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Lactococcus lactis Release from Calcium Alginate Beads†

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Cell release during milk fermentation by *Lactococcus lactis* immobilized in calcium alginate beads was examined. Numbers of free cells in the milk gradually increased from 1×10^6 to 3×10^7 CFU/ml upon successive reutilization of the beads. Rinsing the beads between fermentations did not influence the numbers of free cells in the milk. Cell release was not affected by initial cell density within the beads or by alginate concentration, although higher acidification rates were achieved with increased cell loading. Coating alginate beads with poly-L-lysine (PLL) did not significantly reduce the release of cells during five consecutive fermentations. A double coating of PLL and alginate reduced cell release by a factor of approximately 50. However, acidification of milk with beads having the PLL-alginate coating was slower than that with uncoated beads. Immersing the beads in ethanol to kill cells on the periphery reduced cell release, but acidification activity was maintained. Dipping the beads in aluminum nitrate or a hot $CaCl_2$ solution was not as effective as dipping them in ethanol. Ethanol treatment or heating of the beads appears to be a promising method for maintaining acidification activity while minimizing viable cell release due to loosely entrapped cells near the surface of the alginate beads.

Immobilized cell technology is increasingly being considered for biotechnological processes. The advantages of using immobilized cells over the traditional free-cell systems include continuous utilization, retention of plasmid-bearing cells, prevention of interfacial inactivation, stimulation of production, and excretion of secondary metabolites, protection from a turbulent high-shear environment, and higher fermentation speeds (4, 21). This technology has thus interested the dairy sector, and the use of immobilized lactic acid bacteria has been proposed for a variety of fermentations (7, 14). High bacterial densities (10^{11} CFU/g) can be reached in calcium alginate beads (A beads) (19), but cell release occurs (16). Up to 3% of the total bacterial population in a system can be comprised of free cells (19). In instances where the A beads are used to continuously inoculate yogurt (17) or milk used in cheesemaking (14, 16), cell release is desirable. However, in applications such as cream fermentation for the production of cultured butter, cottage-cheese dressings (6), or psychrotrophic bacterial inhibition in raw milk (3), the release of cells is undesirable.

Cells are released into the surrounding medium once the matrix space in gel beads has been occupied (9). It has been shown that cell release can be influenced by bead load in the reactor (15) and by bead size (1). Limiting the release of yeast cells from A beads has been successfully reported for champagne production (8). However, there are no reports that this has been achieved with calcium-alginate-immobilized lactic acid bacteria.

The aim of this work was to evaluate the effects of cell density, coating the A beads, and killing of cells on the periphery of the beads on the levels of cell release in subsequent milk fermentations.

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MATERIALS AND METHODS

Biological material. *Lactococcus lactis* subsp. *cremoris* CRA-1 was maintained on 12% reconstituted nonfat dry milk. Two transfers per week were performed by inoculating milk at a 1% (vol/vol) concentration and incubating it at 23°C for 16 h. The pH after the incubation of the culture was 4.6. The culture was held at 4°C between transfers. Prior to bead formation, 200 ml of M17 broth (Difco, Detroit, Mich.) was inoculated with 0.2 ml of the milk-grown culture and incubated at 23°C for 22 h.

Media and matrix. (i) Skim milk. Nonfat dry milk (Agropur, low heat type) was reconstituted at either 9 or 12% (wt/wt) solids and sterilized at 112°C for 10 min. The 9% milk was used for the fermentation processes, while the 12% milk served to transfer the mother culture.

(ii) Alginate. A beads were formed with solutions of 2 or 5% (wt/vol) sodium alginate (BDH, Montréal, Québec, Canada). Alginate coating was applied with a 0.17% sodium alginate solution. The alginate solutions were sterilized at 121°C for 15 min.

(iii) Poly-L-lysine. Three different molecular weights of poly-L-lysine (Sigma, St. Louis, Mo.) were studied, namely, 1,000 to 4,000, 15,000 to 30,000, and 150,000 to 300,000. The poly-L-lysine solutions were filter sterilized (0.22 μm; Millipore, Montréal, Québec, Canada).

Cell immobilization. (i) Preparation of A beads. The M17-grown cells were recovered by centrifugation at $5,000 \times g$ for 10 to 15 min. The pellet was resuspended in 5 ml of 0.1% sterile peptone solution, and this suspension was mixed with an alginate solution to yield a final alginate concentration of 1, 2.5, or 4% (Table 1). The alginate-cell suspensions were added dropwise in a 0.1 M $CaCl_2 \cdot 2H_2O$ solution under 50-rpm agitation (magnetic bar). The drops solidified upon contact with the $CaCl_2$ solution, thus entrapping the cells. After 30 min of incubation in the calcium chloride solution to permit hardening, the beads were recovered and washed

TABLE 1. Method of preparing calcium alginate beads

Vol of cell suspension (ml)	Sodium alginate solution		Vol of $CaCl_2$ (0.1 M) solution (ml)	Beads obtained:	
	Vol (ml)	% (wt/vol)		% Alginate	Wet wt (g)
5	5	2	50	1.0	4.2
5	5	5	70	2.5	4.6
5	20	5	100	4.0	10.0

once with 0.1% peptone. The beads had an average size of 2 mm. They were stored overnight at 4°C in a 0.1% peptone solution to which 0.01 M $CaCl_2 \cdot 2H_2O$ was added. Unless otherwise stated, the beads having a 1% alginate concentration were used.

(ii) Coating of the A beads. Two types of coating were applied to the A beads, poly-L-lysine (AP beads) or poly-L-lysine and alginate (APA beads).

Procedures for the preparation of AP and APA beads were based on the methods of King et al. (13) and Sun and O'Shea (20). AP beads were prepared by suspending 3.4 g of A beads in 10 ml of a 0.05% poly-L-lysine solution (unless otherwise stated) and mixing at 50 rpm with a magnetic bar. After 20 min of contact, the poly-L-lysine solution was discarded and the resulting AP beads were rinsed with a 0.1% peptone solution. For the preparation of APA beads, a second layer was applied by adding 10 ml of 0.17% alginate to the AP beads. After 20 min at 50 rpm, the alginate solution was discarded and the resulting APA beads were rinsed with the peptone solution. This resulted in the preparation of a double coating around the original A bead, in which poly-L-lysine was sandwiched between alginate.

(iii) Surface treatment of the beads. Three treatments were applied with the objective of killing the cells within the bead periphery. The beads were dipped in either 60% ethanol for 2.5 min, sterile $Al(NO_3)_3$ (0.1 M) for 1 min, or sterile $CaCl_2$ (0.1 M) at 55°C for 25 min. Beads were rinsed with 0.1% peptone after surface treatment. Treatment times or concentrations varied for some experiments as noted below.

Fermentations. All of the fermentations were performed in 100 ml of 9% milk at 30°C for 2 h, by using a Cellgro stirrer system (Thermolyne) equipped with 125-ml Magnaflex jars (Wheaton) and adjusted to 30 rpm. Such 2-h fermentations can serve as a model for the prefermentation of cream in the manufacture of cultured butter or cottage cheese (6).

After the 2-h incubation period, the fermented milk was removed by decantation and 100 ml of milk (30°C) was added to the beads. The same beads could thus be used for as many as five consecutive fermentations. After each 2-h fermentation period, samples of the fermented milk or of the beads were taken for pH determination and/or cell counts (CFU). In one series of assays, AP beads were rinsed twice with 100 ml of peptone solution between fermentations.

Analyses. The pH measurements were taken at the end of each fermentation and were performed by use of Corning model 140 pH meter. In the studies on poly-L-lysine adsorption to the alginate beads or hydrolysis by the lactococci, residual poly-L-lysine was estimated by using a Pierce (Rockford, Ill.) protein assay kit.

Results are the average of at least three independent trials. Statistical analyses were performed on SAS (Cary, N.C.) software by using Duncan's multiple-range variance test.

Bacterial counts of the fermented milk or of liquefied beads were obtained by plating appropriate dilutions (0.1% peptone) on Elliker agar (Difco) and incubating them at 30°C for 48 h.

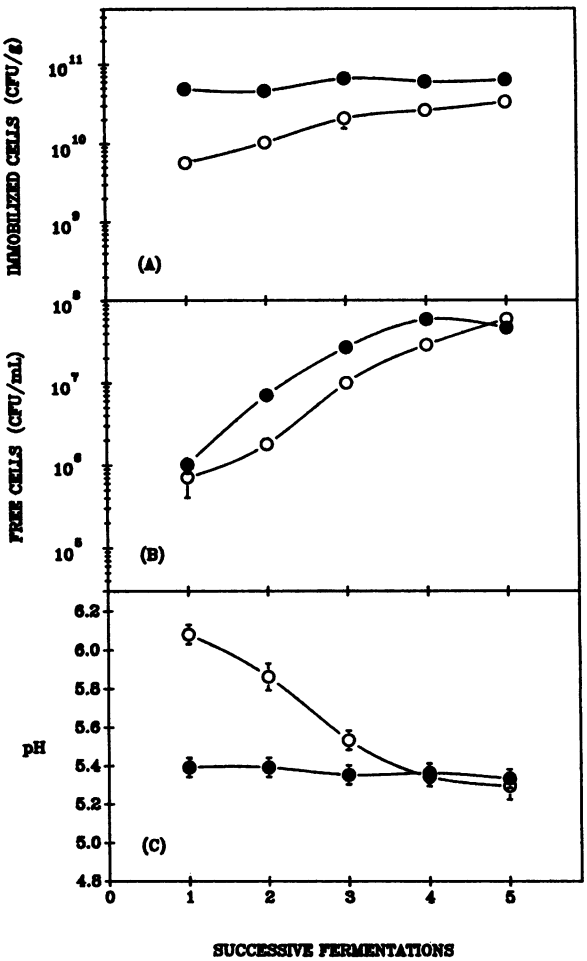

FIG. 1. Effect of initial density of *L. lactis* in the alginate on growth of the immobilized cells, cell release from the beads, and fermented milk acidity upon five successive fermentations with the same beads. Data represent fermentations with *L. lactis* at an initial count of 1×10^9 (○) and then at 2×10^{10} (●) CFU/g of beads. Bars indicate standard deviation from the mean on the basis of three replicate experiments.

Beads were liquefied by aseptically adding 1 g (wet weight) of beads to a sterile 1% sodium citrate solution (pH 6.0).

RESULTS

The addition of alginate-immobilized lactococci to milk resulted in acidification of the medium, with the initial pH of 6.5 dropping to 4.2 after 6 h of incubation. The fermentation was normally stopped by removing the beads when the milk pH dropped to 5.5, which occurred after approximately 2 h. This 2-h fermentation simulates cream fermentations required in the manufacture of cultured butter or fermented cottage-cheese dressings (6). The effect of multiple use of the same lactococci-containing beads was examined, with the results representing the experimental conditions at the end of each successive fermentation.

Initial cell density. A beads were prepared with bacterial populations of 1×10^9 or 2×10^{10} CFU/g. Growth was observed in beads containing the lower initial population, while no change in cell density occurred with beads containing 10^{10} CFU/g (Fig. 1A). The lower initial cell density

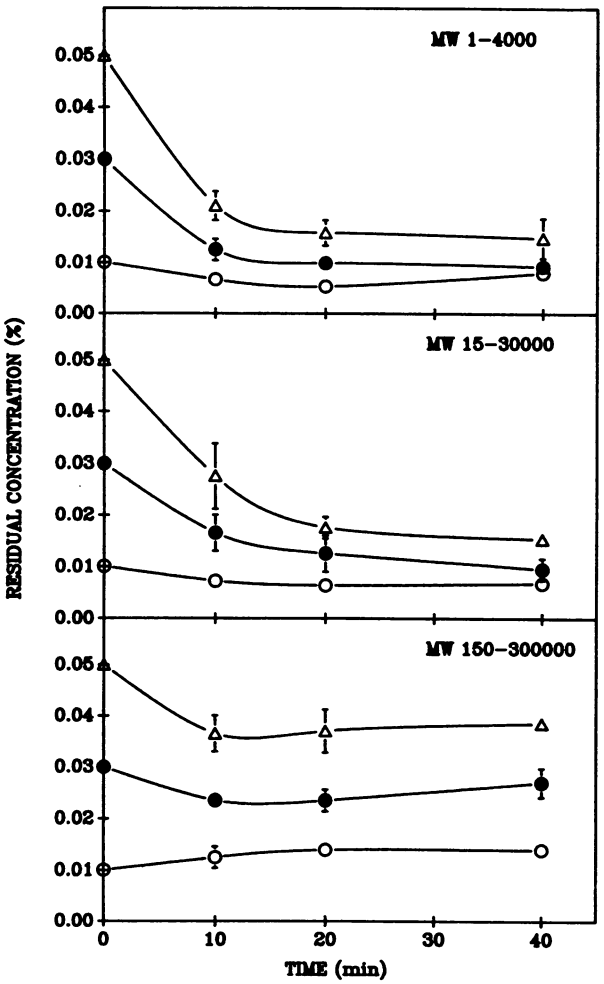

FIG. 2. Effect of poly-L-lysine molecular weight (MW) on its adsorption to A beads. Values are of residual levels of poly-L-lysine after immersion of the A beads in the poly-L-lysine solutions. The standard deviation from the mean is based on at least three replicate experiments.

resulted in slower acidification of milk during the first three fermentation periods (Fig. 1C). With repeated use of the beads, the acidities of the milk samples after the 2-h incubation period became similar, irrespective of initial bead cell density.

We observed an increase in free-cell density as the beads were used for successive fermentations (Fig. 1A). The numbers of free cells in the fermented milks were initially lower when low-cell-density beads were used (Fig. 1B), but this difference gradually decreased as the beads were reutilized. After five fermentations, the total free-cell population in the fermentor was 6×10^9 CFU/100 ml while the total immobilized cell population reached 2.5×10^{11} CFU/4.2 g. The free-cell population accounted for 2.4% of the total cell count in the bioreactor.

When the more concentrated cell suspension was used, the pH values of fermented milk and the immobilized cell population were stable over successive fermentations (Fig. 1C); therefore, high-cell-density beads were used for most of the subsequent experiments.

Alginate concentration. The preparation of beads at various alginate concentrations did not significantly influence the

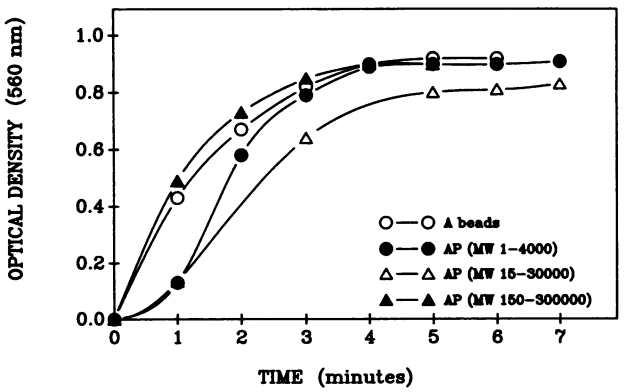

FIG. 3. Bacterial release from A or AP beads of various molecular weights (MW) when immersed in a 1% sodium citrate solution.

numbers of free cells found in the fermented milks. Milk acidification was greater (pH, 0.25 unit lower) with beads prepared from 4% alginate cell suspensions. Although the initial bacterial populations in the beads were identical for all assays, the amount of beads obtained from the 4% alginate preparation was twice that of the other preparations (Table 1). Since the preparation of 1% A beads was easier and since free-cell numbers were not influenced by alginate content, 1% A beads were used for the subsequent assays.

Coating of the beads with poly-L-lysine. Adsorption of poly-L-lysine to the surfaces of the A beads resulted in depletion of the poly-L-lysine from solution (Fig. 2). Lower-molecular-weight poly-L-lysine was bound to a greater extent than the higher-molecular-weight form. The quantity of poly-L-lysine adsorbed was related to the initial concentration (Fig. 2). Thus, when the initial concentration was 0.05%, almost twice as much poly-L-lysine was adsorbed as that observed when the initial concentration was 0.03%. For this reason, 0.05% solutions were used for coating of the beads in subsequent experiments. The contact time was limited to 20 min, since little adsorption occurred between 20 and 40 min.

The enumeration of the immobilized cells became more difficult when the A beads were coated with poly-L-lysine (i.e., the AP beads). AP beads coated with the polymer of 15,000 to 30,000 molecular weight did not completely dissolve after 3 h of undisturbed incubation in the sodium citrate solution, and only 60% of the initial bacterial population could be recovered. In contrast, only 1 h was required to completely recover the immobilized cell population in the A beads. Slight agitation of the citrate-treated AP beads ruptured the fragile capsules, and "ghosts" of the poly-L-lysine alginate membranes were observed. It was thus necessary to use a blender to release the cells from the AP beads. All cells could be recovered after 4 min of blending when the AP beads were coated with the poly-L-lysine of 1,000 to 4,000 or 150,000 to 300,000 molecular weight (Fig. 3). Cells entrapped in the AP beads coated with the poly-L-lysine of 15,000 to 30,000 molecular weight could not be completely dispersed even with blender (Stomacher) homogenization. When compared with a standard method (25 stokes per 30-cm area per 7 s), such a homogenization was found not to influence viable counts from a CRA-1 culture.

There were no significant differences ($P > 0.05$; average of four trials) in acidification activity or cell release between the various types of poly-L-lysine. The use of AP beads did not influence the acidification of milk nor its number of released

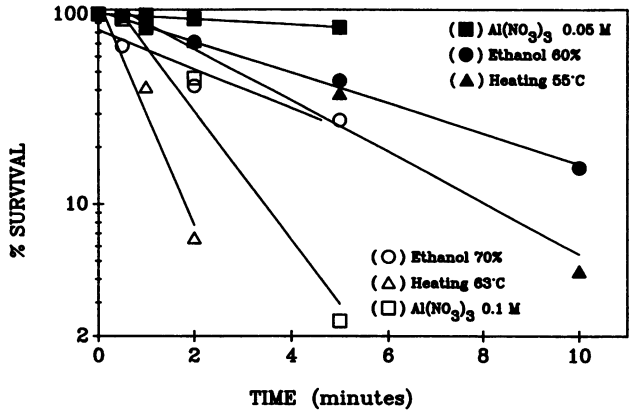

FIG. 4. Viability of *L. lactis* cells immobilized in A beads after immersion in ethanol, $Al(NO_3)_3$, or hot 0.1 M $CaCl_2$ solutions.

cells. These results were not related to loss of the poly-L-lysine coating during the course of the fermentations. AP beads that had been incubated for 10 h under agitation demonstrated the same properties as fresh AP beads in regards to stability to dissolution in citrate solutions. The population reached in the AP beads varied between 6.1×10^{10} and 7.5×10^{10} CFU/g, after the fifth consecutive fermentation, and was thus not different from values observed with A beads (Fig. 1A).

The effect of adding an alginate coat to the AP beads, thus sandwiching the poly-L-lysine between alginate layers, was evaluated. The free-cell count was 1 log lower when APA beads were used. Less acidification (by 0.2 pH unit) was also observed when APA beads were used to inoculate the milk. The acidification of milk with APA beads increased in the successive fermentations. The pH after the third fermentation with APA beads was similar to that found with A or AP beads at the end of the first fermentation. Increased cell release also occurred with repeated use of APA beads and, as was observed with acidification, the free-cell count at the third APA fermentation was similar to that found after the initial fermentation with A or AP beads.

Complete removal of the fermented milk from the bioreactor between the successive fermentations was difficult. Approximately 3% residual milk remained adsorbed to the beads or reactor, which may contribute to the free-cell loading in subsequent fermentations. To estimate this effect, the A and AP beads were rinsed twice with 30 ml of peptone water between each fermentation. Significant differences between numbers of free cells in rinsed beads and numbers in unrinsed beads were not observed. These results suggest that the main source of free cells in the bioreactor was cell release from the beads, with a possible second source being subsequent growth.

Bead immersions. Immersing the beads in ethanol, $Al(NO_3)_3$, or hot calcium chloride solutions resulted in an exponential reduction in cell viability (Fig. 4). The mortality rate was high at 63°C and with 70% ethanol. Slower death rates were desirable to better control the treatment, resulting in the use of 60% alcohol or immersion at 55°C for subsequent experiments. Cell death would presumably occur mainly within the surface of the bead, with the experimental objective of achieving a 25% kill. The same objective was reached by immersing the beads in 0.1 M $Al(NO_3)_3$ for 1 min (Fig. 4).

Since only a small fraction (25%) of the cells were killed by the immersions, the immobilized cell populations were not found to be significantly different from those of the control after the first fermentation (Fig. 5A). The heated beads showed a slightly lower acidifying activity than that of the control during the first fermentation (Fig. 5C), but no significant effect of immersion on acidification when the beads were reused for subsequent fermentations could be detected.

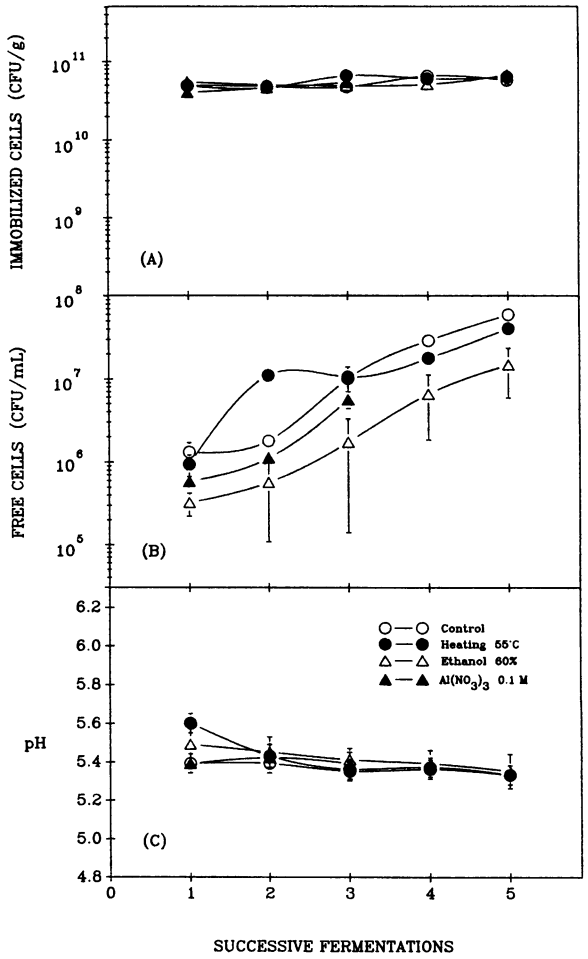

FIG. 5. Effect of heating (55°C) or of immersing A beads containing *L. lactis* cells in ethanol (60%) or in 0.1 M $Al(NO_3)_3$ solutions on the subsequent growth of the immobilized cells, cell release from the beads, and pH of the fermented milk after five successive fermentations using the same beads. The standard deviation from the mean is based on at least three replicate experiments.

Immersion of the beads did influence the numbers of cells released. There was a significant increase in cell release during the second fermentation when the beads had previously been heated (Fig. 5B). The lower free-cell counts obtained from beads treated with $Al(NO_3)_3$ did not prove to be statistically significant. However, when the beads had been treated with ethanol, there were significantly ($P < 0.05$) smaller numbers of free cells in the fermented milks (Fig. 5B).

DISCUSSION

Prévost and Divies (18) demonstrated cell growth in A beads, with bacterial populations eventually reaching 3×10^{11} CFU/ml. In the present study, immobilized cell popu-

lations increased to 7×10^{10} CFU/ml, near the maximum level observed by Prévost and Divies. Growth was observed when a lower level of inoculum was used for immobilization; however, little growth occurred in the beads formed with the concentrated suspension of lactococci. The cell density of beads with initially small numbers of cells remained lower than that of high-cell-density beads even though cell growth occurred in the former case. In both cases, numbers of free cells released from the beads to the external milk medium were identical after five successive fermentations. Since most growth in A beads occurs within the bead surfaces (12), the surface cell densities may be similar after successive fermentations even though the total populations in the beads are different (Fig. 1). This may explain why the acidification levels and numbers of free cells were ultimately identical in high- and low-cell-density cultures, even though the overall bacterial densities in the beads were different.

Champagne and Côté (6) proposed the use of immobilized lactic acid bacteria for cream fermentations in cultured-butter manufacture or in the preparation of fermented dressings for cottage cheese. One of the advantages in the use of immobilized cells for these applications is the small number of free cells in the fermented cream. In the present study, 10^6 CFU/ml of milk was observed after a first fermentation. However, repeated use of the beads leads to a gradual increase from the initially low level as a result of cell release from the beads. Free-cell counts of 8×10^7 CFU/ml of fermented milk were reached after several fermentations, similar to the counts observed by Prévost and Divies (18), who reported a free-cell population of 15×10^7 CFU/ml under steady-state conditions. The repeated use of alginate-immobilized cells slightly reduces its advantage for these processes upon reutilization of the beads. Since rinsing of the beads did not significantly influence the number of cells released, other means of preventing cell release were investigated.

Previously, it was shown that the alginate concentration, up to a maximum of 4%, does not influence diffusion of low-molecular-weight substrates (11) or fermentation rates (4, 5). In the present study, use of 4% alginate in bead formation doubled the mass of beads in comparison to those formed when 1 and 2.5% solutions were used. The increased acidifying activity appeared to be related to the increased bead volume. In addition, Johansen and Flink (10) demonstrated that surface-to-volume ratios influence fermentation rates. Although increasing the bead volume did not influence cell release, the use of larger quantities of 4% A beads reduced the fermentation time required to obtain a pH of 5.5. It remains to be determined if a shorter fermentation time would reduce the amount of free cells in the fermented milk.

The poly-L-lysine alginate coating of the beads was effective in limiting cell release but at the expense of reduced acidifying activity. Upon reutilization of the beads, the acidifying activity improved but free cell release also increased. In the presence of a physical constraint, immobilized bacterial cells can exert forces of comparable magnitudes against the constraint (11). The coatings did not appear to be sufficiently strong to resist the elevated internal (turgor) pressures that can be developed by bacterial cells (up to 3 to 5 atm [1 atm = 101.29 kPa]). Attempts to immobilize bacteria in ultrafiltration membranes have resulted in fiber rupture unless fermentation conditions uncoupled growth and acid production (2).

Aluminum nitrate treatment reduced cell release in a previous study (14), but the mild treatment (0.1 M for 1 min) used in the present study did not have a significant effect. Increasing the exposure of the beads to $Al(NO_3)_3$ for 5 or 10 min resulted in much lower acidification levels (data not shown), and this approach was not adopted. Immersing the beads in ethanol did reduce the free-cell counts, but cell release increased upon reutilization of the beads albeit at a lower level. Since growth in A beads occurs mainly at the surface, the population that was killed by ethanol appears to have been partially replaced. Repeated immersions in ethanol after a given number of fermentations could be performed to maintain the free-cell counts at low levels. However, repeated destructions of the bacterial population at the bead surface could result in a high density of dead cells. This may hinder diffusion of the substrates into the beads (9), which would result in a gradual loss of acidifying activity. However, cell release could potentially still occur since there has been no demonstration of an inhibitory effect of cell crowding on cell growth (11). Thus, ethanol treatment is a promising avenue towards reducing cell release, but work remains to be done on the effect of periodic treatments.

Since a fermentor containing the immobilized cells cannot be sterilized, the successive reutilization of A beads under industrial conditions raises the potential for contamination of the system. The results presented here suggest that treating the surfaces of the beads with ethanol or heating the beads could be performed to eliminate a surface contaminant. The beads could gradually recover their activity upon reutilization.

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