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Use of an immobilized cell bioreactor for the continuous inoculation of milk in fresh cheese manufacturing

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A system was developed to continuously acidify and inoculate skim milk for the production of fresh cheese. Four strains of mesophilic lactic acid bacteria were entrapped separately in κ-carrageenan/locust bean gum gel beads and used in a stirred bioreactor operated at 26°C with a 25% (v/v) gel load. The pH in the reactor was controlled at 6.0 by adding fresh milk using proportional integrated derived regulation. The bioreactor was operated during 8-h daily cycles for up to 7 weeks with different milks (heat treatment, dry matter content) and differing starting procedures. The heat treatment of the milk was an important factor for process performance: a dilution rate increase of 57% and an inoculation level decrease of 63% were observed with sterilized UHT skim milk (142°C – 7.5 s) compared with pasteurized skim milk (72°C – 15 s). The dry matter content of the milk (8–13% w/w) had no detectable effect on these parameters. A convenient starting procedure of the system was tested; steady-state was reached in less than 40 min following an interruption period of 16–60 h. These results combined with our published data on process performance show the feasibility of using an integrated immobilized cell bioreactor for milk prefermentation in cheese manufacture.

Keywords: lactic acid bacteria; fresh cheese; milk; immobilization; continuous inoculation; κ-carrageenan gel beads

Introduction

The use of an immobilized cell bioreactor for the production of fermented milk products has been reported by several authors. Kim *et al* [5,6] and Gobetti *et al* [4] proposed an immobilized cell bioreactor configuration to continuously acidify cheese-making milk. In these studies, inoculation of the preacidified milk was performed batchwise. Other authors proposed the use of an immobilized cell bioreactor to simultaneously acidify and inoculate milk for the production of yoghurt [14,15,17] or fresh cheese [12,16,20]. These studies have reported high performance of immobilized cell bioreactors with extensive inoculation of milk and high dilution rates. For instance, in an earlier study [20], we observed dilution rates and milk inocula levels up to 30 h^{-1} and 10^8 CFU ml^{-1}, respectively, with an immobilized cell bioreactor operated at 30°C, pH 6.2, a bead load of 25% (v/v) and a mixing rate of 120 rpm. The higher rate of inoculation of milk, typically 10 times higher than in a classical batch inoculation [8], enables a considerable reduction in total fermentation time (more than 50% for fresh cheese manufacture [16,20]).

The effects of some operating parameters on process performance include biochemical parameters such as temperature [6,15,18,20] and pH [6,15,20]; physical parameters such as gel ratio [6,15,18,20], agitation rate [1,20] and bead size [6,15]; microbiological parameters such as the strains used [4,12] and the entrapment procedure [6,17,18,19]. No study, however, has been performed on the effect of milk composition on process performance. Different physical treatments can be applied to cheese-making milk, eg heat treatment, preconcentration. Cheese plants do not normally operate 24 h a day. Therefore, in order to be used for milk prefermentations without changing the working schedule, the immobilized cell system should permit periodic interruptions of its operation. There is no published information on interruption and starting procedures of prefermentations and their effect on process performance.

The aim of this study was therefore to investigate the possible integration of an immobilized cell bioreactor in an industrial scale fresh cheese process. The effect of milk composition and of operating procedures (interruption and start) on the process performance were evaluated.

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Materials and methods

Chemicals

κ-carrageenan (Satiagel MR150) and locust bean gum were obtained from Sanofi Bio Industries (CECA, Villacoublay, France). The sunflower oil used for entrapment was a commercial grade product.

Sterilized UHT (142°C – 7.5 s) skim milk was provided by Candia (Lyon, France) and pasteurized (75°C – 15 s) skim milk by La Ferme Expérimentale de Grignon (Grignon, France). For the specific study of the effect of milk dry matter content on process performance, a pasteurized (78°C – 18 s) reconstituted (8–13% w/w) skim milk (Crino-Agropur, Québec, Canada) was used.

Bacterial strains

Four strains of mesophilic lactic acid bacteria were used: *Lactococcus lactis* subsp *lactis* CNRZ144, *Lactococcus*

lactis subsp *cremoris* E8 (Centre National de Recherches Zootechniques, Jouy-en-Josas, France), *Leuconostoc mesenteroides* X2 (Moorepark Research Center, Cork, Ireland) and *Lactococcus lactis* subsp *lactis* biovar *diacetylactis* CDI1 (Centre de Recherche International André Gaillard, Yoplait, Ivry-sur-Seine, France). The strains were kept frozen in skim milk at –20°C. They were reactivated and grown for 8 h at 30°C before use. The lactococci were routinely cultivated in M17 broth (Biokar, Beauvais, France) and *L. mesenteroides* X2 in MRS broth (Biokar).

Cell immobilization

Cells in the late logarithmic growth phase were recovered by centrifugation at $5000 \times g$ for 10 min at 4°C prior to immobilization to provide a concentrated inoculum. The immobilization procedure in κ-carrageenan and locust bean gum gel beads (2.75% and 0.25% w/w, respectively) was based on a two-phase dispersion technique [7]. The strains were immobilized separately in different gel beads. Beads with diameters in the range of 1–2 mm were obtained by wet sifting using a 0.2 M KCl solution. The beads were incubated in M17 containing lactose (50 g L^{-1}) and KCl (0.2 M) during two successive batch cultures of 16 h and 6 h, as described earlier [20]. The immobilized population increased from 4.4 $(\pm 2.5) \times 10^8$ CFU g^{-1} to 3.3 $(\pm 1.1) \times 10^{11}$ CFU g^{-1} during this procedure. Consequently, the initial count in beads was 3.3 $(\pm 1.1) \times 10^{11}$ CFU g^{-1} at the beginning of each prefermentation experiment.

Prefermentation procedure

The continuous prefermentation of milk was performed during 2–7 weeks in a pilot- or laboratory-scale bioreactor.

For the majority of experiments, the prefermentation was carried out at 26°C in a 2-L stirred bioreactor (LSL, Saint-Germain-En-Laye, France) with a working volume of 1 L [20]. The bioreactor was inoculated with 25% (v/v) gel beads. The volumetric proportions of colonized gel beads containing specific bacterial populations were 35% of *L. lactis* subsp *lactis* CNRZ144, 35% *L. lactis* subsp *cremoris* E8, 25% *L. lactis* subsp *lactis* biovar *diacetylactis* CDI1 and 5% *L. mesenteroides* X2. These proportions were selected as being representative of the composition of mesophilic starters used for fresh cheese production. The pH was measured by a pH electrode (Ingold, Paris, France), connected to a pH controller (Setric GI, Toulouse, France). Proportional integrated derived (PID) regulation which controlled the flow rate of the feeding peristaltic pump was used to maintain the pH at 6.0 by addition of fresh milk to the reactor. The experiments were conducted with UHT or pasteurized skim milk. The reactor was monitored daily for 8-h cycles.

The experiment on the effect of milk dry matter content was carried out in a specially designed custom-made small flat-bottomed bioreactor (Verbec, Québec, Canada) with a working volume of 120 ml. A thin pH electrode (catalog number 13 620.293, Fisher Scientific, Fairlawn, NJ, USA) was used. Mixing was provided by a magnetic stirrer (250 rpm). Temperature control, PID pH control by addition of fresh milk and data acquisition were performed through a computerized fermentation system (Biogenie, Industriel 8 version, Sainte-Foy, Québec, Canada). The experiment was performed with reconstituted (8–13%) skim milk. Only two strains were used in this case, in the same proportions: *L. lactis* subsp *lactis* CNRZ144 and *L. lactis* subsp *lactis* biovar *diacetylactis* CDI1, which were the more representative strains of the mixed culture. Four experimental reconstitution levels, corresponding to 8.3%, 10.2%, 11.3%, and 13.0% dry matter in the milk, were tested in duplicate during the same continuous fermentation.

Samples of prefermented milk were withdrawn daily for microbiological and chemical analyses. The milk flow rate was monitored continuously by recording the power supply of the feed pump and was periodically estimated by volumetric measurements using a graduated cylinder.

Interruption and starting procedure

The prefermentation was monitored for 8-h daily cycles. The fermentation was stopped and the bioreactor washed every day. After rinsing the reactor four times with peptone water (peptone at 0.1%, w/v) containing KCl 0.2 M and citrate buffer 0.03 M, the beads were kept in the rinsing solution. The buffer (pH 5.6) prevented loss of activity of the immobilized cells resulting from the low pH that would have been attained in unbuffered storage solution [2]. Following the interruption period of 16–60 h, the fermentation was started again after heating (26°C) and rinsing the bioreactor with milk (four times the working volume) to eliminate the storage solution. Two different starting procedures were then tested. The first starting procedure used a variable milk flow rate which was set by the PID pH controller acting on the feed pump, similar to the continuous prefermentation of milk. The second starting procedure used a prefixed milk flow rate set at 15 L h^{-1} during the first 30 min of operation. This value corresponded to the flow rate observed in steady-state conditions for the tested prefermentation conditions (26°C, pH = 6.0, 120 rpm and a gel ratio of 25%). After this initial starting period, PID regulation, which controlled the flow rate of the feed pump, was activated.

Lactic acid concentration and productivity

Lactic acid concentration was determined by HPLC analysis using a Waters system (Millipore, France) with Bio-rad Aminex HPX-87H or Phenomenex Ions-3000 (Hercules, CA, USA) ion exchange columns, and 0.01 N H_2SO_4 as eluent at a flow rate of 0.6 ml min^{-1}. Prefermented milk samples (1.8 ml) were mixed with 40% trichloroacetic acid (120 μl) and centrifuged at $2000 \times g$ for 30 min at 4°C before injection. Analyses were performed in duplicate. Volumetric lactic acid productivity (g h^{-1} L^{-1}) was calculated from dilution rate (h^{-1}) and lactic acid concentration in milk (g L^{-1}). Specific lactic acid productivity (g h^{-1} CFU^{-1}) was obtained by dividing the volumetric productivity (g h^{-1} L^{-1}) with the cell load in the bioreactor (CFU L^{-1}). Only the bacterial counts in gel beads (CFU g^{-1}) were taken into account: the cells in suspension in the bioreactor were ignored because of their low concentration compared with immobilized cells ($< 2\%$). We assumed a gel bead density of 1000 g L^{-1}.

Cell enumeration and productivity

Bacterial counts in prefermented milks and in gel beads (expressed in CFU ml^{-1} or CFU g^{-1}) were obtained by plating out the appropriate dilutions. For microbial enumerations in prefermented milk, samples were first treated with an Ultra-Turrax (IKA-Labortechnik, Staufen, Germany) for 30 s at 20 000 rpm to break up the chains of lactococci. For microbial enumeration of gel beads, about 1 g of beads in 9 ml of peptone water were homogenized with the Ultra-Turrax in ice for 2 min. The plating was carried out with a Spiral-Plater (Interscience, Saint-Nom-La-Bretèche, France) using 14.5-cm diameter Petri dishes. All analyses were carried out in duplicate. Specific lactic acid bacteria enumeration was carried out by plating onto the following selective media: M16 agar, Kempler and McKay agar, and M17 agar supplemented with vancomycin (50 mg ml^{-1}). The distinction of the four strains in these media was as previously described [20].

Results and discussion

Effect of the pasteurized versus UHT milk on process performance

Prefermentation experiments were carried out for 3 and 7 weeks under the same conditions in the 1-L working volume pilot bioreactor, using UHT or pasteurized skim milk. The determination of bioreactor's performance (composition of prefermented milk, dilution rate, lactic acid and cell productivities) was carried out daily after 7 h of operation (Table 1) of the continuous fermentation. The pasteurized milk was inoculated with three times more biomass than UHT milk (4.5×10^8 compared to 1.5×10^8 CFU ml^{-1}). The equilibrium of the released populations (populations ratio) was the same with the two milks. The strain *L. lactis* subsp *lactis* biovar *diacetylactis* CDI1 dominated, accounting for approximately 80% of the total released population over the entire experimental period. Strains *L. lactis* subsp *lactis* CNRZ144 and *L. lactis* subsp *cremoris* E8 accounted for about 12.5% and 6.5%, respectively, of the released population and the strain *L. mesenteroides* X2 remained lower than 1% of the total population. The lactic acid concentration was the same (1.0 ± 0.2 g L^{-1}) for UHT or pasteurized milk. This result may indicate that the buffering properties of the two tested milks were similar at the same pH value (pH 6), which was corroborated by analysing the milk for total protein content, which was the same for the two milks (26.0 ± 0.9 g L^{-1}). The dilution rates recorded at pseudo steady-state during the prefermentation of UHT or pasteurized milk were significantly different ($P < 0.01$); they varied from 9.9 h^{-1} (pasteurized milk) to 15.5 h^{-1} (UHT milk), respectively, which represented an increase of about 60%. As a consequence, the volumetric and specific lactic acid productivity increased by 59% and 170%, respectively, with UHT milk compared to pasteurized milk (Table 1). The volumetric cell productivity was approximately two-fold higher with pasteurized milk compared to UHT milk as a result of the higher free cell population in the pasteurized milk. Mean growth rates were the same with the two milks.

The higher lactic acid productivity (specific or volumetric) and consequently the higher dilution rate observed with UHT milk might be explained by a better acidifying activity of the entrapped lactic acid bacteria in this medium compared with pasteurized milk. The destruction of natural inhibitory substances of the milk (lactoperoxidase, agglutinins) occurs only at a temperature of 82°C during 15 to 20 s [3]. The pasteurized (72°C –15 s) milk used still contained these natural antimicrobial substances and was less favourable to bacterial activity than UHT (142°C – 7.5 s) milk. Due to the high heat treatment received, UHT milk probably contains greater quantities of nitrogenous compounds of small size (peptides, amino

Table 1 Performance of the pilot immobilized cell bioreactor operated with UHT and pasteurized skim milk (pH 6.0; 26°C; 120 rpm; 25% gel/reactor volume ratio)

	Pasteurized skim milk	UHT skim milk
Composition of prefermented milk		
total cell counts (CFU ml^{-1})[a]	$4.1 \pm 1.4 \times 10^8$ (100)	$1.5 \pm 0.3 \times 10^8$ (100)
strain CNRZ144 (CFU ml^{-1})[a]	$5.1 \pm 2.4 \times 10^7$ (12.4)	$1.9 \pm 0.7 \times 10^7$ (12.7)
strain E8 (CFU ml^{-1})[a]	$2.9 \pm 1.5 \times 10^7$ (7.0)	$9.5 \pm 8.6 \times 10^6$ (6.3)
strain CDI1 (CFU ml^{-1})[a]	$3.3 \pm 1.2 \times 10^8$ (80.2)	$1.2 \pm 0.2 \times 10^8$ (80.0)
strain X2 (CFU ml^{-1})[a]	$1.4 \pm 0.6 \times 10^6$ (0.4)	$1.5 \pm 3.0 \times 10^6$ (1.0)
Dilution rate (h^{-1})	9.9 ± 1.7	15.5 ± 1.2
Lactic acid volumetric productivity (g h^{-1} L^{-1})[b]	9.7 ± 1.2	15.4 ± 2.1
Lactic acid specific productivity (g h^{-1} CFU^{-1})[c]	$2.7 \pm 0.6 \times 10^{-13}$	$7.3 \pm 1.8 \times 10^{-13}$
Cell volumetric productivity (CFU h^{-1} L^{-1})[d]	$4.0 \pm 0.1 \times 10^{12}$	$2.2 \pm 0.5 \times 10^{12}$
Cell mean growth rate (h^{-1})[e]	$10.8 \pm 4.0 \times 10^{-2}$	$10.2 \pm 3.3 \times 10^{-2}$
Immobilized cell counts in beads (CFU g^{-1})	$1.5 \pm 0.3 \times 10^{11}$	$0.8 \pm 0.1 \times 10^{11}$
Bioreactor cell counts (CFU L^{-1})[f]	$3.8 \pm 0.8 \times 10^{13}$	$2.0 \pm 0.3 \times 10^{13}$

[a]Relative values are given in brackets.
[b]Calculated from dilution rate (h^{-1}) and lactic acid concentration in milk (g L^{-1}).
[c]Calculated by dividing the volumetric productivity (g h^{-1} L^{-1}) with the total cell count in bioreactor (CFU L^{-1}).
[d]Calculated from dilution rate (h^{-1}) and cell concentration in milk (CFU L^{-1}).
[e]Calculated by dividing the volumetric productivity (CFU h^{-1} L^{-1}) with the total cell counts in bioreactor (CFU L^{-1}).
[f]Calculated assuming that gel density was 1000 g L^{-1} and that the contribution of free cells in suspensions was negligible (less than 1% of the total cell counts).

acids) compared with pasteurized milk. Law *et al* [9] and Lorient *et al* [10] demonstrated that the activity of lactic acid bacteria starters was stimulated by increasing the quantity of peptides in milk, which could be the result of elevated heat treatment [10]. The large difference in the free cell population in pasteurized milk compared with UHT milk can be explained by the higher reactor cell load (about two-fold higher) and by the lower dilution rate, which allowed a longer residence time of milk inside the bioreactor.

Effect of the milk dry matter content on process performance

As a result of seasonal, regional, nutritional and health state differences, milk used for cheese-making has a varying composition and its dry matter or protein content is usually standardized by the addition of dairy powders or concentrates. This standardization has a direct effect on the buffering characteristics of milk and eventually on its ability to support starter culture growth and activity. A continuous prefermentation experiment was therefore conducted in a laboratory scale bioreactor using a mixed culture of two strains and skim milk reconstituted to different dry matter contents. The experiment aimed at assessing their effects on the performance of the immobilized cell bioreactor and on the composition of the prefermented milk.

The composition of the prefermented milk and the bioreactor productivity were not significantly different ($P > 0.01$) for the four milks tested (Table 2). These results are somewhat different from the values obtained with UHT or pasteurized milk prefermented in the pilot bioreactor (Table 1). This might be explained by the different bioreactor design and by the different milk quality. A high inoculation of milk was observed with the 120-ml bioreactor compared with the pilot bioreactor (Table 1). The mixing conditions and the shear stress in the small-volume laboratory scale bioreactor, which contained different probes, were increased and may have positively influenced mass transfer and cell release by mechanical action on the bead surface. This would lead to a higher milk inoculation than with the pilot bioreactor [1]. As a consequence, the cell volumetric and specific productivities were high (Table 2). In addition, the lower dilution rate and longer residence time of milk in the pilot bioreactor also led to increased inocula levels in the milk.

Performance was not affected by the dry matter content of milk (8–13%). The activity of entrapped bacteria may be favoured by milk containing a higher dry matter content (13%) because the mass transfer in gel beads with such milk is better. Limitation of mass transfer in the immobilization matrix is a crucial problem with immobilized systems [21]. On the other hand, the buffering capacity of milks with high dry matter content increases [11]. The quantity of lactic acid necessary to lower the pH of milk to 6.0 was thus higher for the 13.0%-reconstituted milk (2.32 ± 0.59 g L^{-1}) than for the 8.3%-reconstituted milk (1.25 ± 0.02 g L^{-1}). The combination of these two antagonistic effects may be balanced and explain the absence of a significant difference in dilution rate among the four milks tested.

Table 2 Performance of the laboratory scale immobilized cell bioreactor operated with reconstituted (8.3–13%) skim milk (pH 6.0; 26°C; 250 rpm; 25% gel/reactor volume ratio)

Composition of prefermented milk	
total cell counts (CFU ml^{-1})	$6.4 \pm 3.1 \times 10^{8}$
strain CNRZ144 (CFU ml^{-1})	$3.0 \pm 1.1 \times 10^{8}$
strain CDI1 (CFU ml^{-1})	$3.4 \pm 1.3 \times 10^{8}$
Dilution rate (h^{-1})	8.4 ± 0.5
Lactic acid volumetric productivity (g h^{-1} L^{-1})[a]	14.8 ± 4.0
Lactic acid specific productivity (g h^{-1} CFU^{-1})[b]	$5.1 \pm 2.5 \times 10^{-13}$
Cell volumetric productivity (CFU h^{-1} L^{-1})[c]	$5.4 \pm 2.1 \times 10^{12}$
Cell mean growth rate (h^{-1})[d]	$21.1 \pm 8.9 \times 10^{-2}$
Immobilized cell counts in beads (CFU g^{-1})	$1.3 \pm 0.4 \times 10^{11}$
Bioreactor cell counts (CFU L^{-1})[e]	$3.3 \pm 0.9 \times 10^{13}$

[a]Calculated from dilution rate (h^{-1}) and lactic acid concentration in milk (g L^{-1}).
[b]Calculated by dividing the volumetric productivity (g h^{-1} L^{-1}) with the total cell counts in bioreactor (CFU L^{-1}).
[c]Calculated from dilution rate (h^{-1}) and cell concentration in milk (CFU L^{-1}).
[d]Calculated by dividing the volumetric productivity (CFU h^{-1} L^{-1}) with the total cell counts in bioreactor (CFU L^{-1}).
[e]Calculated assuming that gel density was 1000 g L^{-1} and that the contribution of free cells in suspensions was negligible (less than 2% of the total cell counts).

Management of prefermentations operated on 8-h daily cycles

Two starting procedures for continuous prefermentations in the pilot bioreactor were tested with UHT milk during a 3-week experiment. In the first starting procedure, the milk flow rate was allowed to fluctuate (six repeated trials) and in the second, the milk flow rate was held constant at 15 L h^{-1} (three repeated trials) for 30 min; this corresponded to the pseudo steady state dilution rate observed with UHT milk in the selected prefermentation conditions.

Typical pH and dilution rate changes observed during an 8-h operating cycle with variable or fixed initial flow rate are reported in Figures 1 and 2, respectively. For the starting procedure with a variable flow rate (Figure 1), the dilution rate fluctuated between extreme values (0 and 24 h^{-1}) at the beginning and stabilized at approximately 15 h^{-1} in less than 30 min. For the other starting procedure with flow rate held constant for 30 min (Figure 2), the amplitude of the variations in dilution rate, after starting the pH PID control on the feeding pump, was much

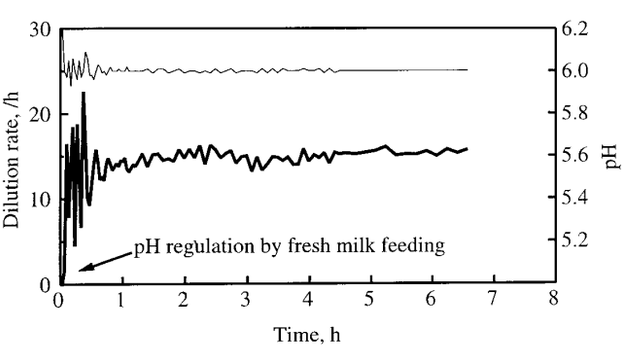

Figure 1 Typical pH (———) and dilution rate (——) changes as a function of time during a prefermentation operated on 8-h cycles using the starting procedure with an initial variable milk flow rate.

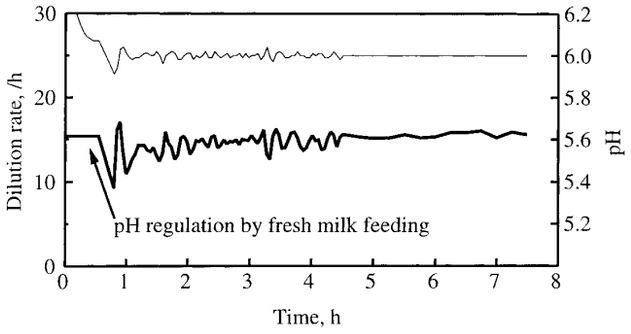

Figure 2 Typical pH (———) and dilution rate (——) changes as a function of time during a prefermentation operated on 8-h cycles using the starting procedure with an initial fixed milk flow rate.

smaller, but the overall time to reach steady state was approximately 1 h. The initial pH changes were quite different for the two procedures used. With an initial flow rate that varied, the pH reached the set value (pH 6.0) in less than 5 min and then remained stable, at 6.0 ± 0.1 during the first 30 min, and at 6.00 ± 0.05 thereafter. An even closer control of pH during the initial 30-min period could probably be obtained by adjusting some parameters of the PID regulation. When the initial flow rate was held constant, the pH in the reactor reached the set value after approximately 30 min. As a consequence, the prefermented milk was acidified, with pH changing from 6.3 to 6.0 during the first 30 min. However, the pH and the dilution rate were remarkably stable after 1 h of operation for the two procedures tested. The starting period of the system was very reproducible for both protocols. Figure 3 reports the inoculation level during the daily 8-h cycles for both procedures. Milk inoculation was extensive (about 10^8 CFU ml^{-1}) and stabilized rapidly (in less than 1 h) with an initial flow rate that fluctuated. On the contrary, when the initial flow rate was held constant, milk inoculation was lower during the initial period with 3.1×10^7 CFU ml^{-1} after 30 min of operation and only reached a high and stable value after 4–5 h.

The starting procedure using a variable flow rate allowed a stricter control of the bioreactor pH and prefermented milk quality during the first hour of the prefermentation

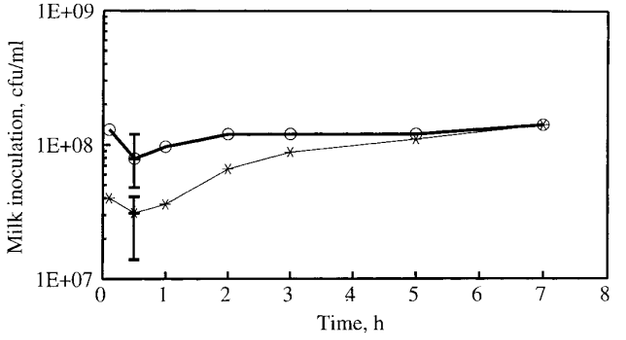

Figure 3 Milk inoculation changes as a function of time during a prefermentation operating 8-h cycle using the starting procedure with variable (—○—) or fixed (—✳—) initial milk flow rate. Means are calculated from respectively six and three repetitions. The mean standard deviation (reported on figure) are respectively of 3.8×10^7 CFU ml^{-1} and 1.7×10^7 CFU ml^{-1}.

cycle. With this procedure, a prefermented milk that is correctly acidified to pH 6.0 ± 0.1 and extensively inoculated (more than 8×10^7 CFU ml^{-1}) can be collected. Two operating cycles were carried out after a 60-h interruption with an initial variable flow rate. Stable high values of flow rate (15 h^{-1}) and inoculation level (10^8 CFU ml^{-1}) were obtained after only 40 min. This starting procedure is therefore appropriate for night or week-end interruption of the continuous prefermentation processing of milk. Prévost [13] demonstrated daily operating cycles for an immobilized cell bioreactor used for continuous milk prefermentation with a strain of *Lactococcus lactis* subsp *lactis* biovar *diacetylactis*, without stopping the prefermentation activity during night periods. During this stage, the reactor was cooled to 7–8°C and continually fed with milk to maintain pH control in the reactor with decreased acidifying activity. Productivity reached its steady state in less than 2 h after increasing the temperature to its original value (25°C). This procedure involved some disadvantages however, such as the production of significant quantities of cold prefermented milk during night periods, the risk of plugging up outlet tubes and the risk of psychotrophic flora contamination of the bioreactor while operating at a reduced flow rate.

The bacterial population equilibria observed in the prefermented milk, for the daily cycles, after 1 h and 7 h of continuous prefermentation, are reported in Table 3. The inoculation level increased between the first and the seventh hour but the strain equilibrium did not change. *L. lactis* subsp *lactis* biovar *diacetylactis* CDI1 was always the major strain of the mixed culture, representing about 80% of the total bacterial population. The percentage of the other strains remained relatively stable, considering the limited accuracy of the specific enumeration method [20].

These data are in agreement with our previous observation [19] on microbial balance and performance of the immobilized cell bioreactor used for continuous UHT milk prefermentation, obtained during 6–8 weeks continuous operation of the system (without night interruption). We observed the same general results: a dilution rate of 16.4 h^{-1} inoculation level of 2.2×10^8 CFU ml^{-1}, and a population equilibrium equal to 88.4%, 9.2%, 2.3%, 0.1% (*Lactococcus lactis* CD11, *Lactococcus lactis* CNRZ144, *Lactococcus lactis* E8, and *Leuconostoc mesenteroides* X2, respectively).

Conclusions

This study confirms the industrial feasibility of a continuous milk prefermentation process for fresh cheese manufacture using immobilized cells. Milk with a high dry matter content (13%) can be used for continuous prefermentation with the proposed immobilized cell bioreactor configuration without any negative effect on process performance compared to regular skim milk (9% total solids). Use of milk which has been treated at an elevated temperature could increase the high dilution rate. Indeed, milk used for fresh cheese production is highly heat-treated (95°C, 2–5 min). The use of the prefermentation pilot bioreactor for daily 8-h cycles and 5 days a week is feasible using a simple pro-

Table 3 Total and specific bacterial population observed after 1 h and 7 h of continuous prefermentation of UHT skim milk with the pilot scale bioreactor, monitored with daily operated 8-h cycles using a variable milk flow rate starting procedure. Relative values of the specific populations are given in brackets. The means are calculated from six repeated trials

CFU ml^{-1} for	Time	
	1 h	7 h
Total cell counts (CFU ml^{-1})	$9.8 \pm 0.4 \times 10^7$ (100)	$1.6 \pm 0.2 \times 10^8$ (100)
Lactococcus lactis CNRZ144 (CFU ml^{-1})	$1.4 \pm 0.6 \times 10^7$ (14.3)	$2.1 \pm 0.7 \times 10^7$ (12.7)
Lactococcus lactis E8 (CFU ml^{-1})	$4.6 \pm 2.7 \times 10^6$ (4.7)	$1.2 \pm 1.0 \times 10^7$ (7.2)
Lactococcus lactis CDI1 (CFU ml^{-1})	$7.8 \pm 3.7 \times 10^7$ (80.0)	$1.3 \pm 0.7 \times 10^8$ (78.3)
Leuconostoc mesenteroides X2 (CFU ml^{-1})	$9.8 \pm 5.2 \times 10^6$ (1.0)	$3.1 \pm 3.1 \times 10^6$ (1.9)

cedure for starting and interrupting the prefermentation system, which could be easily automated.

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