorbed biocatalyst providing complete substrate utilization and stable productivities over repeated cycles under conditions of product inhibition.

A comparison of different lactic acid production systems has been made earlier by several investigators (Goncalves et al., 1992; Mehaia and Cheryan, 1987; Norton et al., 1994a). The volumetric productivities of immobilized cell systems are generally higher for continuous fermentations (ranging from about 3 to 40 g/L · h) than for fermentations run in a batch mode (about 0.5 to 4.0 g/L · h). The productivity values are also, of course, dependent on the fermentation medium and the production organism. The higher productivities during continuous fermentations have invariably been accompanied by the use of low substrate concentrations and/or incomplete substrate utilization (Buyukgungor et al., 1984; Goncalves et al., 1992; Krischke et al., 1991; Nomura et al., 1987; Norton et al., 1994a). On the other hand, in continuous fermentations with membrane cell recycling, extremely high productivities (e.g., 85 to 150 g/L · h), even at high sugar concentrations and complete substrate utilization, have been achieved (Mehaia and Cheryan, 1986, 1987; Ohleyer et al., 1985a). This is a result of much higher cell concentrations obtained in these systems with lower diffusional constraints for the reactants. The performance of these systems over a long period is, however, affected by the decline in flux caused by membrane fouling, high broth viscosity, etc., and ultimately results more comparable with the immobilized systems are obtained (Hjorleifsdottir et al., 1990; Xavier et al., 1991).

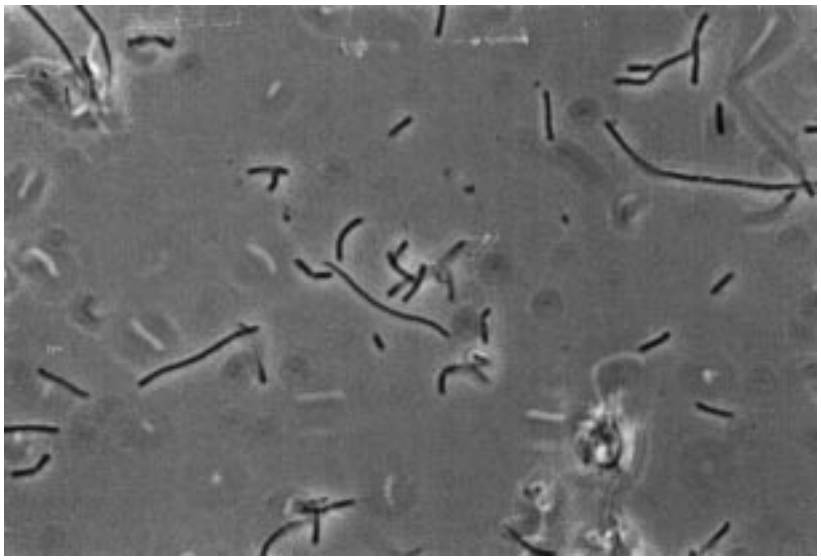
The major problem faced in running repeated recycle batch fermentations was due to the free cells. With increasing number of fermentation cycles, the free cells started to appear as elongated and branched structures (Fig. 6a), first near the lower end of the column, but gradually observed covering the major part of the column. The elongated appearance of the cells has been routinely observed during continuous lactic acid fermentation at high dilution rates (Goncalves et al., 1992; Kwon et al., 1996), and has been attributed to a condition of stress like nutrient limitation. In the present system, the filamentous structures formed kind of a network (Fig. 6b) which clumped the beads together, and ultimately the flow of the medium through the column was channeled and reduced. This limited the number of fermentation cycles possible to 8 and 12 for beads coated with 1% and 2% PEI, respectively. The problem of bead clumping was also encountered during repeated batch fermentations in biofilm reactors with plastic-composite supports (Demirci and Pometto, 1995).

Effect of Recirculation Rate

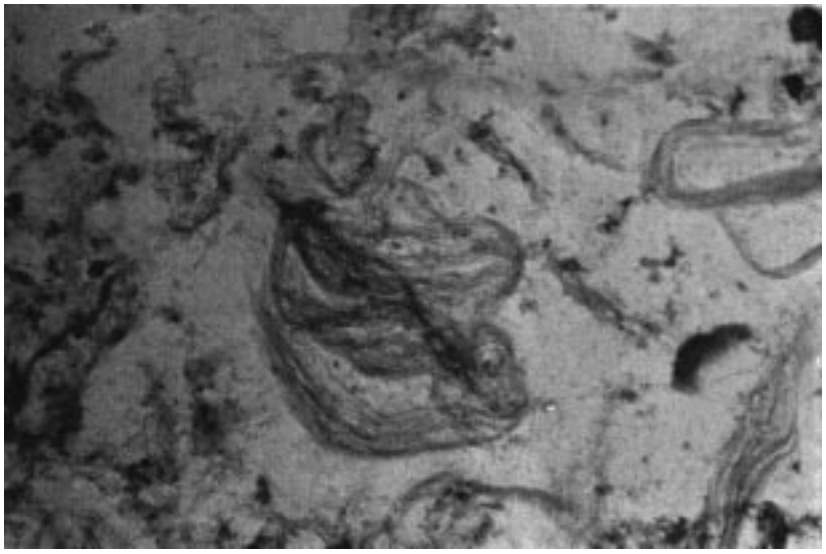
A reactor with a recirculation mode having a provision for pH control (Goncalves et al., 1992; Yang et al., 1995) is likely to reduce the formation of large pH gradients in a packed bed of immobilized cells, which would otherwise create nonoptimal conditions for the cells for product formation (Boyaval and Goulet, 1988). The pH of the culture broth at both ends of the column was monitored at different time periods during fermentation with *L. casei* immobilized to PEI (1%) coated beads in the recycle batch reactor operating at a recirculation rate of 50 mL/min. A drop in pH to 4.6–5.0 was observed at the outlet of the column as compared to pH 6.0 at the inlet of the column. The recirculation rate of the culture medium was further varied between 20 mL/min and 70 mL/min to study the effect on lactic acid production. The fermentations were repeated two or three times at each recirculation rate. As shown in Table I, productivity as well as density of free cells increased concomitantly with the increase in the rate of recirculation. This would imply that a larger proportion of the immobilized cells in the column experience an unfavorable environment at lower flow rates, thus reducing efficiency. The increase in productivity was significant up to the recirculation rate of 50 mL/min. A further increase in flow rate to 70 mL/min was accompanied by a reduction in the product yield from 93% to 87.7% and a relatively larger increase in free cell density, which may suggest that more substrate was diverted for biomass production. The increased free cell density led to a situation in which the number of fermentation cycles possible was reduced.

Increasing the Culture Medium Volume for Fermentation by the Immobilized Biocatalyst

The data obtained on recycle batch fermentation was obtained with the medium-to-biocatalyst volume ratio of about 2:1. The effect of scaling up the medium volume on the fermentation efficiency in the immobilized cell bioreactor was further investigated. For this study, a column (5 cm i.d. × 15 cm height) filled with 2% PEI-coated beads (350 mL) and stirred reactor vessels of varying working volumes were used. The fermentation was initiated as previously indicated using the medium-to-biocatalyst volume ratio of 2:1. Three fermentation runs were performed before increasing the medium volume; the stirred vessel was also changed to suit the medium volume. Prior to changing the reactor vessel, it was filled with 0.9% saline and the solution was al-



(a)



(b)

Figure 6. Microscopic view of *Lactobacillus casei* cells released from PEI-coated Pora-bact A beads by washing with 0.9% NaCl solution. Magnification (a) ×1560, and (b) ×30.

lowed to recirculate several times over the beads to wash away the loosely attached cells.

The medium-to-biocatalyst volume ratio was increased to 10:1, while the initial concentration of sugar was maintained at 100 g/L. Complete substrate utilization was achieved in all cases and the product yield (~93%) was also not affected. However, the increase in the medium volume was accompanied by a gradual increase in free cell density and decrease in productivity (Table II). As the biocatalytic capacity of the reactor is resident in the immobilized cells, the lower productivity with increased medium volume at a constant recirculation rate could be expected to be due to the longer time needed for the whole medium to pass through the column resulting in less effective substrate-to-cell contact. At the same time, the longer residence time of the medium in the stirred vessel under the controlled pH environment is favorable for multiplication of the free cells which still constitute only a minor fraction of

Table I. Effect of medium recirculation rate in the immobilized cell reactor on lactic acid productivity and free cell density.

Recirculation rate (mL/min)	Productivity (g/L·h)	Final free cell density (OD 620 nm)
20	3.1	9.0
35	3.5	9.4
50	5.0	10.2
70	5.1	13.6

Experimental details are given in the text.

Table II. Effect of increasing culture medium volume on lactic acid productivity and free cell density in a recycle batch reactor with immobilized *L. casei.*

Culture medium: Immobilized biocatalyst	Productivity (g/L · h)	Final free cell density ($OD_{620\ nm}$)
2:1	4.4	9.0
3:1	3.9	9.4
4:1	3.7	10.0
6:1	3.6	10.9
8:1	3.5	11.2
10:1	3.4	11.4

the total biomass in the reactor with a less significant role in promoting the fermentation.

Although the difference in productivities with increasing medium volume was not very drastic, the problem was the decrease in the number of cycles due to higher free cell density. The higher number of filamentous cells in the column enhanced the chaneling effects, etc., and the number of fermentation cycles decreased from 12 to 6 runs with an increase in ratio from 2:1 to 6:1. At the ratio of 10:1, only three repeated batches were possible.

Effect of Yeast Extract Concentration

Batch fermentation with free *L. casei* cells in untreated or unsupplemented whey led to very poor productivity as well as incomplete sugar utilization, as reported earlier (Krischke et al., 1991), and also seen in the present studies (data not shown), thus necessitating some means of providing assimilable nitrogen. Both enzyme (cost of Neutrase: 16 $US/kg) pretreatment of whey and yeast extract (about 23 $US/kg) supplementation add to the raw material costs for lactate production. Reduction in either or both of the components is desirable for lowering fermentation costs.

Lactate fermentations in unsupplemented whey medium with other strains of lactic acid bacteria have been reported. Keller and Gerhardt (1975) produced a high protein feed supplement by cultivating *Lactobacillus bulgaricus* on unsupplemented whey in a two-stage continuous reactor, with lactic acid productivity of 1.9 g/L · h at near 100% lactose (48 g/L) conversion. More recently, productivity of 5 g/L · h was reported at total lactose (37 g/L) conversion during continuous lactate fermentation in unsupplemented acid whey using *Lactobacillus helveticus* immobilized to a fibrous bed reactor (Silva and Yang, 1995). These organisms were able to hydrolyze the whey protein to some extent, thus taking care of some of their nutritional demands from whey. But, in whey concentrates containing higher sugar concentrations, substrate utilization was incomplete or the retention time of the medium in the reactor needed to be extremely high to achieve near-complete substrate utilization. In both cases, as well as in other reports (Aeschlimann and von Stockar, 1990; Norton et al., 1994b), the productivity has been improved by supplementing the medium with yeast extract.

In the present work, so far, a high concentration of sugar was completely utilized with high product yields by the immobilized organism in the medium based on whey treated with 10 mL/L Neutrase and supplemented with 2.5 g/L yeast extract. Further studies on the effect of reducing the yeast extract concentration, while maintaining the enzyme treatment, on the reactor performance were done. The first batch of fermentation and cell adsorption was done with a yeast extract concentration of 2.5 g/L to provide adequate nutrients for initial cell multiplication and colonization of the lactic acid bacteria.

The yeast extract concentration was then reduced stepwise; three fermentation runs were performed at each concentration. As seen in Table III, reactor productivity was reduced from 4.6 g/L · h to 2.8 g/L · h with decrease in yeast extract from 2.5 to 0 g/L; however, the effect on productivity was not significant until a yeast extract concentration of 1 g/L was reached. The substrate utilization and product yield were unaffected. The lower concentration or elimination of yeast extract had an additional advantage in that a higher number of fermentation runs was possible without encountering the problem of clogging of the column due to the lower amount of suspended biomass obtained (Table III). At least 20 cycles with an average productivity of more than 4 g/L · h could be operated with whey hydrolysate medium containing 1 g/L of yeast extract.

Yet another effect of decreased yeast extract concentration was reduction in the content of D-isomer of lactic acid (Table III). According to DeBruyn et al. (1988), higher levels of amino acids present in a higher yeast extract dosage may constitute a good source to be converted to D-lactic acid. It was also observed that maintaining the yeast extract concentration at 2.5 g/L during repeated

Table III. Effect of reducing the yeast extract concentration during repeated fermentations with *L. casei* adsorbed to PEI coated Porabact A beads.

Yeast extract concentration (g/L)	Final density of free cells (OD_{620})	Total lactic acid (g/L)	D-lactate (% of total)	Productivity (g/L · h)
2.5	8.3	92.5	7.1	4.6
1.0	8.0	92.9	6.3	4.4
0.5	6.2	93.5	6.0	3.6
0	5.2	93.5	5.4	2.8

The concentration of PEI used for coating was 2% (w/v).

Fermentation at each concentration of yeast extract was performed 3 times. All the values given are an average of the three runs.

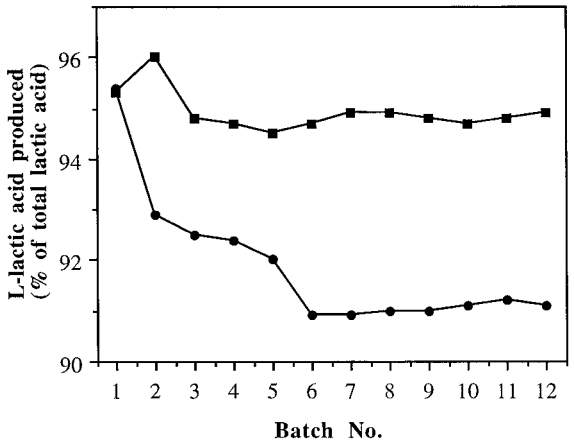


Figure 7. Yield of L-lactic acid in percent of total amount of lactic acid produced during repeated recycle batch fermentation with immobilized *L. casei* in the medium supplemented with 1 g/L (■) and 2.5 g/L (●) of yeast extract, respectively.

fermentations caused a continuous reduction in L-lactic acid levels up to six runs, but at 1 g/L of yeast extract these levels remained steady at around 95% of the total lactate produced (Fig. 7).

It seems that addition of yeast extract to whey hydrolysate medium was not a prerequisite for lactate fermentation with complete sugar utilization. It has been suggested previously (Norton et al., 1994b) that the immobilized, viable, nondividing cells have modest nutritional demands for their survival, thus allowing the fermentations at low nutrient levels. It may also be logical to assume that, as there is a continuous turnover of cells in the biofilm, the dead cells contributing to meeting some of the nutrition requirements (Blenkinsopp and Costerton, 1991; Guoqiang et al., 1991). Studies on use of another bacteria that can hydrolyze whey protein simultaneously with lactate fermentation (Keller and Gerhardt, 1975; Silva and Yang, 1995), and screening of even other inexpensive nitrogenous materials, would be of interest for further studies.

Start-Up of a New Column of Immobilized Cells

It is apparent from these studies that clogging of the reactor limits the operation of the recycle batch fermentations. Once the column was clogged, it was drained of the culture broth and then disconnected from the stirred vessel, and replaced with a new column (having the same dimensions) packed with PEI-coated Porabact A beads. The culture broth present in the vessel was circulated through the new column for about 2 h. The density of free cells in the broth decreased after the circulation (OD_{620} of 8.9 vs. 10.8 prior to circulation) due to cell adsorption to the PEI-coated beads.

The broth was later withdrawn from the reactor system and the column was thoroughly rinsed with 0.9% saline prior to pumping in the fresh medium containing 100 g/L of sugar into the reactor assembly. Fermentation was performed with continuous recirculation as described previously. The suspended biomass content increased gradually and reached a maximum (OD_{620nm} of 5.9) after a period of 40 h (Fig. 8); the entire substrate was also used up at this time with a product yield of 93%. This is in contrast to the 74 h of time required for fermentation with 40 g/L of sugar when the overnight culture (OD = 8.2) of *L. casei* from a batch fermentor was used as the inoculum (5% v/v) (Fig. 4).

The higher fermentation rate in the former case could be due to the use of a higher amount (about 66%) of the dense inoculum and/or the inoculated cells, which were already accustomed to the culture conditions and were in active state to metabolize the substrate. To test the first possibility, the medium (40 g/L of sugar) in the reactor system was inoculated with the overnight bacterial culture (5% v/v) and the cells in the stirred

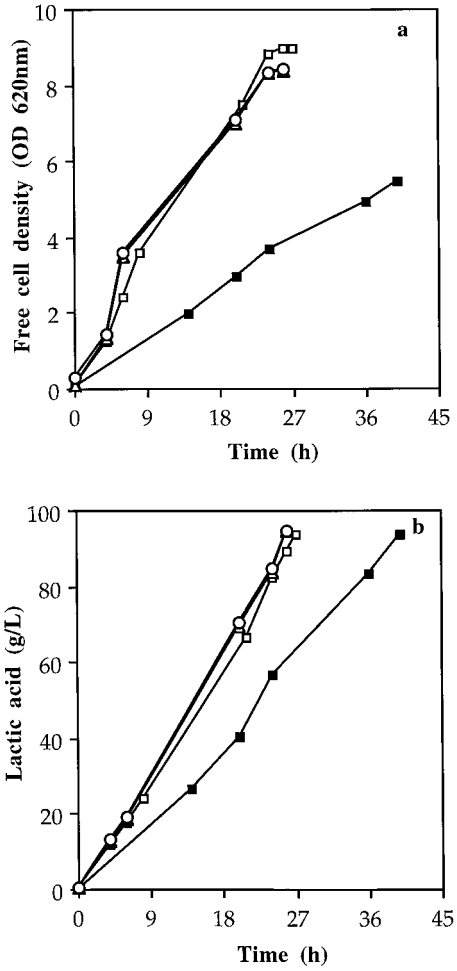


Figure 8. Profiles of free cell density (A) and lactic acid formation (B) during repeated recycle batch fermentations in a bioreactor, which was inoculated by circulating the culture broth from an actively running recycle batch reactor. Fermentation cycles: first (■), second (❑), third (●), and fourth (❍). Experimental details given in the text.

vessel were allowed to grow to an optical density of 10.8 before starting the recirculation through the bead-packed column. The fermentation time was reduced to only 64 h, thus indicating that large, dense inoculum was not the main reason for the higher fermentation rate.

The results suggest that, once the reactor operation is initiated, the suspended biomass can serve as a continuous inoculum without the need for dismantling the whole system. During further operation of the column inoculated with suspended cells of an active recycle batch reactor, the fermentation time was reduced to 27, 26, and 24 h for the second, third, and fourth cycles, respectively (Fig. 8), and ultimately stabilized around the time needed in the previous reactor.

Lactic Acid Recovery

The manufacture of lactic acid currently uses ion-exchange adsorbents for demineralization of crude lactic acid (Evangelista et al., 1994). Several ion-exchange adsorbents are also being screened for recovery of lactic acid from the fermentation broth (Evangelista et al., 1994; Kaufman et al., 1995; Vaccari et al., 1993; Zihao and Kefeng, 1995). We used Amberlite IRA-400 for adsorption of lactic acid in the present studies. About 186 g of lactic acid could be adsorbed per kilogram of resin at pH 6 and above; however, adsorption from the clarified, decolorized fermentation broth was only about 126 g/kg.

Treatment with 2N HCl resulted in elution of 98% of the bound acid (eluate volume of 500 mL/kg of resin). The eluate was concentrated by flash evaporation to an acid concentration of 80% (w/v). The final recovery of lactic acid was 82%, equivalent to 76.3 g from a 1-L fermentation broth. HPLC analysis of the product showed the absence of byproducts like formic acid, acetic acid, and sugars. After appropriate regeneration, the resin was recycled. In the present work, the resin was used at least 15 times for lactic acid adsorption without loss in capacity. The ion-exchange resins are normally used for several hundreds of cycles and thus do not contribute significantly to the overally material costs (Kaufman et al., 1995). But, it would be more desirable to use a resin with a higher binding capacity (Kaufman et al., 1995; Zihao and Kefeng, 1995).

The potential of adsorption for *in situ* recovery of lactate from the broth with a stimulating effect on the fermentation rate has been demonstrated elsewhere (Kaufman et al., 1995; Srivastava et al., 1992). One may expect, however, coadsorption of cells to the matrix during recovery from culture broths. On the whole, the method shows more promise than the alternative separation techniques—that is, extraction and electrodialysis. Extraction is handicapped by the poor solubility of lactic acid in most organic solvents, and toxicity of the extractants to the bacterial cells (Hano et al., 1993; Honda et al., 1995; Silva and Yang, 1995; Yabannavar and Wang, 1991a, 1991b). The main problem with electrodialysis is membrane fouling by the cells and the fermentation medium (Cytko et al., 1987; Hongo et al., 1986; Silva and Yang, 1995); its performance is improved by combination with ultrafiltration and/or cell immobilization (Boyaval et al., 1987; Cytko et al., 1987; Nomura et al., 1987).

CONCLUSION

The above results show that the recycle batch mode of fermentation and adsorptive immobilization of lactic acid bacteria allowed complete utilization of high sugar concentrations and stable productivities during repeated cycles. Sequential batch operation of the reactor further eliminates the inoculum preparation and lag phase for each cycle, characteristic of free cell fermentations. The immobilized cells in the bioreactor were more tolerant to inhibition by high product concentrations, and their nutrient requirements could be limited during repeated fermentations. Maintaining a low density of free cells during fermentation can significantly increase the operational lifetime of the biocatalyst column, also making it possible to increase the volume of the medium to be processed without much loss in productivity. It seems that nitrogen supplementation would be a major contributing factor to the total product cost (Tejayadi and Cheryan, 1995). The whey hydrolysate served as a useful nitrogenous base for lactic acid fermentation with high sugar concentrations. There is, however, a further need to screen the effect of other inexpensive materials on the reactor performance.

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