ROLE AND CONTROL OF STARTER LYSIS IN CHEESE PRODUCTION AND RIPENING

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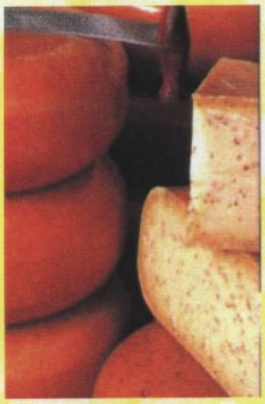
Introduction

Lysis of lactic acid bacteria plays a crucial role in dairy fermentations. Especially in cheese the energy reserves of the starter bacteria are quickly depleted during the maturation. This prevents the translocation of extracellularly produced large peptides into the cell. Therefore it is generally assumed that lysis of the starter bacteria enhances involvement of the intracellular bacterial enzymes in the gradual process of flavour formation by conversion of the milk proteins and their breakdown products. We have studied different aspects related to the phenomenon of lysis:

1. The development of rapid and sensitive methods to analyse and quantify lysis *in situ*, in cheese, by making use of fluorescent probes.
2. The selection of industrial starter strains which have a high lysis efficiency.
3. The effect of bacteriocin-immunity on the cell wall composition and stability (1).



Acknowledgements

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Results

In situ analysis of lysis

To study the activity and integrity of the lactic acid bacteria *in situ*, we are working on a non-destructive method using fluorescent techniques. A *Lactococcus lactis* strain, harboring the lytic genes *lytA* and *lytH* from bacteriophage fUS3 under strict control of the *nisA* promotor (2), was used to obtain controlled lysis of the starter in M17 medium (Figure 1)

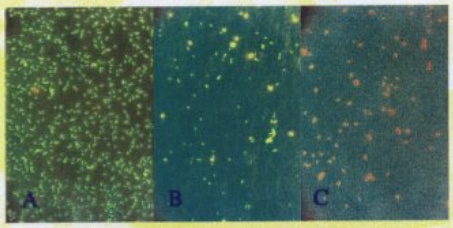

Figure 1. *Fluorescent labelling of live (green) and dead (red) cells with respectively probe SYTO 9 and PI, in a culture of strain NZ3900, harboring pNZ8038F, before (A), during (B) and after full induction of lysis (C) with different doses of nisin.*

These experiments show that the induced lysis results in 1) large reduction in total cell count due to complete desintegration of the majority of the cells and 2) complete permeability and hence PI staining of the remaining cells. Currently the procedure is being implemented in cheese model systems where lysis is expected to result in mainly permeabilization and not desintegration.

Selection of lysis-sensitive strains

Approximately 200 *Lactococcus lactis* strains were isolated from different sources and compared in cell stability and sensitivity of the cell walls for the lytic enzyme mutanolysin (Figure 2).

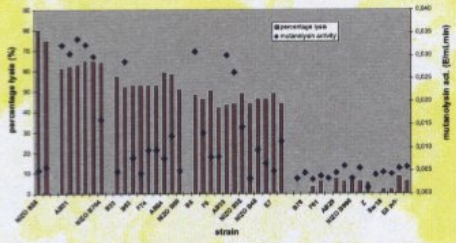

Figure 2. *Lysis of several L. lactis strains in M17-medium with 0.3% glucose after 21 days incubation at 30°C (bars), as well as the sensitivity of the cells (harvested at OD 0.4 at 600nm) for mutanolysin (20U/ml) at 37°C (◆).*

Cells with high stability always showed a low sensitivity to mutanolysin. The reverse was not always the case, indicating that not only cell-wall composition is involved in lysis behaviour.

Effect of bacteriocin-immunity on the cell stability

Nisin-immune starters were developed by introduction of the nisin-sucrose transposon Tn*5276*-NI containing a deficiency in a structural gene for nisin production (3). Transconjugants of *L. lactis* SK110 showed increased stability in cheese (Figure 3). Cheese manufactured with the transconjugant was more bitter than cheese manufactured with strain SK110 (data not shown). This can be explained by the reduced release of debittering enzymes, due to the higher stability of the transconjugant.

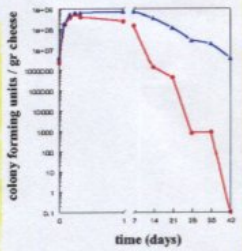

Figure 3. *Lysis of strain SK110 (●) and its nisin-immune transconjugant SK110: Tn5276-NI (▲) during Gouda cheese production.*

The sensitivity of purified SK110 peptidoglycan for mutanolysin was higher than the cell walls of the transconjugant (Figure 4), which is due to differences in the amount of cross-links in the peptidoglycan structure (data not shown). This suggests that a gene encoded by transposon Tn*5276*-NI affects the peptidoglycan composition.

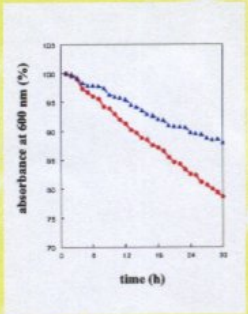

Figure 4. *Relative changes in absorbance (600 nm) after treating 1.4 mg/ml isolated cell walls of strain SK110 (●) and its transconjugant (▲) with mutanolysin (10U/ml) at 37°C.*

Conclusions

- Fluorescent labeling gives good opportunities to develop a rapid and sensitive method to analyse lysis *in situ.*
- Lysis efficiency is extremely variable between different *L. lactis* strains.
- Introduction of transposon Tn*5276*-NI alters the susceptibility of the host strain to lysis via a changed peptidoglycan composition.

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ROLE AND CONTROL OF STARTER LYSIS IN CHEESE PRODUCTION

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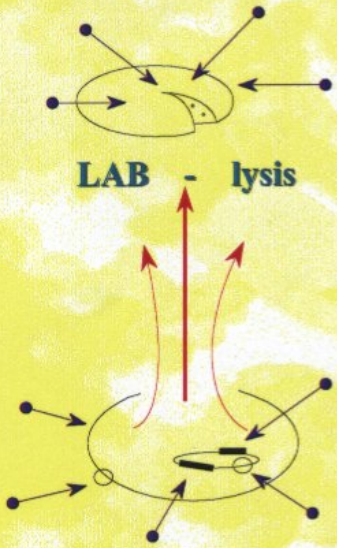
Introduction

Lysis of lactic acid bacteria plays a crucial role in dairy fermentations. Bacterial lysis ensures the involvement of the intracellular starter enzymes in the gradual process of cheese flavour formation. For eventually controlling the process of starter lysis we are studying different aspects related to the phenomenon of lysis:

- The development of rapid and sensitive fluorescence methods to monitor lysis in cultures (1) and in cheese.
- The comparison of highly stable and unstable strains in cheese manufacture to establish the role of starter lysis in flavour formation, e.g. debittering capacity.
- The role of growth conditions on the cell wall stability of starter cells and its implication for cheese manufacture (2).



Results

***In-situ* analysis of lysis**

To study the activity and integrity of the lactic acid bacteria *in situ*, we are working on a non-destructive method using fluorescent techniques. For this purpose commercially available LIVE/DEAD staining in conjunction with CSLM was chosen. The technique was proven effective in two weeks old Gouda-type of cheese, manufactured with the undefined-mixed strain starter NIZOSTAR BOS (Figure 1).

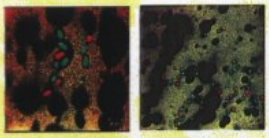


Figure 1. *Fluorescent labelling of live (green) and dead (red) cells with respectively probe PI and SYTO 9, in a two weeks old Gouda-type cheese manufactured with starter culture NIZOSTAR BOS.*

The results demonstrate the strength of *in-situ* viability staining in cheese. Currently, the procedure is statistically being optimised by making use of sophisticated image-analysis technology.

Selection of lysis-sensitive strains

Approximately 200 *Lactococcus lactis* strains were isolated from different sources and compared in cell stability and sensitivity of the cell walls for the lytic enzyme mutanolysin (Figure 2).

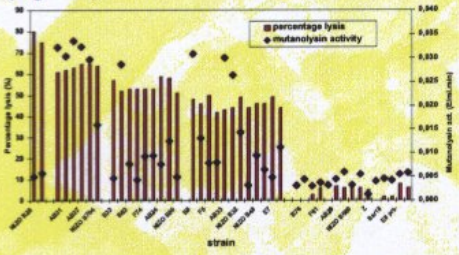


Figure 2. *Lysis of several L. lactis strains in M17-medium with 0.3% glucose after 21 days incubation at 30°C (bars), as well as the sensitivity of the cells (harvested at OD 0.4 at 600nm) for mutanolysin (20U/ml) at 37°C (◆).*

Cells with high stability always showed a low sensitivity to mutanolysin. The reverse was not always the case, indicating that not only the cell-wall composition determines the lysis efficiency.

Lysis of bacterial cells will result in the release of intracellular debittering enzymes. To evaluate the suitability of the different strains as debittering starter, bacterial cells were incubated with a bitter tasting C-terminal part of the ß-casein as substrate in an HPLC method. Strains which showed high degradation of the C-peptide (up to 75% after 4 hours of incubation) appeared to be the strains with high instability and high sensitivity to mutanolysin (data not shown).

To evaluate the exact role of the different intracellular peptidases in debittering, strain *L. lactis* NCDO 712 and different mutants with single and mutiple peptidase knock-outs (3) were used in the debittering assay (Figure 3).

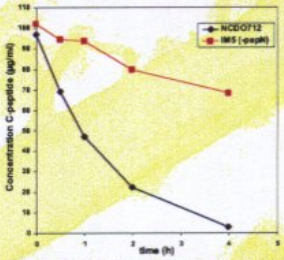


Figure 3. *Relative changes in concentration C-peptide (aa 193-207 of ß-casein) after incubation with strain L. lactis NCDO 712 and IM5 (knock-out in the structural pepN gene) at 30°C in 0.2 M Na-acetate, 3% NaCl at pH 5.4 for 4 hours.*

Release of the peptidase PepN, as a consequence of lysis, is the crucial factor for debittering activity of a starter culture. Bitter intensity of a cheese manufactured with the rapid-lysing strain *L. lactis* B33 was significantly lower than cheese manufactured with the highly stable strain *L. lactis* AB8.

Effect of growth rate on the cell stability

To study the effect of different physiological conditions on the sensitivity to lysis, two *L. lactis* strains, the lysis sensitive strain AB28 and the highly stable strain AB8, were cultivated in chemostats under different growth rates (Figure 4).

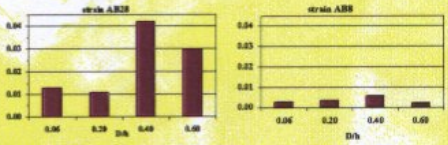


Figure 4. *Relative changes in absorbance (600 nm) after treating cells of strains L. Lactis AB28 and AB8, grown at different growth rates in chemostat cultures in M17 medium with 0.5% lactose at 30°C, with mutanolysin (10U/ml) at 30°C.*

Cells of strain AB28, as well as its cell wall composition, became increasingly stable at lower growth rates. The stability of strain AB8 was not visibly affected by changing growth rates. Currently, the exact role of the peptidoglycan composition on the cell wall susceptibility to lytic enzymes is investigated.

Conclusions

- Fluorescent labeling with permeable stains is a rapid and sensitive method to analyse lysis *in situ*.
- Lysis efficiency is extremely variable within the *L. lactis* species. By selecting the right strains, tailor-made, debittering, starter cultures can be developed
- The release of the intracellular peptidase PepN is crucial for debittering activity.
- The process of lysis can be controlled by suitable growth conditions, as *e.g.* the growth rate

Acknowledgements

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