# Kinetic study of continuous whey permeate fermentation by immobilized *Lactobacillus helveticus* for lactic acid production

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*A pH-controlled continuous lactic acid fermentation of whey permeate supplemented with yeast extract was carried out with* Lactobacillus helveticus *entrapped in κ-carrageenan–locust bean gum gel beads at various dilution rates and pH set points. A kinetic study of the immobilized cell continuous fermentation in a 1-l stirred-tank reactor containing 33% (v/v) gel beads was carried out with an original experimental device, consisting of a 110-ml second-stage free-cell fermenter. This second stage was assumed to reflect the physiological behavior of the free-cell phase in the immobilized-cell bioreactor. A maximum lactic acid production rate of 28.5 g $l^{-1}$ $h^{-1}$ was measured at pH 5.5 and dilution rate 1.21 $h^{-1}$ for the immobilized-cell bioreactor. The overall specific growth rate and the specific lactic acid production rate of entrapped cells were 12–18% those of free cells in the reactor. This low activity may result from the presence of high inhibition in the central part of the beads as a result of substrate, product, and pH gradients. However, entrapped cells were responsible for 75–85% of the lactic acid and biomass productions within the immobilized cell fermenter at pH 4.7–6.3 and close to 90% at pH 4.3.*

**Keywords:** *Lactobacillus helveticus*; whey permeate; continuous fermentation; immobilized cells; lactic acid

## Introduction

Whey is a by-product of cheese production. Whey can be separated by ultrafiltration into high-grade proteins and permeate. The whey permeate contains almost all the lactose of milk and represents an important part of the polluting effluents of dairy industries, due to its high biological oxygen demand (BOD > 25 g $l^{-1}$)[1]. An interesting way to upgrade this effluent could be as a substrate for fermentations. Thus, lactic acid production by lactic acid bacteria could be an alternative processing route for whey lactose. The ammonium lactate produced could be used in a concentrated form as a cattle feed supplement[2,3] or purified to different quality grades according to the recovery process em- ployed.[4] *Lactobacillus helveticus* was chosen in this work because it appears to be one of the most productive lactic acid bacteria used for lactic acid production from lactose media.[5] Furthermore, it is a thermophilic and acidophilic bacterium, which implies that it will grow in conditions inhibitory for most contaminants.

The use of immobilized cell technology to improve lactic acid fermentation processes has been proposed by several authors.[6–8] The entrapment of bacterial cells in natural polysaccharide gel beads allows for high cell density continuous fermentations, and may result in improved productivity. A summary of published data on lactic acid production by entrapped cells is given in *Table 1*. In most cases, lactic acid productivity was limited by factors such as nonuniform pH control and clogging of the column reactors, destabilization of the alginate gel by Ca chelating lactates, and loss of biocatalyst activity. Mechanical stability of the beads and diffusion limitations of substrate and product within the gel bead matrix appeared as the main problems encountered by previous researchers, particularly during continuous fermentation. Thus, the success of such processes could rely on the optimization of all fermentation parameters in order to achieve high stability along with high productivity and low operating and capital costs. A clear understanding of the effects of entrapment on lactic acid bacteria kinetics is required to reach this objective.

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Received 15 October 1992; accepted 15 December 1993

**Table 1** Summary of reported batch and continuous lactic acid production using entrapped cells.

| Bioreactor | Organism | Substrate | Supplement concentration ($g\ l^{-1}$) | Productivity ($g\ l^{-1}\ h^{-1}$) | Sugar utilization (%) | Ref. |
|---|---|---|---|---|---|---|
| **Batch fermentations** | | | | | | |
| Agar | *L. casei* | Lactose[a] | 0 $g\ l^{-1}$ | 0.55 | 73 | (49) |
| | | | MC[b], 60 | 0.64 | 94 | |
| Alginate | *L. casei* | Glucose | YE[c], 10 | 1.6 | 99 | (50) |
| Alginate | *L. casei* | Lactose[a] | YE, 4 | 0.86 | 94 | (51) |
| κ-Carrageenan | *L. lactis* | | | 0.79 | 91 | |
| Polyacrylamide | Coimmobilized | | | 0.78 | 84 | |
| Alginate | *R. orizae* | Glucose | 0 | 2.6 | 72 | (52) |
| Pectate | *Lactobacillus* sp. IMET 11466 | Glucose | MRS YE, 15 | 24[e] | — | (53) |
| **Continuous fermentations** | | | | | | |
| CSTR[d]-alginate | *A. awamori* *S. lactis* | Starch | YE, 5 + tryptone, 5 | 0.4 | 66 | (54) |
| CPBR[f]-alginate | *L. delbrueckii* subsp. *delbrueckii* | Glucose | YE, 10 | 3 | 97 | (55) |
| CPBR-alginate | *L. helveticus* | Lactose[a] | YE, 15 | 4 | 68 | (40) |
| CPBR-alginate | *L. helveticus* | Lactose[a] | YE, 15 | 8 | 82 | (8) |
| CSTR-κ-carrageenan | *L. helveticus* | Lactose[a] | YE, 10 | 28.5<br>41.3[g] | 46<br>60[g] | (56)<br>(44) |

[a]Whey permeate; [b]mustard oil cake; [c]yeast extract; [d]continuous stirred-tank reactor; [e]peak value in standardized conditions; [f]column packed-bed reactor; [g]54% (v/v) gel beads is the immobilized cell reactor

Kinetic parameters of the two discrete phases (immobilized and free cells) of entrapped cell systems are difficult to segregate. The free-cell phase in an entrapped-cell bioreactor originates from cell leakage from the gel bead surface and intrinsic free cell growth. Cell leakage from the gel into the surrounding medium takes place when beads are fully colonized, and cell release rate is dependent upon *in situ* cell growth.[9,10] Analytical models[10–13] require the determination of numerous parameters and many assumptions on the nature of product, substrate, biomass, and activity gradients within the beads. Many investigational techniques have been used to determine these internal gradients, such as electron microscopy,[14] flow cytometry,[15] *in vivo* NMR,[16] scanning microfluorometry,[11,17] and autoradiography.[18] However, these complex techniques require sophisticated equipment, and it is still difficult to accurately measure the real activity of entrapped biomass during fermentations. Mathematical modeling of immobilized *Lactobacillus delbrueckii* revealed that the substrate transport in the immobilization matrix was not likely to be a limiting factor, but inhibitory product buildup and pH decrease were significant within the gel beads.[19] However, direct measurement of internal pH, lactic acid, or substrate gradients within the gel to validate the model was not attempted.

Audet *et al.*[20] assumed in their calculations that the specific growth rate ($\mu$) and lactic acid production rate ($P_{SA}$) of the free-cell phase in an immobilized-cell continuous stirred-tank reactor (CSTR) could be estimated from the rates determined in batch fermentation for the same lactic acid concentrations. However, cells released from the gel bead matrix may have been physiologically altered by immobilization, and may exhibit a different activity than free cells generated in conventional pH-controlled batch fermentations. These released cells may represent an important part of the free-cell phase in an immobilized-cell lactic acid bioreactor, depending on the operational parameters.[20,21]

In this paper we report the stable operation of a continuous immobilized-cell reactor for the production of lactic acid from a supplemented whey permeate medium by *Lactobacillus helveticus*. The effect of pH control set point and dilution rate was determined. To verify the assumption of Audet *et al.*,[20] an original analytical free-cell second-stage bioreactor was developed to experimentally determine the specific growth and lactic acid production rates of the free-cell phase in the immobilized-cell bioreactor. This method is described, and the effects of pH and lactic acid concentration on kinetic properties of cell populations in free-cell batch fermentations and in both phases of immobilized-cell continuous fermentations will be compared and discussed in detail. Emphasis will be made on the possibility of industrial application of this fermentation process.

## Materials and methods

### *Microorganism and medium preparation*

A KCl- and yeast extract-supplemented whey permeate medium (SWPM) was used for fermentations. It was obtained by rehydration of a whey permeate powder (6% w/v, Ridgeview Inc., La Crosse, WI, spec. no. 162), first adjusted to pH 5 by addition of HCl and autoclaved at 121°C for 15 min to induce thermal precipitation of the remaining proteins. After filtration (AP prefilter, Millipore, Bedford MA), KCl (0.3 M, ACP Chemicals, Montréal, Canada) and yeast extract (10 $g\ l^{-1}$, Rosell Institute, Montréal, Canada) were added. The role of KCl was to improve the mechanical stability of the κ-carrageenan–locust bean gum gel beads during continuous lactic fermentations.[22,23] For continuous fermentations, 16 l of supplemented medium were autoclaved at 121°C for 15 min in 20-l Nalgene bottles, then stored at 4°C. For batch fermentations, the medium was sterilized at 121°C for 15 min in

the bioreactor (1 l). In both cases, initial lactose concentration was 48–50 g $l^{-1}$.

*Lactobacillus helveticus* L89 (Rosell Institute, Montréal, Canada) was reactivated and maintained in MRS broth (Rosell Institute, Montréal, Canada). Inoculum for batch fermentations came from a 16-h culture grown at 42°C in the SWPM.

## *Immobilization*

The inoculated gel beads were produced by a two-phase dispersion process.[9,24] The polymer solution (2.75% w/v κ-carrageenan and 0.25% w/v locust bean gum) selected from Arnaud *et al.*,[22] was inoculated at 45°C with 2% of an *L. helveticus* suspension in MRS broth. The inoculated polymer was poured into warm sterile soya oil under agitation, and the temperature was progressively reduced to 30°C. The resulting polymer gel beads were soaked for 1 h in 0.3 M KCl solution to allow the gel to harden. Beads with a diameter of 1–2 mm were selected by wet sieving (Brinkmann stainless steel sieves). κ-Carrageenan (Satiagel MR 150) and locust bean gum were obtained from Satia, Ceca sa., Villacoublay, France.

## *Fermentation techniques*

All fermentations were carried out in a 1-l Bioflow III bioreactor (New Brunswick Scientific Co. Inc., Edison, NJ) at 42°C with mild agitation (75 rev $min^{-1}$) (*Figure 1*). The pH was controlled in the 4.3–6.3 range by addition of 12 N, 2 N, and 6 N $NH_4OH$ respectively in the first-stage immobilized-cell continuous fermenter, the second-stage continuous free-cell analytical reactor, and the free-cell batch fermentations. The choice of ammonia concentration was based upon the precision of the pH regulation. Dropwise addition of a very concentrated alkaline solution in lightly acidifying and buffered fermentation broth, such as in the initial part of a batch fermentation, will result in wide undesirable pH variations. Therefore, ammonia was used in a more diluted form for batch fermentations and for the second-stage continuous analytical bioreactor (small operating volume) than for the first stage, where acidification rates were high. Batch fermentations were carried out with a 2% (v/v) inoculum. Samples for optical density and high-performance liquid chromatography (HPLC) analyses were taken hourly.

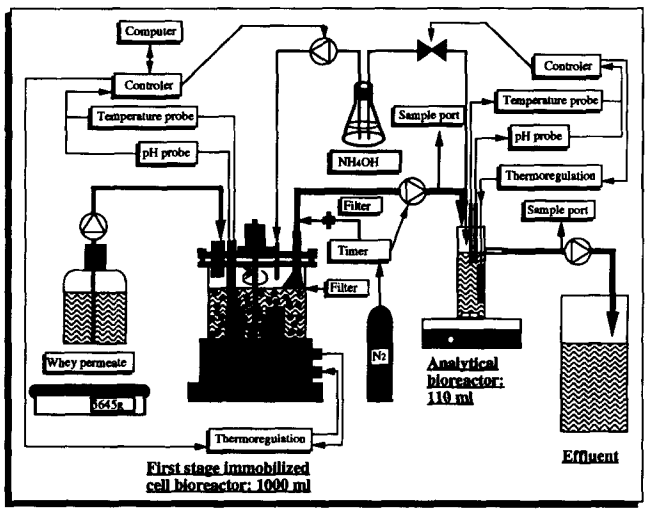


**Figure 1** Experimental set-up of the continuous immobilized-cell fermentation process

For immobilized-cell continuous fermentations, fresh beads were first incubated for three successive pH-controlled batch fermentations in MRS broth in order to increase the population within the gel matrix from 5 × $10^6$ to 5 × $10^8$ CFU $ml^{-1}$ of gel. The bioreactor was then inoculated with 33% (v/v) of colonized gel beads and was operated continuously for an 81-day period at dilution rates ($D$) ranging from 0.11 to 1.6 $h^{-1}$ and pH from 4.3 to 6.3. $D$ was controlled by set point adjustment of the peristaltic pump (Watson Marlow 503U/RL, Falmouth, UK) feeding the bioreactor with SWPM. Actual flow rates of SWPM for accurate determination of $D$ were measured using a Mettler PM34 electronic balance. Fermentation broth was withdrawn from the immobilized-cell bioreactor by a peristaltic pump (Masterflex variable speed, cat. no. 7553-00, Cole Parmer Instruments Co., Chicago, IL) set at a higher flow rate than the feeding pump. A nylon mesh filter (0.5-mm pore diameter) was used to retain gel beads in the fermenter. Periodic nitrogen pulses (10 s every 5 min) were sent via an electrovalve controlled by a timer in order to avoid clogging of the filter surface by the beads (*Figure 1*). All fermentation parameters were controlled and stored by an IBM PS2 computer, using the Advanced Fermentation Software (New Brunswick).

The analytical free-cell fermentation stage in series was made of a 110-ml glass cylinder with external control loops for pH and temperature. It was continuously inoculated with free cells released from the first stage. Temperature and pH were controlled at the same set points as in the first stage. Modifications of operating parameters ($D$, pH) were randomized. After a change in these parameters, the system was allowed a minimum time (about 24 h) to reach a pseudo-steady state, defined as a steady $NH_4OH$ consumption during a 4- to 5-h period. Three samples were then taken from each stage at 30-min intervals and used for optical density (OD) and HPLC analyses.

## *Analytical techniques*

**Cell concentration.** Free-cell biomass measurements were estimated from OD readings at 625 nm (Spectronic 710, Bausch and Lomb, Rochester, NY), using a standard curve to correlate OD and dry cell concentration (biomass). Biomass was measured by centrifuging 200 ml of fermentation broth at 16,300*g*, washing the pellet three times with distilled water, and oven drying at 104°C for 24 h.[25]

Measurement of final biomass within the beads was carried out by oven-drying whole beads.[26] Colony forming units per milliliter (CFU $ml^{-1}$) were determined in duplicate by pour plating in MRS agar.[27] Entrapped cell plating was performed after dissolving 1 ml gel bead samples by strong agitation in sterile peptone water at 45°C in the presence of glass beads.[9]

**Sugar and acid concentrations.** Lactic acid production rate ($P_A$) was estimated on line (estimated error of 5% compared to HPLC) by using the instantaneous volume of $NH_4OH$ delivered by the pH control peristaltic pump and correcting for the molarity of the neutralizing agent. Lactose, galactose, glucose, and lactic acid concentrations were determined by HPLC analysis using a Waters system (Milford, CT), with an Ion 300 column and IonGuard polymeric guard column (Interaction Chemicals Inc., Mountain View, CA). Organic acids were detected by UV at 210 nm and refractive index was used for sugar detection. The mobile phase was 0.0049 N $H_2SO_4$.[28] Accurate $P_A$ estimates were calculated from lactic acid concentrations determined by HPLC, and actual flow rates.

## *Determination of kinetic parameters for free-cell batch fermentations*

Modeling of *L. helveticus* growth was done according to the logistic equation of Moraine and Rogovin:[29]

$$\frac{dX}{dt} = \mu_{max} X\left(1 - \frac{X}{X_{max}}\right) \quad (1)$$

where $X$ is the biomass concentration (g $l^{-1}$), $\mu_{max}$ is the maximum (initial) specific growth rate ($h^{-1}$), and $X_{max}$ is the maximum biomass concentration (g $l^{-1}$). The integrated form of the equation, with $X_0 = X(t = 0)$, gives a sigmoidal curve that empirically represents both an exponential and a stationary phase:[30]

$$X(t) = \frac{X_0 e^{\mu_{max}(t - t_0)}}{(1 - \frac{X_0}{X_{max}})\,[1 - e^{\mu_{max}(t - t_0)}]} \quad (2)$$

where $t_0$ represents the lag time. The following product formation model, developed by Luedeking and Piret,[31] was used to describe the lactic acid production curves:

$$\frac{dLA}{dt} = m\frac{dX}{dt} + nX \quad (3)$$

where $LA$ is the instantaneous lactic acid concentration (g $l^{-1}$), and $m$ and $n$ are respectively the growth- and non-growth-associated lactic acid production constants. Both $m$ and $n$ are dependent upon the strain, growth medium, and fermentation conditions. Procedures described by Weiss and Ollis[30] were used to linearize experimental data for the determination of parameters $\mu_{max}$, $m$, and $n$.

Parameters $\mu_{max}$, $X_0$, $X_{max}$, $m$, and $n$ were determined for each batch fermentation. For each treatment (pH set point), the average parameters were calculated from triplicate experiments. Equation (2) was used to generate a complete biomass data set from an average pH-controlled batch fermentation, and equations (2) and (3) were used to model lactic acid production. Maximum specific lactic acid production rate ($P_{SAmax}$) could be estimated from modeled data from the $Y$-axis intercept of the curve $P_{SA}$ versus lactic acid concentration.

## *Determination of kinetic parameters for immobilized-cell continuous fermentations*

The specific lactic acid production rate ($P_{SA}$) of free cells in the second-stage reactor was determined as follows:

$$P_{SA2} = D_2\frac{LA_2 - LA_1}{X_2}, \text{ g(lactic acid) } h^{-1} \text{ g(biomass)}^{-1} \quad (4)$$

where subscripts 1 and 2 refer to first and second stages. The actual specific lactic acid production rate of the free-cell phase in the first stage bioreactor was unknown. However, considering that the free-cell phase volume at equilibrium in the first-stage immobilized-cell bioreactor was close to 670 ml, the residence time in the first stage was 6 times longer than in the analytical second stage. Furthermore, lactic acid concentration was only 1–2 g $l^{-1}$ higher in the second stage than in the first one. Therefore, it can be assumed that $\mu$ and $P_{SA}$ for free cells in the two stages were very similar.

Therefore, the validity of this approach for $P_{SA}$ determination was largely dependent upon the sensitivity of HPLC analysis. A second-stage fermenter with a larger volume would have resulted in higher lactic acid concentration differences between the two stages and less experimental error in the measurement of this difference. However, the residence time in the second-stage bioreactor had to be minimized so that substrate and product concentrations in the first and second stages were very close and free-cell activity was comparable in both reactors. The precision of the HPLC analysis (0.1 g $l^{-1}$) was suitable for the accurate determination of differences in lactic acid concentration in the 110-ml second-stage bioreactor. Nevertheless, sample preparation (dilution, centrifugation, filtration) generated an error in the overall concentration of sugars and acids on the order of 1–2% (0.15–1 g $l^{-1}$). Therefore, a method based on mass balance and statistical correction was developed to decrease the error on lactic acid concentrations and $P_{SA}$.

A mass balance for products and substrate can be expressed as follows:

$$lac_0 = \frac{LA}{Y_{P/S}} + lac + gal + glu, \text{ g } l^{-1} \quad (5)$$

where $lac_0$ is the initial lactose concentration in the SWPM and *LA, lac, gal,* and *glu* are respectively lactic acid, lactose, galactose, and glucose concentrations in a sample of the fermentation broth. The theoretical yield of lactose conversion into lactic acid ($Y_{P/S}$ is 105%.[32] However, the average experimental yield we have determined for the homofermentative microorganism *L. helveticus* was 95%. Glucose was always present as trace and galactose was generally in the 0.5–3 g $l^{-1}$ range.

An estimated initial lactose concentration $lac_{0E}$ was calculated for each sample using equation (5) with *LA, lac, gal,* and *glu* concentrations as determined by HPLC and the average $Y_{P/S}$ of 95%. Since most of the experimental error of HPLC measurement resulted from dilutions performed during sample preparation, *LA* was linearly related to $lac_{0E}$ for each treatment. A covariance analysis was carried out for each experimental treatment ($D$, pH control set point), with *LA* as independent variable and $lac_{0E}$ as covariate.[33] Adjusted means of *LA* were taken for the calculation of differences in lactic acid concentration between the first- and second-stage bioreactors. This statistical correction of the data allowed for an average threefold reduction in standard deviation for lactic acid concentrations (from 1–2% to 0.3–0.8% of the mean values), and resulted in a reliable estimation of the specific lactic acid production rate in both stages.

Logically, calculation of the specific growth rate ($\mu$) in the second stage could be performed as follows:

$$\mu = D_2\frac{X_2 - X_1}{X_2} \quad (6)$$

However, the coefficient of variation in biomass measurements by OD was on the order of 5%. This error did not allow the accurate determination of biomass differences between the first and second stages. Nevertheless, the intercept and slope of the regression curve between the lactic acid/biomass ratio ($R_{LA/X}$) in the free-cell phase of the first and second stages were not significantly different from 0 and 1, respectively ($\alpha = 0.001$). Therefore, for each treatment, $R_{LA/X}$ could be assumed to be identical between the two stages and $\mu$ was estimated by the formula:

$$X_i = \frac{LA_i}{R_{LA/X}} \qquad i = \text{1st or 2nd stage} \quad (7)$$

Then, equation (6) becomes:

$$\mu = D_2\frac{LA_2 - LA_1}{LA_2} \quad (8)$$

The determination of specific rates in the free-cell phase of the immobilized-cell bioreactor permits calculation of an average $\mu_E$ and $P_{SAE}$ for the entrapped biomass, using the following balances:

$$P_{SAE} = \frac{(P_{AT}V_T) - (P_{SA2}X_FV_F)}{V_EX_E}, \text{ g(lactic acid) g(biomass)}^{-1}\ h^{-1} \quad (9)$$

$$\mu_E = \frac{(P_{XT}V_T) - (\mu_2X_FV_F)}{V_EX_E} \quad (10)$$

where $V_T$ is the total volume, $V_E$ is the bead volume, and $V_F$ is the volume of the free-cell phase in the immobilized-cell bioreactor. These specific rates, determined by difference between the total production of the immobilized-cell bioreactor and the free-cell phase, did not include any assumption on the nature of the gradients of product, substrate, biomass, and activity within the gel beads, but treated gel beads as homogeneous biocatalysts.

### *Statistics*

Three repetitions were carried out for the free-cell batch fermentations. A second-order polynomial regression analysis was carried out for the determination of the optimal pH corresponding to a maximum in curve.[34]

Four continuous immobilized-cell fermentations were performed. The first three preliminary fermentations lasted approximately 2 weeks, whereas the fourth fermentation was operated continuously for 81 days. Only the last fermentation is presented in this paper. However, the three preliminary experiments exhibited similar tendencies (unpublished data). During the last continuous fermentation, there was no systematic repetition of treatments (pH and dilution rate). However, the smoothness of result curves, made with data points randomized on a 2-month period, is in itself a guarantee of statistical significance.

The comparison of $\mu$ and $P_{SA}$ for immobilized-cell continuous fermentations and free-cell batch fermentations, as well as of $R_{LA/X}$ between the first and second stage in the immobilized-cell continuous process, was tested by a first-order regression analysis.[34] The specific growth rate and specific lactic acid production rate of the free-cell phase in the immobilized-cell continuous fermentations were determined experimentally, whereas for free-cell batch fermentations, these data were calculated for the same lactic acid concentration, using equations (2) and (3). The intercept and slope of the regression were used to compare the two sets of data. A slope and an intercept not significantly different ($\alpha < 0.001$) from 0 and 1 respectively[34] indicated that the evolution of the considered parameter was identical for both treatments.

## Results and discussion

### *Free-cell batch fermentations*

Kinetic parameters of free-cell batch fermentations for various pH control set points are presented in *Table 2*. A regression analysis showed a significant quadratic effect of pH control set point on the maximum specific growth rate ($\mu_{max}$) of *L. helveticus* and its maximum specific lactic acid production rate ($P_{SAmax}$). The optimum pH of both second-order regression equations was very close to 5.5, with $\mu_{max}$ and $P_{SAmax}$ equal to 0.66 $h^{-1}$ and 7.57 $g\,l^{-1}\,h^{-1}$, respectively (*Table 2*). All the available lactose was consumed during 15- to 20-h batch fermentation with pH controlled in the 4.7–6.3 range. At pH 4.3, lactic acid inhibition occurred before completion of the fermentation, with a final lactic acid concentration of approximately 30 $g\,l^{-1}$. It has been shown that inhibition is mainly due to the undissociated form of lactic acid.[35–37] The critical lactic acid concentration was lower at low pH, since the proportion of the undissociated form of lactic acid increased compared to higher pH.[38]

Aeschlimann and Von Stockar[39] found the same optimum pH (5.5) with *L. helveticus* in a similar SWPM. The shape of $\mu_{max}$ as a function of pH as reported by Roy *et al.*[40] under similar conditions was comparable to the data determined in this work. However, the optimum pH was somewhat higher (5.9). The *L. helveticus* strain used in the present study exhibited high activity for pH values as low as 4.7–5.1 (*Table 2*). This characteristic may be advantageous for an industrial production of lactic acid, since it is desirable to operate under conditions which are highly inhibitory for potential contaminants.

The growth-associated lactic acid production constant $m$ was always higher than the non-growth-associated lactic acid production constant $n$ by at least a factor of 10 (*Table 2*). This suggested that lactic acid production was strongly linked to biomass production. Furthermore, the ratio $m/n$ increased with decreasing pH as reported in the literature.[31,40] For pH 4.3, non-growth-associated lactic acid production was nil.

### *Immobilized-cell continuous fermentations*

Total lactic acid production rate ($P_A$) and total biomass production rate ($P_X$) in the first-stage immobilized-cell bioreactor are shown respectively in *Figures 2* and *3* as a function of dilution rate for different pH control set points. $P_A$ and $P_X$ increased with $D$ up to a maximum above 1 $h^{-1}$. $P_A$ reached up to 28.5 $g\,l^{-1}\,h^{-1}$ at pH 5.5 and a dilution rate of 1.21 $h^{-1}$, which was more than 9 times higher than the lactic acid production rate reported for an industrial free-cell batch fermentation.[4] As observed for batch data, the pH effect was limited in the 5.1–6.3 range. However, the optimum pH for lactic acid production shifted from pH 5.5 for batch fermentation to pH 6.3 for immobilized cell fermentation. Nevertheless, the optimum for biomass production was unchanged as compared to batch fermentations, i.e., pH 5.5. Lactic acid and sugar concentrations were dependent upon dilution rate ($D$). When $D$ was lowered below 0.2 $h^{-1}$, almost all sugars were consumed at all pH values except 4.3 (*Figure 4*), and lactic acid concentration was close to its maximum level of 45 $g\,l^{-1}$. However, the

**Table 2** Kinetic parameters estimated from free-cell batch fermentations of SWPM with *Lactobacillus helveticus*. Reported data are an average of three replicates

| pH | $X_0$[a] ($g\,l^{-1}$) | $X_{max}$[a] ($g\,l^{-1}$) | $\mu_{max}$[b] ($h^{-1}$) | $R^2$[c] | $m$[d] ($g\,g^{-1}$) | $n$[d] ($g\,g^{-1}h^{-1}$) | $R^2$[c] | $P_A$[e] ($g\,l^{-1}\,h^{-1}$) | $P_{SAmax}$[e] ($g\,g^{-1}\,h^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| 6.3 | 0.01 | 3.48 | 0.56 | 0.96 | 10.5 | 0.90 | 0.98 | 2.78 | 6.87 |
| 5.9 | 0.01 | 3.51 | 0.63 | 0.99 | 10.3 | 0.83 | 0.99 | 2.94 | 7.10 |
| 5.5 | 0.01 | 3.55 | 0.66 | 0.95 | 10.2 | 0.80 | 0.97 | 3.10 | 7.57 |
| 5.1 | 0.02 | 3.30 | 0.64 | 0.98 | 11.0 | 0.57 | 0.99 | 2.93 | 7.03 |
| 4.7 | 0.01 | 2.94 | 0.51 | 0.96 | 12.5 | 0.30 | 0.98 | 2.14 | 5.90 |
| 4.3 | 0.03 | 2.00 | 0.30 | 0.91 | 15.0 | 0.01 | 0.94 | 0.90 | 4.39 |

[a]Determined experimentally; [b]calculated from the model of Moraine and Rogovin,[29] using equations (1) and (2); [c]average coefficient of correlation between experimental and estimated values; [d]calculated from the model of Luedeking and Piret,[31] using equation (3); [e]calculated from modeled data

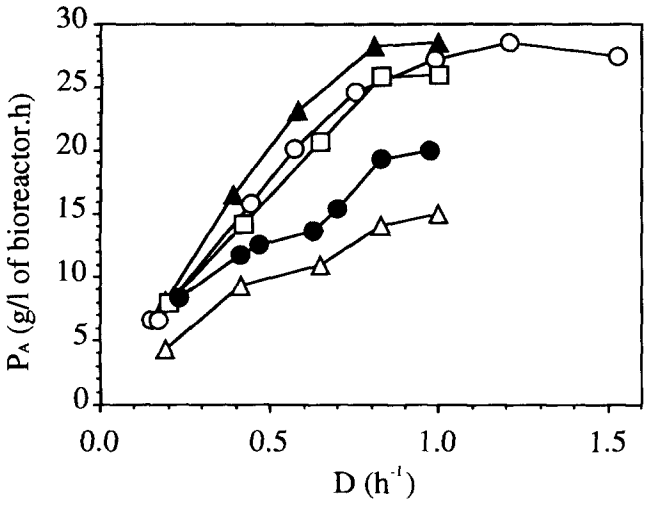


**Figure 2** Total lactic acid production rate ($P_A$) versus dilution rate ($D$) for various pH control set points in the immobilized-cell continuous bioreactor. pH 6.3 (▲), pH 5.5 (○), pH 5.1 (□), pH 4.7 (●), and pH 4.3 (△)

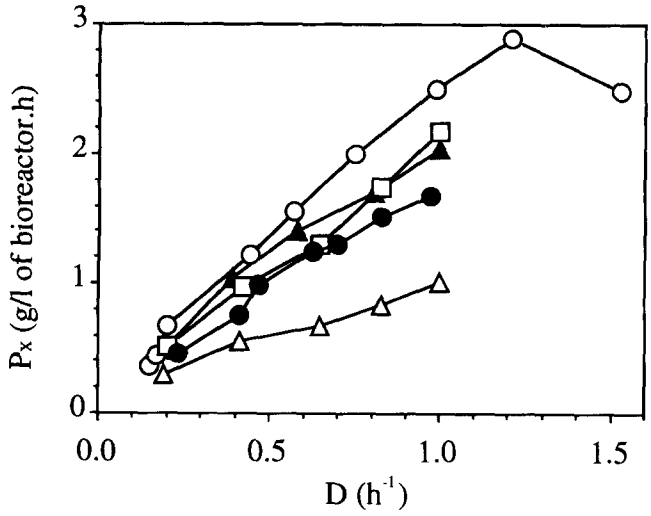


**Figure 3** Total biomass production rate ($P_X$) versus dilution rate ($D$) for various pH control set points in the immobilized-cell continuous bioreactor. pH 6.3 (▲), pH 5.5 (○), pH 5.1 (□), pH 4.7 (●), and pH 4.3 (△)

highest lactic acid concentration reached at pH 4.3 was only 24 g $l^{-1}$ at a $D$ of 0.11 $h^{-1}$, which was slightly lower than for batch fermentations at the same pH (30 g $l^{-1}$).

Biomass within the gel beads was almost constant at 125 g $l^{-1}$ of gel throughout the fermentation after a colonization phase of about 7 days.[26] This value was about 50 times higher than the average biomass concentration (2.5 g $l^{-1}$ of broth) obtained in the free-cell phase for the optimum pH. The biomass concentration in the free-cell phase of the immobilized cell bioreactor was slightly lower than the final biomass of a free-cell batch fermentation for the same pH, even at a low dilution rate (*Table 2*).

The lactic acid production rates obtained in the present study were higher than those reported for *L. helveticus* in supplemented whey permeate of similar composition. Maximum lactic acid production rates reported for free-cell continuous fermentations were 8.27[41] and 9.7 g $l^{-1}$ $h^{-1}$.[7] For a cell-recycle bioreactor, a maximum lactic acid production rate of 15.8[42] and 4.6 g $l^{-1}$ $h^{-1}$ [43] was obtained, while for alginate-entrapped systems, it was only 4[7] and 8 g $l^{-1}$ $h^{-1}$.[8] Entrapped cell processes using different substrates and strains also showed lower productivities (*Table 1*). However, the high $P_A$ obtained in the immobilized-cell system described in this paper was achieved at the expense of relatively high residual sugar concentrations in the effluent from the first immobilized-cell bioreactor (*Figure 4*). At pH 5.5, residual sugar concentration (including lactose and galactose) was 26.5 g $l^{-1}$ for the maximum $P_A$ of 28.5 g $l^{-1}$ $h^{-1}$ at a dilution rate of 1.21 $h^{-1}$. In order to attain a low residual sugar concentration of 1 g $l^{-1}$, which is acceptable for industrial production of lactic acid,[4] the dilution rate had to be reduced to 0.15 $h^{-1}$ and the resulting $P_A$ was lowered to 6.7 g $l^{-1}$ $h^{-1}$. This drawback could be eliminated by the use of a multistage bioreactor.[25]

### *Comparison of specific rates*

*Figures 5* and *6* present the evolution of the specific growth rate ($\mu$) and lactic acid production rate ($P_{SA}$) versus lactic acid concentration, for free cells in the second-stage bioreactor and free-cell batch fermentations. These specific rates were measured during continuous fermentations at pseudo-steady state, and were compared to the same rates determined during free-cell batch fermentations (unsteady state), using modeled data for the same lactic acid concentration. The variations of lactic acid concentrations corresponded to variations of dilution rate for immobilized-cell continuous fermentations and the fermentation time for free-cell batch fermentations. Except for pH 6.3, specific rates from immobilized-cell continuous fermentations

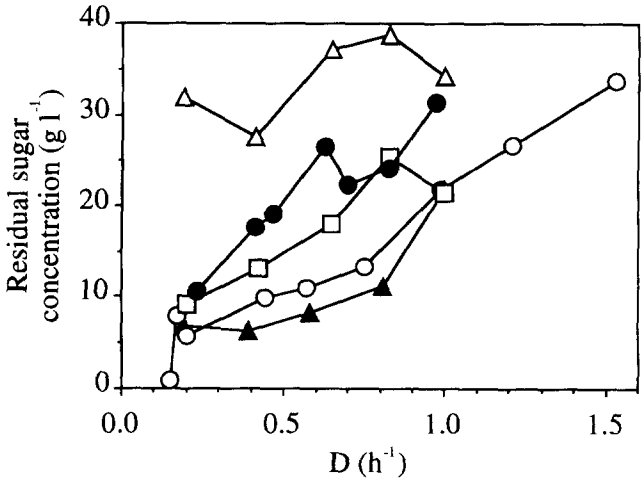


**Figure 4** Residual sugar concentration (lactose and galactose) versus dilution rate ($D$) for various pH control set points in the immobilized-cell continuous bioreactor. pH 6.3 (▲), pH 5.5 (○), pH 5.1 (□), pH 4.7 (●), and pH 4.3 (△)

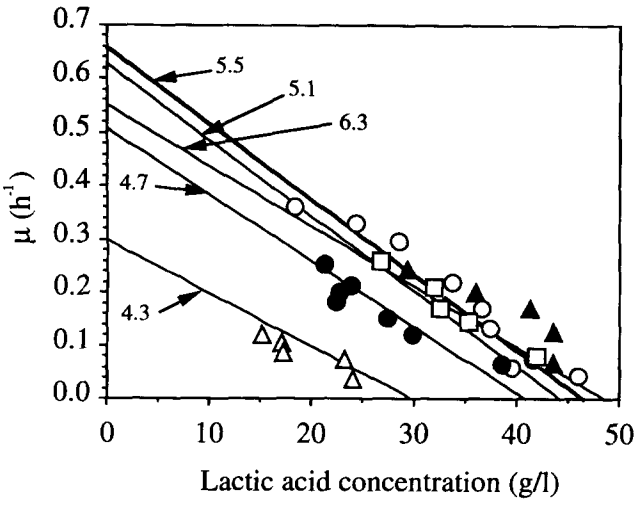


**Figure 5** Specific growth rate of *Lactobacillus helveticus* versus lactic acid concentration, in modeled free-cell batch fermentation (continuous lines) and in the free-cell phase of the immobilized-cell continuous bioreactor at pseudo-steady state (symbols), for various pH control set points. pH 6.3 (▲), pH 5.5 (○), pH 5.1 (□), pH 4.7 (●), and pH 4.3 (▲)

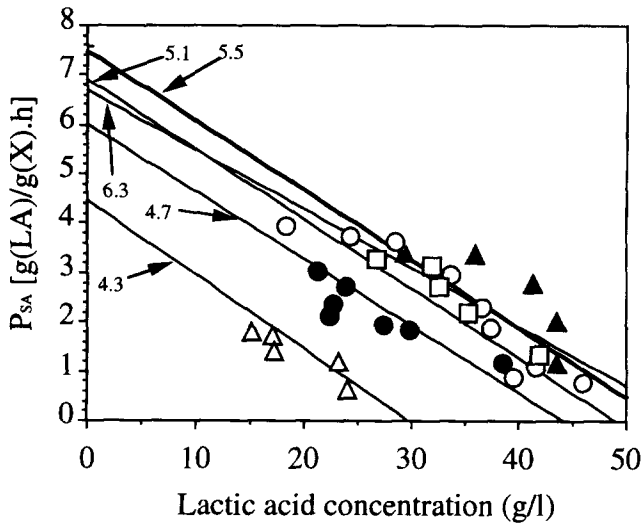


**Figure 6** Specific lactic acid production rate of *Lactobacillus helveticus* versus lactic acid concentration, in modeled free-cell batch fermentation (continuous lines) and in the free-cell phase of the immobilized-cell continuous bioreactor at pseudo-steady state (symbols), for various pH control set points. pH 6.3 (▲), pH 5.5 (○), pH 5.1 (□), pH 4.7 (●), and pH 4.3 (△)

appeared to agree well with rates determined for batch fermentations. Since whey permeate contained 48–50 g $l^{-1}$ of lactose, the maximum lactic acid concentration attainable was approximately 45 g $l^{-1}$. On the other hand, lower lactic acid concentrations could have been achieved in immobilized-cell continuous fermentations with $D$ higher than 1.6 $h^{-1}$.

A linear regression analysis was carried out on $\mu$ and $P_{SA}$ data for the free-cell batch (dependent variable) and the free-cell phase of immobilized-cell continuous fermentations (independent variable) (*Table 3*). Free-cell batch rates were calculated from equations (1) and (3) using parameters in *Table 2*, for the exact lactic acid concentration of the continuous immobilized-cell fermentation data points.[44] Correlation coefficients were generally higher for $\mu$ than for $P_{SA}$. It was interesting to note that for both rates, intercepts were not significantly different from zero ($\alpha < 0.05$). However, some slopes appeared to be significantly different from 1 (*Table 3*). This was the case with pH 4.3 for $\mu$ and $P_{SA}$, and pH 4.7 for $P_{SA}$ with slopes significantly below 1. This meant that for low pH, the free cells in the immobilized-cell bioreactor were more inhibited than free cells in batch fermentations for the same lactic acid concentration. On the other hand, slopes of pH 5.1, 5.5, and 6.3 data were greater than 1 (*Table 3*). However, due to the nonlinearity of data for pH 6.3, the only significant difference was recorded for $P_{SA}$ at pH 5.5

This displacement of pH-related activity could be due to the concomitant presence of a proton and lactic acid gradient within gel beads.[19] At low pH, there is a synergistic effect of external high lactic acid and proton concentrations on intracellular pH.[45] Therefore, the progressive buildup of both lactic acid and protons as a result of diffusional limitations within gel beads would result in a higher inhibition effect on entrapped cells than on free cells. Conversely, this accumulation of protons within gel beads would be favorable at high pH. Overall results for the immobilized-cell reactor appeared to confirm this hypothesis. Hence, the optimum for lactic acid production shifted from pH 5.5 to

**Table 3** Linear regression of specific growth and lactic acid production rates of free-cell phase in immobilized-cell bioreactor (dependent variable) versus the same parameters determined for free-cell batch fermentations at the same lactic acid concentration

| pH control set point | $\mu$ ($h^{-1}$) | | | $P_{SA}$ (g $l^{-1}$ $h^{-1}$) | | |
|---|---|---|---|---|---|---|
| | Intercept | Slope | $R^2$ | Intercept | Slope | $R^2$ |
| 6.3 | 0.056 | 1.04 | 0.767 | −0.09 | 1.32 | 0.600 |
| 5.5 | 0.004 | 1.11 | 0.909 | −0.24 | 1.05[a] | 0.839 |
| 5.1 | 0.044 | 1.02 | 0.940 | 0.45 | 1.06 | 0.899 |
| 4.7 | 0.017 | 0.90 | 0.932 | 0.62 | 0.64[a] | 0.808 |
| 4.3 | 0.006 | 0.78[a] | 0.939 | 0.26 | 0.71[a] | 0.834 |

$R^2$, Coefficient of correlation between batch and continuous data
[a]Slope significantly different from 1.0 ($\alpha = 0.001$)

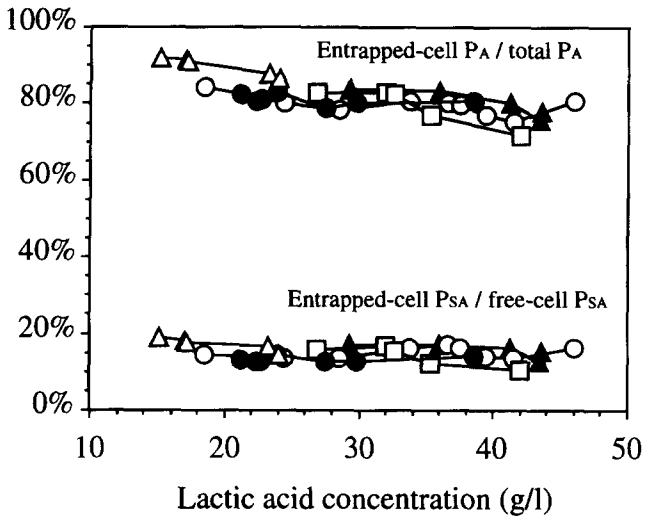


**Figure 7** Ratio of the mean specific lactic acid production rates for entrapped and free cells ($P_{SAE}/P_{SAF}$) and of lactic acid production rates for entrapped cells and the whole immobilized-cell bioreactor ($P_{AE}/P_{AT}$) as a function of lactic acid concentration at various pH control set points. pH 6.3 (▲), pH 5.5 (○), pH 5.1 (□), pH 4.7 (●), and pH 4.3 (△)

6.3 for $D$ up to 1 $h^{-1}$ (*Figure 2*). Moreover, the maximum lactic acid concentration attainable at pH 4.3 was lower for immobilized-cell than for free-cell batch fermentations, indicating an earlier inhibition.

The effects of immobilization on kinetic parameters in response to the pH were very limited. In an applied perspective, the response of the immobilized-cell system to pH variations could be considered as not significantly different from that of a free-cell system. For example, the increase in optimal pH may be considered a drawback for an industrial application of this process, since an acidic fermentation is less susceptible to contamination. Nevertheless, the effect of pH in the 5.1–6.3 range on fermentation kinetics was not very important, and any pH in this range should be considered as equivalent. Moreover, high dilution rate and low average residence time may decrease the susceptibility to contamination.

The estimated lactic acid production rate of entrapped cells ($P_{SAE}$) was 12–18% that of free cells ($P_{SAF}$) in the first-stage fermenter for all pH set points studied (*Figure 7*). Similar values were obtained for the specific growth rates (data not shown). The low overall activity of the entrapped biomass could be explained by the creation of concentration gradients of either substrates or products within the beads, which eventually resulted in the formation of a dead volume in the core of the beads and decreased the effectiveness factor.[10,21,46–48]

The low effectiveness factor of the gel beads has emphasized the importance of size reduction for those biocatalysts, in order to improve volumetric productivity of immobilized-cell bioreactors.[46] Thus, a reduction in bead size increases the surface-volume ratio and results in a higher active biomass concentration in the bioreactor. However, reducing the size of polysaccharide gel beads below 1 mm in diameter may result in separation problems at the outlet of a bioreactor operated at high $D$ (clogging of the filter) and in mechanical instability of the gel during continuous fermentations in a stirred-tank reactor.[20] Therefore, the choice of polysaccharide gel bead diameter should be the result of a compromise between productivity and separation characteristics. As a matter of fact, in spite of their low specific activity, the 1- to 2-mm beads used in this work were still responsible for 75–85% of the lactic acid production ($P_{AE}/P_{AT}$) in the immobilized-cell fermenter (*Figure 7*) and 75–90% of the biomass production (data not shown).

## Conclusion

The immobilized-cell CSTR used in this work allowed for a much higher lactic acid productivity as compared to previously reported processes (*Table 1*). The κ-carrageenan–locust bean gum mixed gel matrix showed a high stability over the 3-month continuous fermentation. Furthermore, in spite of nonsterile conditions (frequent bioreactor openings and tubing connection changes), no contamination of the bioreactor was recorded. This demonstrates that proper selection of the carrier and operating parameters can enhance the performance of an immobilized-cell bioreactor to meet the requirements of commercial fermentation. A more detailed evaluation of this process for industrial applications will be published shortly.

An immobilized-cell system for the production of primary metabolites such as lactic acid operates ideally at high dilution rates (great availability of substrate and relatively low concentration of inhibiting products). Under these conditions, it can express all its potential of high productivity but show incomplete sugar utilization. This was observed in this research where a very high productivity of up to 28.5 g $l^{-1}$ $h^{-1}$ was recorded at pH 5.5 and $D$ = 1.21 $h^{-1}$, but resulting in a high residual sugar level. Nevertheless, the development of industrial applications of immobilized-cell systems for lactic acid production would require low final residual sugar concentrations. A two-stage system was successfully used to achieve both high total productivity and low residual sugar concentration (to be reported).

Finally, the method described in this paper proved to be reliable for the measurement of both free and entrapped cell specific rates in an immobilized-cell continuous fermentation, over a wide range of dilution rates. In this research, the specific lactic acid production and growth rates of the free cells in the immobilized-cell bioreactor were close to those determined in free-cell batch fermentations, indicating that released cells were not physiologically altered by immobilization, as compared to free cells. The optimal pH of entrapped *L. helveticus* was close to the pH 5.5 determined for batch fermentation.

## Acknowledgements

This work was supported by the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (government of Québec). Special thanks to Céline Paquin for her precious help and advice.

## Nomenclature

CSTR continuous stirred-tank reactor
$D$ dilution rate, $h^{-1}$

| | |
|---|---|
| *gal* | galactose concentration, $g\,l^{-1}$ |
| *glu* | glucose concentration, $g\,l^{-1}$ |
| *LA* | lactic acid concentration, $g\,l^{-1}$ |
| *lac* | lactose concentration, $g\,l^{-1}$ |
| $lac_0$ | initial lactose concentration, $g\,l^{-1}$ |
| $lac_{0E}$ | initial lactose concentration predicted from equation (5), $g\,l^{-1}$ |
| $\mu$ | specific growth rate, $h^{-1}$ |
| $\mu_{max}$ | maximum (initial) specific growth rate, $h^{-1}$ |
| $m$ | growth-associated lactic acid production constant, $g(LA)\,g(X)^{-1}$ |
| $n$ | non-growth-associated lactic acid production constant, $g(LA)\,g(X)^{-1}\,h^{-1}$ |
| $P_A$ | lactic acid production rate, $g(LA)\,l^{-1}\,h^{-1}$ |
| $P_{SA}$ | specific lactic acid production rate, $g(LA)\,h^{-1}\,g(X)^{-1}$ |
| $P_{SAmax}$ | maximum specific lactic acid production rate, $g(LA)\,h^{-1}\,g(X)^{-1}$ |
| $P_X$ | biomass production rate, $g(X)\,l^{-1}\,h^{-1}$ |
| $R_{LA/X}$ | lactic acid/biomass ratio, $g(LA)\,g(X)^{-1}$ |
| SWPM | supplemented whey permeate medium |
| $t$ | time, h |
| $t_0$ | lag time, h |
| $V$ | volume, l |
| $X$ | biomass concentration, $g\,l^{-1}$ |
| $Y_{P/S}$ | product yield, [(*LA* produced) (*lac* consumed)$^{-1}$] |
| Indices | |
| 1 | first-stage immobilized-cell bioreactor |
| 2 | second-stage analytical bioreactor |
| F | free-cell phase of the first-stage immobilized-cell bioreactor |
| E | entrapped-cell phase of the first-stage immobilized-cell bioreactor |
| T | value for the whole immobilized-cell bioreactor |

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