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Full Paper

Growth and morphology of thermophilic dairy starters in alginate beads

Laurence Lamboley,[1] Daniel St-Gelais,[1,*] Claude P. Champagne,[1] and Maryse Lamoureux[2]

[1] *Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600, Casavant Blvd. West, Saint-Hyacinthe, Quebec, Canada J2S 8E3*

[2] *Agropur, 510, Rue Principale, Granby, Quebec, Canada J2G 7G2*

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The aim of this research was to produce concentrated biomasses of thermophilic lactic starters using immobilized cell technology (ICT). Fermentations were carried out in milk using pH control with cells microentrapped in alginate beads. In the ICT fermentations, beads represented 17% of the weight. Some assays were carried out with free cells without pH control, in order to compare the ICT populations with those of classical starters. With *Streptococcus thermophilus*, overall populations in the fermentor were similar, but maximum bead population for (8.2×10^9 cfu/g beads) was 13 times higher than that obtained in a traditional starter (4.9×10^8 cfu/ml). For both *Lactobacillus helveticus* strains studied, immobilized-cell populations were about 3×10^9 cfu/g beads. Production of immobilized *Lb. bulgaricus* 210R strain was not possible, since no increases in viable counts occurred in beads. Therefore, production of concentrated cell suspension in alginate beads was more effective for *S. thermophilus*. Photomicrographs of cells in alginate beads demonstrated that, while the morphology of *S. thermophilus* remained unchanged during the ICT fermentation, immobilized cells of *Lb. helveticus* appeared wider. In addition, cells of *Lb. bulgaricus* were curved and elongated. These morphological changes would also impair the growth of immobilized lactobacilli.

Key Words—cell immobilization; cell morphology; *Lactobacillus bulgaricus*; *Lactobacillus helveticus*; *Streptococcus thermophilus*

Introduction

Lactic acid bacteria (LAB) are widely used in the manufacture of dairy products, such as cheese and fermented milks. During cheese making, through their production of lactic acid from lactose they stabilize the curd by coagulating the protein and expelling moisture (Sandine, 1985). Some strains may contribute to texture and viscosity formation of fermented milks through the production of polysaccharides (Zourari et al., 1992). Lactic acid bacteria also participate in flavor and aroma of dairy products, either directly, via the production of aroma compounds, or indirectly, by releasing proteolytic or lipolytic enzymes in the curd during cheese ripening (Sandine, 1985). By the production of inhibitory compounds, such as bacteriocins, or by lowering pH, lactic acid bacteria inhibit the growth of pathogenic microorganisms (Gilliland, 1985a).

The traditional production of LAB starter for cheese vat inoculation requires several subcultures, from mother culture to bulk starter. These steps are time consuming, require proper equipment and skilled personnel. The use of concentrated starters made it possible to inoculate directly either the bulk starter vat or

* Address reprint requests to: Dr. Daniel St-Gelais, Food Research and Development Centre, Agriculture and Agri-Food Canada, 3600, Casavant Blvd. West, Saint-Hyacinthe, Quebec, Canada J2S 8E3.

E-mail: stgelaisd@AGR.GC.CA

the milk itself (Gilliland, 1985b). Usually, the biomass production step of concentrated starters is achieved by batch fermentation.

Immobilized cell technology (ICT) is being proposed to the food industry, for the production of biomass, metabolites, enzymes, or fermented products, such as wine, beer or yoghurt (Groboillot et al., 1994). In the dairy industry, continuous inoculation and prefermentation of milk for yoghurt (Prévost and Diviès, 1988) or fresh cheese (Sodini et al., 1998) as well as batchwise cream fermentation (Prévost and Diviès, 1992) can be carried out using LAB starter immobilized in polysaccharide beads. Regarding starter production, mesophilic and thermophilic LAB starter, along with propionic starters were produced continuously using ICT (Audet et al., 1992; Lamboley et al., 1997). Since beads populations can reach almost 10^{11} cfu/ml, ICT was proposed for the production of concentrated starter suspensions in alginate beads (Champagne et al., 1993, 2000b; Morin et al., 1992). These suspensions might be used as starters, or added to frozen dessert to provide live bacteria to the consumer (Sheu and Marshall, 1993). Using ICT to produce concentrated LAB has many advantages, such as ease of cell recuperation, better survival against freeze-drying (Champagne et al., 1994), increased survival against freezing in dairy desserts (Sheu et al., 1993), increased survival under gastric conditions (Lee and Heo, 2000) as well as protection against bacteriophages (Champagne et al., 1992; Steenson et al., 1987). ICT cultures can thus hold many advantages over free-cell cultures and, from an economic perspective, there is a need to examine how high bacterial yields can be obtained in the beads.

Conflicting results are reported concerning thermophilic immobilized starter population yields: *Streptococcus thermophilus* bead population extended from 1×10^7 cfu/ml gel to 1×10^{10} cfu/g beads (Audet et al., 1988; Champagne et al., 2000b), while *Lactobacillus delbrueckii* ssp. *bulgaricus* bead population varied between 1.9×10^7 cfu/ml gel and 3.1×10^{10} cfu/g beads (Masson et al., 1994). Furthermore, a high level of cell release can occur in some instances (Champagne et al., 1993). Therefore more data is needed with respect to the effect of species or strain of LAB on the production of concentrated ICT cultures.

The aim of the research was to examine the production of thermophilic LAB biomass in alginate beads using pH-stat immobilized-cell batch fermentations. Strains of *Streptococcus salivarius* ssp. *thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* were studied and morphological cell changes following immobilization were also evaluated.

Materials and Methods

Bacterial strains. Three strains of *Lactobacillus* were studied: one strain of *Lactobacillus delbrueckii* ssp. *bulgaricus* (*Lb. bulgaricus*) 210R (Waterford, Gist Brocades, Millville, UT), and two strains of *Lactobacillus helveticus* (*Lb. helveticus*) R211 (Rosell, Montreal, Canada) and HR17 (Chr. Hansen, Mississauga, Canada). One strain of *Streptococcus thermophilus* (*S. thermophilus*) HC15 (Chr. Hansen) was also investigated.

Chemicals. Alginate FD155 was purchased from Multi Chem, Inc. (Boucherville, Canada). Milk powder (Crino, low heat) was obtained from Agropur (Granby, Canada). MRS, M17, PCA, Bacto peptone and methylene blue were bought from Difco (Becton Dickinson, Sparks, MD). Glacial acetic acid and ammonia were obtained from Fisher (Fair Lawn, NJ). Calcium chloride dehydrate was purchased from Laboratoire Mat (Beauport, Canada). Anachemia (Lachine, Canada) supplied lactose and ACP, Inc. (St Léonard, Canada), sodium citrate dihydrate.

Inoculum preparation. The inoculum was prepared on an Agropur broth medium (ABM), a proprietary formulation developed by Agropur. Prior to fermentation, all strains were propagated by inoculating ABM with a frozen stock culture (1%, v/v) and incubating 6 h at 41°C.

Immobilization procedure. Milk having 12% nonfat milk solids and 0.1 M $CaCl_2$ was prepared. An ABM-grown culture was added to 2% sodium alginate, previously autoclaved (121°C, 15 min) at a ratio of 1 : 1. The bacterial suspension was extruded drop wise in the $CaCl_2$ supplemented milk, where the beads were kept for 30 min for gel strengthening. The bead ratio to $CaCl_2$ milk was adjusted approximatively to 17% (200 g of beads in 1,000 g of medium).

Fermentations. For each strain, free- and immobilized-cell batch fermentations were conducted in triplicate. For immobilized-cell fermentations, beads and $CaCl_2$ milk were poured in a double-jacketed bottle. Temperature was controlled at 37°C by recirculating water. pH was maintained at 5.7 for *Lactobacillus* and 6.2 for *Streptococcus*, by addition of 7 N NH_4OH using

a Radiometer Titrator system consisting of a PHM84 Research pH meter, a TTT80 titrator and a MNV1E valve (Radiometer, Copenhagen, Denmark). Immobilized-cell fermentations lasted 8 to 10 h. For cell enumeration, beads samples were withdrawn regularly, while milk samples were taken at the beginning (t0) and at the end of the fermentation.

Some classical (no pH control) free-cell fermentations were carried out to determine if major differences could be noted between a traditional propagation procedure, and the modern ICT fermentation under pH control. For free-cell fermentations, 1,200 ml 12% total solids skim milk was inoculated with 12 ml of an ABM-grown inoculum. Free-cell fermentations lasted 8 to 12 h, without pH control. Milk samples were taken regularly for cell enumeration, from the beginning (t0) until the end of fermentation.

Morphological study of Lb. bulgaricus 210R. Tests were conducted to study the influence of medium composition and medium physical state (liquid or solid) on strain 210R morphology. *Lb. bulgaricus* 210R was first propagated twice in milk (12% SNF) or MRS broth (1% v/v inoculation, 6 h at 41°C incubation) and then streaked on milk agar (PCA agar+10% of 10% SNF reconstituted milk) or MRS agar. The plates were incubated anaerobically for 48 h at 37°C. To investigate the influence of cations on *Lb. bulgaricus* 210R morphology, this strain was propagated twice (1% v/v inoculation, 6 h at 41°C) in MRS broth containing 30 mM $MgSO_4$. Microscopic examinations were done with cells from the second culture in milk or MRS broth, with or without magnesium, and from colonies from MRS agar or milk agar.

Analyses. Cell enumerations were performed on milk and bead samples. Milk samples were first diluted 1/100 in peptonized water (1 g/L) and then homogenized with an Omni 2000 homogenizer (Omni International, Inc., Gainesville, VA) for 30 s at 20,000 rev/min. Bead samples were diluted 1/10 in 1% sodium citrate solution (pH 6.0) and left under agitation for 5 min using a rotator (Roto-Torque, model 7637, Cole Parmer Instrument Co., Chicago, IL). The solution containing the dissolved beads was then homogenized 30 s at 25,000 rev/min, agitated 10 min and again treated 30 s with the homogenizer. Subsequent dilutions were done in peptonized water. Adequate dilutions were pour-plated in duplicate. MRS agar, acidified to pH 5.4 with glacial acetic acid was used for lactobacilli, and M17 agar, supplemented with 50 g/L lactose was used for streptococci. The plates were incubated anaerobically for 48 h at 37°C.

For each strain, microscopic preparations were realized from milk samples withdrawn during free-cell fermentations and from homogenized beads. They were stained with methylene blue (5%), observed using a Leitz Laborlux D microscope (Leitz Wetzlar GmbH, Wetzlar, Germany) and photographed. Slides from the study of strain 210R morphology were stained and photographed the same way. *Lb. bulgaricus* 210R colonies were observed and photographed using a Stereomicroscop (Photomakroscop M400, Wild Heerbrugg, Switzerland).

Results

Fermentations

Figure 1 shows the growth of the four strains in ICT fermentations. Except for *Lb. bulgaricus* 210R, the strains demonstrated an increase in their populations during the fermentation. Growth curves for immobilized-cell fermentations showed a lag phase, which lasted between 2 and 4 h, except for strain 210R. The populations obtained in the beads are shown in Table 1. During immobilized-cell fermentations, cell release from the beads occurred, leading to the inoculation of milk. At the end of the immobilized-cell fermentations, the released-cell population in the ICT system was al-

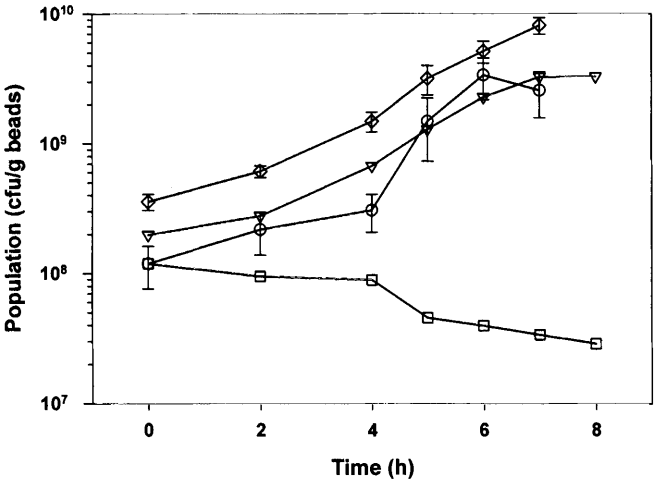

Fig. 1. Growth of immobilized cells in alginate beads. Means and standard errors calculated from the triplicates. ○, *Lb. helveticus* HR17; ▽, *Lb. helveticus* R211; □, *Lb. bulgaricus* 210R; ◇, *S. thermophilus* HC15. Error bars represent the standard errors of the means. To improve presentation error bars for R211 and 210R strains were not presented but were similar to HC15 strain.

Table 1. Distribution of total population in the bioreactor, at the end of the immobilized-cell fermentation under pH control, and final populations for traditional free-cell milk-grown starters grown without pH control.

Strain[a]	Immobilized cell fermentations						Traditional free-cell starters	
	Per g beads	Total in beads[b]	Released cells per ml of medium	Total released cells in medium[a]	Total in fermentor	% of cells in the beads	Per ml of medium	Total in fermentor[a]
R211	3.3×10^{9}	7.9×10^{11}	1.3×10^{6}	1.2×10^{9}	7.9×10^{11}	99.8	9.2×10^{8}	1.1×10^{12}
HR17	2.6×10^{9}	6.2×10^{11}	2.3×10^{6}	2.2×10^{9}	6.2×10^{11}	99.6	1.0×10^{9}	1.2×10^{12}
210R	3.1×10^{7}	7.4×10^{9}	3.7×10^{4}	3.5×10^{7}	7.4×10^{9}	99.5	3.3×10^{8}	4.0×10^{11}
HC15	8.2×10^{9}	2.0×10^{12}	8.2×10^{7}	7.9×10^{10}	2.0×10^{12}	96.2	6.1×10^{8}	7.3×10^{11}

[a] R211 and HR17 = *Lactobacillus helveticus*, 210R = *Lactobacillus delbrueckii* ssp. *bulgaricus*, HC15 = *Streptococcus thermophilus*.
[b] Based on fermentations of 1.2 kg of total mass. In the immobilized cell system, the beads represented 17%.

ways lower than 10^{8} cfu/ml (Table 1). Taking into account that the fermentor contained 200 g of beads and 1,000 ml of medium, it was possible to determine the total free-cell population in the medium as well as the population that was entrapped in the alginate beads (Table 1). By adding the total number of cells in the beads with the total number of cells in the liquid phase, the total population in the ICT system was established (Table 1). Since the free-cell counts in the ICT system were low, the total population in the system was basically that of the beads (over 95% in all cases). The percentage of immobilized cells was slightly inferior for *S. thermophilus* HC15 (96.2%), indicating that cell release was slightly higher for this strain.

In the traditional free-cell fermentation, no lag phase was observed (data not shown). The populations obtained in the classical method were between 3.3 and 10×10^{8} cfu/ml (Table 1), which is typical of milk-grown starters propagated in milk without pH control (Champagne et al., 1995). This data is thus in agreement with the literature.

Populations in beads were 3 to 13 times higher than those reached in the medium with the classical milk-grown fermentations, except for *Lb. bulgaricus*. This suggests that ICT cultures generate higher yields. It must be remembered, however, that the beads represent only a fraction of the fermentor volume. Thus, in order to have an idea if the ICT process gave high yields, the total populations in the traditional and ICT systems were compared. It was found that, for all lactobacilli, higher cell counts in a fermentor unit were obtained in the traditional fermentations (Table 1). The opposite was found with *S. thermophilus* HC15, where higher total cfu counts in the fermentor were obtained when the cultures was grown in the beads. Although comparing free and immobilized cell physiology was not the aim of this study, comparisons in total fermentor populations are useful in estimating the value of the ICT population yield. However, the comparison suffers from the fact that the traditional starters were prepared without pH control, while those obtained with ICT were. It has been shown that conducting pH control in milk enables higher yields of both lactobacilli and streptococci (Champagne et al., 1993).

Morphology of the cells

Figure 2 (a, b, c) and Fig. 3a show typical strain morphologies of cells in alginate beads. The *Lb. helveticus* HR17 (Fig. 2a) and R211 cells (Fig. 3a) were curved, relatively short rod-shaped cells, while the *Lb. bulgaricus* 210R culture (Fig. 2b) demonstrated elongated curved large cells, between 30 and 50 μm long. The gel-entrapped *S. thermophilus* HC15 culture (Fig. 2c) appeared as cocci, associated in pairs or short chains which was similar to free-cell starters.

When compared to traditional starters, ICT cultures had different cell morphologies. The *Lb. helveticus* R211 ICT-grown cells (Fig. 3a) were twice as wide as those in the traditional starters (Fig. 3b). However, the length of the *Lb. bulgaricus* 210R cell chains in the traditional starter (Fig. 4a) were 5 times shorter than when micro-entrapped (Fig. 2b). The traditional and ICT streptococci were similar in microscopic appearance.

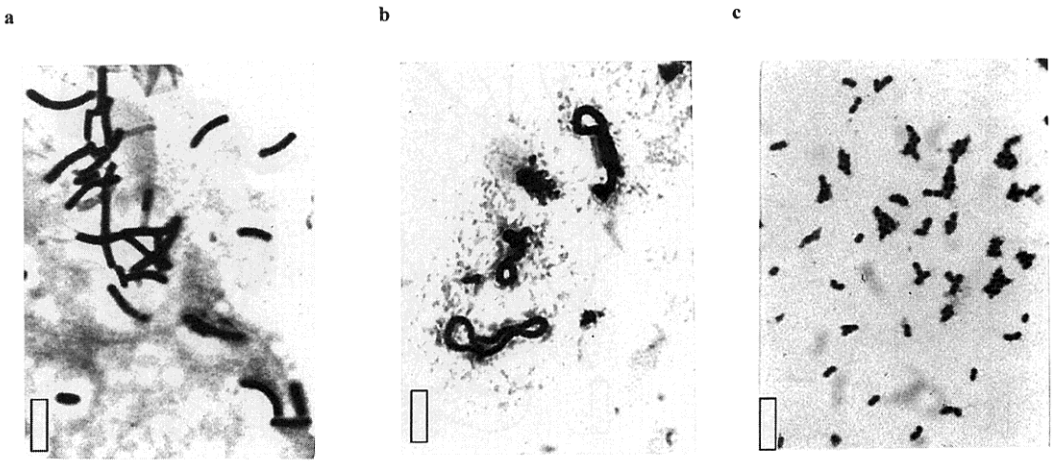

Fig. 2. Photomicrographs of the strains immobilized in alginate beads following fermentation in milk. (a) *Lb. helveticus* HR17, (b) *Lb. bulgaricus* 210R, (c) *S. thermophilus* HC15. Bars represent 10 μm.

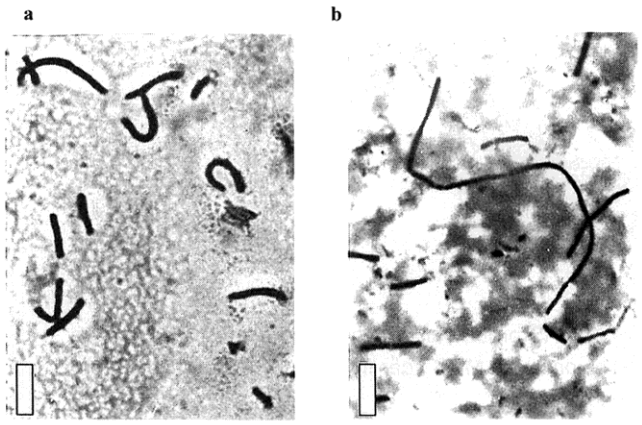

Fig. 3. Photomicrographs of the *Lb. helveticus* R211 strains (a) immobilized in alginate beads following fermentation in milk and (b) in free cell fermentation in milk.
Bars represent 10 μm.

It was not determined if production under pH control or microentrapment in the alginate gel were responsible for the morphological changes of the *Lb. helveticus* strains, but further assays were carried out with *Lb. bulgaricus* 210R.

Lactobacillus bulgaricus 210R morphological studies

When *Lb. helveticus* 210R strain was cultivated in milk, it formed rod-shaped cell strands measuring 10±4 μm, with intracellular granules (Fig. 4a). Very long chains of cells, up to several hundred μm long, were observed for 210R cultivated in MRS broth (Fig. 4b). However, a short rod-shaped morphology was observed in Mg-supplemented MRS broth (Fig. 4c). Strain 210R colonies displayed the same unusual morphology on MRS agar, when streaked from either milk (data not shown) or MRS broth (Fig. 4d). The colonies were rough and translucent, similar to that observed by Wright and Klaenhammer (1981) for the *Lb. bulgaricus* rough colonial mutant 1243F. *Lb. bulgaricus* 210R exhibited the same aspect on milk agar (data not shown), whether streaked from milk or MRS culture. The colony morphology was thus independent of the composition of the agar medium. It was also not influenced by the cell morphology in broth: both individual cells from milk culture (Fig. 4a) and chains from MRS broth (Fig. 4b) yielded the same type of colonies. When slides were prepared from an MRS agar colony (Fig. 4e) or a milk agar colony (data not shown), cells appeared as long strands, several hundred μm long, organized in main strands of parallel filaments. Some cells showed intracellular granules.

Discussion

Production of cell-containing alginate beads is typically carried out by adding the alginate-cell suspension to a $CaCl_2$ solution; the beads are then recovered and added to the fermentation medium. One novel feature of this study is the production of the beads directly in the growth medium (milk). Although milk is rich in calcium ions, its concentration is insufficient to enable adequate bead formation. Addition of 0.1 M $CaCl_2$ to milk was sufficient to enable satisfactory bead production. The $CaCl_2$ in the growth medium also serves to maintain bead integrity during the subsequent fermentation, since lactic acid produced during fermentation tends to weaken alginate gels (Roy et al., 1987). With such an approach, it can be envisioned that a droplet-produc-

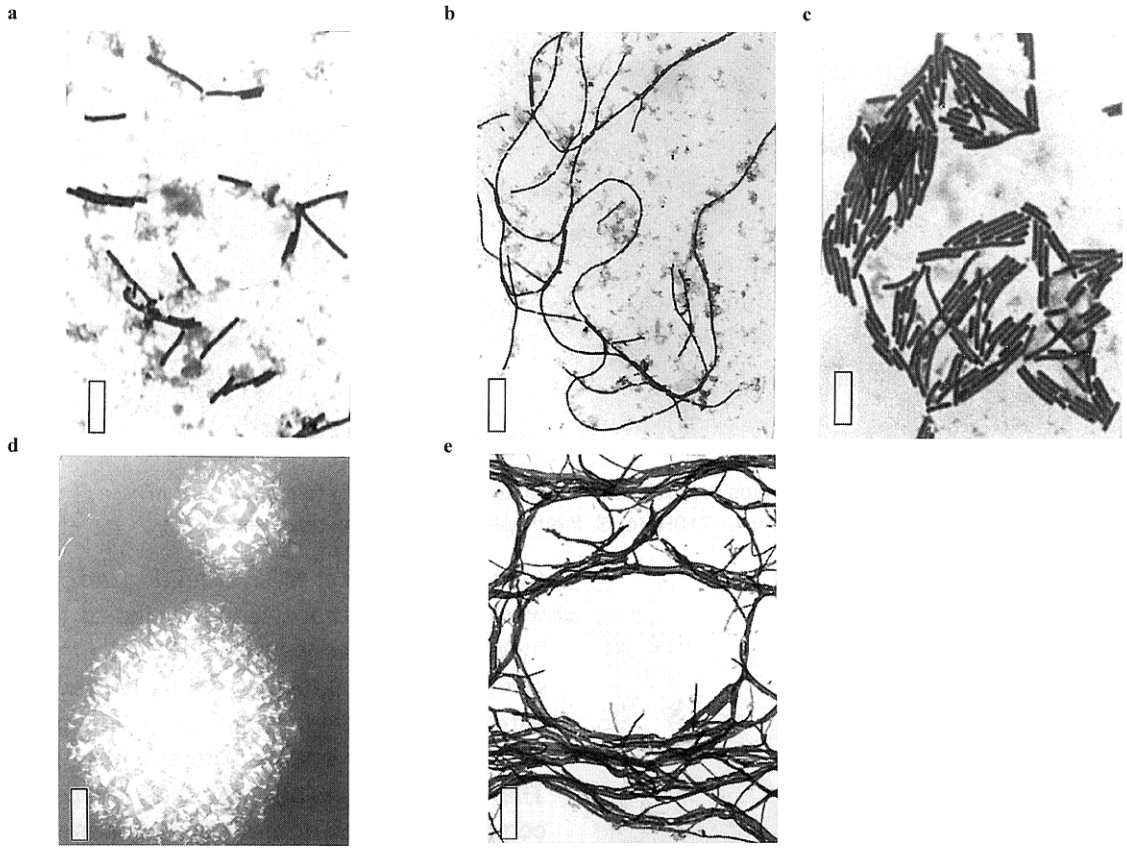

Fig. 4. Photomicrographs of *Lb. bulgaricus* 210R.
(a) 210R grown in milk, bar represents 10 μm; (b) 210R grown in MRS broth, bar represents 25 μm; (c) 210R grown in Mg-supplemented MRS broth, bar represents 10 μm; (d) 210R colonies on MRS agar from an MRS culture, bar represents 1 mm; (e) 210R cells from an MRS agar colony, bar represents 25 μm.

ing unit could be placed inside the fermentation vessel, thus eliminating the bead-transfer step from an exterior $CaCl_2$ bath. Many units could be adapted for this purpose: Inovatech (Dottikon, Switzerland) or Nisco (Zurich, Switzerland) multi-nozzle systems, the vortex-bowl system (Champagne et al., 2000a) or jet-cutting system (K-Vorlop, Braunschweig, Germany).

One of the advantages of immobilization is the possibility of obtaining higher cell densities in gel beads than in fermented milk or broth (Champagne et al., 1994). Although total populations in free-cell and ICT fermentors were similar, it was indeed possible to generate concentrated cultures of *S. thermophilus*, since immobilized-cell populations in beads were 13 times higher than in the traditional free-cell starters. Therefore, this study confirms the usefulness of ICT to produce concentrated cultures without the use of centrifugation of filtration. This could be critical for cultures that are sensitive to these technological steps.

Maximum *S. thermophilus* bead cell count obtained in this study was 8.2×10^9 cfu/g beads, which is in accordance with the population achieved by Champagne et al. (2000b), of 1×10^{10} cfu/g beads. However, both results were well above those reported by Audet et al. (1988), at 10^7 cfu/ml gel, for a *S. thermophilus* immobilized in carrageenan/locust bean gum gel beads. This shows the considerable variability in the literature with respect to cell counts of lactic cultures in gels.

Cell retention in beads were high for both lactobacilli and streptococci. In ICT systems designed for the continuous inoculation of milk, the free cell population typically represents less than 5% of the total population in the system (Prévost and Diviès, 1988). Therefore results of this study are in agreement with the literature on continuous-culture conditions. However, the free-cell levels of the ICT system were lower than those reported Champagne et al. (1993), who obtained between 40 and 60% of free-cells following batch fermentations. Since batch fermentations were performed in this study, it was expected that free-cell levels in the

ICT fermentors would therefore be higher. The reason for such a discrepancy has not been established. One possible explanation is that this study was carried out with Ca-supplemented milk, in order to prevent bead dissolution, which would reduce cell release from the alginate beads. Klinkenberg et al. (2001) have examined the effect of high pH values on cell release, but more data is needed on this aspect, because free-cell release is unwanted when ICT is used for the production of concentrated starters; parameters that affect cell release in batch fermentations need to be better controlled.

The ICT was effective for preparing high cell densities for the streptococci, but not as much for lactobacilli. Lower lactobacilli cell yields in ICT systems had been reported (Champagne et al., 1993) and the data from this study add to the literature on this subject. Differences in bead cell counts were observed depending on the immobilized strains. Among lactobacilli, *Lb. helveticus* yielded the highest cell counts (3×10^9 cfu/g beads). This population was well below that reported by Masson et al. (1994) of 3.1×10^{10} cfu/g beads for a *Lb. helveticus* strain immobilized in carrageenan/locust bean gum gel beads. The discrepancy could be explained by the effect of strains or of the type of fermentation carried out. Beads obtained from ICT systems operating for extensive periods under continuous fermentations have higher cell densities than those obtained from single batch fermentations (Champagne et al., 1993; Prévost and Diviès, 1988, 1992).

Many studies report good growth of *Lb. bulgaricus* in alginate beads (Champagne et al., 1993), and that their cell counts can be as high as 1.2×10^{10} cfu/g beads. However, bead cell counts lower than those reported here were obtained by Audet et al. (1988), at 1.9×10^7 cfu/ml gel for a *Lb. bulgaricus* immobilized in carrageenan/locust bean gum. The *Lb. bulgaricus* 210R strain may be atypical in its sensitivity to alginate, but these data show that some lactobacilli apparently cannot grow well in alginate gels, and attempts were made to determine the reasons for the low cfu values in certain conditions/strains.

Results from this study suggest that low cell counts obtained with immobilized lactobacilli might be related to the morphological cell changes observed following entrapment in the gel. While no morphological modification for immobilized cells of *S. thermophilus* HC15 was observed, all immobilized *Lactobacillus* strains displayed cells larger than in free-cell fermentation. Furthermore, strain 210R exhibited the strangest morphology, with curved elongated cells. Thus, microscopic examination suggests that growth of strain 210R did occur, but the long cell chains resulted in an absence of increase in the cfu counts (even if the methodology included a chain-breaking homogenization step). Lactobacilli are known to be pleomorphic. The morphological modifications commonly reported are chain or cell elongation, globule or bleb formation and spheroplast- or protoplast-like cells (Norton et al., 1993; Pavlova et al., 1993).

Growth conditions are recognized to affect the morphology of lactic lactobacilli. Several studies (Norton et al., 1993; Wright and Klaenhammer, 1983) mention that chain length of a *Lb. helveticus* strain increases when pH increases. Rhee and Pack (1980) demonstrated that the synthesis of the dechaining enzyme required to disconnect newly formed cells of a *Lb. helveticus* strain is lower at high pH. During continuous fermentation with free cells, increasing dilution rate causes *Lb. helveticus* to become thinner and longer (Aeschlimann et al., 1990; Roy et al., 1987). Erratic changes in *Lb. bulgaricus* morphology with increasing dilution rate are also reported (Stein et al., 1989). Moreover, cell immobilization has an impact on chain length (Norton et al., 1993): immobilized *Lb. helveticus* demonstrates medium-sized chains, which are only slightly affected by pH.

Composition of broth medium is also reported to affect lactobacilli morphology. Deficiency in vitamin B_{12} (Kitahara and Kusaka, 1959) or formic acid (Suzuki et al., 1986) causes cell elongation of *Lb. bulgaricus*. Minerals contents of broth medium affect lactobacilli cell morphology in different ways. They are thought to be required for cell division and cell wall synthesis. Wright and Klaenhammer (1983) showed that *Lb. bulgaricus* needs Mg^{2+} in addition to Ca^{2+} or Mn^{2+} for the transition from filamentous chains to short bacilloid rods. These cations are required for dechaining. The authors also observed at low level of calcium or manganese, spherical protrusions on the cell surface which disappear when calcium is added to the broth. Wright and Klaenhammer (1983) related the blebs with autolysin-induced wall damage that calcium is thought to minimize. Pavlova et al. (1993) observed similar round forms with *Lb. helveticus* cultivated in mineral-depleted broth. Moreover, they observed round cells attached to mother cells, spherical particles and rod-shaped spheroplast-like cells. The authors assumed that these

abnormalities are associated with an irregular cell wall synthesis and showed that supplementation of the medium with Mg^{2+} restores the rod shape morphology.

The differences in mineral environment between milk and MRS broth could explain the different aspects of strain 210R. Minerals such as calcium, magnesium and manganese are found in both milk and MRS broth. However, in MRS broth, some of them could be partially chelated by potassium phosphate. Chelation of divalent cations in phosphated milk was proved to alter the morphology of *Lb. bulgaricus*. In phosphated milk, the strain existed as long, tangled chains of cells, instead of rods in milk without phosphate (Wright and Klaenhammer, 1984). Our data are in accordance with this since *Lb. bulgaricus* 210R appeared as rods in milk and as long tangled chains in MRS broth. However, addition of 30 mM $MgSO_4$ in MRS was sufficient to restore the rod-shaped morphology of the strain.

Immobilization in alginate beads could very well change the mineral environment of the immobilized cells. Czaczyk et al. (1997) demonstrated that alginate gel adsorbs divalent cations such as Mg^{2+} and Mn^{2+} from a casein broth medium supplemented with various divalent cations. The most striking morphological alteration was that observed for strain 210R in alginate beads, which existed as long tangled cells. Indeed, the appearance of *Lb. bulgaricus* 210R in alginate beads was very similar to that of strain *Lb. bulgaricus* 1243F in a phage inhibitory medium, as demonstrated by Wright and Klaenhammer (1984). In both cases, the cells were in severely cation-deprived environments. Cell elongation caused by mineral depletion is accompanied by severe growth inhibition (Wright and Klaenhammer, 1983, 1984), which could explain the low cell counts obtained for immobilized *Lb. bulgaricus* 210R. Although Ca, Mg and Mn contents of the beads were not determined, the encapsulated cells could be in a mineral-deficient environment, which might be responsible for modifications in immobilized cell morphology. On the other hand, the fact that long cell chains of strain 210R were seen in a milk agar points to an effect of immobilization "per se."

The doubling of cell width observed for all immobilized lactobacilli could also be linked to a cation deficiency caused by alginate. Pavlova et al. (1993) observed rod-shaped spheroplast- or protoplast-like cells of a strain of *Lb. helveticus*, with a rough surface in cation-depleted broth. The spheroplast-like cells were about 1 μm width, which is twice the width of the cells in a mineral-rich medium. Unlike cell elongation, cell enlargement is not associated with growth inhibition (Pavlova et al., 1993).

The *Lb. bulgaricus* 210R colony morphology on MRS agar was very different from that of the other lactobacilli used in this study. Instead of round, opaque and smooth colonies, *Lb. bulgaricus* 210R yielded rough colonies. Cells from colonies appeared as very long filaments, which were longer than those observed in the beads. Chain elongation might be caused by an interaction between cells and a solid support. Such an interaction could also occur in alginate beads. Thus, in alginate beads, 210R growth could be impaired by a deficiency in Mg^{2+} and an interaction between the cells and a solid support.

Conclusion

Suspensions of lactobacilli in alginate beads do not reach cell counts as high as streptococci. Some lactobacilli show much longer cell chains when in alginate gels, which could partially explain the lower cfu counts. Furthermore, lactobacilli growth in alginate beads might be impaired by the combination of their higher cation sensibility and the cation-reduced micro-environment in alginate beads. Further work will be needed to confirm this hypothesis. To increase intrabead cation concentration, carrageenan or galactomanan gels could be substituted to alginate, since these gels have a lower cation affinity than alginate. However, regarding industrial application, alginate is a convenient polysaccharide for cell immobilization, because cell encapsulation can be done at low temperature. Moreover, alginate beads can easily be dissolved using sodium citrate, allowing cell recovery in a liquid state. Supplementation of the growth medium with high levels of cations, such as calcium, magnesium or manganese might also prove useful, as long as the increase in cation concentration in broth is translated by an increase in cation concentration in beads.

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