Production of the autolytic culture *Lactobacillus delbrueckii* ssp. *lactis* FAM-10991 using continuous culture with immobilized cells

S. Koch[1], M. Wettstein, E. Eugster-Meier, L. Meile and C. Lacroix[1] *
[1] ETH Zurich, Schmelzbergstr. 7 8092 Zurich, Switzerland
* (christophe.lacroix@ilw.agrl.ethz.ch)

Introduction

Lactic acid bacteria, such as lactobacilli, are widely used as starter cultures for the production of cheese. These cultures are mainly produced in frozen or dried forms. For dried cultures, starter bacteria have to withstand drying conditions during lyophilization. Next to stress resistance, high cell viability and high acidification activity of starter cultures are required. Cell immobilization showed many advantages for biomass and metabolite production as well as increased tolerance to environmental stresses (Doleyres & Lacroix 2005). For example, using immobilized cell technology, high cell yield and productivity was reported for *B. longum* (Doleyres, 2002) and a progressive increase in the tolerance of cells to various environmental stresses (acidity, oxygen, gastric conditions, freeze drying, antimicrobials, etc) was shown (Doleyres, 2005).

An important contribution of starter cultures to cheese ripening is through their autolytic activity and subsequent release of intracellular enzymes which are responsible for cheese flavor development. Intracellular enzymes are usually peptidases or lipases or enzymes from amino acid catabolism. Nowadays high autolytic strains of lactobacilli or lactococci are commonly used during cheese production to control and accelerate the ripening process. However autolytic cultures usually exhibit low survival during processing as autolysis is induced by environmental stresses resulting in large viability loss. We recently showed that application of sublethal stress during fermentation, such as a high osmotic stress (2.5% salt) during the exponential growth phase, can be used to increase survival of autolytic culture to subsequent stress as encountered during lyophilisation. However this stress application limits cell yield during fermentation.

In this study the drying-sensitive high autolytic *Lb. delbrueckii* ssp. *lactis* FAM-10991 (Koch, 2006a) was produced in a two stage continuous fermentation system with immobilized cells while resistance to lyophilization and autolytic properties were investigated.

Material and methods

Lb. delbrueckii ssp. *lactis* FAM-10991 was immobilized in Gelrite gellan gum and xanthan gum as previously described by Cinquin (2004) using an 2% (v/v) inoculum. Beads with diameter in the range from 1 to 2 mm were collected by wet sieving. Continuous fermentation was carried out for 20 days in a continuous two stage fermentation system as previously described (Doleyres, 2004a). The first 500 ml bioreactor (F1) inoculated with 30% (v/v) beads (culture volume of 240 ml) was connected in series with a 1.5 l bioreactor (F2). The cultures were performed at 37°C and pH 5.5 (F1) or 6 (F2) using 5N NaOH until day 17, with stirring at 150 (F1) and 200 rpm (F2) and CO_2 in the headspace. After day 17, the pH control in F2 was stopped (pH was reduced from 5.5 on day 17 to 4.7 on day 20) to induce acid stress in F2. The culture medium (MRS medium supplemented with 65g/l glucose) was pumped at a flow rate of 340 ml/h, for dilution rates (D) of 1.5 h^{-1} (F1) and 0.24 h^{-1} (F2). Effluent samples were analysed for cell counts, optical density (OD_{650}) and morphology; dry biomass, autolytic activity, PepX activity, survival to lyophilization and glucose and lactate concentrations. Cell count concentration in beads was measured on day 4, 10 and 17.

Reversibility studies were performed in three successive batch fermentations at pH 5.5, 37°C and 150 rpm. The first batch culture was inoculated (0.1% v/v) with cells of F1 and F2 sampled on day 17 and kept in glycerol at -80°C. The second batch culture was inoculated with cells from the first batch culture and so on. At the end of each culture, viable cell, dry biomass, glucose and lactate concentrations, autolytic and PepX activities and survival to lyophilization as well as acidifying properties were determined. A control batch culture under the same conditions was carried out using a fresh inoculum (1% v/v) similar to that used for immobilization. Successive batch and control fermentations were carried out in duplicate and means were reported.

Results and Discussions

Continuous fermentation

Viable cell counts measured in the effluent medium of the two continous reactors reached 1.5 and 6.1 x 10^8 cfu/ml in F1 and F2 after 4 days of fermentation (Fig. 1A).

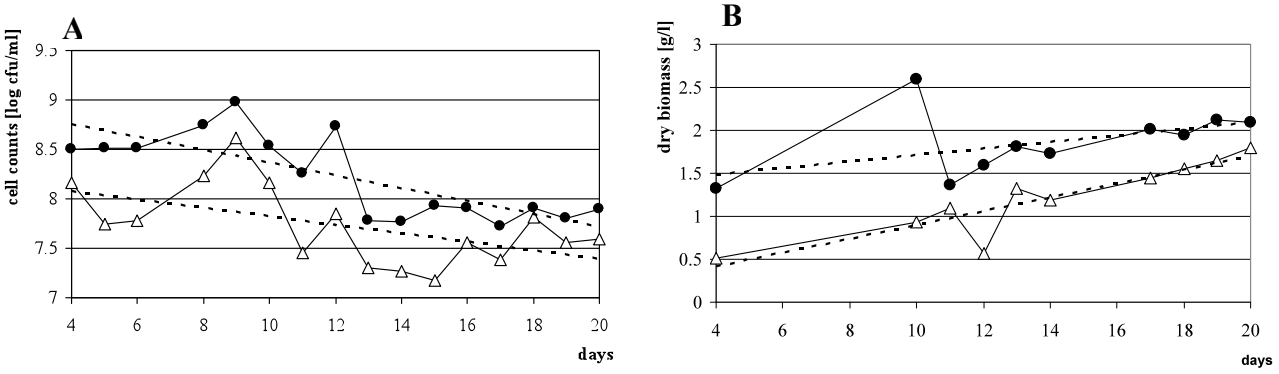

Fig. 1. Viable cell counts (A) and dry biomass concentration (B) during continuous production of *Lb*. *delbrueckii* ssp. *lactis* FAM-10991 in a 2 stage fermentation system (F1 (open symbols) and F2 (close symbols)) using ICT.

At the beginning of the continuous fermentation, cell morphology showed rod shaped individual cells or short chains (1-4 cells) of approximately 10 to 30 µm in both reactors (picture not shown). In the course of fermentation, chain length increased to approximately 50 µm in both reactors and after day 10, cluster formation was observed next to chains of endless length. Viable cell counts in both F1 and F2 progressively decreased with fermentation time ($p<0.05$, Fig. 1A). On the other hand, the dry biomass concentration (Fig. 1B), optical density and volumetric productivity significantly increased with time. The observed decrease in viable cell counts in F1 and F2 can be explained by chain and aggregate formation of *Lb. delbrueckii* ssp. *lactis* FAM-10991 which was also observed by Bergmaier (2005) for *Lb*. *rhamnosus* during continuous fermentation with immobilized cells.

Statistical analysis showed that there was no significant influence of the fermenters F1 and F2 for autolytic activities. Autolytic activity determined at the first day was significantly higher compared to all other fermentation times (Fig. 2A). Autolytic activity decreased during the first 8 days and then remained constant. There was no influence of the fermenter on cell survival rates after lyophilization (Fig. 2B). The mean survival rate was approximately 5% in the first 11 days and increased to high value of 48 % on day 20 ($p<0.05$).

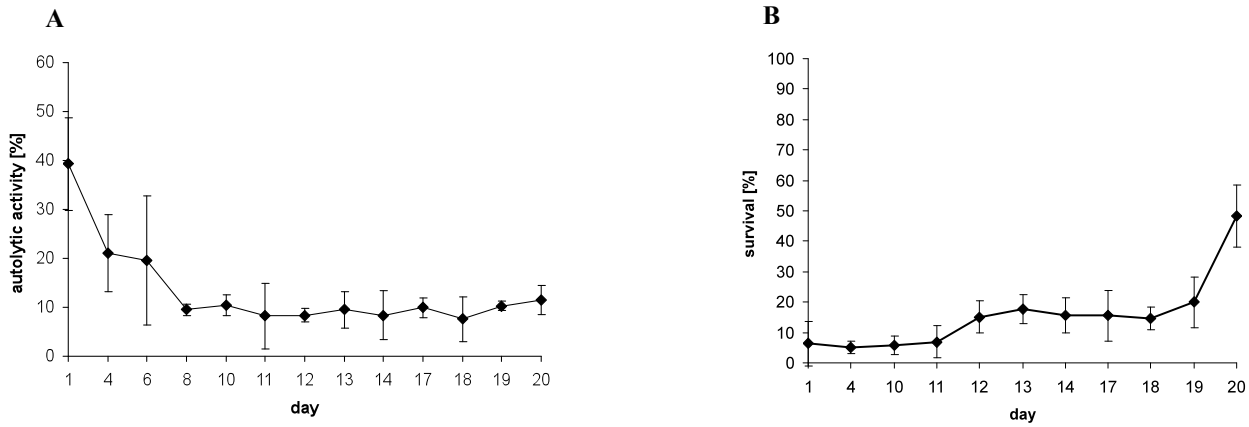

Fig. 2. Autolytic activity (A) and survival after lyophilization (B) during continuous production of *Lb. delbrueckii* ssp. *lactis* FAM-10991 in two stage fermentation system.

Reversibility studies

The properties of cells produced during three successive batch fermentations inoculated with cells from day 17 of F1 (batch F1) or F2 (batch F2) continuous culture were compared to those of cells at day 17 of the continuous fermentation and cells from control cultures. Viable cell counts at the end of control batch cultures were significantly higher compared to counts at the end of the successive batch cultures (3-fold and 3.3-fold for batch F1 and batch F2, respectively) and to cells from continuous culture at day 17 (60-fold and 36-fold for F1 and F2, respectively) (Tab. 1). Autolytic activity of cells grown in F1 continuous fermentation was 3.3-fold lower ($p<0.05$) compared to control cells. Autolytic activities of cells grown in successive batch fermentations were all in the same range close to 15% and lower than control cells (although generally not significant) (Tab. 1).

Fermentation	Cell counts [x10^8 CFU/ml] F1 / F2	Autolytic activity [%] F1 / F2
Continuous (day 17)	0.3^{a} / 0.5^{a}	8.6^{a} / 11.3^{a}
Batch 1	4.0^{b} / 5.8^{b}	14.9^{ab}/ 14.9^{a}
Batch 2	6.0^{b} / 7.3^{bc}	13.5^{a} / 15.7^{ab}
Batch 3	6.0^{b} / 5.5^{b}	18.7^{ab} / 20.8^{ab}
Control	18.0^{c}	28.2^{b}

Table 1. Viable cell counts and autolytic activities of *Lb. delbrueckii* ssp. *lactis* FAM-10991 produced during control batch culture, continuous culture F1 and F2 at day 17, and three successive batch cultures for reversibility study.

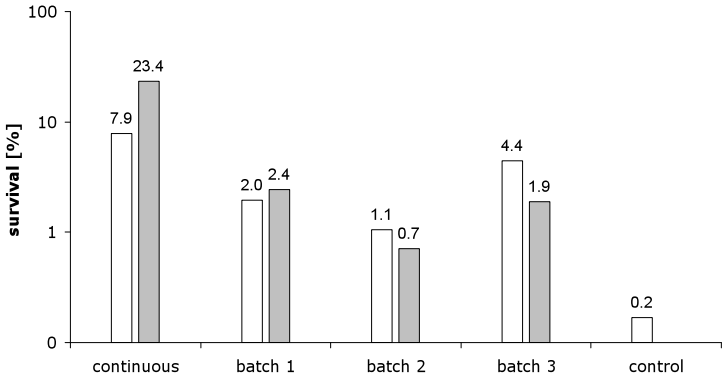

Fig. 3. Survival rates during lyophilisation of *Lb. delbrueckii* ssp. *lactis* FAM-10991 produced during control batch culture, continuous culture F1 (white bar) and F2 (grey bar) at day 17, and three successive batch cultures for reversibility study.

Survival rates to lyophilization of cells from F1 and F2 continuous cultures after 17 days were approximately 40- and 120-fold higher, respectively, than for cells from control culture (Fig. 3). A decrease of cell survival to lyophilization was observed during the successive batch cultures (not significantly) compared to cells from F1 continuous culture. A two-fold increase in the survival to

lyophilization of *B. longum* produced during continuous immobilized cell fermentation was reported by Doleyres (2004b).
Biomass volumetric productivity was approximately 3-fold higher for the two-stage continuous culture (0.41 g/l h) ($p<0.05$) than for control or successive batch cultures (0.14 g/l h for 24 h culture).

The reversibility studies showed that resistance to lyophilization of cells produced during continuous immobilized cell culture is partly lost during successive three batch fermentations. On the other hand, the high autolytic activity of the culture which is important for cheese production was largely recovered during successive batch fermentations.

Conclusions

In this study we confirmed that cell immobilization and continuous culture lead to important changes in cell physiology. Important factors for the starter culture production like survival to lyophilization and autolytic properties were changed during continuous production with immobilized cells compared to cells produced with classical free cell batch cultures. Reversibility studies also showed that these physiological changes are reversible during successive batch fermentation which were performed to simulate industrial application of starter cultures in food fermentation processes.
The increased cell survival rate to lyophilization and biomass volumetric productivity for immobilized cell technology and the recovered high autolytic activity after successive batch cultures could be of special interest for industrial autolytic starter culture production.

References

Bergmaier, D., et al. (2005). *Growth and exopolysaccharide production during free and immobilized cell chemostat culture of lactobacillus rhamnosus rw-9595m*. Journal of Applied Microbiology, 98, 272-284.
Cinquin, C., et al. (2004). *Successful immobilization of infant fecal flora and utilization in an in vitro colonic fermentation system*. Microbial Ecology, 48, 128-38.
Doleyres, Y., et al. (2004a). *Continuous production of mixed lactic starters containing probiotics using immobilized cell technology*. Biotechnology Progress, 20 (1), 145-150.
Doleyres, Y., et al. (2004b). *Increased stress tolerance of Bifidobacterium longum and Lactococcus lactis produced during continuous mixed-strain immobilized-cell fermentation*. Journal of Applied Microbiology, 97, 527-539.
Doleyres, Y., et al. (2005). *Technologies with free and immobilised cells for probiotic bifidobacteria production and protection*. International Dairy Journal, 15, 973-988.
Doleyres, Y., et al. (2002). *Bifidobacterium longum atcc 15707 cell production during free- and immobilized-cell cultures in MRS-whey permeate medium*. Applied Microbiology and Biotechnology, 60 (1-2), 168-173.
Koch, S., et al. (2006a). *Effects of strain and growth conditions on autolytic activity and survival to freezing and lyophilization of Lactobacillus delbrueckii ssp. lactis isolated from cheese*. International Dairy Journal, submitted.
Koch, S., et al. (2006b). *Salt sublethal stress increases cell yield, autolytic properties, and survival to lyophilization of Lactobacillus delbrueckii ssp. lactis*. International Journal of Food Microbiology, submitted.