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Production of *Lactococcus lactis* biomass by immobilized cell technology

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SUMMARY

Calcium alginate beads containing *Lactococcus lactis* cells were used for three batch fermentations of milk or a commercially available growth medium (Gold Complete, Nordica) with the aim of producing concentrated cultures. Repeated fermentations did not significantly increase bead CFU counts which were between $3.3–7.8 \times 10^{10}$ CFU/g. During the second and third fermentations, which lasted 6 h each, the bead populations decreased if the incubation was extended over 2 h. There was cell release from the beads. Fermentation media and fermentation time all had an effect on free cell counts, but none of these factors statistically interacted. Free cell counts were higher at the end of fermentations 2 and 3 than in the first fermentation and approximately 50% of the population was in the free state. Free cell counts were higher when the beads were incubated in Gold Complete than in milk. Although the total bacterial population of a standard free cell fermentation was always higher than those having immobilized cells, immobilized cell technology did enable the production of dense cultures.

INTRODUCTION

The fermentation of milk or whey has traditionally been performed with free cells [5]. Immobilized cell technology (ICT) has been proposed for various dairy fermentations [7] mostly on calcium-alginate gels. Many are based on continuous fermentations, and are aimed at the production of metabolites such as lactic acid [7] or for continuous inoculation of milk [10]. Under continuous fermentation, the bacterial cell density in the matrix can reach 10^{11} CFU/g. There is thus potential for ICT in the production of concentrated lactic acid bacteria (LAB), particularly when strains are sensitive to the classical concentration procedures [4] or are difficult to retrieve from milk [9].

Biomass production from ICT has been reported under continuous fermentation. There are no studies, however, on the use of multiple batch fermentations for the purpose of biomass production of LAB in alginate gels. In this work, we report the effect of three consecutive batch fermentations on biomass obtained, and compare population yields obtained from ICT to those of a classical free cell system.

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MATERIALS AND METHODS

Microorganism

Lactococcus lactis ssp. *cremoris* CRA-1 was maintained on 12% reconstituted non-fat dry milk (Agropur, low heat type, sterilized at 112 °C, 10 min) and transferred twice per week by inoculating milk (1%, v/v) and incubated at 23 °C for 16 h. After incubation, culture pH was 4.5.

Immobilization

Inoculum was produced by adding 0.5 ml of the milk grown culture to 50 ml of M17 broth (Difco), and incubating without agitation at 23 °C for 22 h.

Five ml of this culture were mixed with 5 ml of sodium alginate 2% (BDH, Montréal) sterilized at 121 °C for 15 min. Using a Pasteur pipet, the mixture was added dropwise to an agitated (70 rpm) sterile solution of $CaCl_2 \cdot 2H_2O$ (0.1 M). Alginate beads so formed were kept in the $CaCl_2$ solution for 30 min for gel strengthening before being rinsed with sterile peptone (0.1%) and used in the fermentation.

Fermentations

Two media supplemented with 1% $CaCO_3$ were studied: reconstituted skim milk, 12% solids (Agropur, Québec) and Gold Complete, 6% solids (Nordica, Sioux Falls) which is a milk and whey-based commercially

available growth medium. The media were sterilized at 112 °C for 10 min and their pH adjusted to 5.6 after sterilization. $CaCO_3$ was added to both media at a 1% level prior to sterilization. Alginate beads obtained from 5 ml of the M17 grown inoculum were added to 150 ml of medium. One control medium was obtained by inoculating reconstituted skim milk with 5 ml of free cells. During fermentation, pH level was maintained constant at 5.6 by adding NH_4OH 5.0 N using a Radiometer system (Copenhagen, pH M84, TTT80 Titrator, MNV1E valve) or a Chemcadet unit (Cole-Parmer, Chicago) connected to an Atto peristaltic pump. The medium was agitated constantly at 30 rpm with a Bellco Quad Drive system (Vineland, NJ, model Sci Era) and incubated at 23 °C. Three consecutive fermentations were performed with the same alginate beads: the first fermentation lasted 18 h, while the other two were reduced to 6 h. Bead and medium samples were taken at the end of the first fermentation and at 2-h intervals during the last two fermentations. Between the first and second fermentation, the medium was removed from the fermenter and extracted unrinsed beads were added to 150 ml of freshly prepared medium for the second fermentation. Following the latter, beads were kept in a sterile peptone solution (0.5%) at 4 °C for 18 h, until the last fermentation. Free and immobilized cell counts per gram were obtained in all samples and total biomass was measured at the end of each fermentation. The series of three fermentations was replicated thrice. The selection of media, pH 5.6 and 18 h incubation time was the result of a preliminary study using response surface methodology (data not shown).

Microbiological analyses

Bead samples (0.1–0.2 g) were weighed aseptically and put in 99 ml of a sterile sodium citrate solution (1%, pH 6.0). When depolymerization was complete (1 h at 23 °C), samples were diluted in sterile peptone solutions (0.1%). Cell counts were obtained by plating on Elliker agar (Difco). Incubation was at 30 °C, for 48 h. Total counts and mortality levels were assessed through direct microscopic counts.

Statistical analyses

To stabilize the variance and normalize the residuals, CFU counts were transformed to their base 10 logarithms before analysis. Mean CFU counts are expressed and presented on the logarithmic scale, but, for simplicity, will be referred to as CFU counts nonetheless.

The variances of Log_{10} CFU and biomass were analyzed according to the experimental plan. The design was a split-split-plot with the six preparations as primary units (two media × three replicates); three fermentations of each preparation formed the secondary units and samples withdrawn from the bioreactor at 2-h intervals, the tertiary units. These samples were considered independent and it was checked that assumptions of normality, independence, and homogeneity of the variance of the error vector were reasonable [8].

Both free and immobilized cell counts were obtained from the samples. The treatment structure was highly unbalanced with seven fermentation numbers and sampling time combinations crossed with two media and a three-level error structure corresponding to the three types of experimental units. Error terms could be reduced to two for bead cell counts and to one for free cell counts. Free cell counts for the first replicate of the Gold Complete medium were missing.

There were six degrees of freedom for the seven fermentation-number and fermentation-time combinations. A contrast was constructed to investigate the difference between the first fermentation and fermentations 2 and 3 at 6 h. Other orthogonal comparisons investigated differences among fermentations and sampling times and corresponding interactions with media. In the analysis of bead cell counts, the imbalance of the media-fermentation-sampling time structure required that denominators for the F-tests on media, fermentation and their interaction be obtained as linear combinations of the two error mean squares; degrees of freedom for the adjusted mean squares were computed using Satterthwaite's approximation. Adjusted means and their standard-errors were obtained following methods described by Milliken and Johnson [8].

RESULTS AND DISCUSSION

Bead populations

Many cell immobilization techniques are available [6], but the entrapment of bacteria in calcium alginate beads is widely used since this is not toxic to the cells [12], and because alginate is an approved food additive. It has been shown that fermentative activity increases when the same alginate beads are used in successive fermentations [2]. We thus attempted repeated use of the same beads for three successive fermentations in the hope of increasing biomass in the alginate gel. While the first fermentation lasted 18 h, the second and third fermentations were only performed during 6 h. Since lactococci have a generation time of approximately 2.5 h at 20 °C [3], the 6-h fermentation enabled to theoretically multiply the cell population by a factor of four. Beads were harvested following a first 18-h fermentation, and added to fresh sterile medium. Multiple fermentations did not significantly increase bead CFU counts in milk ($P = 0.8216$, Table 1) where they remained relatively stable over fermentations with a final count between 10.52 (3.3×10^{10}) and 10.72 (5.2×10^{10})

TABLE 1

Results of the analysis of variance in the immobilized cell system

Source of variation	Bead population			Free cells		
	DF[a]	Mean square	P[b]	DF	Mean square	P
Media	1	0.1828	0.15413[c]	1	0.5911	0.0001
Fermentation	1	0.0072	0.8216	1	0.3116	0.0010
Media × Fermentation[d]	1	0.0498	0.5547	1	0.0791	0.0681
Error A	12	0.1067	0.0001	Pooled with Error B		
Time	2	0.0569	0.0080	2	2.7591	0.0001
Fermentation × Time	2	0.0246	0.0867	2	0.0077	0.7005
Media × Time	2	0.0266	0.0728	2	0.0089	0.6649
Fermentation × Time × Media[e]	2	0.0407	0.0242	2	0.0266	0.3087
Error B	16	0.0086		21	0.0214	
Total	1			34		

[a] DF = degrees of freedom.
[b] P = probability that a given source of variation does not have a significant effect.
[c] Denominator degrees of freedom and mean squares: media (M): 12.74, 0.0796; fermentations (F) and M × F: 11.57, 0.1347.
[d] Media × Fermentation: analysis of a possible interaction between two variables (in this instance, media and fermentation).
[e] Analysis of a possible triple interaction.

CFU/g on the logarithmic scale (Table 2); this was so in both media. More specifically, final bead cell counts did not differ significantly, between the first and two subsequent fermentations ($P = 0.6624$) in both media ($P = 0.7340$ for the interaction between this contrast and media) or between the last two fermentations ($P = 0.5927$) in both media ($P = 0.3092$ for the interaction). Under continuous culture, it has been shown that the bacterial population in beads stabilizes to approximately 10^{11} CFU/g [10,11]. Our results are thus in agreement with these observations. Although there is considerable information on bacterial populations in gels, the aims of the published processes are to promote cell release for milk inoculation [7,10,11] or to produce

TABLE 2

Adjusted means of $\log_{10}$ CFU/g (bead population) or $\log_{10}$ CFU/ml (free cells) in the immobilized cell systems

Fermentation (No.)	T (h)	Bead population		Free cells		
		Milk	Gold Complete	Milk	Gold Complete	Mean
1	18	10.64	10.83	9.10	9.48	9.29
2	2	10.73	10.84	8.40	8.65	8.52
	4	10.74	10.83	9.06	9.43	9.24
	6	10.72	10.72	9.32	9.76	9.54
3	2	10.87	10.86	8.16	8.44	8.30
	4	10.51	10.89	8.99	8.98	8.99
	6	10.52	10.79	9.32	9.47	9.39
Standard error		0.053	0.053	0.084	0.103	0.067

metabolites [1,13], and most are based on continuous culture. No studies were performed with the aim of producing high bacterial densities in the gels while limiting cell release, and under batch fermentation conditions.

On average, bead cell counts decreased with sampling time in the last two fermentations ($P = 0.0080$) an there was some indication that the difference in the decrease rate between the second and third fermentations was not the same in milk and in Gold Complete ($P = 0.0242$ for the second order interaction between media, fermentations, and sampling times). In the second milk fermentation, bead CFU counts were stable over time, but dropped from 10.87 after 2 h of the third fermentation to 10.51 and 10.52 after 4 and 6 h, respectively, whereas in Gold Complete, the rate of decrease was much the same in fermentations 2 and 3. It thus appears that the second and third fermentations should not be extended over 2 h.

Free cells in the immobilized cell system

Fermentation, media, and fermentation time all had an effect on free cell counts ($P = 0.0001$, 0.0010, 0.0001, respectively, Table 1), but none of these factors interacted ($P > 0.06$ for all interactions). Free cell counts were higher at the end of fermentations 2 and 3 than in the first fermentation ($P = 0.0432$) but did not differ at the end of the two 6-h fermentations ($P = 0.1364$). Free cell counts were also higher in Gold Complete than in milk ($P = 0.0001$, Table 1).

In our bioreactor, cell counts were about 25 times higher in the beads than in the surrounding medium (Table 2). Prevost et al. [11] reported that, under steady state, the number of free cells in a bioreactor ranged from 1 to 4% of that of immobilized cells. These results apply to systems having a heavy load of calcium alginate beads. Under the present experimental conditions, beads occupied only 5% of the volume. Although free cell counts were of the order of 10^9 CFU/ml, their biomass made up 44 to 66% o the total. This high percentage is related to both cell release from the beads and growth of released cells in the medium. Bead harvesting recovers only about half the bacterial population from the system. Further work must be undertaken to reduce the amount of free cells in the bioreactor. Bead coating with poly-L-lysine or chitosan [6] will be attempted towards this aim.

Immobilized vs classic free cell fermentations

In a classic free-cell fermentation in milk under the same conditions of pH control and $CaCO_3$ addition, the population after 18 h of incubation was of 8.7×10^9/ml. Thus in 150 ml of milk we obtained 13.1×10^{11} CFU. The total cell populations in the immobilized cell systems were always inferior to that value (Table 3). The immobilized cell population on the average was 4×10^{11} and 5.3×10^{11} CFU for milk and Gold Complete, respectively. Thus approximately half of the cells were in the immobilized state. Although this bead-contained population is easily recuperated without filtration or centrifugation, the biomass yield was only half that of a classical system. Uncoupling between growth and acid production was observed in the immobilized cell system, since acidification occurred during fermentations 2 and 3, without significant bacterial growth. Uncoupling between growth and acidification has been observed with free [14] and immobilized [13] lactic acid bacteria. Lower overall biomass yields are thus to be expected in an immobilized state due to more rapid uncoupling between growth and acid production than in free cell fermentations.

TABLE 3

Total cell populations ($\times 10^{11}$ CFU) in the medium (150 ml) and beads (8 g)

Fermentation (No.)	T (h)	Medium	
		Gold Complete	Milk
1	18	9.9	5.3
2	2	6.2	4.7
	4	9.4	6.1
	6	12.8	7.3
3	2	6.2	6.1
	4	7.6	4.1
	6	9.3	5.8

We recuperated approximately 8 g of beads at the end of the fermentations. The highest populations reached were 7.4×10^{10} and 7.7×10^{10} (per gram of bead) for milk and Gold respectively. These were about nine times higher densities than for the classic fermentations. Thus although total cell yields are lower, immobilized cell technology could be a convenient method of producing concentrated cultures that cannot be produced in a classic fashion because of their sensitivity to centrifugation or filtration [4], or because they are difficult to separate from the growth medium [9]. In these cases, there would not seem to be a big advantage of using multiple batch fermentations. However, a second fermentation was useful in increasing the total cell population in the system (Table 3), but this was mostly related to increased levels of free cells.

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