Continuous Production of Lactic Acid from Whey Permeate by Free and Calcium Alginate Entrapped *Lactobacillus helveticus*  

**ABSTRACT**

*Lactobacillus helveticus* strain *milano* was used for the continuous fermentation of lactic acid in cheese whey-yeast extract permeate medium. The best productivity of lactic acid was with the free cell system, which was 9.7 g/L per h at a dilution rate of .352 h\(^{-1}\). Under such conditions, lactose conversion was 87.5%, based on the lactose concentration of 37.4 g/L in feed. Under high dilution rates, the cells were elongated to several times their normal size, resulting in wall growth. The cell growth on the fermentor wall caused system instability; however, it prevented cell wash-out under high dilution rates. The packed bed column system using Ca alginate entrapped cells is not suitable for practical application. Nonuniform pH control, plugging of the column by leaked cells, and decalcification of Ca alginate beads were major drawbacks of the packed bed system.

**INTRODUCTION**

Milk permeate and whey permeate (WP) are by-products of the ultrafiltration of milk and cheese whey. The permeates contain mainly lactose (and salts), which could be used as a carbon source for certain fermentation processes. Previous studies in our laboratory have demonstrated that WP can be transformed into a nitrogen-enriched wort, which in turn, can be used as culture medium for the fermentation process in the production of baker's yeast (2, 5). This nitrogen-enriched wort was prepared by fermentation of lactose into lactic acid with *Lactobacillus bulgaricus* or *Streptococcus thermophilus*, and then by transforming lactic acid into ammonium lactate through neutralization with ammonia. Parallel work on the screening of thermophilic lactobacilli revealed a strain of *Lactobacillus helveticus*, which consistently yielded highest concentrations of lactic acid in milk (Jacques Goulet and Renée Roy, Internal Report, Centre de Recherche en Nutrition, Laval University, June, 1980). Furthermore, *L. helveticus* is able to ferment galactose, a breakdown product in the fermentation of lactose (12). In contrast to other thermophilic lactobacilli, which produce dextrorotary D-lactic acid only, *L. helveticus* produces a racemic DL-lactic acid (L-lactic acid is a more readily metabolizable form (1). Because of the advantageous properties of *L. helveticus*, it would be of interest to investigate the use of this microorganism for the production of lactic acid from WP. Recent studies have been conducted in batch fermentations under controlled pH for yielding optimum pH, the setting of an inoculum preparation procedure, and the discovery of the best nitrogen growth factor source (11). Therefore, these optimum conditions have been applied to continuous fermentations of WP. This paper will report the experimental results using two systems: the free cell system in a conventional bioreactor and the Ca alginate entrapped cell system in a packed bed column. Comparative assessments of the results will be presented.
LACTIC ACID FROM WHEY PERMEATE

MATERIALS AND METHODS

Culture Media

Skimmed milk (SM) medium was prepared by 12 g of nonfat dried milk solids (low heat grade) rehydrated in 88 g of distilled water and autoclaved at 121°C for 10 min.

The lactose synthetic (LS) medium contained (g/L) lactose (ACS standard grade) 50; yeast extract (Difco) 30; MgSO₄ .6; FeSO₄ .03; MnSO₄ .03; Na acetate 1.0; KH₂PO₄ .5, and K₂HPO₄ .5.

Cheese whey permeate was prepared by spray-dried cheese whey powder (Kraffen, Kraft) rehydrated at 6.5% wt/vol in a 1.5% (wt/vol) yeast extract (Difco, technical grade) solution, then ultrafiltered using a Romicon HF2SSS system equipped with 2 HF PMS0 cartridges. The resulting whey-yeast extract permeate (WYEP) in 20-L carboy was brought to pH 5.0 with concentrated HCl before being autoclaved at 121°C for 1.5 h. The sterile WYEP medium was then cooled to room temperature and brought to pH 5.5 to 6.1 with 3 N NaOH.

Test Microorganism and Inoculum

Lactobacillus helveticus strain milano was obtained from the Institut Rosell, Montréal, Québec, Canada. Stock cultures were maintained in autoclaved SM and stored in liquid nitrogen.

Working cultures were propagated by weekly transfer (2% vol/vol) in still cultures in a LS medium.

Inocula for free-cell fermentations and for Ca alginate entrapped cells were prepared from working cultures (at 2% vol/vol inoculum), which were cultivated for 20 h at 42°C.

Cell Entrapment Method

Free cells were grown in 200 ml LS medium, then harvested in the late exponential growth phase by centrifugation at 16,300 × g for 30 min. Cells were then resuspended in 200 ml of 2% sodium alginate (Sigma, Type IV) that had been autoclaved previously (121°C, 15 min). The homogenous suspension was extruded drop by drop into a sterile solution of 0.5 M CaCl₂ using a syringe. Beads of approximately 2 mm of diameter were allowed to harden for 30 min in the CaCl₂ solution before being aseptically transferred to a sterile column.

Culture Conditions

Free-cell continuous culture experiments were conducted in a 750 ml working volume fermentor (B. Braun, Biostat, Model M) that was inoculated at 1% (vol/vol) under the following conditions: 200 rpm agitation speed, 42°C automatic pH control at 5.9 with 3 N NH₄OH. The fermentations were started batchwise until the end of exponential growth phase was reached (o.d. ≈ .9). Then, the fresh medium was fed into the fermentor and, simultaneously, the whole fermented medium was withdrawn from the fermentor using two peristaltic pumps. The continuous fermentations were conducted at different dilution rates (e.g., feed rate per unit volume of culture medium in the fermentor) and the results reported are the values at steady state only.

The entrapped cell fermentation system consisted of a three-stage packed bed column (Figure 1). Each stage consisted of a jacketed column of 2.6 cm i.d. and 30 cm height (LKB 2137 column) containing 200 ml of alginate beads. Temperature was controlled at 42°C by circulating water in the column jackets. The medium was fed to the top of the first column by means of a peristaltic pump (LKB 2132 MicroPerpex). The effluent from each stage was readjusted to optimal pH 5.9 in a holding chamber by automatic addition of 3 N NH₄OH (Radiometer pH meter, Copenhagen, Titrator II, Model 51) before entering the next stage. The holding chambers were 200 ml working-volume New Brunswick Scientific Microferm fermentors. The effluent in the holding chambers was kept at 50°C to prevent the growth of leaked cells.

Figure 1. Packed bed continuous fermentation system. WYEP = Whey-yeast extract permeate medium.
Analytical Methods

The lactic acid concentration was determined by HPLC (9) using a 300 × 7.8 mm Aminex HPX-87H column, Micro-Guard Ion Exclusion Cartridge (Bio-Rad), an ultraviolet (UV) detector (Waters, Model M-490). Glucose, galactose, and lactose concentrations were determined by GLC (Pierce Catalog 1985-86, method No. 18, page 110) using a 15 m × 0.25-mm silica capillary column and flame ionization detector in a Model 5880 Hewlett-Packard Gas Chromatograph. Cell densities were estimated by optical density (OD) measurements at 620 nm on a Bausch & Lomb Spectronic 21 Spectrophotometer. Cell elongation was observed by microscopic examination after Gram stain.

RESULTS AND DISCUSSION

Free Cell Continuous Fermentation

Maximum lactic acid productivity of *L. belveticus* under continuous free cell fermentation in WYEP medium varied from 9.3 to 9.7 g/L per h at dilution rates around 0.33 and 0.48 h⁻¹ (Figure 2). The lactic acid productivity dropped significantly beyond the dilution rate of 0.48 h⁻¹. The measurement of steady state values of biomass, lactic acid, and lactose concentrations at dilution rate higher than 0.48 h⁻¹ (as shown by dashed line in Figure 2) was not accurate, because it was difficult to reach stability in the system.

During continuous fermentation, wall growth of *L. belveticus* in the fermentor (deposit of bacterial cells on the inner wall of the fermentor jar at the liquid-air interface) became more and more significant with increasing dilution rates. This wall growth had not been observed either in batch fermentation experiments, under the same conditions, or in continuous fermentation at very low dilution rates (< 0.1 h⁻¹).

This wall growth was associated with the morphological change of *L. belveticus* cells under high dilution rate fermentations. Under such conditions, the cells elongated to several times their normal size (Figure 3). The longest cells were obtained in fermentation experiments at the highest dilution rate (0.67 h⁻¹).

Neither the wall growth nor the cell elongation was observed in continuous fermentation experiments using LS medium. The cell elongation observed in WYEP, especially at high dilution rates, might be the result of the limitation of certain nutrients under such conditions. Wall growth and cell elongation, however, has beneficial effects in the continuous fermentation of lactic acid from WYEP medium because there was no danger of cell wash-out at high dilution rates. Indeed, the maximum lactic acid productivity in WYEP medium occurred at D = 0.48 h⁻¹ (Figure 2) as compared with only D = 0.31 h⁻¹ in LS medium (results not shown).

Other microorganisms called "homofermentative lactic acid bacteria" such as *L. delbrueckii*, *L. lactis*, *L. bulgaricus*, *L. casei*, and *S. lactis* produced other organic compounds found for

![Figure 2](https://example.com/figure2.png)
Figure 3. Morphological change of *Lactobacillus helveticus* in WYEP medium: A) batch fermentation at 10 h; B) continuous fermentation at $D = 0.35 \text{ h}^{-1}$; C) continuous fermentation at $D = 0.45 \text{ h}^{-1}$. $\times 1744$. Arrow indicates cell separation.
heterofermentative lactobacilli (such as acetic acid, formic acid, ethanol) depending on fermentation conditions (continuous versus batch; high dilution rates versus low dilution rates (4, 6, 8). Analyses of organic acids produced in all samples obtained with the continuous fermentation experiments shown in Figure 2 revealed that lactic acid was obtained as the sole product. This confirms the true homofermentative nature of *L. helveticus*. Thus, for the manufacture of lactic acid, the use of *L. helveticus* represents a great advantage over other lactobacilli because the substrate is not wasted in producing undesirable by-products under fluctuating fermentation conditions.

Results from a batch fermentation under the same conditions were plotted in Figure 2 (open symbols at zero dilution rate) for purposes of comparison. Continuous fermentation had the same values of maximum biomass concentration, maximum product concentration and minimum residual sugar. However, the maximum productivity of lactic acid in continuous fermentation (at dilution rates of 0.33 to 0.48 h⁻¹) was four times that obtained in batch fermentation.

**Packed-bed Continuous Fermentation**

Results of the continuous fermentation of lactic acid using packed bed system are shown in Figure 4 in terms of lactic acid productivity (lactic acid concentration P × dilution rate D) versus dilution rates. The dilution rates are calculated as D = F/V; F/2V and F/3V for the system of one, two, and three packed bed stages, respectively, where F is the fresh medium feed rate to the system. The volume of Ca alginate beads changed (expansion followed by shrinkage) during the start-up of the continuous experiments and stabilized at about two-thirds of its initial volume under steady state condition. Therefore, the dilution rates could be expressed on the total volume of column V = V_C; on the steady state bed volume V = V_T or on the reactive volume V = V_R (which is the void volume of the bed volume V_T).

During continuous fermentation, the fresh medium was fed to the top of the first stage and the effluent of each stage was fed to the top of the next stage. This feeding mode, resulting in a down flow type operation, was essential to avoid plugging (13). Under such conditions, about one third of the volume of each column stage was not used for fermentation (dead zone), because there was no contact between the medium and the cells (in beads). Therefore, the lactic acid productivities calculated from the total column volume V_C are lower than those calculated from the steady-state bed-volume V_T (Figure 4A). The productivity-dilution rate curves calculated from V_C and V_T are identical for each stage. However, the presence of the dead volume in each stage caused displacement of productivity (based on V_T) toward lower values (based on V_C) on the same curves.

The beneficial effect of using a multistage column is shown in Figure 4A. Under the same

![Figure 4](https://via.placeholder.com/150)

**Figure 4.** Continuous fermentation of *Lactobacillus helveticus* in packed-bed system: A) lactic acid productivity based on total column volume (V_C, closed symbol) and on steady state bed volume (V_T, open symbol); B) effluent pH from each stage. Most experimental data are average values from two repetitive runs.
dilution rate, significantly higher lactic acid productivity was obtained with a column of many stages. This is because the maximum pH gradient in such a column is lower than that in the fewer stage column. Indeed, under the same dilution rate, for instance, at $D = 0.1 \text{ h}^{-1}$, the pH gradients in one-stage, two-stage, and three-stage columns were (5.9 at feed - 3.7 at first stage effluent =) 2.2; (5.9 at inlet - 4.1 at outlet of second stage =) 1.8 and (5.9 at inlet - 4.7 at outlet of third stage =) 1.2 pH units, respectively. This means that columns of many stages operated more closely to the optimal pH of 5.9 for lactic acid fermentation. The lower maximum pH gradient associated with the column of many stages is quite understandable. In the one-stage column, there was neither control of pH nor readjustment of pH between the inlet and the outlet of the column. This typifies fermentation without control of pH. In the two-stage column, the medium pH was readjusted to 5.9 once between the inlet and the outlet of the column. In the three-stage column, the medium pH was brought back to 5.9 twice between the inlet and the outlet of the column. Therefore, a multistage column typifies fermentation with a more uniform pH control along the column.

Lactic acid productivity of *L. helveticus* in WYEP medium, in batch fermentation, was very sensitive to pH. Within the controlled pH range studied, the maximum lactic acid productivity was reduced by 50% for each decrease of medium pH of one unit (11).

Residual sugars in the culture medium effluents are shown in Table 1. At the same dilution rates within the range of 0.036 to 0.144 h$^{-1}$ consumption of lactose is higher with the free cell system. It could be because in the free cell system, pH is better controlled, thereby preventing inhibition by lactic acid (10). The ratio of residual galactose to residual glucose was approximately equal for the free cell system regardless of dilution rate. Such a ratio was higher, increasing from 3 to 15, corresponding to the decrease in dilution rates, in the entrapped cell system. This suggests that *L. helveticus* cells do not have a preference for utilizing glucose or galactose in a free cell system; however, for an unknown reason, galactose is less metabolizable when the same microorganism is entrapped in Ca alginate gel, especially at low dilution rates.

### Evaluation of the System for Lactic Acid Production

Under our experimental conditions, lactic acid productivity was better with the free cell system for the same dilution rate. The lactic acid productivity versus dilution rate curves of the Ca alginate entrapped-cells (effluent from each stage of the packed bed column; Figure 4) were below that obtained with the free cell continuous culture (Figure 2). However, the former has a tendency to approach the latter, under the same dilution rate, with addition of column stages, e.g., with more closely controlled pH. This suggests that, in the packed

### TABLE 1. Residual sugars in culture medium.

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>Glucose (g/L)</th>
<th>Galactose (g/L)</th>
<th>Lactose (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Free cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>.032 to .328</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>.352</td>
<td>.86</td>
<td>1.41</td>
<td>4.69</td>
</tr>
<tr>
<td>.416</td>
<td>1.53</td>
<td>2.00</td>
<td>7.71</td>
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<tr>
<td>.480</td>
<td>1.15</td>
<td>1.49</td>
<td>9.21</td>
</tr>
<tr>
<td><strong>Entrapped cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>.036</td>
<td>.57</td>
<td>8.92</td>
<td>6.54</td>
</tr>
<tr>
<td>.072</td>
<td>.24</td>
<td>1.59</td>
<td>3.86</td>
</tr>
<tr>
<td>.144</td>
<td>1.02</td>
<td>3.60</td>
<td>7.09</td>
</tr>
</tbody>
</table>

*Based on total volume of column for the entrapped cells.*
bed column system, if the pH could be vigorously controlled along the column to preclude pH gradients, lactic acid productivity would probably reach the same level as that obtained with the free cell system.

The pH gradient problem in the packed bed column exists at two different levels: the outside environment of the beads (the extragel pH) and the inside of the beads themselves (the intragel pH). The extragel pH can be easily controlled by appropriate fermentor design and operation. For instance, the fluidized-bed column would be more suitable than the fixed-bed column to control extragel pH. It is expected that the intragel pH is lower than the extragel pH, because lactic acid is produced inside the gel and because there is no convection flow of medium inside the beads. Therefore, the intragel pH can be controlled only by use of an appropriate technique for cell immobilization. For instance, entrapped cells in very thin film of polymeric material would be suitable to avoid the intragel pH gradients.

Plugging was a major problem with the Ca alginate entrapped cells of _L. helveticus_ in the packed bed system. Such plugging was caused by: 1) the decalcification of Ca alginate by lactic acid (13). As a consequence, the beads were softened and had a tendency to compress into a solid mat, and thus, blocked the column. This phenomenon usually occurred before the 5th d of operation; and 2) the cells leaking into the medium as a result of cell overgrowth in the beads. Thus leaked cells accumulated in large quantities at the later stages of the column, causing the most plugging of the column. Such phenomenon was usually observed after the 5th d of fermentation. The plugging problem happened more rapidly when high dilution rates were applied to the packed bed system. For this reason, no experimental data could be obtained at dilution rates higher than .144 h⁻¹ (based on total volume \( V_C \)) in Figure 4.

Another drawback of the Ca alginate entrapped cell system is that high cell concentrations were initially required in the bioreactor. Indeed, according to the procedure for immobilizing the cells, the initial inoculum concentration (_L. helveticus_ cells per liter of medium volume in the bioreactor) in the packed bed system was approximately 180 times that used to seed the free cell system. However, the

![Table 2: Comparison of lactic acid productivity of thermophilic lactic acid bacteria in continuous fermentation.](image)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Feed lactose concentration (g/L)</th>
<th>Process</th>
<th>Lactose conversion (%)</th>
<th>Lactic acid productivity (g/L per h)</th>
<th>Dilution rate (h⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. bulgaricus</em></td>
<td>50</td>
<td>Two-stage</td>
<td>98</td>
<td>1.77</td>
<td>.032</td>
<td>(7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>continuous</td>
<td>51</td>
<td>5</td>
<td>.17</td>
<td>(14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Continuous</td>
<td>62</td>
<td>4.44</td>
<td>.136</td>
<td>(15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dialysis</td>
<td>48</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td><em>L. bulgaricus</em></td>
<td>242</td>
<td>Continuous</td>
<td>48</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Packed bed</td>
<td>50</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>242</td>
<td>Continuous</td>
<td>48</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Packed bed</td>
<td>50</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td><em>L. helveticus</em></td>
<td>38.3</td>
<td>Continuous</td>
<td>38.3</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey permeate</td>
<td>38.3</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yeast extract</td>
<td>38.3</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whey permeate</td>
<td>38.3</td>
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<td>.085</td>
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<tr>
<td></td>
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<td>Yeast extract</td>
<td>38.3</td>
<td>1.2–1.6</td>
<td>.085</td>
<td>(16)</td>
</tr>
</tbody>
</table>

1. Based on total volume of column.
2. Gel entrapped cells; otherwise free cells.
3. WYE = Whey extract permeate medium.
productivity in the packed bed system was lower than that obtained in the free cell system.

Calcium alginate entrapped cells in a packed bed column are not suitable for lactic acid fermentation when compared to continuous fermentation using free cells. Among the continuous fermentations reported for different microorganisms, media, and processes (Table 2), the free cell system of L. helveticus yielded the highest lactic acid productivity and lactose conversion simultaneously. Such criteria of high productivity and high conversion in the same time are desirable for industrial process.

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REFERENCES