Development of a Spiral Mesh Bioreactor with Immobilized Lactococci for Continuous Inoculation and Acidification of Milk[1]

FLAVIA M. L. PASSOS and HAROLD E. SWAISGOOD[2]
Southeast Dairy Foods Research Center
North Carolina State University
Raleigh 27695-7624

ABSTRACT

A laboratory-scale bioreactor with lactococci immobilized in calcium alginate gel was developed for continuous acidification and inoculation of milk. Cells were entrapped in a calcium alginate film coating a spiral mesh and placed in a column through which milk was recirculated from a reservoir. Steady-state conditions were achieved by addition of fresh milk using a pH controller to maintain the pH at 5.7 and acidified milk was continuously removed during operation periods up to 5 d. Immobilized and free cell bioreactors were compared using both proteinase-positive and proteinase-negative strains of *Lactococcus lactis* ssp. *lactis* C2. Productivities were 1.5- to 3.5-fold larger with immobilized cell bioreactors than with free cell bioreactors because of higher cell densities, although specific productivities were lower for immobilized cells. Productivity increase was larger for proteinase-negative cells, which do not grow as well as free cells in milk. However, high densities can be immobilized, resulting in productivities of immobilized proteinase-negative cells that were similar to those of proteinase-positive cells. Free proteinase-negative cells responded to amino acid and peptide supplementation by increasing productivity (5-fold), but the immobilized cells did not respond proportionally, suggesting that free cell activity was limited by substrate availability but that immobilized cells were limited by product inhibition.

(**Key words**: immobilized lactococci, continuous acidification, immobilized cell bioreactor, continuous inoculation)

Abbreviation key: **FCB** = free cell bioreactor, **Lac$^+$** = lactose-positive, **Prt$^-$** = proteinase-negative, **Prt$^+$** = proteinase-positive, **RSM** = reconstituted skim milk, **SMICRCB** = spiral mesh immobilized cell recirculating column bioreactor.

INTRODUCTION

A potential advantage of cell immobilization in traditional fermentation processes is the possibility of increasing cell population per unit of volume beyond that allowed by the growth rate of free cells, which is limited by the environment in both transient batch or in continuous processes. In continuous processes, steady-state cell density is dictated by the dilution rate, which, to avoid washing the culture out with the effluent, cannot be higher than the maximum growth rate of the cells. Cell immobilization potentially can uncouple dilution rate from growth rate, thus permitting continuous flow operation of a reactor at high cell density and higher volumetric reaction rate than a chemostat. This concept has been exploited in a variety of fermentation processes (11, 12, 16, 23).

Entrapment of cells in calcium alginate beads has been the most common immobilization technique because of maintenance of high cell viability and high cell concentration per gram of support. However, the success of this method depends on compensating for mass transfer limitations associated with alginate

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[2]Corresponding author.

gels by increasing the loading of biocatalyst in the bioreactor.

Continuous milk preacidification and inoculation for the processing of fermented milk products with immobilized lactic acid cultures has been studied. Although some results have been satisfactory (28, 29), difficulties with bioreactor performance (20) and loss of cell-specific activity (25, 29, 35) have been reported. Several factors affecting successful immobilization of lactic cultures, such as severe product inhibition because of limited diffusion of lactate from the matrix, have not been addressed with milk cultures because some factors governing growth and activity of suspended lactic cultures in milk have not been completely established (5, 17). Immobilized cells have been regarded as self-regenerating and self-proliferating biocatalysts (22), and immobilization of lactic cultures has been considered to be an alternative to the use of frozen and lyophilized inocula (30).

To achieve the full advantage of cell immobilization for cultured milk processes, we have designed a system that specifically takes into account the properties of both milk and the fermenting cells. This study describes the development and characterization of a laboratory-scale immobilized cell bioreactor for continuous acidification of milk intended for manufacturing cultured milk products.

MATERIALS AND METHODS

Bacteria and Culture Conditions

Lactose-positive (Lac^+), proteinase-positive (Prt^+) *Lactococcus lactis* ssp. *lactis* C2 and its spontaneous Lac^+, proteinase-negative (Prt^-) derivative C2S (21) were obtained from T. R. Klaenhammer (Department of Food Science, North Carolina State University, Raleigh). The cells were cultured in M17 broth at 30°C (37) with .5% lactose and stored in the same medium containing 20% glycerol at –20°C.

Preparation of Cultures for Immobilization

The calcium alginate biocatalysts were prepared according to the following procedure. Cells were propagated overnight (22°C) in 11% reconstituted skim milk (RSM) (previously steamed for 45 min), inoculated (1%) into 60 ml of milk, and incubated for 4 h at 30°C. Milk proteins were solubilized by addition of 120 ml of ice cold 1% Na_2-EDTA, pH 10. Cells were harvested by centrifugation (6000 × *g* for 10 min at 4°C), washed twice with 200 m*M* potassium phosphate buffer, pH 7, and resuspended 1:12 (vol/vol) in .1% peptone dissolved in water. This cell suspension was mixed with 100 to 500 ml of a 4% solution of sodium alginate (Protanal LF 10/60; Protan a/s, Drammen, Norway) that had been previously steamed at 100°C for 30 min. Total viable colony-forming units in the alginate-cell mixture were enumerated in Elliker's agar (Difco Laboratories, Detroit, MI).

Cell Immobilization and Effect of Surface Area

Lactococcus lactis ssp. *lactis* cells were entrapped in small beads (2-mm diameter), large beads (4-mm diameter), or in a layer of alginate gel that coated a 20.5-cm^2 stainless steel mesh with .89-mm openings. The same weight of the mixture (alginate and cells) required to coat the mesh (i.e., .56 g) was used to prepare small and large beads. Small beads were formed by dropwise addition of the alginate and cell mixture into a 2% $CaCl_2$ solution using a 3-cc syringe with 23-gauge needle held close to the surface of the solution. Large beads were prepared similarly, except that a Pasteur pipet was held 5 cm above the $CaCl_2$ solution instead of the needle. The gel film was prepared; the mesh was dipped into the alginate and cell mixture to form an adhering liquid film that was subsequently gelled by being dipped into a 2% $CaCl_2$ solution. The biocatalysts were incubated in 30 ml of 11% RSM at 30°C with shaking in a thermostated water bath (Blue M Electric Company, Blue Island, IL). To minimize the activity of cells that were released from the biocatalyst, milk was removed every 30 min and replaced with fresh milk at pH 6.6. The pH decrease over each 30-min interval was recorded.

Bioreactor Development

Initially, different bioreactor configurations were examined, including packed beds and fluidized beds of 2-mm immobilized cell beads and a coated stainless steel mesh cylinder attached to a stirring rod. None of these configu-

rations operated satisfactorily in milk, primarily because of the inability to achieve a high ratio of surface area to bioreactor volume without clumping of the beads because of milk clotting.

Therefore, a spiral stainless steel cylindrical mesh (408-cm^2 surface area) coated with a thin film of calcium alginate-immobilized cells was characterized in this study. The cylindrical spiral mesh with the film of immobilized cells was inserted into a column [spiral mesh, immobilized cell recirculating column bioreactor (**SMICRCB**); see (Figure 1] that was placed external to a 250-ml stirred reservoir from which milk was recirculated through the column at a rate of 1 L/min. The spiral mesh was coated with a thin film of calcium alginate-immobilized cells as the mesh was dipped into a sodium alginate and cell mixture to form an adhering liquid film, drained of the excess liquid, and the film subsequently gelled by immersion in 2% $CaCl_2$ solution. The gel was allowed to equilibrate in the $CaCl_2$ solution for 20 to 30 min to ensure mechanical stability.

Steady-state pH in the SMICRCB was maintained using a dual channel pH controller (model 4000-120-60; New Brunswick Scientific Co., Inc., Edison, NJ) connected to an Ingold pH electrode (Wilmington, MA) that emits an on-off signal to a feedstock peristaltic pump that controls the rate of dilution with fresh milk (11% RSM steamed 45 min to simulate pasteurization), thus maintaining the pH. The SMICRCB volume was fixed by using a continuously operating peristaltic pump connected to the outflow tube (Figure 1). Prior to steady-state lactic acid production and release of free cells from the calcium alginate surface, the immobilized cells grew to fill the gel pore volume; this period typically required 24 to 30 h. After steady state was reached, the flow rate through the SMICRCB was constant, and the SMICRCB could be operated without the pH controller while the preset pH was maintained. Typically, these SMICRCB were operated continuously for roughly 60 h; however, they were sometimes operated continuously for 5 d. Continuous operation of the entire system was regulated by the pH controller set at pH 5.7. The other pH controller channel was connected to free cell bioreactor (**FCB**) operated as a pH stat for continuous acidification of milk with a suspended free cell culture. Thus, SMICRCB and FCB were set to operate simultaneously to produce acidified milk under identical conditions at 30°C.

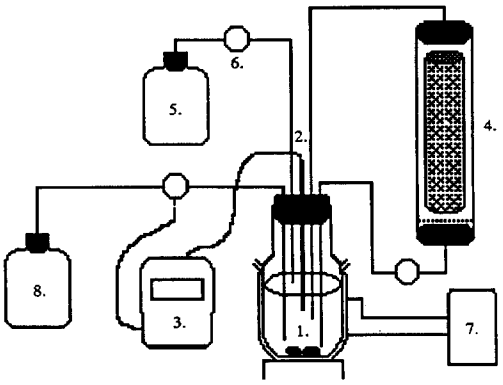


Figure 1. Schematic diagram of the spiral mesh immobilized cell recirculating column bioreactor 1) stirred flask, 2) pH electrode, 3) pH controller, 4) bioreactor column and immobilized cell spiral, 5) milk feedstock, 6) peristaltic pump, 7) water bath, and 8) acidified and inoculated milk.

Characterization of the SMICRCB

The Lac^+ Prt^-, *L. lactis* ssp. *lactis* C2S was cultured in 500 ml of Elliker broth. At late exponential growth phase, the culture was centrifuged (8000 rpm, 15 min, 4°C) and resuspended in 20 ml of 1% sterile peptone water. An inoculum of .5% concentration from a 100-fold dilution was introduced into a pH stat FCB fed with 11% RSM. The remaining 19-ml cell suspension was mixed with 500 ml of alginate solution. A film of the cells entrapped by calcium alginate was formed on a 408-cm^2 spiral mesh as previously described. The spiral mesh was placed in the SMICRCB, where RSM was continuously acidified under identical conditions of pH and temperature. To determine whether the activity of immobilized Prt^- cells was affected by substrate limitation or product inhibition, FCB and SMICRCB were also operated with RSM supplemented with .25% casamino acids (Difco Laboratories).

Growth Kinetics of *L. Lactis* ssp. *lactis* C2 in Milk

The specific growth rate (μ) of *L. lactis* ssp. *lactis* C2 in milk was determined in batch and

continuous fermentation processes at various concentrations of lactic acid. For batch processes, cells previously grown in milk were inoculated to about 10^3 cfu/ml in 50-ml samples of RSM containing 0, .45, .90, 1.57, and 3.15 g/L of lactic acid. Every 30 min, colony-forming units were determined in Elliker agar. During 4-h incubations, the pH conditions were unchanged because of the low inoculum. The initial growth rate was determined by linear regression analysis of a logarithmic plot of colony-forming units per milliliter versus time. In continuous processes, an SMICRCB connected to a pH controller was operated at various pH set points. At steady state, the dilution rate was recorded as the specific growth rate. Data collected from batch experiments were used to determine the growth rate equation.

Analytical Methods and Cell Counting

At steady-state conditions, i.e., when the effluent pH and dilution rate were constant, samples were withdrawn from the SMICRCB and analyzed for lactose, lactate, acetate, primary amino group concentration, and viable cell counts. Lactose and organic acids were determined by HPLC with an ion-exchange column (Bio-Rad HPX-87H, Richmond, CA) using a refractive index detector with .01*N* H_2SO_4 as eluent at a flow rate of .8 ml/min. Milk samples (.5 ml) were mixed with .5 ml of acetonitrile, centrifuged at 7000 × *g* for 5 min, and filtered with a .45-μm filter (Acrodisc, Gelman, Ann Arbor, MI) before injection into the system. The concentration of primary amino compounds was measured by the *o*-phthaldialdehyde spectrophotometric assay as described by Church et al. (10). Calcium alginate gels were solubilized by exposure to .1 *M* EDTA, and viable colony-forming units per milliliter were determined on Elliker agar. Preliminary experiments indicated that this treatment did not affect cell viability.

RESULTS

Bioreactor Development and Preliminary Characterization

Figure 2 shows the effect of surface area of the biocatalyst on the specific rate of pH decrease in milk that is due to fermentation by *L. lactis* ssp. *lactis* C2 entrapped in small beads (2-mm diameter), large beads (4-mm diameter), and in a layer of alginate gel that coated a 20.5-cm^2 stainless steel mesh. The rate of pH drop increased during the first 8 to 10 h. Subsequently, a steady state was reached; the pH decrease per hour was constant, and the biocatalyst had attained its maximum activity

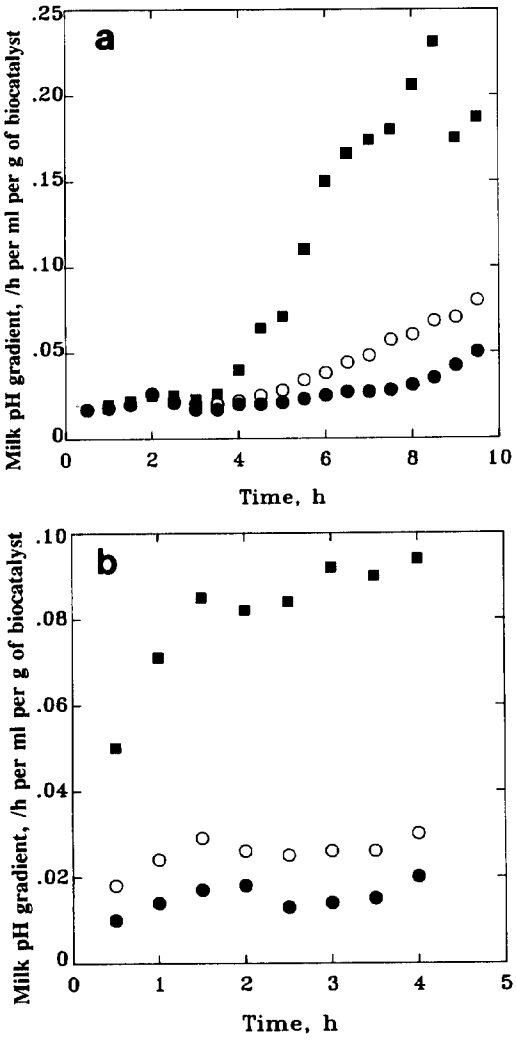


Figure 2. Effect of the surface area of the biocatalyst on the specific rate of pH decrease that is due to fermentation by *Lactococcus lactis* ssp. *lactis* C2 immobilized in 2-mm diameter beads (○) (13.3 cm^2), 4-mm beads (●) (8.6 cm^2), and .89 mm open-width mesh (■) (41 cm^2). a) Immediately after cell immobilization and b) after an activation time (12 h) in milk at 22°C.

(Figure 2b.) The mesh exhibited the greatest activity, followed by that of 2- and 4-mm beads, respectively. This behavior was attributed to the higher surface area of the mesh because the activity per unit of surface area proved to be the same for all three biocatalysts: $.8 \times 10^{-3}$ pH units/h per ml per cm^2 for large beads, 1.0×10^{-3} pH units/h per ml per cm^2 for small beads, and 1.2×10^{-3} pH units/h per ml per cm^2 for the mesh at steady state. Characteristics of the three biocatalysts used in this experiment are listed in Table 1. The cells grew in the gel such that an initial cell population of approximately 10^6 increased to a final cell population of approximately 10^8. The final immobilized cell population in the large beads was about 2-fold lower than that in the small beads or in the film on the mesh; the latter two were very similar.

Both fluidized and packed bed reactors with gel beads proved to be infeasible for operation in milk because of their inability to retain beads in the reactor with the fluidized bed and because of milk clotting and varied fluid flow with the packed bed. The cylindrical mesh attached to a stirring rod did not provide a sufficient ratio of biocatalyst surface area to bioreactor volume to yield a dilution rate higher than the growth rate (24). Therefore, the SMICRCB was designed and characterized in this study.

Characterization with Lac+ Prt+ *L. lactis* ssp. *lactis* C2

Steady-state characteristics of the SMICRCB and FCB operating under identical conditions are shown in Table 2. The SMICRCB allowed operation with a dilution rate that was higher than the growth rate of the freely suspended culture, which is numerically equal to the dilution rate of free cells in continuous culture. Immobilization provided for maintenance of about 100 times more cells in the SMICRCB than in a suspended continuous culture. However, the total gain in productivity with the SMICRCB was only 1.5-fold higher than with the FCB. Hence, specific productivity of *L. lactis* ssp. *lactis* C2 appeared to be dramatically affected by immobilization. If the productivity of free cells in the SMICRCB was assumed to be equal to the productivity of cells in an FCB, then, in a freely suspended state, free cells produce about 45 times more acid than when in an immobilized state. Lactose and organic acid analyses of samples at steady state indicated that only about 2.5% of the lactose in milk, present at concentrations of 48 to 52 g/L, was consumed by the SMICRCB. Lactate was the primary end product of the fermentation.

Growth Kinetics of Lactococci in Milk

To understand better the behavior of immobilized *L. lactis* ssp. *lactis* C2, the kinetics of freely suspended cells in milk was investigated (Figure 3). The specific growth rate was determined at different lactate concentrations. The results indicate that growth of *L. lactis* ssp. *lactis* C2 in milk was limited by product formation. Data were fitted to a linear relationship that gave

$$\mu = 1.33(1 - .2207 \times P)$$

($R^2 = .968$), where .2207 is an inhibition constant representing the reciprocal of the terminal acidity (i.e., the acid concentration that stops *L. lactis* ssp. *lactis* activity: $\mu = 0$), P is the product concentration in grams per liter of lactic acid, and 1.33/h is the maximum growth rate.

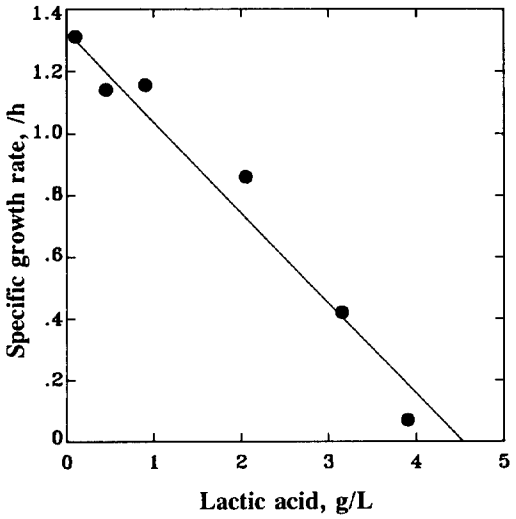
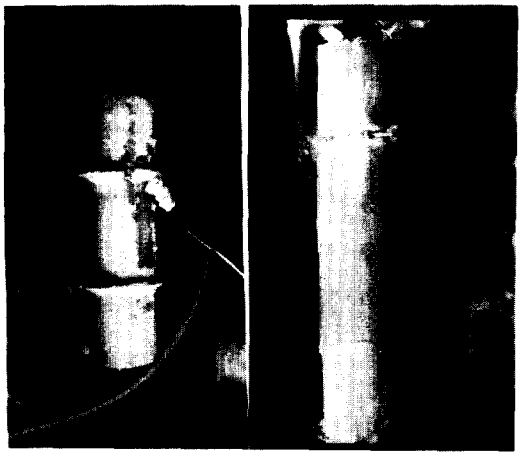


Figure 3. Growth kinetics of *Lactococcus lactis* ssp. *lactis* C2 in milk.

SMICRCB: Characterization with Prt⁻ *L. lactis* ssp. *lactis* C2S

After 4 d of continuous operation with the Prt⁺ strain, clotting of milk protein was observed on the spiral-mesh surface, resulting in formation of an extra layer over the calcium alginate film (Figure 4). To eliminate this problem, we examined the performance of the Lac⁺ Prt⁻ derivative C2S in the same bioreactor. Formation of the protein precipitate was not observed with the Prt⁻ variant of *L. lactis* ssp. *lactis* C2S. Steady-state characteristics of the SMICRCB with Prt⁻ cells are presented in Table 3. The dilution rate of the SMICRCB running with the Prt⁻ strain was 3-fold higher than that of the FCB. Moreover, the gain in bioreactor productivity with immobilization of the Prt⁻ variant of *L. lactis* ssp. *lactis* C2S was higher than that previously noted for the Prt⁺ strain; approximately a 4-fold increase. The specific productivity of free cells was still larger than that for immobilized cells, but the difference was not as great as that for *L. lactis* ssp. *lactis* C2 (4-fold vs. 45-fold); compare Tables 2 and 3.

The activity of *L. lactis* ssp. *lactis* C2S in milk is limited by the amount of free amino acids present in the milk. Supplementation of milk with a mixture of amino acids and peptides (at .25%) increased the productivity of SMICRCB and FCB, but not to the same extent. The productivity of the FCB increased about five times; the productivity of SMICRCB doubled (Table 4; Figure 5). The supplementation had a direct effect on the population and activity of free cells. However, although the cells grew to a higher maximum cell population inside the gel, supplementation did not have a further positive effect on the specific activity of the immobilized cells. As shown in Figure 6, supplementation of RSM with .25% amino acids and peptides more than doubled the specific productivity of the free



A B

Figure 4. Spiral mesh after 4 d of continuous operation in the spiral mesh, immobilized cell recirculating column bioreactor. A) *Lactococcus lactis* ssp. *lactis* C2 (proteinase-positive) B) *Lactococcus lactis* ssp. *lactis* C2S (proteinase-negative).

TABLE 1. Characteristics of the biocatalysts inoculated in 30 ml of milk.

Parameter	Large beads	Small beads	Mesh
Mass, g	.560	.560	.560
Number of beads	18	133	. . .
Diameter,[1] mm	3.9	1.8	. . .
Film thickness,[2] μm	. . .	. . .	273
Area,[3] cm²	.478	.100	20.5
Total area, cm²	8.6	13.3	41.0
Relative area	1.00	1.5	4.7
Initial immobilized cells, cfu/g	5.4×10^6	5.4×10^6	5.4×10^6
Final immobilized cells, cfu/g	3.8×10^8	9.4×10^8	8.3×10^8

[1]Estimated from $V = N(4/3)\pi r^3$ and a density of 1.0 g/ml for a gel made from 3.6% alginate, determined experimentally by measuring the volume displaced by a weighed amount of drained beads. N is the number of beads, and V is the total volume.

[2]Estimated from V = area × thickness.

[3]Estimated area for beads from $S = 4\pi r^2$.

TABLE 2. Steady-state parameters for immobilized and free cell proteinase-positive lactococci bioreactor systems in the continuous acidification of milk.

Parameter	Immobilized cells[1]	Free cells
Volume, L	.45	.30
Flow rate, L/h	.42	.18
Dilution rate, /h	.93	.60
Free cells,[2] cfu/ml	3.0×10^8	6.0×10^8
Immobilized cells,[2] cfu/ml	2.8×10^{10}	. . .
Total cells, cfu	1.3×10^{13}	1.8×10^{11}
Bioreactor productivity,[2,3] g/h per L	1.46	.94
Specific productivity $\times 10^{10}$ mg/cfu per h		
Total	.51	15.7
Immobilized[4]	.35	
Lactate,[2] g/L	1.57	1.57
α-NH_2, m*M*	2.4	1.7

[1]*Lactococcus lactis* ssp. *lactis* C2 immobilized in a calcium alginate film formed on a stainless steel spiral mesh (420 cm^2).

[2]Volume in these calculations refers to the total bioreactor volume.

[3]Productivity in grams of lactate per hour per liter of milk.

[4]Calculated by subtracting the productivity contribution of free cells from the total bioreactor productivity assuming the same specific productivity for free cells as observed in the free cell bioreactor.

cells. However, upon supplementation, the specific productivity of the immobilized cells was even lower (1.7 times) than that without supplementation. Remarkably, an extra layer over the gel surface formed when milk was supplemented with amino acids and peptides similar to that formed with the Prt^+ *L. lactis* ssp. *lactis* C2.

DISCUSSION

A period of initial growth of entrapped cells inside calcium alginate beads has been previously reported (8, 19, 27). An increase in the rate of pH drop in milk by immobilized lactococci during the first 8 to 10 h reflects this growth. After this activation period, a steady

TABLE 3. Steady-state parameters for immobilized and free cell proteinase-negative lactococci bioreactors in the continuous acidification of milk.

Parameter	Immobilized cells[1]	Free cells
Volume, L	.510	.185
Flow rate, L/h	.40	.042
Dilution rate, /h	.78	.23
Free cells,[2] cfu/ml	3.1×10^8	3.4×10^8
Immobilized cells,[2] cfu/ml	3.5×10^9	. . .
Bioreactor productivity,[2,3] g/h per L	1.13	.32
Specific productivity $\times 10^{10}$ mg/cfu per h		
Total	2.96	9.6
Immobilized[4]	2.38	. . .
Lactate,[2] g/L	1.44	1.44
α-NH_2, m*M*	.96	.82

[1]*Lactococcus lactis* ssp. *lactis* C2S immobilized in a calcium alginate film formed on a stainless steel spiral mesh (408 cm^2).

[2]Volume in these calculations refers to the total bioreactor volume.

[3]Productivity in grams of lactate per hour per liter of milk.

[4]Calculated by subtracting the productivity contribution of free cells from the total bioreactor productivity assuming the same specific productivity for free cells as observed in the free cell bioreactor.

state was reached in which the pH decrease per hour was constant and the biocatalyst had attained its maximum cell density. Biocatalysts with different ratios of surface area to volume achieve similar final cell populations; however, some of the immobilized cells in lower surface area beads are not as active as when they are in gel films with a higher surface area, suggesting that limitations of substrate, product mass transfer, or both occur throughout the gel matrix in the lower surface area biocatalyst. This conclusion is in agreement with the concept that cells on the gel surface, which have easy access to substrate, grow faster, thus hindering substrate from entering and the product from leaving deeper regions of the alginate beads (19, 26, 27). The estimated thickness of the calcium alginate film coating the mesh was roughly 400 μm. Because both surfaces of the film have contact with milk feedstock, the maximum mass transfer depth for each surface is 200 μm, which is within the range of 50 to 200 μm reported (26, 27, 32, 36) to be the outer layer thickness of alginate beads where activity of immobilized cells is observed.

The primary advantage of cell immobilization is the increase in volumetric productivity by increased cell population because cell population is limited in a suspended cell, continuous culture by the specific growth rate of the cells used. The activity of immobilized cells not only depends on a higher population, but also on the proximity of the cells to the surface such that mass transfer is facilitated. Growth of immobilized cells near the biocatalyst surface and increased activity with reduction of bead diameter have been widely reported (9, 31, 33).

To compensate for diffusion limitations and to obtain higher volumetric productivity for calcium alginate beads, a large amount of biocatalyst should be loaded in a bioreactor (42). Consequently, fixed bed reactors have been suggested (41, 42) as a first choice. However, preliminary analyses in this study confirm previous reports (3, 20) that fixed bed reactors have limited application with milk as a feedstock because of clotting of milk protein, which restricts fluid flow. The fluidized bed reactor could be an alternative if the density of the alginate beads could be increased so that they become greater than the density of milk. Nevertheless, as with a continuous stirred tank reactor, a very high ratio of gel volume to fluid volume is not feasible in the fluidized bed reactor. Immobilizing cells in a thin alginate-cell film on a mesh not only reduces diffusion limitations, but also permits more possibilities for bioreactor configuration. The SMICRCB containing entrapped cells in a film coating a stainless steel spiral mesh allowed higher ra-

TABLE 4. Steady-state parameters for immobilized and free cell proteinase-negative lactococci bioreactors in the continuous acidification of milk supplemented with .25% casamino acids.

Parameter	Immobilized cells[1]	Free cells
Volume, L	.37	.19
Flow rate, L/h	.439	.161
Dilution rate, /h	1.187	.849
Free cells,[2] cfu/ml	1.5×10^8	7.5×10^8
Immobilized cells,[2] cfu/ml	1.5×10^{10}	. . .
Bioreactor productivity,[2,3] g/h per L	2.37	1.69
Specific productivity $\times 10^{10}$ mg/cfu per h		
Total	1.57	22.6
Immobilized[4]	1.37	
Lactate,[2] g/L	2.0	2.0

[1]*Lactococcus lactis* ssp. *lactis* C2S immobilized in a calcium alginate film formed on a stainless steel spiral mesh (408 cm^2).

[2]Volume in these calculations refers to the total bioreactor volume.

[3]Productivity in grams of lactate per hour per liter of milk.

[4]Calculated by subtracting the productivity contribution of free cells from the total bioreactor productivity assuming the same specific productivity for free cells as observed in the free cell bioreactor.

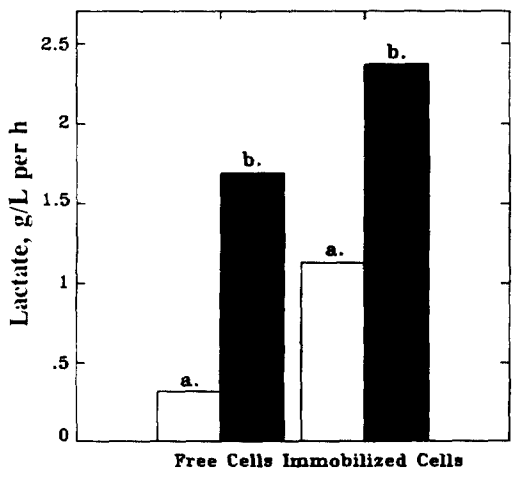


Figure 5. Lactate productivity of immobilized and free cell lactococci bioreactors in the continuous acidification of 11% reconstituted skim milk (RSM) (a) and RSM supplemented with .25% casamino acids (b).

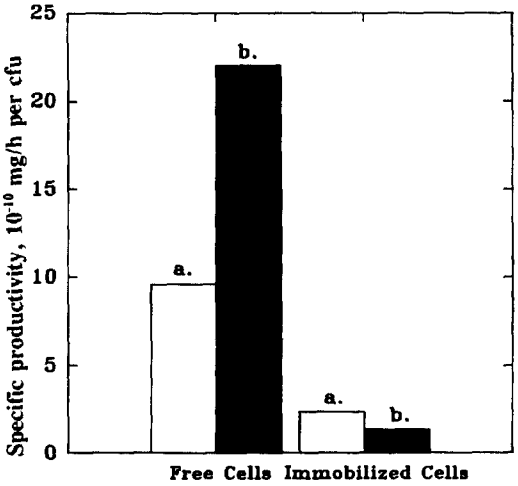


Figure 6. Specific lactate productivity of immobilized and free cell lactococci bioreactors in the continuous acidification of 11% reconstituted skim milk (RSM) (a) and RSM supplemented with .25% casamino acids (b).

tios of surface to volume and operation with dilution rates higher than the growth rate of lactococci suspended in milk. Productivity of free cells in the SMICRCB is small compared with that of the immobilized cells. The high volumetric productivity is provided by a high cell population in the surface layer on the spiral mesh. Productivity in either the SMICRCB or the FCB can be increased by increasing cell numbers. To increase cell numbers in an FCB, the volume must be increased because the number of cells per unit of volume is constant and is a function of the specific growth rate of that particular strain under the defined conditions. However, in an immobilized cell system, the surface layer cell population does not depend on the specific growth rate. Hence, cell populations per unit of bioreactor volume can be immobilized that are higher than that population given by the defined conditions in a continuous suspended culture. The superficial population of immobilized cells is limited only by the reactor configuration. Present results indicate that the spiral mesh is a promising choice because the working volume of the SMICRCB could be reduced in order to more than double the achieved volumetric productivity.

The specific productivity of immobilized cells was low compared with that of free cells, suggesting that mass transfer limitation still existed. In this case, low specific productivity was probably due to limitation of lactate diffusion, thus concentrating lactate inside the gel and inhibiting the activity of the cells as characterized by product inhibition kinetics. Cells that exhibit this characteristic are most susceptible to influence of product diffusion limitations in immobilized forms. The kinetic equation obtained for *L. lactis* ssp. *lactis* C2 growing in milk may be used to describe a batch or continuous milk fermentation with suspended and with immobilized cells, because no evidence indicates that physiological alteration occurs beyond that manifested by the higher local acid concentrations inside the gel. The HPLC chromatograms did not reveal any other organic acid in samples withdrawn from the SMICRCB under steady-state conditions, which indicates that the cells remain homofermentative and that a switch of the main carbohydrate pathway did not occur, as would be expected under limiting sugar conditions (39). Other investigators (2) have reported a similar conversion of lactose to lactate by both free and immobilized lactococci.

A possible limitation of the SMICRCB with *L. lactis* ssp. *lactis* C2 for preacidification and inoculation of milk is the buildup of a clotted

milk film over the alginate gel after 3 d of continuous operation. This film most likely results from a combination of a localized higher lactate concentration near the gel surface; the resulting pH is close to the isoelectric point of casein, and the proteolytic activity of the cells on the surface is high. However, proteolytic activity of cells within the gel is expected to be low because casein micelles cannot penetrate the pores of alginate gels (34, 35).

The formation of a protein layer on the beads also has been reported (1, 20). In the spiral mesh, formation of the proteinaceous layer did not cause reduction in the SMICRCB productivity during the period of observation. However, productivity probably increased because of expansion of a highly active associated cell layer resulting from inclusion of cells in a porous protein clot. Nevertheless, formation of the protein cell film could become a limitation if flow through the SMICRCB is compromised by continued buildup of the layer. Bioreactors that form films may have a schedule of intermittent operation with periodic cleaning of the mesh similar to that used by Linko (25) in which the bioreactor was operated daily and then cleaned, and the biocatalyst was washed and stored overnight with a 5% lactose solution circulating at room temperature.

A film of clotted protein did not form on the spiral mesh with immobilized *L. lactis* ssp. *lactis* C2S, a Lac^+, Prt^- strain. In spite of the low growth rate of these cells and their low productivity in the continuous acidification of milk by free cells, immobilization provides a means to achieve high volumetric productivity. The immobilized Prt^- cells exhibit a high productivity that resembles Prt^+ productivity. The specific productivity of the immobilized cells is still lower than that of free cells, perhaps because of limiting free amino acids available in milk to support growth of a high population of immobilized cells, to product diffusion limitations, or both. For the Prt^- strain, immobilization evidently resulted in a change from substrate limitation kinetics to product inhibition kinetics. At the higher dilution rate allowed by the immobilized system, amino acids are not limiting because cell production in beads by immobilized Prt^- cells resembles that of immobilized Prt^+ cells. Reduction of nutritional limitations by increasing the dilution rate also occurred in high cell density cultures of *Lactobacillus casei* (18) and *Lactococcus cremoris* (4, 5).

However, supplementation of milk with amino acids and peptides resulted in overgrowth of the surface-immobilized cells and an internal region of high lactic acid concentrations, thus accentuating product inhibition and decreasing the specific productivity of the immobilized cells. Nevertheless, high cell populations compensate for lower specific productivity. In addition, although lactate is a growth-associated product, the immobilizing conditions favor uncoupling of growth from acid production as noted (6, 7, 13, 15, 38, 40) in acidic and other stressful conditions.

Upon supplementation of the milk, a film also formed with Prt^- *L. lactis* ssp. *lactis* C2S, although the film was thinner and more sandy than that formed with the Prt^+ strain. This film could result from a high activity of the surface cells because of availability of amino acids and peptides, thus yielding larger pH gradients and resultant isoelectric precipitation of casein on the surface. The film would, in turn, hinder product diffusion and eventually culminate in an overall decay of bioreactor performance.

The Prt^- lactococci are favored for immobilization for continuous milk acidification because they can be maintained at high cell populations to ensure high volumetric productivity and minimal growth, which are ideal conditions previously recommended for cell immobilization (22). In addition, Prt^- lactococci have been frequently associated (14, 38) with desired characteristics in cultured milk products, such as reduction of bitter flavor and increases in cheese yield, that are due to limited proteolysis.

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