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Sugar Utilization and Acid Production by Free and Entrapped Cells of *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactococcus lactis* subsp. *lactis* in a Whey Permeate Medium

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Cells of *Streptococcus salivarius* subsp. *thermophilus* and *Lactococcus lactis* subsp. *lactis* entrapped in κ-carrageenan-locust bean gum gel performed similarly to free cells in the conversion of lactose to lactic acid. Bead diameter influenced the fermentation rate. Cells entrapped in smaller beads (0.5 to 1.0 mm) showed higher release rates, higher lactose, glucose, and formic acid utilization, higher galactose accumulation, and higher lactic acid production than did cells entrapped in larger beads (1.0 to 2.0 mm). Values for smaller beads were comparable with those for free cells. Immobilization affected the fermentation rate of lactic acid bacteria, especially *Lactobacillus delbrueckii* subsp. *bulgaricus*. Entrapped cells of *L. delbrueckii* subsp. *bulgaricus* demonstrated a lower lactic acid production than did free cells in batch fermentation. The kinetics of the production of formic and pyruvic acids by *L. lactis* subsp. *lactis* and *S. salivarius* subsp. *thermophilus* are presented.

Alginate and κ-carrageenan gels have been widely used for lactic bacteria entrapment (14, 17). Due to the porous nature of these gels, cell growth takes place in the gel beads. Preferential cell growth at the surfaces of the beads results in high cell release in the medium (1). This creates a steady inoculum for continuous food processing, as in yoghurt (14) and cheese making (13). However, alginate gels suffered instability with phosphate and lactate ions. This polymer was reported not to be resistant to the growth of lactic acid bacteria due to chemical modification of the gel. Calcium ions which stabilize this type of gel are displaced by lactate ions produced by lactic bacteria (5, 17). κ-Carrageenan produced brittle gels which are not able to withstand the stresses of internal bacterial growth and the shear of the agitated reactor (J.-P. Arnaud, L. Choplin, and C. Lacroix, submitted for publication). A synergistic effect between κ-carrageenan and locust bean gum led to more flexible gels due to specific interaction between carrageenan and galactomanan chains (10, 16; J.-P. Arnaud, L. Choplin, and C. Lacroix, submitted for publication). These gels demonstrated greatly improved rheological properties during lactic fermentation (Arnaud et al., submitted).

The most commonly used method for cell entrapment is extrusion of the cell-polymer suspension through a needle by using air or mechanical force. The drops fall into a hardening salt solution and form beads (5, 17, 23). Mean diameter can be varied from 2 to 4 mm, resulting in important diffusional problems (4, 18).

To prevent bead disruption and to promote diffusion throughout the beads, a special cell entrapment procedure was used. It was based on a dispersion process in a two-phase system which allowed production of beads with a mean diameter of between 0.25 and 4 mm (2, 11; P. Audet and C. Lacroix, manuscript in preparation). Bead sieving permitted the selection of desired bead diameters to minimize mechanical and diffusional problems (4, 18).

The present report compares the performance of free and entrapped cells of *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactococcus lactis* subsp. *lactis* for the production of different acids and sugar utilization. Results on cell release rates, lactose utilization, and lactic acid production were presented in an earlier paper (1). A special high-pressure liquid chromatography (HPLC) technique has been used to monitor the various metabolites during batch fermentations of a whey permeate medium (G. Doyon, Y. Beaulieu, and G. Gaudreau, submitted for publication).

MATERIALS AND METHODS

Chemicals. κ-Carrageenan, Satiagel MR 150, and locust bean gum were obtained from SATIA, Ceca sa., Vélizy, Villecoublay, France. Commercial soybean oil was used.

Microorganisms. An *S. salivarius* subsp. *thermophilus* (*Streptococcus thermophilus*) strain isolated from Delisle yoghurt, *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus*) 5085, and *Lactococcus lactis* subsp. *lactis* (*Streptococcus lactis*) 2432 obtained from Rosell Laboratories, Montréal, Canada, were used in this work. (Bacteria names in parentheses correspond to the old nomenclature [9]). Cells of *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were grown at 42°C for 5 h, and *L. lactis* subsp. *lactis* was grown at 30°C for 16 h in a whey permeate medium supplemented with 0.5% glucose and yeast extract. Stationary cells were immobilized.

Immobilization procedure. The immobilization procedure was developed and presented previously (1). All immobilization steps were performed under sterile conditions with sterilized solutions. A 7-ml whey permeate medium containing lactic acid bacteria (10^8 cells per ml) was mixed with 350

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ml of a 3% (wt/wt) solution of total polymer (κ-carrageenan/locust bean gum ratio, 2:1) at 45°C.

The polymer solution was prepared by suspending the appropriate amount of powder in warm 0.85% saline water. It was then autoclaved at 121°C for 15 min for sterilization and deaeration. The cell-polymer suspension was poured rapidly into the reactor, which contained sterile soy oil (650 ml at 40°C), and stirred for 2 min at 700 to 1,000 rpm. A marine impeller was used to induce a strong vortex without air incorporation.

The cell-polymer dispersion was then cooled to 25°C to allow the gelation of the small drops formed during the process. The beads were then washed and soaked in a sterile 0.3 M KCl solution for 2 h. They were separated to obtain two ranges of diameter (small beads, 0.5 to 1.0 mm, and large beads, 1.0 to 2.0 mm) by wet sieving with sterile 0.3 M KCl solution in Brinkmann stainless steel sieves. To increase the entrapped-cell population, the beads were incubated in a whey permeate-supplemented medium for 16 h at 37°C before fermentation.

Fermentation procedures. Fermentations were carried out in triplicate in 500-ml vertical reactors, a Bioflo model C-30 and a Multigen model F-1000–F-2000 (New Brunswick Scientific Co.). Agitation with a flat-bladed impeller was done at 50 rpm. The temperature was maintained at 42°C for *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and at 30°C for *L. lactis* subsp. *lactis*. After falling to the desired value, the pH of the medium was controlled by using a Radiometer pH M 84 titrator adjusted to pH 5.8 and coupled with a magnetic valve dispensing 3 N NH_4OH. The pH-controlled fermentations and maintenance of lactic bacteria were conducted in the same whey permeate-supplemented medium. The same volume of inoculum (10 ml) was used for free and entrapped cells; free cells were cultured in the whey permeate-supplemented medium for 6 h at 42°C for *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* and for 6 h at 30°C for *L. lactis* subsp. *lactis*. Prior to fermentation, inoculated beads were incubated before use as previously described and washed in 0.1% peptonized water. Samples were taken for enumeration and HPLC analysis after 0, 2, 4, 5 or 6 h of each experiment.

Analytical methods. Sugars and organic acids were measured by HPLC with a Waters HPLC unit (column ion, 300 Mandel), with 0.0049 N H_2SO_4 as the mobile phase and a UV detector combined with a refractive index detector.

The analytical procedure followed was developed by the Centre de Recherches Alimentaires de St.-Hyacinthe, Agriculture Canada (Doyon et al., submitted).

Cell enumeration. Cell enumeration was carried out by the pour plate technique. *L. delbrueckii* subsp. *bulgaricus* was enumerated on Lactobacilli MRS agar after 48 h at 42°C, *S. salivarius* subsp. *thermophilus* was enumerated on Elliker agar after 24 h at 42°C, and *L. lactis* subsp. *lactis* was enumerated on Elliker agar after 48 h at 30°C. Contaminants were counted on tryptic soy agar after 24 h at 37 or 30°C.

For bacterial enumeration in the gel beads, the beads were washed in 0.1% peptonized water, and about 1 ml (measured by displacement in a 10-ml graduated cylinder) was soaked in 9 ml of 0.85% saline water and shaken with glass beads for 15 min at 45°C to suspend the immobilized cells. Serial dilutions were done, and counts were expressed in cells per milliliter of gel. This technique allowed bead dissolution without affecting cell viability.

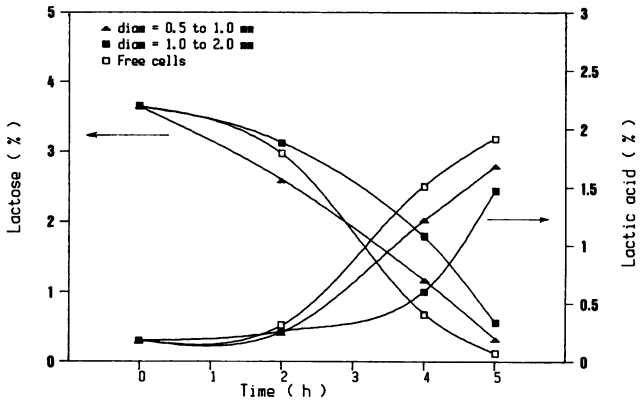

FIG. 1. Lactose utilization and lactic acid production by free and entrapped *S. salivarius* subsp. *thermophilus*.

RESULTS AND DISCUSSION

All free or entrapped lactic acid bacteria grew in a medium containing 0.5% glucose and a nonlimiting amount of lactose (4%); they simultaneously used these two components.

Free and entrapped cells of *S. salivarius* subsp. *thermophilus* exhausted lactose in the medium at a similar rate (Fig. 1). When limited lactose amounts (~0.1 to 0.2%) remained after 5 h, galactose utilization could be observed (Table 1). Cells used lactose in the presence of glucose; galactose accumulation took place during the first step of the fermentation (Table 1). These results agree with *S. salivarius* subsp. *thermophilus* metabolism in M 17 broth reported by Hutkins and Morris (7), Thomas and Crow (20), and Tinson et al. (22).

The highest amount of lactic acid production was obtained with free cells, and the next highest amounts were obtained with small and large beads, respectively (Fig. 1). HPLC analyses allowed the determination of metabolite formation during fermentation. The production of pyruvic and formic acids by free and entrapped cells is illustrated in Table 1.

TABLE 1. Glucose utilization, galactose accumulation, and formic acid and pyruvic acid production during fermentation of whey permeate medium by free and entrapped cells of *S. salivarius* subsp. *thermophilus*

Cell condition and fermentation time (h)	Glucose (%)	Galactose (%)	Formic acid (%)	Pyruvic acid (%)
Free				
0	0.482	0.060	0.053	0.000
2	0.471	0.183	0.074	0.003
4	0.376	1.283	0.076	0.007
5	0.204	1.366	0.095	0.007
Entrapped (bead diameter, 0.5 to 1.0 mm)				
0	0.482	0.060	0.053	0.000
2	0.319	0.235	0.026	0.005
4	0.367	1.040	0.052	0.006
5	0.189	1.155	0.089	0.005
Entrapped (bead diameter, 1.0 to 2.0 mm)				
0	0.482	0.060	0.053	0.000
2	0.456	0.136	0.051	0.0015
4	0.289	0.537	0.043	0.008
5	0.328	1.228	0.055	0.009

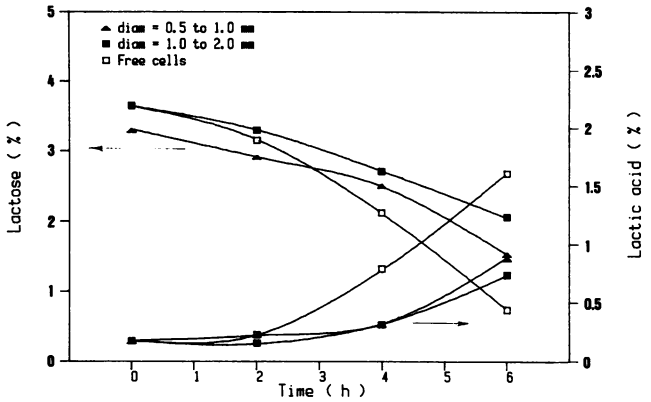

FIG. 2. Lactose utilization and lactic acid production by free and entrapped *L. delbrueckii* subsp. *bulgaricus*.

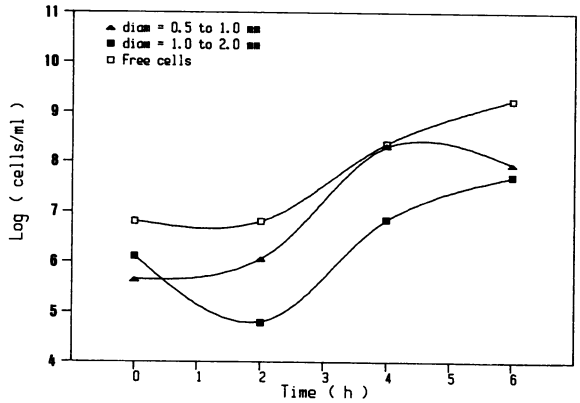

FIG. 3. Cell release rates for free and entrapped *L. delbrueckii* subsp. *bulgaricus*.

These two metabolites are reported to enhance lactic acid production and cell growth of *L. delbrueckii* subsp. *bulgaricus* (19).

Recently, Juillard et al. (8) claimed that formic acid kinetics by *S. salivarius* subsp. *thermophilus* had never been reported and suspected its importance in cooperative growth with *L. delbrueckii* subsp. *bulgaricus*. The experiments indicated that formic acid was produced by free and entrapped *S. salivarius* subsp. *thermophilus*. The effect was particularly notable with small beads in the whey permeate medium. Formic acid production (0.05%) was higher than amounts generally needed to promote growth of *L. delbrueckii* subsp. *bulgaricus* (<0.01%) (19). Fumaric acid production was observed in small amounts (maximum, 0.004%). Butyric acid was initially present in the medium (0.1%) and decreased during fermentation by 10-fold. Fumaric, L-malic, oxaloacetic, and α-ketoglutaric acids could be substituted for pyruvate as an enhancer for *L. delbrueckii* subsp. *bulgaricus* growth in the medium (15).

Entrapment in κ-carrageenan-locust bean gum gel greatly influenced lactose utilization and galactose accumulation by *L. delbrueckii* subsp. *bulgaricus* (Fig. 2 and Table 2).

After 6 h of fermentation, free cells nearly exhausted the medium (the residual lactose level was close to 0.7%). Residual lactose was about 1.5 and 2.1% for entrapped cells in small- and large-diameter beads, respectively. Free and entrapped *L. delbrueckii* subsp. *bulgaricus* simultaneously used lactose and glucose (Fig. 2 and Table 2), resulting in galactose accumulation in the medium (6). The variations which were observed in carbohydrate utilization could be explained by a diffusional problem linked to the size of the beads. This problem could probably be minimized by increasing agitation rates during fermentation.

The highest lactic acid production was obtained with *L. delbrueckii* subsp. *bulgaricus* (Fig. 2). This could be related to cell population during fermentation (Fig. 3); free cells reached 10^9 cells per ml instead of $\leq 10^8$ cells per ml for entrapped cells. Significant amounts (0.4 to 0.5%) of butyric acid were produced during fermentation without any evidence of contamination; such results were also reported for milk by Oner and Erikson (12). HPLC analysis did not detect acetic acid formation or formic acid production (Table 2).

The population growths for free and entrapped *S. salivarius* subsp. *thermophilus* and *L. lactis* subsp. *lactis* are presented in Fig. 4 and 5. Free cells yielded the same or higher population growth even when fewer numbers of cells were used at the beginning of the fermentation. In all cases, the number of cells released from the beads was equal to or

TABLE 2. Glucose utilization, galactose accumulation, and formic acid production during fermentation of whey permeate medium by free and entrapped cells of *L. delbrueckii* subsp. *bulgaricus*

Cell condition and fermentation time (h)	Glucose (%)	Galactose (%)	Formic acid (%)
Free			
0	0.482	0.060	0.053
2	0.569	0.197	0.057
4	0.443	0.607	0.057
6	0.283	1.238	0.053
Entrapped (bead diameter, 0.5 to 1.0 mm)			
0	0.482	0.060	0.053
2	0.446	0.213	0.051
4	0.471	0.353	0.037
6	0.293	0.752	0.040
Entrapped (bead diameter, 1.0 to 2.0 mm)			
0	0.482	0.060	0.053
2	0.487	0.109	0.046
4	0.564	0.246	0.041
6	0.485	0.649	0.048

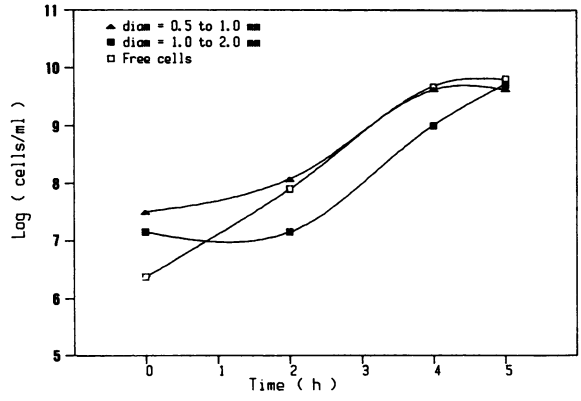

FIG. 4. Cell release rates for free and entrapped *S. salivarius* subsp. *thermophilus*.

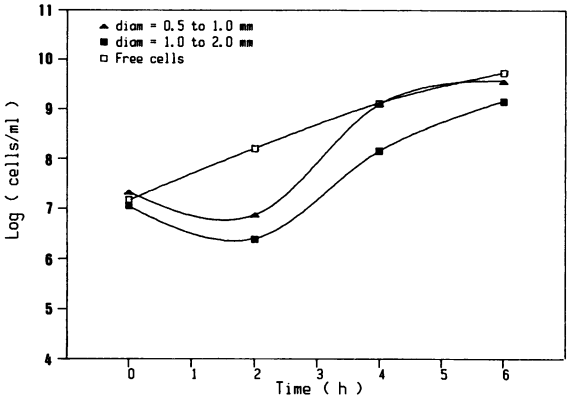

FIG. 5. Cell release rates for free and entrapped *L. lactis* subsp. *lactis*.

lower than the cell growth in free-cell fermentation. The best results were obtained with streptococcal strains immobilized in small beads, which attained nearly the same population size as did free-cell cultures.

Small beads permitted a better fermentation rate than large beads due to an increase in surface-volume ratio. The increase promoted mass transfer between the beads containing the cells and the surrounding medium.

Unlike *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, *L. lactis* subsp. *lactis* is a mesophilic starter which ferments lactose by the hexose diphosphate pathway (or Embden-Meyerhof pathway) to produce lactic acid with a key enzyme, P-β-galactosidase. Free and entrapped *L. lactis* subsp. *lactis* were shown to utilize approximately the same amount of lactose (0.35 to 0.4%) during the 6-h fermentation (Fig. 6). Two batches of whey permeate medium were used, and some variations of lactose composition were observed at time zero. During the same period (Table 3), *L. lactis* subsp. *lactis* completely consumed the glucose and did not accumulate galactose. Free and entrapped *L. lactis* subsp. *lactis* used galactose as soon as fermentation started. Group N streptococci metabolized galactose via two separate pathways, the D-tagatose-6P pathway and the Leloir pathway (22).

Thomas et al. (21) reported that *L. lactis* subsp. *lactis* growth on galactose could result in homolactic fermentation or, depending on the strains, in production of formate,

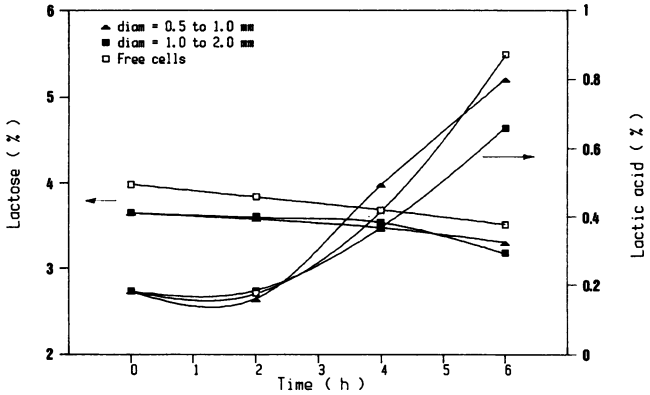

FIG. 6. Lactose utilization and lactic acid production by free and entrapped *L. lactis* subsp. *lactis*.

TABLE 3. Glucose and galactose utilization and formic acid production during fermentation of whey permeate medium by free and entrapped cells of *L. lactis* subsp. *lactis*

Cell condition and fermentation time (h)	Glucose (%)	Galactose (%)	Formic acid (%)
Free			
0	0.482	0.060	0.053
2	0.365	0.053	0.056
4	0.204	0.051	0.060
6	0.000	0.022	0.064
Entrapped (bead diameter, 0.5 to 1.0 mm)			
0	0.482	0.060	0.053
2	0.351	0.027	0.055
4	0.149	0.042	0.060
6	0.000	0.033	0.069
Entrapped (bead diameter, 1.0 to 2.0 mm)			
0	0.482	0.060	0.053
2	0.398	0.052	0.063
4	0.166	0.052	0.055
6	0.021	0.035	0.061

acetate, and ethanol as well as lactate. Our results indicated no production of acetic, butyric, or pyruvic acids, a very small amount of formic acid (Table 3), and a predominant lactic acid production (Fig. 6). During fermentations with free and entrapped cells of *L. lactis* subsp. *lactis*, decreasing amounts of butyric acid were observed. This suggests that butyric acid is used by *L. lactis* subsp. *lactis* for cell metabolism (3).

Free and entrapped lactic acid bacteria in a mixed gel (κ-carrageenan and locust bean gum) demonstrated relatively similar levels of acid productivity and carbohydrate utilization. This type of matrix also permitted high cell release, continuous inoculation, and prefermentation for yoghurt or cheese making. Small-diameter (0.5- to 1.0-mm) beads showed higher fermentation rates compared with large-diameter (1.0- to 2.0-mm) beads, sometimes as high as those shown by free cells. Entrapped-cell productivity could probably be increased by optimizing agitation in the reactor.

Work is in progress to determine the effect of lactic acid bacteria entrapment on the protocooperative growth of these organisms.

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