Long-Term Mechanical and Biological Stability of an Immobilized Cell Reactor for Continuous Mixed-Strain Mesophilic Lactic Starter Production in Whey Permeate

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The aim of this study was to characterize the biological and rheological stability of continuous immobilized cell fermentation for the production of mixed-strain mesophilic starters. Three strains of Lactococcus were immobilized separately in κ-carrageenan-locust bean gum gel beads. Continuous fermentation was carried out in a 1 L pH-controlled stirred tank reactor, operated at 30 °C, pH = 6.2, and D = 2 h⁻¹ in whey permeate medium supplemented with yeast extract (1.5%) and 0.1 M KCl and inoculated with 30% (v/v) bead inoculum (strain ratio 1:1:1). The continuous mixed-strain immobilized cell fermentation demonstrated a high biological stability, and no strain became dominant or was eliminated during the 52 day fermentation. The total and specific free cell populations showed high time stability. All immobilized populations, except MD, were unchanged, but a cross contamination of gel beads initially immobilizing a pure culture occurred, leading to a redistribution of immobilized population in individual beads. After initial modifications of bead rheological properties during colonization batches, the beads demonstrated a high mechanical stability, even with reduced KCl supplementation of the broth medium, in the range 0.1–0.5 M. This work emphasizes the potential of immobilized cell technology for producing mixed lactic starters in continuous fermentation.

Introduction

Lactic acid bacteria perform important functions in cheese making. Through the lactose catabolism and the resulting lactic acid production, lactic acid bacteria help stabilize the curd by coagulating the proteins and expelling moisture (1). Moreover, lactic acid production inhibits the growth of undesirable microorganisms. During cheese making, aromatic lactic acid bacteria produce aromas such as diacetyl, acetaldehyde, and acetone. During cheese ripening, lysed cells of lactic acid bacteria provide proteolytic and lipolytic enzymes which contribute to the development of cheese texture and aroma.

Although lactic starters are traditionally produced by batch fermentation, the accumulation of metabolic end products limits cell growth. The use of continuous fermentations may overcome this limitation, but they are more susceptible to contamination and loss of plasmid-mediated characteristics and they rapidly lead to the disappearance or domination of strains in a mixed-strain continuous fermentation (2–4). One promising alternative is the use of immobilized cell technology to produce mixed lactic starters in continuous fermentation (5). The high immobilized cell concentration results in a very high productivity and decreases contamination risks due to the high dilution and inoculation rates provided by cell release from beads. Immobilization also improves plasmid stability (2, 6, 7).

In a previous study, we examined the production of mixed-strain mesophilic lactic starters during continuous fermentations of a supplemented whey permeate medium, using immobilized cell technology (5). The effect of pH, temperature, and dilution rate and their interactions on the starter composition and activity in the effluent, bead population, and lactic acid production were studied by periodically changing the set points during continuous fermentation. The process showed a high biological stability and cell productivity (maximum cell productivity of 5.3 × 10¹² CFU L⁻¹ h⁻¹) over the tested period exceeding 50 days. By varying pH, D, and T, we could obtain a large range of strain ratios, while starter activity remained constant. However, the enumeration of individual beads, initially immobilizing a single strain, did not reveal a pure bacterial culture after one week of fermentation. Nevertheless, the biomass redistribution led to a strain ratio in beads that was close to the initial bead ratio in the reactor.

When encapsulated cells are used, the matrix stability is of considerable importance for long-term stability of the process. Matrix stability may be estimated indirectly in packed bed column reactors, by comparing the ratio of the flow rate through a column containing gel beads to that of an empty column (8, 9). However, most studies have used rheological measurements of biocatalysts. Because of the small size of gel beads typically used for cell immobilization (diameters less than 3–4 mm) and the heterogeneous size distribution, accurate rheological measurements are difficult. As an alternative, rheological properties of thin gel disks immobilizing cells or cylinders without cells have been examined, using compression
tests (10–14), an oscillatory shear experiment (15), or creep measurement in compression (16). Uniaxial compression tests and rupture tests performed on gel beads sampled from the immobilized cell reactor have been reported in very few studies, some of which used custom-developed complex equipment (16, 17). While some authors have examined the effect of biomass (8, 9, 15) or fermentation medium (13, 14) on gel properties, to our knowledge, changes in rheological properties of beads during long-term fermentations have not been reported yet.

The aim of this study was to characterize the biological and rheological stability of continuous immobilized cell fermentation for the production of mixed-strain mesophilic starters. During a long-term continuous fermentation (8 weeks), operated under a single set of pH, T, and D, the following changes were studied as a function of time: total and specific populations in the effluent and in gel beads, lactic acid production, and activity of the starter. Mechanical characteristics of gel beads were evaluated by a new and accurate 99% compression test. We also studied the effect of different KCl supplementation on bead mechanical properties, to evaluate the possibility of reducing and eventually eliminating whey permeate medium-KCl supplementation which is currently used to stabilize κ-carrageenan-locust bean gum mixed gels. Finally, the influence of weekend interruption on process performance is reported; the shut off/start protocol proposed in this study shows the high flexibility of immobilized cell fermentations.

**Material and Methods**

**Strains.** All strains were provided by Rhone Poulenc (Brampton, ON, Canada). Strains KB and KBP were isolated from the mixed culture MA016 Ezal and further identified as Lactococcus lactis subsp. lactis; strain “MD089 Ezal” (MD) was identified as Lactococcus lactis subsp. lactis biovar diacetylactis (5).

**Chemicals.** Kappa-carrageenan GENUGEL X0909 was obtained from the Copenhagen Pectin Company (Lille Skensved, Denmark). Sandofi Bio-Industrie (Baupte, France) supplied the locust bean gum (LYGOMME, 6HV, no. 425). KCl was provided by BDH Inc (Toronto, ON). MRS medium and yeast extract were supplied by Rosell Institute Inc. (Montreal, QC, Canada). Whey permeate powder was obtained from Wisconsin Dairy (Foremost Ingredient Group, Baraboo, WI) and low-heat skim milk powder (Crino) from Agropur (Granby, QC, Canada).

**Medium.** Whey permeate powder was reconstituted at 5.7% (w/w) total solids and, unless otherwise specified, supplemented with 0.1 M KCl. The pH was adjusted to approximately 10.0 with NH₄OH. The medium was allowed to settle overnight. It was then filtered on a filter press (Fqjet, Buon Vino, Cambridge, ON) with filter no. 2 and heat-treated with a Spiratrehem Model lab (Cherry Burrel Corp., Cedar Rapids, IA) at 135 °C for 8 s. To test the effect of KCl concentration, we prepared the medium as described above, except for KCl addition. An autoclaved 3M KCl solution was then added to adjust the KCl concentration in the final medium in the range 0–0.1 M. The medium was supplemented with 1.6% (w/v) sterile yeast extract (5).

**Immobilization.** KB, KBP, and MD strains were immobilized separately in gel beads of κ-carrageenan and locust bean gum gels (2.75%: 0.25% (w/v)) using a double-phase dispersion process with some modifications (5). The beads, immobilizing a pure culture, were incubated in MRS medium for three successive pH-controlled (pH = 6.0) batch fermentations. To study the effect of these successive batch fermentations on bead rheological properties, we sampled beads containing KB, KBP, and MD 15 min after the beginning of the first fermentation and at the end of the first, second, and third fermentations. Cell enumerations were also performed on these samples, as described previously (5).

**Continuous Fermentation.** To study the effect of a long-term fermentation on biological process stability and bead rheological properties, we conducted a continuous fermentation for 8 weeks in a 1 L stirred tank bioreactor inoculated with 100 mL of beads of each strain as previously described (5). The pH was maintained at 6.2 by adding of NH₄OH (6 M), the temperature was controlled at 30 °C, and the dilution rate was set at 2 h⁻¹, corresponding to the central point of the previous study. At the end of each week, beads were drawn from the bioreactor, washed with peptonized water (0.1%) containing 0.2 M KCl, and then stored for the weekend at 4 °C in the rinsing solution containing citrate buffer (pH 6.0, 0.05 M). To start the fermentation at the beginning of the week, we successively set the dilution rate at 0.5 and 1.5 h⁻¹ for 30 min, except for the first week, where it was successively set at 0.5 and 1 h⁻¹ for 30 min and 1.5 h⁻¹ for 3 h. The bioreactor was operated 5 days per week, except for the first and third week (3 and 4 days, respectively) for 8 weeks. During this period, cell enumerations were performed daily, 4 days a week on three effluent samples taken from the bioreactor at hourly intervals. The samples were kept frozen (−18 °C) for the determination of sugars and organic acids. Population enumeration on 0.5 g beads and 10 individual beads, rheological analysis, and the acidification test were done once during the first week, twice during weeks 2 and 3, and then once a week for weeks 4–7. At the start of each fermentation, 0.5 g beads were also enumerated. During week 8, KCl concentration in the medium was decreased daily from 0.1 to 0.025, 0.012, 0.06, and 0 M KCl. For each concentration tested, rheological measurements were performed on beads sampled from the reactor, which was operated for at least 21 h with the new KCl concentration. Other analyses were performed as for week 7.

**Cell Enumeration.** Enumerations of the different populations in the effluent samples and in gel beads were performed as described previously (5), by plating on KMK agar, a differential medium based on citrate utilization (18). After sampling, rinsing, and sieving, gel beads were dried for 1 min on a sterile filter placed in a Petri dish before proceeding to enumeration.

**Activity Tests.** The acidifying activity of the culture in the effluent from the continuous immobilized cell reactor was determined, using a standardized activity test (5). For each test, the effluent sample was first diluted with sterile whey medium to obtain an absorbance of 0.250 at 625 nm on a Novaspec II spectrophotometer (LKB, Biochrom, Cambridge, England). Erlemeyer flasks containing 100 mL of reconstituted milk (11% w/w), previously autoclaved at 121 °C for 10 min, were inoculated at 1% (v/v) with the absorbance standardized sample. For each sample, four Erlemeyer flasks were inoculated. The pH measurements were done every 45 s during incubation at 30 °C with agitation provided by magnetic stirrers at 150 rpm. Changes in pH versus time were recorded using a software written in Basic (Quick Basic Version 4.5, Microsoft). The pH versus time data were first smoothed using a mobile mean over 15 points, and the first derivative was calculated. Then, the following parameters were calculated: time to reach pH
= 4.9 (T_{4.9}, in min), the acidification rate at pH = 4.9 (V_{5.9}, in milliunits of pH/min), the maximal acidification rate (V_{max}, in milliunits of pH/min), the time corresponding to V_{max} (T_{max}, in min), and the pH at which V = V_{max} (pH_{max}).

**Determination of Sugars and Organic Acid Concentrations.** Concentrations of sugars (lactose, glucose, galactose) and organic acids (lactic acid, acetic acid, formic acid) as well as acetoin and ethanol concentrations were determined by HPLC analysis (Waters, Millipore Co., Montreal, QC, Canada) with a Phenomenex ion column (Phenomenex, Torrance, CA) and H_{2}SO_{4} 0.0064 N as eluent at a flow rate of 0.4 mL/min. The samples were prepared as previously described (S).

**Determination of Cations in Whey Permeate Medium.** The concentration of K^{+} and Ca^{2+} in a KCl–unsupplemented effluent sample was determined by capillary electrophoresis (3DCE, Hewlett-Packard, Kirkland, QC, Canada) with a capillary (length: 72 cm with an internal diameter of 50 μm) and a mobile phase composed of 4 mM CuSO_{4}, 4 mM HCO_{3}, and 4 mM 18-Crown-6 (Sigma, St Louis, MO). The analysis was done at 22.5 °C, 30 mV, and 50 mbar. The sample was acidified to solubilize minerals and centrifuged for 10 min at 22.5 °C, 30 mV, and 50 mbar. The sample was acidified to solubilize minerals and centrifuged for 10 min at 22.5 °C, 30 mV, and 50 mbar. The sample was acidified to solubilize minerals and centrifuged for 10 min at 22.5 °C, 30 mV, and 50 mbar. The sample was acidified to solubilize minerals and centrifuged for 10 min at 22.5 °C, 30 mV, and 50 mbar. The sample was acidified to solubilize minerals and centrifuged for 10 min at 22.5 °C, 30 mV, and 50 mbar.

**Rheological Analyses.** To measure the rheological characteristics of the bioencapsulation beads and their changes during fermentation, we developed an accurate test. Single compressions of the beads, at 99% of their original height, were carried out with texture analyzer TA-XT2 version 5.15 (50 N maximum force, precision of 0.001 N; Stable Micro Systems, Halslemere, Surrey, United Kingdom). A compression speed of 0.2 mm/s was used to obtain an adequate resolution of rupture forces and an accurate automatic detection of the contact of the bead surface (sensitivity set at 0.01 N). The return speed of the cell after compression was 1.0 mm/s. Rupture force (measured in N), percentage of deformation at rupture, and maximum force (N) measured at 99% of deformation were calculated from the rheograms using the XT.RAD Dimension software, version 3.7H from Stable Micro System. To avoid drying, we limited the air exposure of the beads to less than two minutes. Large beads with diameters greater than 1.5 mm were visually preselected, leading to an average bead diameter of 1.8 ± 0.2 mm, estimated on all rheological analyses. Rheological tests were performed on 10–15 individual beads.

**Statistical Analysis.** For statistical analysis, microbial populations were transformed to their base 10 logarithm. All statistical analyses were performed with SAS (SAS System, SAS Institute Inc., Cary, NC). Bead diameter was determined from the rheograms. Provided that the automatic detection of the bead surface by the probe was accurate enough, the bead diameter was calculated using the distance between bead detection and the peak at 99% compression. For rheological analysis, bead diameter was included as a covariate when the rheological parameter was significantly correlated (p < 0.05) with bead diameter. Analysis of variance was done using the GLM procedure to discriminate among the immobilization steps. Beads made with different strains were considered as repetitions. The ANOVA procedure was used to compare bead populations assayed on Mondays with those assayed during the week. Linear regressions were performed with the REG procedure to test for the time changes of free and immobilized populations, acidifying activity, sugar consumption, and lactic acid production, as well as the effect of KCl on bead rheological properties.

**Results**

**Free Cell Populations.** Time changes for total and specific free cell populations during the 8-week continuous fermentation are reported in Figure 1. The missing data points correspond to 2 day weekend interruptions and the first day of operating the bioreactor. The total population in the effluent did not change significantly with time (p < 0.05) and was 1.7 × 10^{6} ± 0.4 × 10^{6} CFU/mL throughout the fermentation. The mixed culture demonstrated a high stability, and no strain became dominant or was eliminated. Free-cell populations of KB increased from 1.0 × 10^{6} CFU/mL at day 2 to 6.2 × 10^{7} CFU/mL at day 7. After this stabilization period, KB free-cell population was slightly correlated with time (R = 0.68, p < 0.001) and slowly increased to reach 1.4 × 10^{8} CFU/mL at the end of the 8 week fermentation. KBP free-cell population increased from 1.3 × 10^{6} CFU/mL at day 2 to 3.6 × 10^{8} CFU/mL at day 3 and then increased slightly with time (R = 0.62, p < 0.001) to reach 7.0 × 10^{9} CFU/mL at the end of the fermentation. MD free-cell population did not demonstrate any stabilization period and decreased slightly but significantly (R = −0.58, p < 0.001) with time during the experiment, from 1.2 × 10^{7} CFU/mL at day 3 to 8.6 × 10^{6} CFU/mL at the end of the fermentation.

**Imnobalized Populations.** Immobilized populations were estimated immediately after the weekend storage periods (2 days) and during the subsequent continuous fermentations during the week. Populations for day 1 were eliminated since bead colonization was not completed. For total immobilized and MD-strain populations, populations estimated following storage periods were significantly lower than those estimated during fermentations (p < 0.05). Mean total immobilized populations after storage period (9.0 × 10^{10} ± 3.3 × 10^{10} CFU/g of bead) and on week days (1.3 × 10^{11} ± 0.4 × 10^{11} CFU/g of bead) were not correlated with time (p > 0.05). The immobilized populations of MD strain (Figure 2) after the storage period and during fermentation were affected by time (p < 0.05) and increased, respectively, from 1.9 × 10^{10} CFU/g of bead on day 6 to 8.1 × 10^{10} CFU/g of bead on day 48, and from 4.8 × 10^{10} CFU/g of bead on day 6 to 8.1 × 10^{10} CFU/g of bead on day 48.  

![Figure 1. Specific and total population counts during the 52 day continuous fermentation with three immobilized lacticocci in the supplemented whey permeate medium (KB, □; KBP, ○; MD, △; total population, ◆).](image333x597 to 543x744)
characterized by on day 50. The and decreased slightly from 480 min on day 3 to 402 min significantly (\( \text{p} < 0.001 \)) from 684 on day 3 to 575 min, and pHm ranging between 400 and 600 min, for the analysis of 10 (a, c, and d) or 20 (b) individual beads during the 8 week continuous fermentation: (a) week 1; (b) week 3; (c) week 6; (d) week 8.

Figure 4. Changes in maximum acidification rate \((V_m)\) during the 52 day continuous fermentation measured by the absorbance standardized (0.250 at 625 nm) activity tests. Bar represents the standard error calculated from four replications.

on day 50. However, pHm for the first peak and \(V_{49}\) were not correlated with time \((\text{p} > 0.70)\) and remained constant at 5.72 \pm 0.09 and \(-3.04 \pm 1.36\) milliunits of pH/min, respectively.

Lactose Utilization and Lactic Acid Production. Lactic acid concentration in the effluent was slightly \((R = 0.64)\) but significantly \((\text{p} < 0.001)\) correlated with time and increased from 5.9 g/L on day 2 to 7.9 g/L on day 52. Lactose utilization also increased slowly but significantly from 5.7 g/L on day 2 to 8.3 g/L on day 52. Glucose, galactose, formic acid, acetic acid, ethanol, and acetone concentrations were below the detection threshold of the HPLC (less than 0.4 g/L) for all experimental points.

Rheological Analysis. Colonization Batches. Beads encapsulating a single strain culture were analyzed after immobilization and at each of the successive batches used for colonization. The rheograms obtained from compressing beads to 99% deformation exhibited one, two, or more clear ruptures (Figure 5); only the first rupture was used for the analysis. The diameter of the beads was used as a covariate, because rheological parameters were correlated with bead diameter \((\text{p} < 0.05)\). Rupture force
(Table 1) decreased significantly \( (p < 0.001) \) from 3.87 to 3.01 N from the beginning to the end of batch 1 and to 2.54 N at the end of batch 2, and then remained constant. The percentage of deformation at rupture (Table 1) decreased significantly after each step, from 79\% to 69\%, from the beginning of batch 1 to the end of batch 3. The maximum force measured at 99\% deformation (Table 1) increased significantly \( (p < 0.01) \) between the beginning of batch 1 (5.89 N) and the end of batch 3 (6.40 N).

**Continuous Fermentation.** The effects of long fermentation time on rheological properties of beads were determined on beads sampled for 7 weeks of fermentation with whey permeate medium supplemented with 0.1 M KCl. Rupture force and force at 99\% compression were correlated with bead diameter \( (p < 0.001) \) which was included in the models, but the percentage of deformation at rupture was not \( (p > 0.10) \). Rupture force was not affected by time \( (p > 0.10) \), averaging 1.54 \( \pm 0.43 \) N. The force at 99\% compression changed with time \( (p < 0.001\) and \( R^2 = 0.65 \) and decreased from 5.89 N on day 2 to 4.44 N on day 44, for a 2 mm diameter bead. The percentage of deformation at rupture increased linearly from 67.7\% at day 2 to 72.8\% at day 24 and stayed constant at approximately 72.9\% from day 24 to day 44 (Figure 6).

**Effect of KCl Concentration.** The KCl concentration was decreased progressively at the end (week 8) of the mixed-strain continuous fermentation from 0.1 to 0 M KCl. Rupture force \( (p < 0.001) \), force at 99\% compression \( (p < 0.001) \), and percentage of deformation at rupture \( (p < 0.01) \) were significantly correlated with bead diameter. For a 2 mm diameter bead, rupture force and force at 99\% compression decreased, respectively, from 1.75 to 1.56 N and from 5.61 to 3.91 N as KCl concentration decreased from 100 to 0 mM. The percentage of deformation at rupture, 72.9\% \( \pm 2.9\% \), was not affected by KCl concentration \( (p > 0.05) \).

**Discussion**

In a previous paper \( (5) \), we studied the production of mixed mesophilic lactic starters, referred to as F1 in the text, using the same strains, culture medium, and bioreactor configuration. The immobilized cell technology proved suitable, because no strain in the mixed culture disappeared or became dominant and the total population, estimated by 8 repetitions of the central points during the 53 day fermentation, was high and stable at \( 1.5 \times 10^6 \pm 0.3 \times 10^6 \) CFU/mL. By varying pH, T, and D, we obtained starters of controlled composition with

![Figure 5](image1.png)

**Figure 5.** Rheograms obtained from compression of beads at 99\% deformation exhibiting 1 (a) or 2 (b) clear ruptures or multiple ruptures (c).

![Figure 6](image2.png)

**Figure 6.** Changes in percentage of deformation at rupture of gel beads sampled during the 52 day continuous fermentation. Datapoints represent means recorded from the analysis of 10–15 beads.

KB, KBP, and MD varying from 0\% to 35\%, 10\% to 79\%, and 12\% to 85\% of total population, respectively, while a constant activity of the absorbance standardized cultures in the effluent was observed \( (T_{abs} = 604 \pm 27 \) min, \( V_{abs} = -3.90 \pm 0.79 \) milliunits of pH/min, \( T_{max} = 482 \pm 83 \) min, \( V_{max} = -5.37 \pm 0.26 \) milliunits of pH/min, and \( pH_{max} = 5.72 \pm 0.24 \) ). Industrial applications require a high biological and mechanical stability of the continuous process over a long time period. It is therefore necessary to study the time changes of the process with a single set of conditions from Lamboley et al. \( (5) \). Data obtained in this condition can then be used to validate the time changes that were observed in the continuous fermentation where operating parameters were periodically changed \( (5) \). Since the mechanical stability of the carrier

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**Table 1. Change of Rheological Properties of Gel Beads during Bead Colonization**

<table>
<thead>
<tr>
<th></th>
<th>before batch 1</th>
<th>after batch 1</th>
<th>after batch 2</th>
<th>after batch 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>% deformation at rupture (%)</td>
<td>79.2 ± 0.65</td>
<td>73.5 ± 0.62</td>
<td>70.8 ± 0.60</td>
<td>69.1 ± 0.60</td>
</tr>
<tr>
<td>% deformation</td>
<td>5.89 ± 0.43</td>
<td>6.22 ± 0.46</td>
<td>6.04 ± 0.44</td>
<td>6.40 ± 0.46</td>
</tr>
<tr>
<td>Rupture force (N)</td>
<td>3.87 ± 0.43</td>
<td>3.01 ± 0.46</td>
<td>2.54 ± 0.44</td>
<td>2.50 ± 0.46</td>
</tr>
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* For each rheological parameter, means with the same letter are not significantly different \( (p < 0.05) \).
The beads initially immobilized a pure culture of one of the three strains, but by the end of the fermentation, the enumeration of individual beads indicated that contamination had occurred. This phenomenon was observed by our group during milk pre-fermentations using four strains of lactic acid bacteria separately immobilized in κ-carrageenan/locust bean gum gel (21, 22); cross contamination among the four types of beads containing different strains occurred as early as the third week of fermentation. Cross contamination was also observed in the previous study (F1) of mixed mesophilic lactic starter production, where beads contained 2 populations at 2 weeks and 3 populations by the end of the fourth week. At the end of fermentation F1, the average bead composition ratios (ratio of one strain to total population) were KB = 20%, KBP = 38%, and MD = 42%, or 1:2:2, which was close to the initial bead ratio of 1:1:1. This cross contamination could not be associated with the formation of a biofilm on the bead surface (5). A theoretical model for cell release that could explain cross contamination was proposed (21). According to this model, the disruption of gel microcolonies near the bead surface might result from biological (cell colony expansion) and mechanical (collision and shearing force in the bioreactor) causes. As a result, cell release occurred and the partially emptied cavities closed again, entrapping a sample of the bulk medium and leading to a new colonization cycle. This would incorporate contaminating flora into the active layer of the beads. The local conditions inside the microcolony, such as concentration of dissociated or nondissociated lactic acid, would influence the competition between strains during the new colonization cycle.

In this study, the changes in cross contamination were different from those in F1. For F2, at week 3 (Figure 3), two groups of beads were distinguished on the basis of their composition. The low KB group might represent beads initially immobilizing pure cultures of MD and KBP, in which cross contamination first occurred with KBP and MD, respectively. Contamination of these beads with KB population appeared later, starting at week 5 (not shown). The second group (high KB) observed at week 3 was located at the apex of the triangle and represented beads containing a high proportion of KB (80–98%) and between 0% and 10% each of KBP and MD. This group is therefore composed of KB beads, which were slightly cross contaminated by MD and KBP strains. These bead groups can still be distinguished at week 8 (Figure 3d). On the other hand, during fermentation F1, all beads exhibited a uniform mixed-strain population corresponding to 20% KB, 38% KBP, and 42% MD after 6 weeks. This difference in the development of cross contamination might be due to the different conditions of operation in fermentation F1, where control set points (pH, D, T) were changed every 24 or 48 h. Since the operation conditions have been shown to influence the starter composition in the reactor effluent, they may also have modified the development of cross contamination by locally changing strain competition. On the contrary, fermentation F2 was operated with constant conditions of pH, temperature, and dilution rate.

Nonetheless, the system exhibited a very high stability in F2. Even though cross contamination resulted in major modifications of strain ratios in individual beads, it had little impact on the overall immobilized population, determined on 1 g of beads. Total KB- and KBP-immobilized populations did not change over the fermentation period, and MD-immobilized population increased only 2-fold (Figure 2). The changes in strain distribution of individual beads did not have major effect on free-cell
composition in the effluent; total population remained constant. From day 7 to the end of fermentation and from day 2 to the end, respectively, KB and KBP populations doubled; while, from day 1 to day 52 MD, free-cell population was multiplied by 1.5. However, the acidifying activity of the absorbance standardized starter significantly increased with time, as demonstrated by the decrease in Vm (Figure 4), Tm (–16%), and Ts (–16%) observed after day 3 of the continuous fermentation. This apparent increase in the specific activity of the culture was also confirmed by the increase in lactic acid production, from 5.9 g/L on day 2 to 7.9 g/L on day 52 and the parallel increase in lactose utilization, from 5.7 to 8.3 g/L, respectively, although the total biomass in the reactor did not change significantly. This observation may be partly explained by the slight increase in the immobilized cell MD population since total KB- and KBP-immobilized populations did not change with time, and by the slight change in starter composition observed with time toward optimal strain ratios for acidifying activity.

Bead rheological characteristics were studied during batch colonization and continuous fermentation until week 7 and for decreasing KCl concentrations during week 8. At the beginning of the first colonization batch, about 50% of rheograms showed multiple (i.e., mostly double) ruptures. This proportion decreased progressively during successive colonizations; after the last colonization batch and during the continuous fermentation, a single rupture point was observed. The multiple ruptures may be explained by the heterogeneous bead structure in κ-carrageenan and alginate gel bead, such as superficial crust, radial microshafts and microchannels, the presence of discrete cavities, concentric gel block layers, amorphous gel block, and randomly distributed fractures, all of which can originate from heterogeneous supramacromolecular structures. These heterogeneities are directly affected by polymer characteristics as well as by parameters of bead fabrication. During batch colonization and subsequent continuous fermentations, the rheological properties of beads were also modified by cell growth inside the beads, mechanical stress in the stirred reactor, and chemical interactions between the polysaccharide gel and the fermentation medium. Cell growth has already been shown to alter carrier stability. It was observed that the polymer lattice of yeast immobilizing alginate beads was destroyed as yeast populations increased. Arnaud et al. (15) noted only a slight effect of cell growth on mechanical properties of thin κ-carrageenan/LBG disks immobilizing S. thermophilus, using oscillatory shear experiments. This attenuated effect was thought to be due to limited cell growth in gel disks (maximal population: 7 × 10⁸ CFU/g of gel) during the batch incubation of the inoculated gel disks. To our knowledge, no other studies on the effect of cell growth on mechanical properties of carriers have been reported. The effect of cell growth was greater between the beginning and the end of the first colonization batch, where the average population in beads increased 240 times, from 3.8 × 10⁷ to 9.2 × 10⁹ CFU/g, and rupture force decreased from 3.87 to 3.01 N. The microscopic observation of bead sections sampled from continuous fermentations showed two distinct concentric parts: an external layer, highly disrupted by cellular growth, and a central part, where local conditions such as inhibitory lactic acid concentrations and pH did not allow bacterial growth. The decrease in rupture force might be partly associated with the formation and expansion of the peripheral layer and the resulting decrease in the radius of the internal core.

During fermentations, interactions between polysaccharide gels and fermentation medium occur and might explain changes in gel bead rheological properties. Czaczek et al. (13) examined the effect of organic acids (propionic and acetic acids) on the mechanical stability of κ-carrageenan and κ-carrageenan/LBG gel disks. They found that the rupture force for gels stored in acid solution decreased as the acid concentration increased. Lactic acid has been reported to interact with κ-carrageenan during diffusion experiments, probably through electrostatic interactions between the negatively charged lactate molecule and the K⁺ ion involved in the network stabilization. During fermentations, NH₄OH is used to neutralize lactic acid produced. The effect of ammonium lactate on rheological properties of mixed κ-carrageenan/LBG gel was studied using uniaxial compressions at 5% and 90% deformation of noninoculated gel cylinders. Ammonium lactate was shown to increase hardness, rupture force, and percentage of deformation at rupture, while cohesion and resilience decreased. The mechanism of this interaction could not be explained by the presence of negatively charged lactate ions, which may interact with K⁺ ions that stabilize the gel network, but probably involves electrostatic interactions between the positively charged ammonium ion and the negatively charged gel. Beads are subject to mechanical stress in the bioreactor due to shocks with the bioreactor walls, probes, and mixing device. The changes in mechanical properties of gel beads may represent the net result of weakening effects of cell growth, chemical interactions with lactic acid produced by immobilized cells with the Ca²⁺ or K⁺ stabilizing ions, mechanical stress, and the strengthening effect of NH₄⁺.

A previous study on κ-carrageenan/LBG mixed gels showed that KCl supplementation (0.3 M) was necessary to avoid bead softening in Lactobacillus selection medium (28) and whey permeate medium (29). However, a reduction in KCl supplementation between 100 and 0 mM had a limited or no effect on rupture force and percentage of deformation at rupture, while force at 99% compression decreased from 5.61 to 3.91 N. It was demonstrated that κ-carrageenan (Satiagel MR 150) used by Lacroix et al. (28) had significantly lower mechanical properties than delonized κ-carrageenan (Genugel X-0909) used in this study, which may explain their different behavior during fermentation (14). Whey permeate-based medium supplemented with yeast extract already contains 85 mM K⁺ and 7 mM Ca²⁺ as determined by capillary electrophoresis. Calcium ions are thought to interact with κ-carrageenan gel made with K⁺. The probable mechanism involves the binding of Ca²⁺ to sulfate groups of adjacent polysaccharide chains and the subsequent increase in the number of rigid internal links between polysaccharide chains. As the number of links increases, not only gel strength but also rigidity and brittleness increases. Because this study tested each KCl concentration for only approximately 21 h, these results cannot be extrapolated to long-term fermentations using reduced supplementation. Nonetheless, it is now clear that KCl supplementation (0.1 M) can be reduced, and further work is currently underway to examine this effect on the rheological properties of κ-carrageenan/LBG gel beads during long-term fermentations.

Although the study of gel disks (11, 13) might provide pertinent information on gel structure, the results are not applicable to beads. Disk have higher mass transfer limitations compared with beads; disk populations are 15–75 times lower than bead populations, which therefore reduces the internal stress resulting from microbial
growth in disks, compared with beads (15). The study of gels in model solutions allowed the determination of specific effects of individual components (12–14). However, the dynamics of fermentations, that is, the combination of biological, chemical, and mechanical changes in gel beads, was not taken into account. Other methods that are used to assess biocatalyst mechanical properties are indirect measurements of the overall bead resistance in packed-bed reactors (8, 9), as well as compression tests with custom-made equipment (17, 31). Only recently has commercial equipment been used for compression tests on beads (16). The rheological method used in this study was simple, required commercial equipment, and permitted an accurate estimation of rheological characteristics of gel bead biocatalysts of small size as affected by fermentation. Arnaud et al. (15) reported coefficients of variation (CV) from 6% to 20% for oscillatory shear measurements on gel disks, while Chen and Huang (24) reported a CV of 6% for stress at fracture on gel beads. When combined with covariance analysis using bead diameter as covariate, our method gave CVs of approximately 7.5% for rupture force, 5% for force at 99% compression, and 1% for percentage of deformation at rupture, all of which are well within an acceptable range of CV for compression tests on gel cylinders (32).

Conclusion

The continuous mixed-strain immobilized cell fermentation with mesophilic lactic acid bacteria demonstrated a high biological stability during the 52 day culture experiment, even though cross contamination of gel beads occurred. Total free-cell population was high and stable at $1.7 \times 10^9 \pm 0.4 \times 10^9$ CFU/mL, while KB and KPB free-cell populations increased slightly after an initial stabilization period. MD free-cell population decreased slightly during the fermentation, but did not demonstrate any stabilization period. Acidification activity of the effluent starter and the immobilized cell reactor increased during the fermentation, as demonstrated by the decrease in $V_m$, $T_m$, and $T_{2.9}$, and by the increase in lactic acid production. Cross contamination of gel beads led to the appearance of two groups of beads with different individual compositions at the end of week 8: low KB with a composition of 5–10% KB, 20–35% KPB, and 60–80% MD; and high KB with a composition of 21–48% KB, 10–16% KPB, and 40–60% MD. Nonetheless, immobilized populations evaluated on 0.5 g of beads were not greatly affected, and the ratio of specific immobilized populations (KB/KPB/MD) changed from 1:1:3 at the beginning of fermentation to 1:1.7 after 8 weeks, for an initial beads ratio (KB/KPB/MD) of 1:1:1.

The rheological method used to evaluate the mechanical stability of beads provided reliable information on changes in bead rheological properties during fermentation. Rheological changes were important during colonization batches and were attributed to the combined effects of increased immobilized populations with chemical and mechanical stresses. The slow rate of change in bead rheological properties during the subsequent continuous fermentations demonstrated the high mechanical stability of the system. In addition, this study showed that a reduction of KCl supplementation in the supplemented whey permeate medium from 0.1 M had a limited effect on rheological characteristics of the beads during fermentation. Further work is currently underway to evaluate the changes in bead rheological properties with reduced KCl supplementation in whey permeate medium.

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References and Notes


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