Effect of Dilution Rate on Bacteriophage Development in an Immobilized Cell System Used for Continuous Inoculation of Lactococci in Milk

M. LAPOINTE,[1] C. P. CHAMPAGNE,[1] J. C. VUILLEMARD,[2] and C. LACROIX
Centre de Recherche en Sciences et Technologie du Lait (STELA),
Université Laval, Québec, QC, Canada G1K 7P4

ABSTRACT

The response to bacteriophage contamination of a free cell and an immobilized cell bioreactor was studied during continuous pH-controlled fermentation of milk with *Lactococcus lactis* ssp. *lactis*. After phage infection (1×10^5 pfu/ml), the phage population reached 10^{10} pfu/ml in a free cell bioreactor operated at a dilution rate of 0.5/h and then declined to about 10^7 pfu/ml as a phage-resistant cell population became established in the system. In the immobilized cell bioreactor operated at dilution rates of 0.5 and 3/h, the phage population continuously increased until reaching 10^{10} pfu/ml where it remained throughout the 48 h of continuous culture. Conversely, phage populations decreased during the first 30 min following contamination at dilution rates of 10 and 15/h but subsequently increased. For all tested conditions in the immobilized cell bioreactor, the phage-resistant population increased to 10^2 to 10^4 cfu/ml, but the effluent milk contained mostly phage-sensitive cells. Analysis of bead populations showed the implantation of the phage as well as a limited population of phage-resistant cells. The effluent biomass from the immobilized cell bioreactor sharply reduced acidifying activity because this biomass was composed mainly of phage-sensitive cells and contained high phage populations.

(**Key words**: *Lactococcus lactis* ssp. *lactis*, bacteriophage, immobilized cells, continuous fermentation)

Abbreviation key: **D** = dilution rate, **FCB** = free cell bioreactor, **ICB** = immobilized cell bioreactor, **PRC** = phage-resistant cells.

INTRODUCTION

Various industrial applications of immobilized lactic acid bacteria have been proposed for the dairy industry (7). Because cells are released from the gel beads during fermentation, immobilized cell technology has been proposed for the continuous production of starter cultures (3) as well as for the continuous inoculation of milk for yogurt (18) and fresh cheese manufacture (17, 21). Immobilized cells exhibit many advantages over free cells, such as maintenance of stable and active cells for extended periods, reuse of biocatalysts, continuous processing without cell washout, high volumetric productivity at high dilution rates (**D**), improved process control, and improved product recovery (4, 20). However, continuous utilization of a lactic acid culture can be limited by contamination with undesirable microorganisms or phages. Despite the many strategies deployed to prevent phage contamination or proliferation (10, 14), phage infections still occur and constitute the major cause of failure in milk fermentations (9). Raw milk is an important source of new phages in a plant (12). Because phages in raw milk are not destroyed by pasteurization (8), contamination of an immobilized cell system used for continuous inoculation, or prefermentation, appears to be inevitable.

Immobilized lactic acid bacteria may reduce susceptibility to contamination by undesirable microorganisms (5), but little information is available on phage contamination. Steenson et al. (22) observed no decrease in the acidifying activity following bacteriophage infection of batch cultures with lactococci that were immobilized in calcium alginate gel beads. Those researchers (22) attributed this protection of immobilized cells to the exclusion of phage particles from the gel matrix. Champagne et al. (6) examined the possibility of eliminating phage contamination by rinsing the beads. A rinsing protocol that reduced the bacteriophage contamination by 4 log cycles prior to fermentation did not avoid phage buildup in the system. However, the activity of the culture was maintained, and results suggested the appearance of phage-resistant cells (**PRC**). Recently, Passos et al. (15) studied the response to phage infection of *Lactococcus lactis* ssp. *lactis* cells immobilized in a film of

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[1]Food Research and Development Center, St-Hyacinthe, QC, Canada J2S 8E3.
[2]Reprint requests.

calcium alginate gel coating a spiral mesh. The spiral mesh was incorporated in a column bioreactor operated in a continuous mode (16). Passos et al. (15) found that, although a D of 2.6/h was insufficient to eliminate the phages completely from the bioreactor during 15 to 28 h of culture, phage buildup was prevented. This result suggests that a high D could be used to eliminate bacteriophages from the immobilized cell bioreactor (**ICB**).

This study examined the effect of high D on bacteriophage and lactococci populations in a continuously stirred tank ICB used for milk prefermentation. Extremely high D, in excess of 40/h, can be obtained using this process for the continuous prefermentation of milk with mixed mesophilic cultures (21). This study also determined the impact of bacteriophages on populations of phage-sensitive cells and PRC and the acidifying activity of the culture in the prefermented milk.

MATERIALS AND METHODS

Culture and Bacteriophage

Lactococcus lactis ssp. *lactis* CRA-1 was provided by the Food Research and Development Center collection (St-Hyacinthe, QC, Canada). Strain CRA-1 was prepared by subculturing twice (16 h at 21°C) from a frozen stock in skim milk (11%, wt/vol; reconstituted NDM autoclaved at 110°C for 10 min). Cultures were maintained at 4°C between transfers. The bacteriophage CRA-1 culture was obtained from an industrial whey sample; this particular strain was isolated from a well-defined lysis zone on M17 agar. The phage stock was prepared every 3 mo by inoculating lactose M17 broth (Unipath, Nepean, ON, Canada), autoclaved at 115°C for 15 min, containing 1% $CaCl_2$ (1 *M*) and 10^7 cfu of starter bacteria/ml plus 10^5 pfu of the corresponding phage/ml. The M17 broth (100 ml) was supplemented by 1 ml of a 1 *M* $CaCl_2$ sterile solution prior to incubation. After 16 h of incubation at 21°C, the broth was filtered (0.22-μm filter; Millipore, Bedford, MA), and the filtrate was stored at 4°C.

Cell Immobilization

The immobilization procedure was based on a two-phase dispersion technique previously described by Arnaud and Lacroix (1). Immobilization was conducted with a 3% (wt/vol) total polymer mixed gel made of 2.75% κ-carrageenan (Satiagel RH; Sanofi Bio Industries, Mississauga, ON, Canada) and 0.25% locust bean gum (Sigma Chemical Co., St Louis, MO). Inoculated gel beads with diameters in the range 1.0 to 2.0 mm were selected for the experiments by wet-sieving with sterile peptone water (0.1%). To increase the cell load after bead manufacture, beads were preincubated in milk at 28°C for 16 h with pH controlled at 6.0 using 5*N* KOH.

Continuous Fermentation Procedure

Continuous fermentations were carried out in a 2-L reactor (Biostat M; B. Braun, Melsungen, Germany). The fermentation medium was low heat skim milk powder (Crino; Agropur, Granby, QC, Canada) rehydrated to 9% solids (wt/vol), heated at 112°C for 15 min in a 200-L bioreactor (Setric Génie Industriel, Toulouse, France), and kept at 5°C until use. During fermentation, once pH 6.0 had been reached, pH was controlled by external addition of 5*N* KOH. Mixing was provided by a marine impeller set at 80 rpm, and temperature was maintained at 30°C. Two peristaltic pumps were used for continuous feeding of the reactor and harvesting the prefermented milk. A screen of 0.5-mm mesh size was installed at the outlet pipe to prevent washout of gel beads. Gaseous N was automatically injected in the effluent grid for 5 s at 5-min intervals to prevent plugging of the outlet mesh.

For free cell fermentations, 1 L of skim milk was inoculated with 1% (vol/vol) of a fresh culture of *L. lactis* ssp. *lactis* CRA-1 and incubated 16 h at 24°C in batch mode with pH controlled at 6.0 by addition of 5*N* KOH. The continuous culture was then initiated at a D of 0.5/h, which was selected to avoid cell washout.

For immobilized cell fermentations, 200 ml of preincubated beads, measured by water displacement in a graduated cylinder, were placed in the reactor for a total fermentation volume of 1 L. Four D (0.5, 3, 10, and 15/h) were tested with the ICB. Two replicates of the entire experiment were carried out for each D except D at 10/h.

Bacterial and Phage Counts

The total bacterial population (phage-sensitive and phage-resistant cells) was determined; cells were plated on lactose-M17 agar supplemented with 0.75% K_2HPO_4 and 0.25% KH_2PO_4. Phosphates were added to lactose-M17 agar to bind the calcium required for phage infection of the CRA-1 strain. This addition prevented the proliferation of phages eventually present in the samples and their possible interference with the enumeration of the sensitive cells, as has

been suggested previously (6). Plates were incubated at 30°C for 24 h. Glass pellets (10 beads of 3-mm diameter) were added to the first dilution solution (0.1% peptone) and then vortexed for 30 s to help disrupt potential lactococcal chains.

The PRC were enumerated using a double agar overlay technique with added lactose-M17 agar containing 1 ml of 1 *M* $CaCl_2$/100 ml of agar after separate sterilization of the two solutions. The surface of the sterile bottom layer agar of lactose, M17, and $CaCl_2$ was covered with 3 ml of melted M17 soft agar (0.5% agar at 45°C) to which 1 ml of a diluted milk sample, 200 μl of phage suspension (10^9 pfu/ml), and 50 μl of $CaCl_2$ (1 *M*) were added. The plates were then incubated 24 h at 30°C.

Enumeration of the phage population was carried out by the spot test method (11). A solidified plate of lactose, M17, and $CaCl_2$ agar was overlaid with 3 ml of lactose, M17, and $CaCl_2$ soft agar (0.5% agar at 45°C) to which was added 0.1 ml of *L. lactis* ssp. *lactis* CRA-1 culture. After solidification for 10 min in a laminar flow cabinet, 10-μl aliquots of diluted milk samples to be tested (five per plate) were dropped on the agar plate and incubated overnight at 30°C. In one series of analyses, the fermented milk was centrifuged for 7 min at 14,000 × *g* to remove the infected cells prior to phage enumeration.

For bacterial and phage enumeration in the gel matrix, the beads were first washed with 0.1% peptonized water, and 1 ml was added to 9 ml of peptonized water with ca. 1 g of glass beads. The mixture was agitated for 15 min at 45°C in a vortex mixer to break up the beads and to resuspend immobilized cells and phages. Because phages might be sensitive to vortexing, this sample preparation procedure could inactivate a portion of the phage population. Thus, data on phage counts in beads could be underestimated.

Results are expressed as colony-forming units or plaque-forming units per milliliter of gel or skim milk. All counts were performed in duplicate, and mean values are reported.

Acidifying Activity Test

The acidifying activity tests were performed with prefermented milk samples (10 ml) collected from the outlet of the bioreactor. The pH was measured before and after incubation (5 h at 30°C), and data were reported as the difference between initial and final pH, corresponding to the mean of duplicate experiments.

Latent Period

To estimate the length of the latent period of phage infection, *L. lactis* ssp. *lactis* CRA-1 (1%, vol/vol) was cultured in milk (pH 6.4 at 30°C). After 1 h of incubation, 2×10^4 phages/ml were added, and samples were enumerated for phage at 5-min intervals.

Statistical Analysis

Fermentations were conducted in duplicate except for the fermentation carried out at the D of 10/h (only one repetition). The general linear models procedure of SAS (19) was used to analyze the data. The homogeneity of variance was verified by the Bartlett's test. Orthogonal contrasts were calculated to evaluate the effect of significant sources of variation. Differences between means were considered to be highly significant at $P < 0.01$ and significant at $P < 0.05$.

RESULTS

Free Cell Fermentations

The evolution of total, phage-sensitive, and phage-resistant populations after phage infection in the free cell bioreactor (**FCB**) operated at a D of 0.5/h is shown in Figure 1. Phage infection (10^5 pfu/ml) had an important effect on total counts of phage-sensitive cells and PRC in the effluent. Within 10 h of infection, total cell population decreased from 1.8×10^9 to 3.7×10^3 cfu/ml, and phage counts increased rapidly to $>10^{10}$ pfu/ml after 2 h. Then, the phage population gradually declined and stabilized around 10^7 pfu/ml. Total bacterial counts increased after 10 h to reach approximately the initial value after 40 h. The gradual increase of total bacteria could be explained by the establishment of a phage-resistant population. This phenomenon is supported by the data on acidifying activity, which were parallel to those data for the total bacterial population after 20 h of continuous fermentation. The initial value was recovered after 40 h of culture, even in the presence of high numbers of phages in milk samples. It is assumed that PRC enumerated were obtained from the parental phage-sensitive strain, but this assumption was not verified genetically.

Microbial and Phage Populations in ICB

Phage counts in the ICB at a low D of 0.5/h rapidly increased after phage infection from 10^5 to 10^{10} pfu/ml after 4 h of culture (Figure 2). However, the phage population did not decline after 4 h as it did in

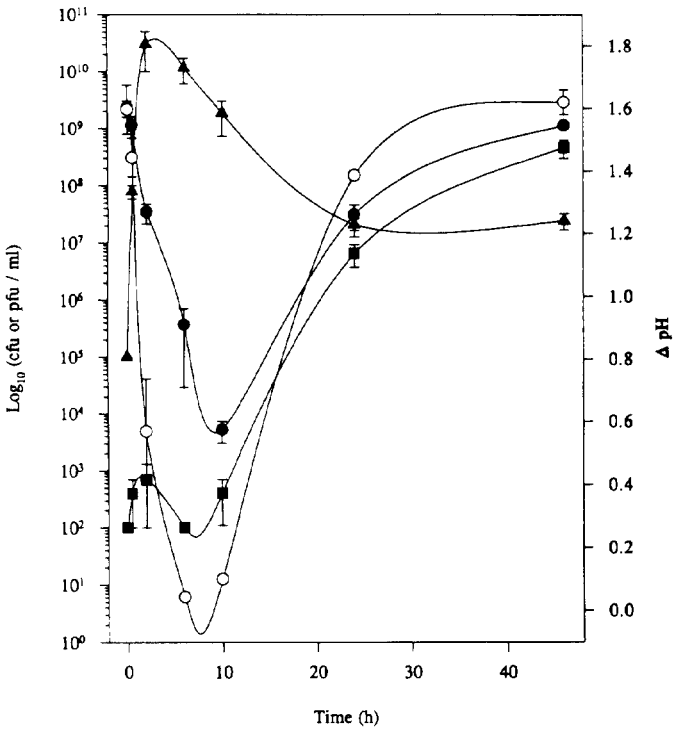

Figure 1. Effect of phage contamination in a free cell bioreactor with *Lactococcus lactis* ssp. *lactis* CRA-1 at a dilution rate of 0.5/h. Time zero corresponds to the time of phage infection (10^5 phages/ml). Error bars represent standard deviation. Total lactococci (●), phage-resistant lactococci (■), bacteriophages (▲), and acidifying activity expressed as change in pH (△ pH) (○).

the FCB for the same D. Total cell counts decreased only by 10^1 in the ICB after the addition of phages compared with a reduction of 10^5 in the FCB (Figure 1), although the phage population remained high at 10^{10} pfu/ml (Figure 2). Differences were detected for phage populations ($P < 0.02$), PRC ($P < 0.01$), and acidification of milk ($P < 0.01$) in the FCB and ICB at D = 0.5/h, but not for total cell counts ($P = 0.10$).

In the ICB, the phage population reached a plateau at ca. 10^{10} pfu/ml after 10 h of culture at the low D tested (0.5 and 3/h; Figures 2 and 3). However, when D was further increased, the pseudo steady-state value for phage population progressively decreased (Figures 3, 4, and 5). The method for direct phage enumeration carried out on effluent samples slightly overestimated the free phage counts, although no difference ($P = 0.17$) was detected when this method was compared with counts obtained from milk samples that were centrifuged to remove infected cells (Figure 5).

Total cell population in the effluent increased to 2.5×10^8, 2.0×10^8, and 3.7×10^7 cfu/ml for D of 3, 10, and 15/h, respectively (Figures 3, 4, and 5), confirming the efficiency of the ICB for the continuous inoculation of milk and its apparent insensitivity to phage attack. The statistical analysis showed an effect of D on the effluent lactococcal populations ($P < 0.01$). However, the effluent lactococcal populations were not affected by time ($P = 0.10$), suggesting a stability of the cell release from the ICB. In these instances, the cell population in the effluent was mainly formed of sensitive cells, but some PRC were also present (Figures 3, 4, and 5).

Phage populations in the ICB were significantly influenced by the D and time ($P < 0.01$). Figure 6 shows the evolution of phage populations during a 5-h period following phage infection as influenced by D. As early as 30 min after the addition of 10^5 phages/ml, differences were obvious between low (0.5 and 3/h) and high D (10 and 15/h). During the first 30 min, the phage populations increased to 10^6 to 10^7 pfu/ml at D of 0.5 and 3/h and decreased to 10^4 pfu/ml at a high D of 10 and 15/h. These observations confirmed the hypothesis of phage washout at high D. However, this washing out of phage particles at high

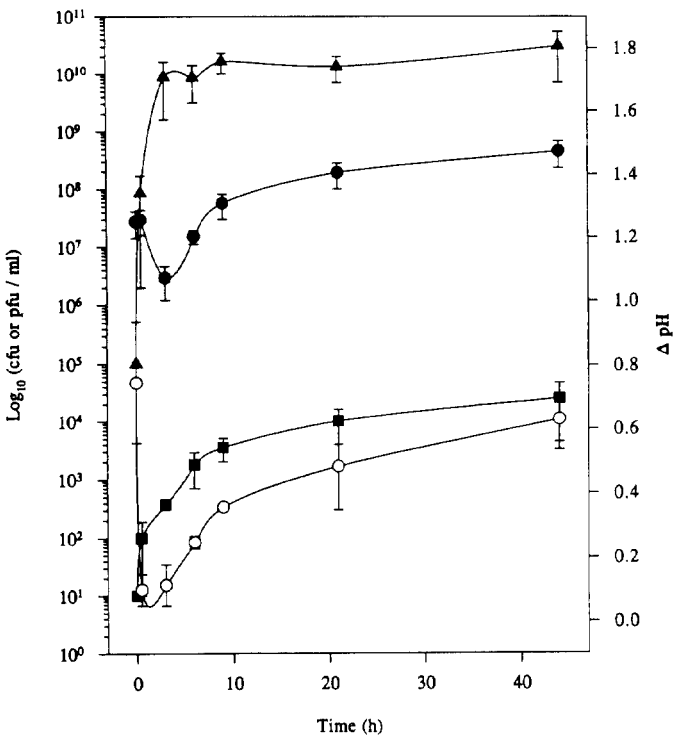

Figure 2. Effect of phage contamination on free cell populations in an immobilized cell bioreactor with *Lactococcus lactis* ssp. *lactis* CRA-1 at a dilution rate of 0.5/h. Time zero corresponds to the time of phage infection (10^5 phages/ml). Error bars represent standard deviation. Total lactococci (●), phage-resistant lactococci (■), bacteriophages (▲), and acidifying activity expressed as change in pH (△ pH) (○).

D was only observed during the first 30 min of incubation, and phage counts subsequently increased.

Microbial Populations in Gel Beads

A PRC population was also detected in the beads. This population varied from 5×10^3 to 8×10^4 cfu/ml according to the D and thus represented only a very minor fraction of the total entrapped bacterial population, which was close to 10^{10} cfu/ml (Table 1). For each D tested, $>10^8$ pfu/ml gel beads were detected. This phage contamination was not the result of a simple adsorption process on the matrix. In a cell-free control, only 2.3×10^2 pfu/ml were located in the matrix after 24 h of exposure to a milk sample containing 10^5 pfu/ml (Table 1). Statistical analysis showed no effect ($P > 0.05$) of D on total bacteria, PRC, and phage in the beads.

Acidifying Activities

The acidifying activity of the culture in the effluent milk is an important feature of the continuous ICB process used for the continuous inoculation and prefermentation of cheese milk (21). With this type of process, a second step final batch fermentation was required to form the coagulum and to attain the final pH in the curd.

At D of 0.5, 3, 10, and 15/h, the effluent populations were 3.7×10^7, 2.0×10^8, 2.5×10^8, and 3.7×10^8 cfu/ml, respectively. In uncontaminated samples, this translated into changes in pH values after 5 h incubation of 0.8, 1.35, 1.40, and 1.60, respectively (results not shown). In the ICB, the total bacterial population remained high, but acidifying activity sharply decreased shortly after phage infection. For the same total cell population, acidifying activities of the ICB samples (Figures 2 to 5) were lower than acidifying activities of uninfected populations. This result was obviously related to the fact that the effluents contained essentially phage-sensitive cells, which were continuously released from the beads. Although bacteria within the gel were protected from phages (15, 22), released cells and those on the bead surface were not. In the presence of phages, released cells were subsequently infected, and, as a result, the acidifying activity decreased. The D had no effect ($P = 0.43$) on acidifying activity, although fermentation time significantly influenced acidifying activity ($P < 0.01$).

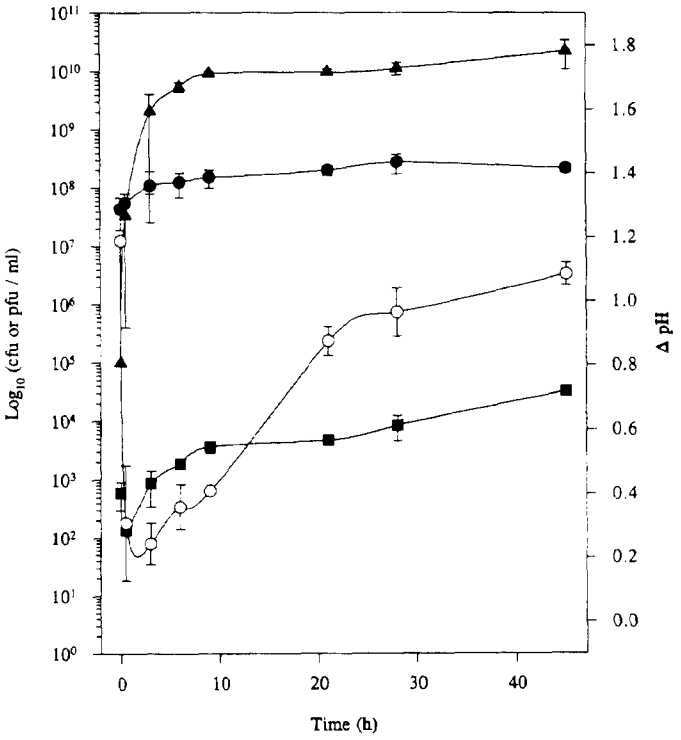

Figure 3. Effect of phage contamination on free cell populations in an immobilized cell bioreactor with *Lactococcus lactis* ssp. *lactis* CRA-1 at a dilution rate of 3/h. Time zero corresponds to the time of phage infection (10^5 phages/ml). Error bars represent standard deviation. Total lactococci (●), phage-resistant lactococci (■), bacteriophages (▲), and acidifying activity expressed as change in pH (△ pH) (○).

TABLE 1. Bacterial and phage counts in the gel beads at the end of immobilized cell bioreactor fermentations at various dilution rates (D).

D	Fermentation	Total bacteria[1]	Resistant bacteria	Phages
(/h)	(h)	(cfu/ml of gel)		(pfu/ml of gel)
0.5	45	3.0×10^{10}	5.0×10^3	2.0×10^8
3	45	7.0×10^9	8.0×10^4	2.0×10^8
10	12	4.4×10^{10}	1.7×10^4	1.2×10^9
15	5	2.0×10^{10}	8.0×10^4	1.0×10^8
Exposure[2]	24	. . .	. . .	2.3×10^2

[1]Include phage-sensitive and phage-resistant bacteria.

[2]Exposure of uncolonized beads to 10^5 pfu/ml for 24 h.

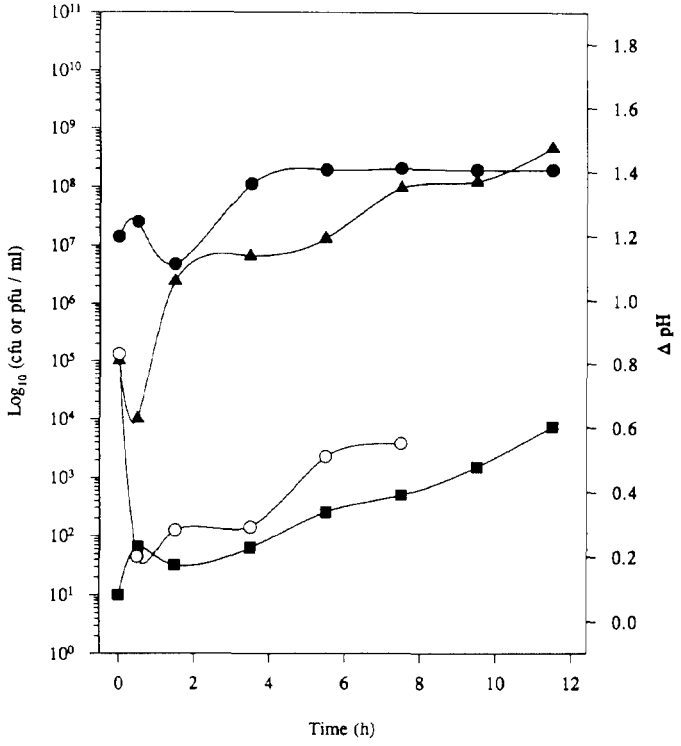

Figure 4. Effect of phage contamination on free cell populations in an immobilized cell bioreactor with *Lactococcus lactis* ssp. *lactis* CRA-1 at a dilution rate of 10/h. Time zero corresponds to the time of phage infection (10^5 phages/ml). Error bars represent standard deviation. Total lactococci (●), phage-resistant lactococci (■), bacteriophages (▲), and acidifying activity expressed as change in pH (Δ pH) (○).

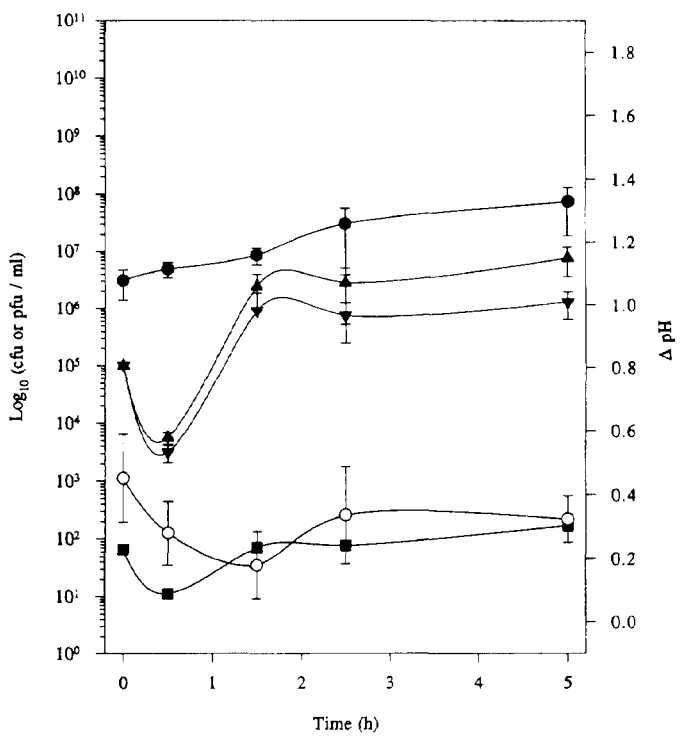

Figure 5. Effect of phage contamination on free cell populations in an immobilized cell bioreactor with *Lactococcus lactis* ssp. *lactis* CRA-1 at a dilution rate of 15/h. Time zero corresponds to the time of phage infection (10^5 phages/ml). Error bars represent standard deviation. Total lactococci (●), phage-resistant lactococci (■), bacteriophages in uncentrifuged samples (▲), bacteriophages in centrifuged samples (▼), and acidifying activity expressed as Δ pH (○).

DISCUSSION

FCB Fermentations

Phage infection in the FCB provoked a sharp decrease in total cell counts after 10 h of culture, followed by a gradual increase to recover the initial value that was observed just before phage addition. It is well known that phage-resistant clones appear following a phage infection, and the bacterial receptor for phage adsorption is usually modified (13). This phenomenon explains the dominance of PRC among the cell population. However, the stable and relatively high (10^7 pfu/ml) phage population in existence after 24 h of culture (Figure 1) was unexpected. Stabilization of the phage population at a high level in the FCB may be associated with the presence of phage-sensitive revertants of the phage-resistant clones, but this assumption was not verified. The subsequent lysis of phage-sensitive revertant cells may explain their low number in the effluent and the dominance of resistant cells.

ICB Fermentations

At D of 0.5/h, the decrease in total bacteria following phage contamination was relatively small in the ICB compared with those in the FCB, corresponding to 10^1 and 10^5, respectively. This result agreed with the data of Champagne et al. (6), who reported a 1000-fold higher free cell count in an ICB system than in the FCB system after phage infection. Because the mean residence times in the FCB (120 min) and in the ICB (96 min, based on extraparticular volume) were higher at D of 0.5/h than the latent period (approximately 30 min), phages were not washed from the continuous reactor and could multiply and infect sensitive cells. Conversely, the limited decrease in released cells in the ICB could be explained by the protection of immobilized cells from phage attack. This observation was confirmed by the high and stable proportion of sensitive cells that occurred among the total released cells, which resulted from growth and release of uncontaminated cells from the beads

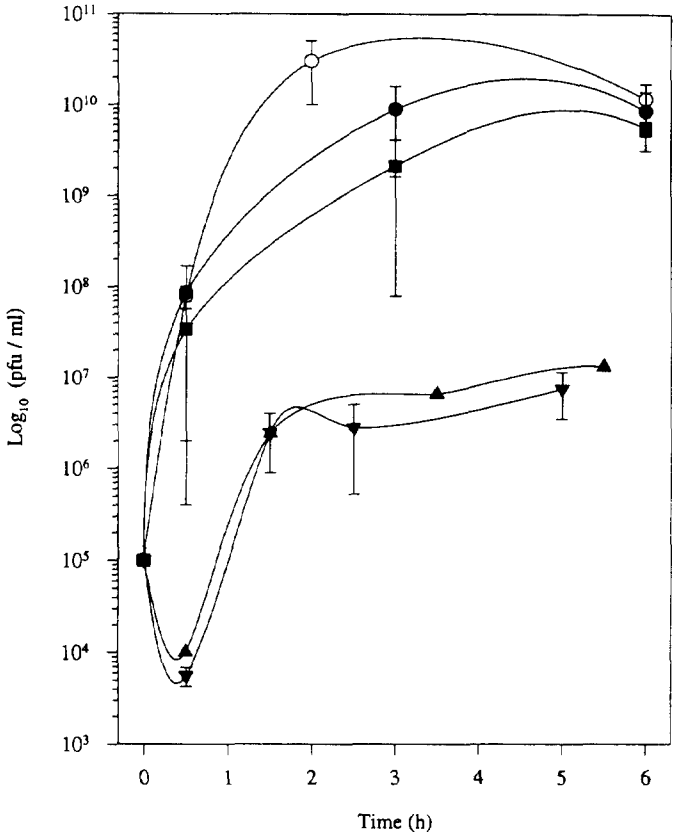

Figure 6. Effect of dilution rate (D) on bacteriophage populations in the free cell bioreactor (FCB) and the immobilized cell bioreactors (ICB) during the 6-h period following phage contamination (10^5 phages/ml). Error bars represent standard deviation. FCB, D = 0.5/h (○); ICB, D = 0.5/h (●); ICB, D = 3/h (■); ICB, D = 10/h (▲); ICB, D = 15/h (▼).

(Figure 2). The high proportion of sensitive cells in the effluent also explained the low acidifying activity of the samples removed from the ICB.

In theory, the latent period of the phage, which was estimated to be 30 min in this study, would preclude development of the virus in the ICB when operated at a high D. Mean residence times calculated on the basis of the extraparticular volume for D of 3, 10, and 15/h are, respectively, 16, 4.8, and 3.2 min. Phage data from the ICB (Figure 6) show that washout conditions occur for a short period of ca. 30 min after phage infection at D of 10 and 15/h. Washout of the phages under these conditions was not complete, and phage concentration increased thereafter to $>10^6$ pfu/ml. At the relatively short residence times at D of 10 and 15/h, the presence of $>10^2$ cfu/ml of PRC in the effluent was abnormally high.

Microbial Populations in Beads

In fermentations conducted at high D (10 and 15/h), the phage population in the medium (Figure 6) was lower than that in the beads at the end of fermentation (Table 1). Therefore, we concluded that phages colonized the beads and resulted in the establishment of PRC population. Because cell growth depends upon substrate and product concentration surrounding the cells, fermentations with immobilized lactococci resulted in the progressive formation of a high density cell layer consisting of microcolonies occupying gel cavities close to the gel surface (1). As a result of cell growth in this active cell layer, cells were continuously released from the gel bead surface in the liquid medium. This phenomenon forms the basis of the continuous inoculation of the bulk medium in ICB. Microscopic observations have clearly shown that the wall of peripheral gel cavities containing the microcolonies is disrupted by forces resulting from cell growth and by shear forces and that cells leak from open gel pores (2). Recently, we suggested that, as a result of the viscoelastic properties of the gel, discharged cavities eventually close, entrapping a sample of the surrounding bulk medium (7, 21). Cell growth in the local environmental conditions allows for recolonization of the cavities by the mixed culture composed of the original immobilized culture in the gel bead and the newly entrapped culture, eventually leading to openings and cell release. These successive cycles of colonization and discharge might explain the cross-contamination of gel beads that occurs during long-term continuous mixed fermentations in the ICB (21). As a result, phages could become entrapped in the surface cavities of the gel beads and multiply in the presence of sensitive cells, which would explain the high level of bead contamination by phages in this study ($>10^8$ pfu/ml of gel beads). The presence of $>10^8$ pfu/ml of gel beads (Table 1) would account for the development of the PRC population in the gel matrix.

Consequently, these results suggest that both bacteriophage and phage-resistant lactococcal populations established themselves on the surface of the beads and that a concomitant release of bacteriophages, phage-sensitive lactococci, and phage-resistant lactococci occurred. Results from this study confirm those of Passos et al. (15) with respect to the continual release of sensitive cells in the presence of contaminating phages. However, some noteworthy discrepancies have been found. Passos et al. (15) did not observe the appearance of PRC clones, and a relatively low D (2.4/h) seemed to initiate phage washout. Whether the differences in lactococcal strain, phage strain, gel matrix, fermenter design,

and analytical methods could be responsible for these differences remains to be determined.

CONCLUSIONS

Our study showed that the entrapment of *L. lactis* ssp. *lactis* in polysaccharide gel beads is effective in maintaining a high milk inoculation when phage contaminations occur. Bacterial cell counts in the effluent remain high, even at high D, because of the stable cell release rate from the gel beads. Thus, the ICB is more stable than the FCB, and phage contamination does not substantially modify the acidification and inoculation of milk within a given culture.

However, the hypothesis stating that a high D washes phages from the reactor was not entirely verified. Indeed, at D of 10 and 15/h, phage washout occurred, but only during the first 30 min following the infection; after 30 min, phage counts increased. This result may be explained by the surface contamination of beads by the phages, occurring even at the high D tested in this study.

The phage contamination level (10^5 pfu/ml) used in this study was relatively high and might not be representative of industrial contamination. Therefore, further work is currently in progress to determine the combined effect of D and phage contamination level on the phage susceptibility of the ICB.

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