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# REVIEW

# Continuous Production of Lactic Acid in a Cell Recycle Reactor

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## ABSTRACT

The production of lactic acid from glucose has been demonstrated using a CSTR (continuous stirred-tank reactor) with cell recycle. Studies were conducted with *Lactobacillus delbrueckii* at a fermentation temperature of 42°C and a pH of 6.25. A cell density of 140 g dry weight/L and a volumetric productivity of 150 g/L·h, with complete glucose consumption, were obtained. It was not possible to obtain a lactic acid concentration above 60 g/L because of product inhibition. A cell purge was not necessary to maintain high viability bacteria culture or to obtain a steady state. At steady state the net cell growth appeared to be negligible. The specific glucose consumption for cell maintenance was 0.33 g glucose/g cells·h.

**Index Entries:** Lactic acid, production from *Lactobacillus delbrueckii;* continuous fermentation, production of lactic acid by; cross-flow filtration, in continuous lactic acid production; lactic acid inhibition, during continuous production; cell concentration during continuous lactic acid production; broth viscosity, during continuous lactic acid production; cell recycle, during continuous lactic acid production; *Lactobacillus delbrueckii,* continuous production of lactic acid from; recycle reactor, continuous production of lactic acid in.

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## INTRODUCTION

Lactic acid is widely used in the food industry as an acidulent, preservative, and precursor for stearoyl-2-lactylates. Production via fermentation presently provides about 50% of the world supply. The remainder is produced by synthetic routes: lactonitrile hydrolysis or reaction of hydrogen cyanide with acetaldehyde (*1*).

The productivity of conventional fermentation is limited by the relatively low concentration of microorganisms in the system. High cell concentrations can provide a much higher volumetric productivity than dilute suspensions. Product inhibition is a major factor limiting conventional fermentation processes. In order to significantly increase the productivity, it is necessary to remove the lactic acid as it is produced. Various cell culture techniques that increase the cell concentration and reduce lactic acid concentration have been investigated. These include immobilized cells (*2*), hollow fiber reactors (*3*), batch dialysis (*4*), continuous dialysis (*5*), rotating filter fermentors (*6*), and ultrafiltration (*7*).

Continuous fermentations of glucose using *Lactobacillus delbrueckii* has been described in the literature. Leudeking and Piret (*8*) obtained a productivity of 6.7 g lactic acid L·h and a residual glucose level of less than 0.1% in a carbon-limited chemostat with a medium containing large amounts of nitrogenous nutrients and 5% glucose. A continuous stirred-tank reactor with cell recycle has was employed by Vick Roy et al. (*7*) and a volumetric productivity of 76 g/L·h was obtained with a residual glucose concentration less than 2 g/L. Stenroos et al. (*2*) immobilized *Lactobacillus delbrueckii* in calcium alginate beads and employed a continuous-flow column reactor to obtain a yield of 97% lactic acid from 4.8% glucose with a residence time of 18 h. Vick Roy et al. (*3*) have immobilized *Lactobacillus delbrueckii* in a hollow fiber fermentor. The productivity was as high as 100 g/L·h, but only 5% of the glucose was converted.

The purpose of the present study was to investigate continuous fermentative lactic acid production with cell recycle. Processes recently described, using a similar cell recycle system, have shown increases of volumetric productivity, conversion efficiency and high cell concentration (*7,9,10*).

## MATERIALS AND METHODS

### Strain

*Lactobacillus delbrueckii* NRRL-B445 was used throughout this study and maintained at 4°C on agar slants containing 0.5% yeast extract (Amber Tastone 154, Universal Food Corp.) and 2% glucose. This organism is a facultative anaerobe, gram positive, homofermentative, lactic acid producer.

## Media

Glucose and mineral solutions were adjusted to pH 6.5 and filter sterilized. The yeast extract was steam sterilized and then mixed with the glucose solution. The media composition consisted of the following (in g/L): Glucose (as stated in Table 1), yeast extract (25, 2.5), $(NH_4)_2SO_4$ (0.5), $MgSO_4$ (0.3), $KH_2PO_4$ (0.2), $K_2HPO_4$ (0.2).

## Inoculum Preparation

The inoculum was prepared by cultivating the bacteria in 20 mL of the fermentation medium at 42°C on a rotary shaker for 24 h. After inoculation, the culture was allowed to grow batchwise anaerobically with pH control of 24 h before starting the cell recycle fermentation.

## Analytical Methods

### Biomass Determination

Cell concentration was determined by optical density measurement at 610 nm. Optical density was calibrated against dry weight. Dry cell weight estimations were made by filtering aliquots of fermentation broth through tared 0.4 μm filters (Millipore Corp.), drying the filters at 70°C for 24 h.

### Culture Viability

The viability of the cultures was measured by the slide culture technique of Postgate (*11*).

### Lactic Acid and Glucose Determination

Samples were taken from the filtrate effluent and from the vessel. Fermentation broth samples were first centrifuged to prevent interference from the biomass. Lactic acid and glucose concentrations were both determined by HPLC. Analyses were performed on an Aminex HPX-87X column (Bio-Rad) using 0.01*N* sulfuric acid as the eluent (flow rate 0.6 mL/min). Detection was performed with a refractive index detector (Waters Assoc.).

### Viscosity Assay

Broth viscosity was estimated with a Couette viscometer (Brookfield) at shear rates of 6, 12, 30, and 60 rpm.

### Protein Assay

Protein concentration was determined by using the method of Bradford (*11*).

TABLE 1
Steady-State Data from Continuous Culture with Cell Recycle

| Run | $D$, $h^{-1}$ | $B$, $h^{-1}$ | $S_0$, g/L | $X$, g/L | L, g/L | S, g/L | Volumetric lactic acid productivity, g/L·h | Specific glucose consumption, g/g·h | Specific lactic acid productivity, g/g·h | Glucose conversion, % | Viable cells, % | Duration of steady state, h | $Y_{L/S}$, g/g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.59 | 0.012 | 58 | 64 | 57 | 0 | 33 | 0.52 | 0.52 | 100 | 92 | 30 | 0.98 |
| 2 | 0.43 | 0.023 | 59 | 46 | 58 | 0 | 25 | 0.55 | 0.54 | 100 | 89 | 32 | 0.96 |
| 3 | 0.43 | 0.032 | 61 | 44 | 59 | 0 | 25 | 0.59 | 0.57 | 100 | 94 | 31 | 0.98 |
| 4 | 0.43 | 0.035 | 85 | 36 | 57 | 27 | 24 | 0.68 | 0.68 | 68 | 96 | 25 | 0.98 |
| 5 | 0.59 | 0.037 | 59 | 48 | 56 | 2 | 33 | 0.67 | 0.69 | 97 | 93 | 26 | 0.94 |
| 6 | 0.59 | 0.065 | 60 | 36 | 50 | 7 | 29 | 0.85 | 0.81 | 88 | 92 | 28 | 0.96 |
| 7 | 0.43 | 0.065 | 83 | 22 | 49 | 32 | 21 | 1.00 | 0.96 | 61 | 96 | 25 | 0.96 |
| 8 | 0.59 | 0.080 | 63 | 26 | 48 | 14 | 28 | 1.10 | 1.08 | 78 | 91 | 32 | 0.97 |
| 9 | 0.25 | 0 | 85 | 17 | 58 | 26 | 14 | 0.87 | 0.85 | 70 | 96 | 37 | 0.98 |
| 10 | 0.36 | 0 | 60 | 38 | 58 | 0 | 21 | 0.56 | 0.55 | 100 | 94 | 35 | 0.96 |
| 11 | 0.40 | 0 | 83 | 40 | 57 | 23 | 23 | 0.60 | 0.57 | 73 | 88 | 28 | 0.95 |
| 12 | 0.59 | 0 | 60 | 41 | 57 | 0 | 34 | 0.86 | 0.82 | 100 | 92 | 45 | 0.95 |
| 13 | 0.90 | 0 | 85 | 51 | 59 | 27 | 53 | 1.02 | 1.04 | 69 | 95 | 18 | 0.98 |
| 14 | 1.06 | 0 | 60 | 72 | 59 | 0 | 63 | 0.87 | 0.87 | 100 | 93 | 21 | 0.98 |
| 15 | 1.67 | 0 | 60 | 115 | 57 | 0 | 96 | 0.83 | 0.83 | 100 | 91 | 33 | 0.95 |
| 16 | 2.45 | 0 | 60 | 106 | 59 | 0 | 144 | 1.37 | 1.35 | 100 | 90 | 13 | 0.98 |
| 17 | 2.55 | 0 | 62 | 118 | 59 | 0 | 151 | 1.32 | 1.28 | 100 | 92 | 18 | 0.95 |
| 18 | 2.80 | 0 | 62 | 140 | 57 | 4 | 160 | 1.15 | 1.14 | 93 | 94 | 14 | 0.98 |

### *Conditions of Growth*

All cultures were conducted at 42°C under a nitrogen atmosphere. The fermenter vessel was equipped with an Ingold Electrodes sterilizable pH probe. The pH was maintained at 6.25 with 4 or 8*N* ammonium hydroxide. An Analytical Measurements model RC recorder controller was used to automatically control the pH.

### *Cell Recycle Continuous System*

Figure 1 shows a diagram of the system, which is similar to the apparatus described by Vick Roy et al. (*7*). Once a sufficient biomass concentration was obtained in batch growth, continuous fermentation with cell recycle commenced. The fermentor consisted of a 1.0-L glass vessel with baffles and impellers. Filtration was performed with a Pellicon Cassette System ultrafiltration device (Millipore Corp.) containing 0.09 $m^2$ of Durapore membrane (Millipore HVLP-OHV 20, pore size 0.5 μm) and maintained at 42°C. The filtration unit was rinsed with 20 L of sterile water prior to use. After each run, membranes were cleaned *in situ* with 6 L of buffer (pH 7) and 6 L of 0.1*N* sodium hydroxide for 1 h and then stored in 0.005% sodium azide. A variable-speed diaphragm pump (AMF Cuno H3791-121) was used to remove the broth from the bottom of the fermentor and provide tangential flow across the membrane to prevent fouling and to maintain an adequate filtration rate. The circulation rate was varied from 0.9 to 1.5 L/min. At this rate the inlet pressure was 6.3 atm at a cell concentration of 140 g dry weight/L. The effluent flow was

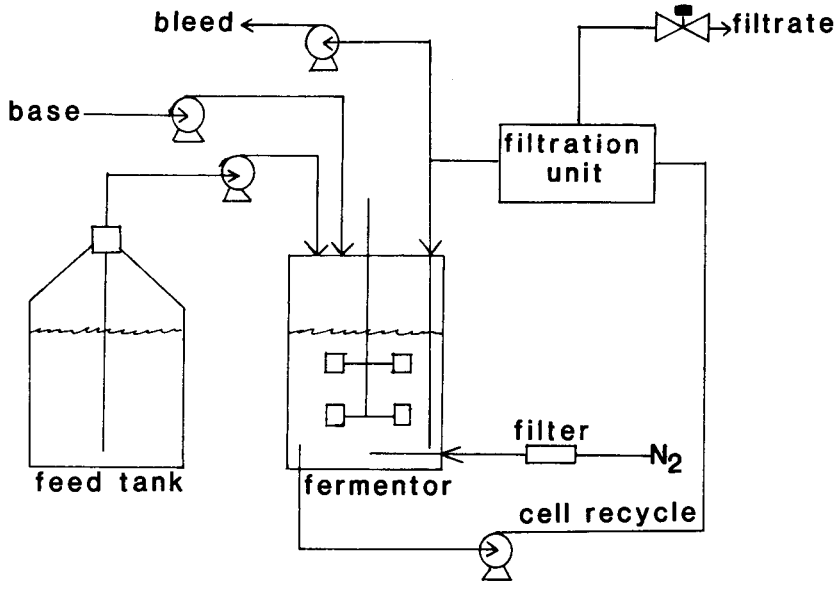


Fig. 1. Schematic representation of the cell-recycle continuous fermentation apparatus.

controlled by an electro-solenoid valve (Skinner Electric V52D8) coupled with an electronic liquid level controller (Dyna Sense 7186). Two valves placed on a filtration unit allowed flow reversal on the membranes. The fermentor and the filtration unit were maintained at 42°C. The operating volume of the continuous fermentation system was maintained at 0.6 L, consisting of 0.45 L in the reactor and 0.15 L in the recycle loop. A bleed from the broth was withdrawn from the recycle loop by using a peristaltic pump (Sigmamotor AL2E).

## RESULTS AND DISCUSSION

The performance of the recycle system was evaluated by varying the dilution rate of the feed, the bleed rate, and the glucose and yeast extract concentrations in the feed. A typical fermentation is shown in Fig. 2 and steady state data are summarized in Table 1. A steady state for the biomass concentration was obtained, even when the system was operated without cell purge, because of endogenous turnover of cells (*13–16*).

### *Steady-State Kinetics*

Material balances for cells and substrate in the cell recycle reactor can be written for the entire system as

$$dX/dt = \mu X - k_D X - BX \quad (1)$$

The substrate mass balance can be written

$$dS/dt = D(S_o - S) - BS - (1/Y_{X/S})\mu X - mX \quad (2)$$

The above equations are based on viable cell concentrations, and viability was determined for each steady state.

During all the runs, the viability of *Lactobacillus delbrueckii* was between 88 and 96%. Using a diaphragm pump to circulate the broth avoids mechanical stresses on the microbes and increases viability (*7*). The use of ammonium hydroxide instead of sodium hydroxide as neutralizing agent also improves the viability (*7,17*). Increasing the normality of ammonium hydroxide from 2 to 8*N* did not decrease cell viability and resulted in less dilution of the feed. For the runs described in Table 1, 4*N* ammonium hydroxide was used.

Some experiments were conducted without cell bleed, and a steady state was obtained, indicating that the growth rate of the cells was balanced by the death rate. An alternative is to consider that cells lysed and passed out of the system through the ultrafiltration membrane. However, microscopic examination revealed no cell debris in the permeate. It is unlikely that cell debris passed through the filter because the same filtration device has been used to separate intracellular enzymes from cell debris (*18*). In addition, if debris passed through the membranes, the

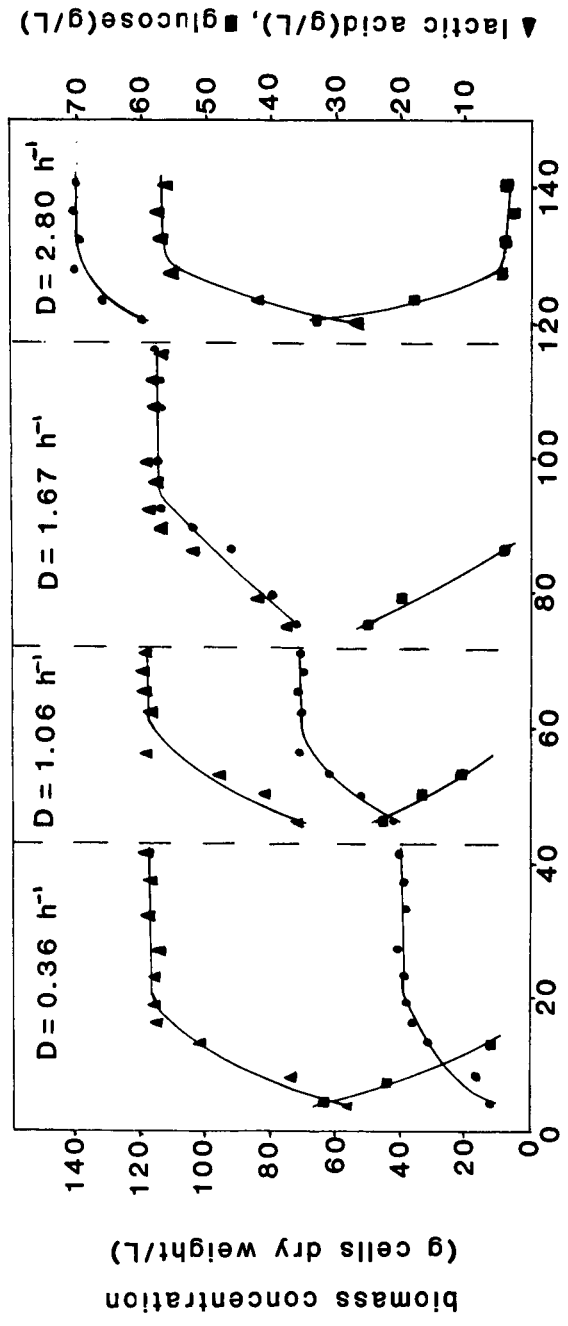


Fig. 2. Biomass, lactic acid, and residual glucose concentrations in the CSTR with cell recycle.

protein concentration would be higher than that in the feed. Table 2 shows that protein concentration in the feed and the effluent remained essentially constant.

Switching from a feed with high nitrogen concentration (25 g/L yeast extract) to one with low nitrogen concentration (2.5 g/L yeast extract) did not affect biomass concentration or viability after the system without a cell bleed had reached steady state, at either low or high dilution rates (Fig. 3). At steady state, very small amounts of nitrogen appeared to be required to maintain a constant biomass concentration. This low nitrogen requirement indicates that little growth occurred once steady state was obtained.

In most fermentation processes with cell recycle, a bleed is used to remove cell mass. Figure 4 shows the effect of a bleed on the cell concentration at steady state. A cell bleed at the flow rate used decreased the biomass concentration and lactic acid production. When operating a closed system with complete cell retention, it is not possible to estimate the growth yield ($Y_{X/S}$). The uptake of glucose can be divided into two parts: that required for cell growth and that required for cellular maintenance. By using a bleed, it is possible to estimate the fraction of the substrate consumed for growth and for maintenance. It has been shown (*6*) that the total rate of substrate utilization is not directly coupled with growth. For lactic acid production with *Streptococcus*, Sortland and Wilke (*6*) observed a nonlinear relationship between the amount of cell growth and the energy released from the glucose catabolism. Two models have been proposed to explain this dissociation of energy production from growth. The first assumes that the energy not used for growth is used for cell maintenance. The second postulates that anabolic pathways become uncoupled from the catabolic processes (*19*), and the additional energy is wasted by some mechanism (*20*). In the present case, the presence of a maintenance energy requirement seems to be more probable. Several authors (*13,15,21*) have demonstrated the validity of the maintenance concept for different microorganisms.

At steady state, Eq. (2) can be rearranged to allow determination of the cell yield and maintenance constants:

$$D(S_o - S)/X = (1/Y_{X/S})\mu + m + BS/X \quad (3)$$

TABLE 2
Protein Concentration in the Feed and the Effluent

| Dilution rate, $h^{-1}$ | Protein concentration, mg/mL |
|---|---|
| 0.59 | 0.089 |
| 1.67 | 0.087 |
| Feed | 0.085 |

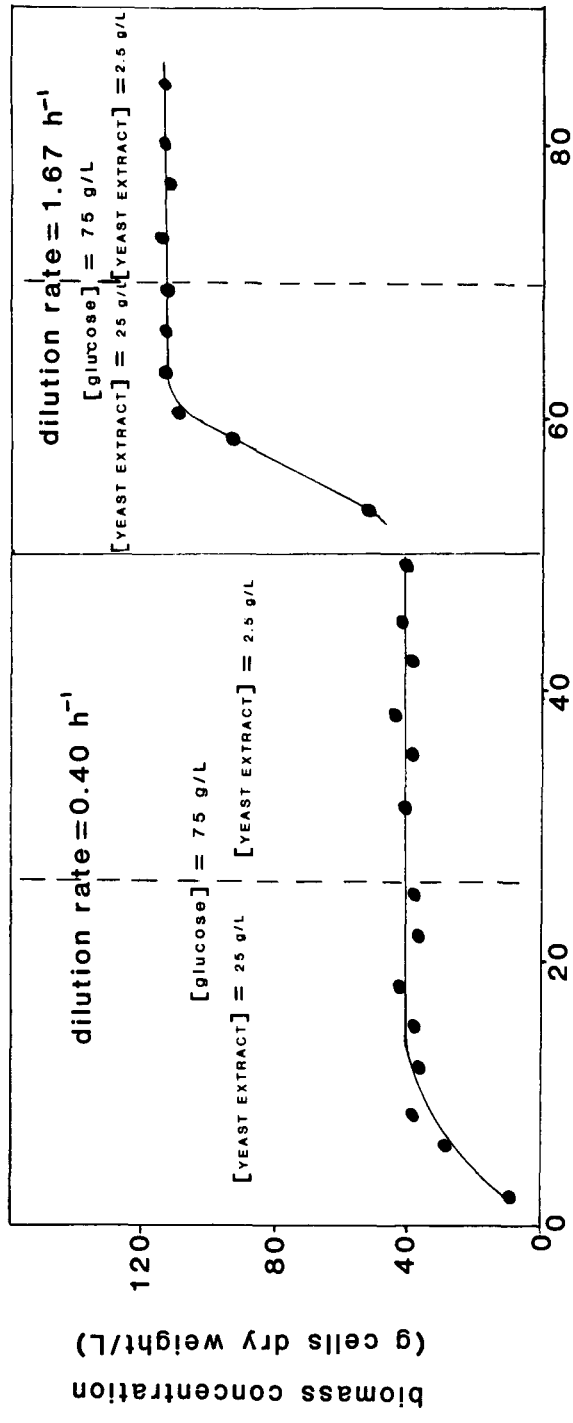


Fig. 3. Influence of nitrogen supply on biomass concentration at steady state.

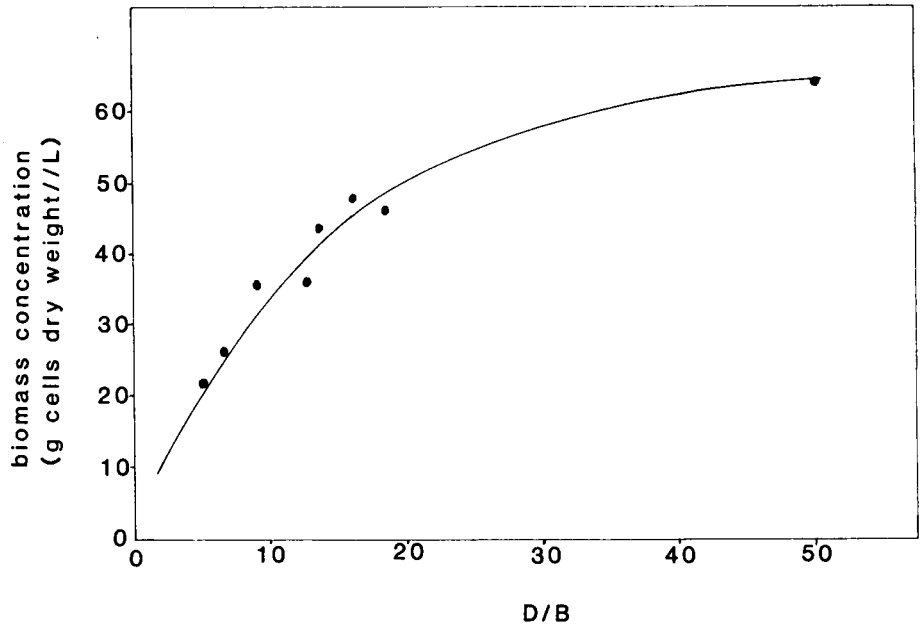


Fig. 4. The effect of bleed on biomass concentration.

if the specific death rate ($k_D$) is small, then from Eq. (1)

$$\mu = B \tag{4}$$

thus

$$D[S_o - S - (BS/D)]/X = (1/Y_{X/S})B + m \tag{5}$$

i.e., the specific glucose uptake rate should be linearly related to the bleed rate. Figure 5 demonstrates this dependence, and indicates that

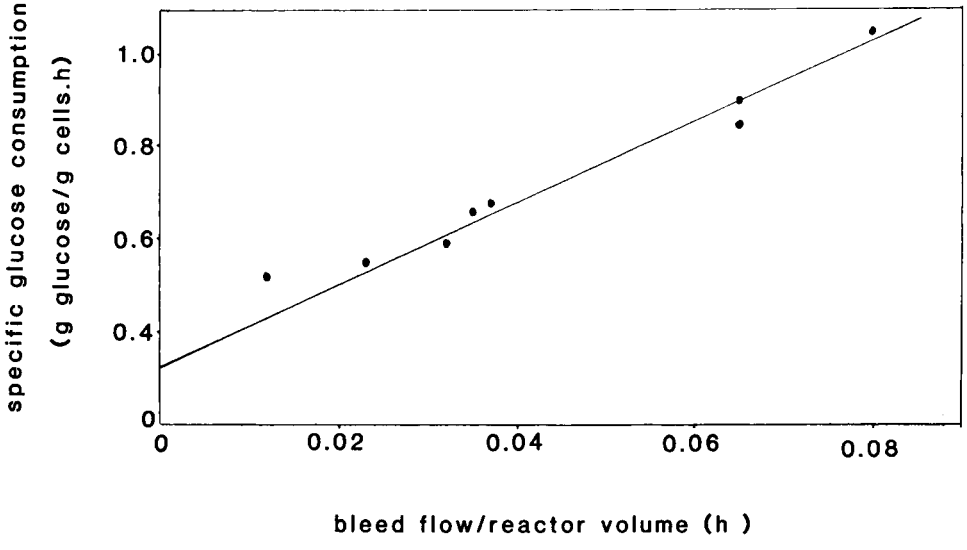


Fig. 5. The specific glucose uptake rate as a function of the bleed rate.

$$m = 0.33 \text{ g glucose/g cells·h}$$

$$Y_{X/S} = 0.114 \text{ g cells/g glucose}$$

Rogers (*16*) found a similar value for $Y_{X/S}$ (0.13 g cells/g glucose), but a lower value for $m$ (0.0045 g glucose/g cells·h). The maintenance term is generally lower for anaerobic culture, perhaps because of the absence of respiration; however, most values reported have been at low cell concentrations.

The specific rate of substrate consumption in the CSTR with cell recycle appeared to be a function of the dilution rate (Fig. 6). Both the specific lactic acid productivity and specific glucose consumption increased with increasing dilution rate (Table 1). This indicates that at low dilution rates there is excess of biocatalytic capacity in the fermentor, resulting in low specific lactic acid production. This has also been noted by Vick Roy et al. (*7*) for a cell-recycle system fermentation using the same bacteria. The relation between the dilution rate, the specific substrate consumption, and the specific lactic acid production was not very clear, and only a trend was observed.

Assuming a linear relationship between the dilution rate and the specific glucose consumption, Eq. (5) indicates that, with or without bleed, the intercept ($m$) should have a constant value. But without bleed, a higher value is found (0.6 g glucose/g cells·h) than when there is a bleed (0.33 g glucose/g cells·h). This difference indicates that when bacterial growth occurs, the requirement for maintenance seemed to be lower than when there is negligible growth. In comparison with other continu-

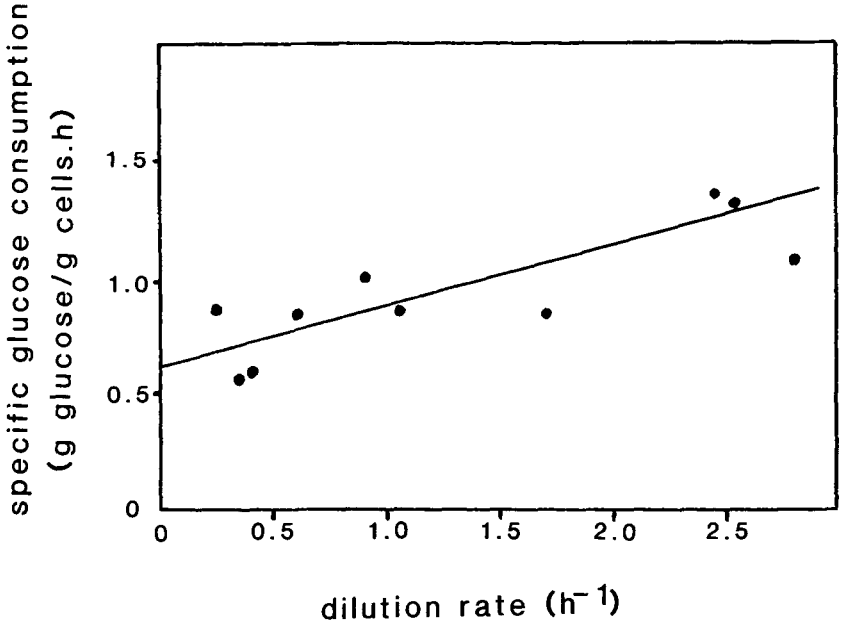


Fig. 6. The effect of dilution rate on specific glucose consumption.

ous fermentation systems, the CSTR with cell recycle has shown higher cell mass concentration, volumetric productivity, and substrate conversion than any other system producing lactic acid (*7*).

### *Product Inhibition and Cell Concentration*

Lactic acid productivity is strongly influenced by pH and lactic acid concentration (*22,23*). However, the effect of lactic acid on cell growth and lactic acid production is not very clear. It has been proposed (*24*) that only the undissociated form of lactic acid is inhibitory, whereas Friedmann (*25*) suggests that the lactate form is the inhibiting species. However, in either case, lactic acid inhibition limits the concentration of glucose that can be completely fermented. In a previous work (*7*) using a CSTR with cell recycle, a 35 g/L glucose solution was completely converted to 35 g/L lactic acid. In order to increase the volumetric productivity, a glucose concentration of 85 g/L was used in this work. At any dilution rate used, the maximum lactic concentration reached was below 60 g/L with only 70% conversion (runs 9, 11, 13; Table 1). By using a glucose concentration of 60 g/L, complete conversion could be obtained (Table 1; runs 1–3,10,12,14–17), except in the case of a high cell-bleed rate (Table 1; runs 6–8). In these cases the biomass concentration was not sufficient to metabolize 60 g/L of glucose concentration. At pH 6.25, the equilibrium $CH_3CHOHCOOH \leftrightharpoons CH_3CHOHCOO^- + H^+$ is shifted almost completely to the right and the lactate ion form predominates. At 60 g/L of total lactic acid plus lactate, there is only 0.24 g/L of the undissociated lactic acid form.

### *Volumetric Productivity*

The volumetric productivity may be increased by raising the dilution rate. The highest productivity obtained with a complete glucose consumption was at a dilution rate of 2.55 $h^{-1}$ (run 17). At this dilution rate, the volumetric productivity was 151 g/L·h, the specific lactic acid productivity was 1.28 g lactic acid/g cells dry weight and the biomass concentration was 118 g cells dry weight/L. The feed containing 60 g/L glucose was completely converted to lactic acid. When the dilution rate was increased to 2.80 $h^{-1}$ (run 18), the volumetric productivity was 160 g/L·h with 4.0 g/L of residual glucose. At this dilution rate, the residence time was too short to ferment the glucose totally. The cell concentration (140 g cells dry w/L) reached a critical point. Assuming that cells contain 75% water and have a density of 1.05 g dry w/L, then a biomass concentration of 140 g cells dry w/L results in maximum cell packing. At this cell concentration the viscosity should rise rapidly, whereas at concentrations between 30 and 110 g cells dry w/L, the viscosity increased only from 6 to 15 cp (Fig. 7).

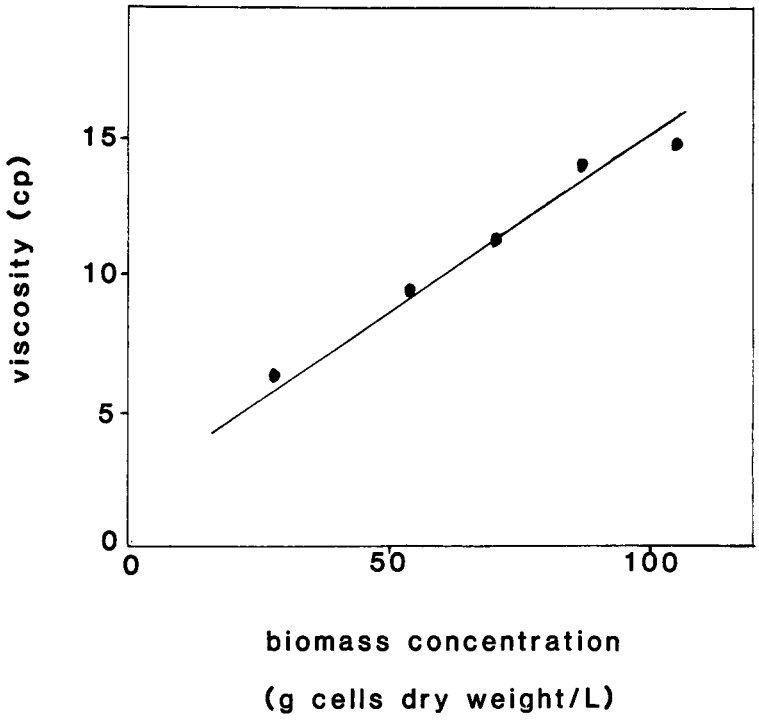


Fig. 7. Viscosity of the *Lactobacillus* broth versus cell concentration.

### *Membrane Efficiency*

The application of cell recycle to industrial processes depends greatly on the cost of filtration membranes. Figure 8 shows the membrane capacity after extensive experimentation, with the transmembrane flux of water plotted against the circulation rate. The membranes were cleaned by backflushing before measuring the flux of water through them. A loss of filtration capacity occurred after a few hours of fermentation, but after 40 h the filtration rate was stabilized. The early loss of capacity might be caused by the formation of an irreversibly adsorbed layer of hydrophobic compounds or the accumulation of insoluble compounds at the membrane surface (*18*), altering its transport properties.

For all continuous fermentation runs, no antifoam agent was used. Foam formation was minimized by recycling the cells back to the bottom of the fermentor.

## CONCLUSIONS

An experimental cell-recycle fermentor using ultrafiltration membranes was used to investigate continuous culture of microorganisms at

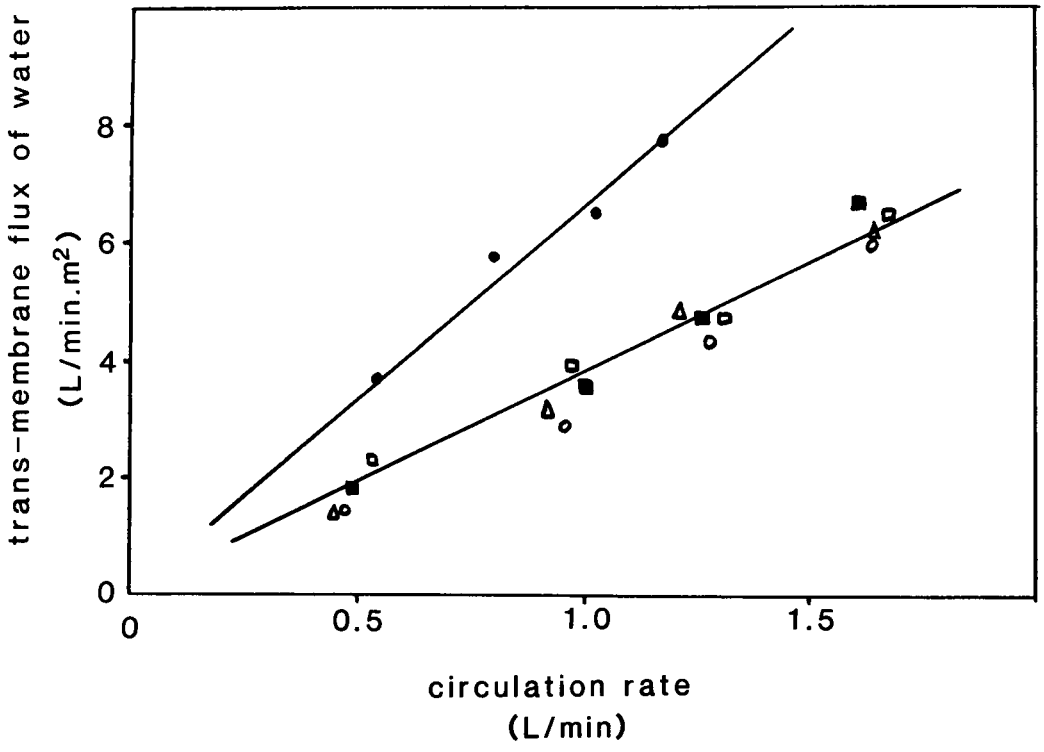


Fig. 8. Transmembrane flux of water versus circulation rate (● new membranes, ○ after 40 h, □ 100 h, △ 145 h, ■ 400 h of run).

high cell density. Tangential flow filtration was successfully used to withdraw cell-free product from the fermentor. Operation with a filtration rate up to 18.5 L/m$^2$·h was accomplished with a cell concentration of 140 g cells dry w/L, close to the theoretical maximum packing volume of cells. No cell purge was required to maintain high density of viable bacteria, and at steady state, zero cell growth was observed. The glucose uptake rate for cell maintenance was 0.33 g glucose/g cells·h. A maximum concentration of 60 g/L lactic acid concentration in the broth was found; beyond this product inhibition was severe.

The fermentation system produced high cell density and high volumetric productivity (150 g/L·h) with complete glucose conversion. Long-term operation under industrial operating conditions needs to be investigated, but the application of this technology to commercial lactic acid production seems promising.

## ACKNOWLEDGMENTS

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## NOMENCLATURE

| | |
|---|---|
| $B$ | Bleed blow/reactor volume ($h^{-1}$) |
| $D$ | Dilution rate ($h^{-1}$) |
| $k_D$ | Specific death rate ($h^{-1}$) |
| $L$ | Lactic acid concentration (g/L) |
| $m$ | Maintenance coefficient (g/g·h) |
| $S_o$ | Initial glucose concentration (g/L) |
| $S$ | Final glucose concentration (g/L) |
| $t$ | Time (h) |
| $\mu$ | Specific growth rate ($h^{-1}$) |
| $X$ | Biomass concentration (g/L) |
| $Y_{L/S}$ | Lactic acid yield (g lactic acid produced/g glucose consumed) |
| $Y_{X/S}$ | Biomass yield (g cells produced/g glucose consumed) |

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