

Biochemical Factors Influencing Autolysis of *Leuconostocs* in Buffer

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Abstract: Factors influencing autolysis of two dairy leuconostocs strains chosen for their moderate and relatively higher autolytic extend were investigated in buffer system. Higher level of autolysis was observed in bacteria that were harvested at earlier exponential phase. Autolysis was greatly influenced in acidic and basic pH values and optimal level was monitored between 6 and 7 values. Optimal incubation temperature to induce lysis was detected at 30°C, which is at the same time optimal growth temperature for leuconostocs strains. Whereas autolysis was activated by the presence of monovalent cations, an important level of inactivation was noted in the presence of divalent cations.

Key Words: *Leuconostoc*, autolysis, biochemical factors, cheese ripening.

Tampon Ortamlarda *Leuconostok* Otolizini Etkileyen Biyokimyasal Faktörler

Özet: Orta ve daha yüksek oranda otolitik özelliğe sahip olan süt orjinli iki adet leuconostok susunun otolizine etki eden biyokimyasal faktörler tamponlanmış ortamda test edildi. Logaritmik büyüme fazının erken safhasında tampon ortamına alınan bakterilerin daha yüksek litik özelliğe sahip oldukları gözlemlendi. Asidik ve bazik ortamlarda aktivitenin azaldığı gözlenirken optimal aktivite için gerekli pH ortamının 6 ile 7 değerleri arasında olduğu saptandı. En uygun inkübasyon ısısı da yine leuconostok susularının optimal ürettiği 30°C olarak belirlendi. Monovalan katyonlar otolizi aktive ederken, divalen katyonların ise inhibe ettiği saptandı.

Anahtar Kelimeler: *Leuconostok*, otoliz, biyokimyasal faktörler, peynir olgunlaşması.

Introduction

Lactic acid bacteria by their enzymatic contents play important roles during cheese ripening. Proteolytic enzymes of these bacteria degrade milk proteins, in particular casein to peptides which are subsequently degraded to amino acids by intracellularly-located peptidases^{2,11}. Amino acids are the precursors of cheese flavour compounds. In cheese technology, strains showing high autolytic activity are preferred since they release their enzymatic contents into the curds during ripening. This phenomenon stimulates the acceleration of ripening by the earlier production of flavour com-

pounds and inhibits the formation of bitter peptides⁷.

Leuconostocs are the heterofermentatif lactic acid bacteria used in dairy industry as co-culture with lactococcal strains⁴. They are able to produce carbon dioxide and diacetyl by metabolizing citrate. Whereas diacetyl is an important flavour compound, carbon dioxide is responsible for the eye formation²¹. Despite their importance in cheese technology the knowledge acquired about this bacterium is very limited.

Bacterial autolysis can be defined as the spontaneous disintegration of the cell wall by

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the enzymes called peptidoglycan hydrolases or autolysins^{3,19}. Many Gram positive and Gram negative bacteria are known to possess these enzymes. Autolysis can be induced by transferring the cells in a buffer solution in which the cells will be in nutritionally starvation condition⁵. In this case, various factors such as growth phase, pH, temperature, activators and inhibitors influencing cell lysis can be studied.

In the present study, biochemical factors influencing autolysis of dairy leuconostocs were characterized by incubating cells in buffer systems.

Materials and Methods

Bacterial strains and growth conditions

The strains *Ln. mesenteroides* subs. *dextranicum* CNRZ 1772 and *Ln. mesenteroides* subs. *mesenteroides* CNRZ 1470 were obtained from CNRZ culture collection of INRA, Jouy-en-Josas / FRANCE. The stock cultures were stored at -20°C in MRS broth⁸ containing 15% glycerol (vol/vol). Preculturing and two subculturing were carried out in MRS broth at 30°C.

Measurement of Autolysis

Bacterial autolysis was determined as described previously⁶. Briefly, unless otherwise stated, early exponential phase cells (OD_{650nm} = 0.5-0.7) were harvested by centrifugation at 3000 g for 10 min at 4°C and washed two times in phosphate buffer (50 mM, pH 6.5). After resuspension in the same buffer the initial OD_{650nm} (Beckman DU Spectrophotometer USA) was adjusted to 0.6 to 0.8 and the tubes were incubated at 30°C. The decrease in OD_{650nm} was followed for 24h. Percentage autolysis was estimated according to the following formulation:

$$\% \text{ lysis} = 100 - (A1/A2) \times 100$$

where A1 is the lowest O.D. value and A2 is the maximal O.D. value measured during incubation period.

Influence of the growth phase on the autolysis was tested on the cells harvested after 3, 6, 12, 15, 18, 28 and 36 h of growth in culture medium. The effect of temperature on early exponential phase harvested cells studied in potassium phosphate buffer at 7, 12, 20, 30, 42 and 50°C. To study the influence of pH on the autolysis, cells were resuspended in sodium acetate buffer (50 mM, pH 4.5, to 5.5), potassium phosphate buffer (50 mM, pH 5.5 to 7.5)

and Tris-HCl buffer (50 mM, 7.5 to 8.5). Influence of mono and divalent cations on the autolysis was tested at 30°C for 24 h. by the addition of a final concentration of NaCl, KCl, MgCl₂, MnCl₂ and CaCl₂ into the potassium phosphate buffer (50 mM, pH 6.5). Chemicals used were obtained from Sigma Co (USA).

Results

Strains *Leuc. mesenteroides* subsp. *dextranicum* CNRZ 1772 and *Leuc. mesenteroides* subsp. *mesenteroides* CNRZ 1470 exhibiting moderate and higher autolytic activity respectively were used to determine the conditions leading to autolysis in buffered media. To determine the relatedness between growth phase and autolysis, the strains were harvested at different points of growth and incubated in buffer. The decrease in turbidity was not constant during all the period of growth (figure 1). The maximum turbidity loss was obtained with the cells harvested at earlier exponential phase of growth. At this stage the strains CNRZ 1772 and CNRZ 1774 showed 27 and 42% turbidimetric decrease respectively. The activity was gradually decreased towards the end of the exponential phase and minimal activity was obtained at stationary phase harvested cells. Early exponential phase cells showing highest activity were used for the subsequent studies.

The influence of the temperature on the activity was studied for a range of temperature varying from 7 to 50°C. Similar observations were obtained for both of the strains. Representative results of the strain CNRZ 1772 were shown in figure 2. Optimal turbidimetric decrease was marked at 30°C. This temperature at the same time is the cultivation temperature for the *Leuconostoc* strains. At 42 and 50°C the rate of the autolysis was higher during the first hours of incubation, but this rate was then reduced sharply probably depending on the denaturation of the autolytic enzyme(s). Conversely, the rate was quite low but linear during all the incubation period at 7 and 12°C.

Autolysis at different pH values was demonstrated in figure 3. Although autolysis was observed at all pH tested, optimum values were obtained between 5.5 and 7.5. Autolysis was lower at high and low pH that indicates inactivation of autolytic enzymes.

As shown in table 1, all the three strains showed an increase in turbidimetric decrease with the addition of monovalent cations Na⁺ and K⁺ to buffer. Meanwhile the activity was in-

creased gradually when the concentration of cations was augmented. Conversely, the presence of divalent cations Mn^{++} , Mg^{++} and Ca^{++} in the incubation had negative effect on the activity thus the activity was reduced at different extends.

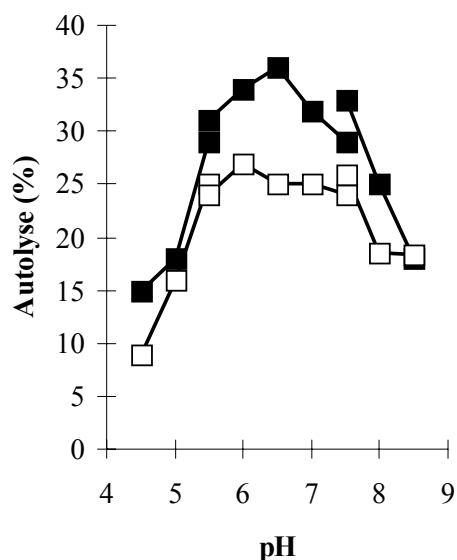


Figure 1.
Autolysis of the strain CNRZ 1772 (\square) and CNRZ 1470 (\blacksquare) as a function of growth phase. Cells were harvested at indicated times and resuspended in potassium phosphate buffer (50 mM, pH 6.5) during 24 h at 30°C.

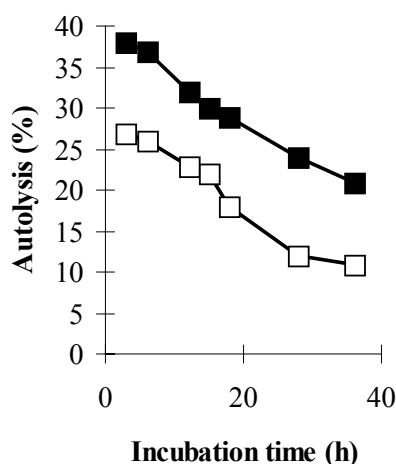


Figure 2.
Autolysis of early exponential phase cells of strains CNRZ 1772 (\square) and CNRZ 1470 (\blacksquare) at different pH values; sodium acetate (50 mM, pH 4.5-5.5), potassium phosphate buffer (50 mM, pH 5.5-7.5) and tris HCl buffer (50 mM, pH 7.5-8.5).

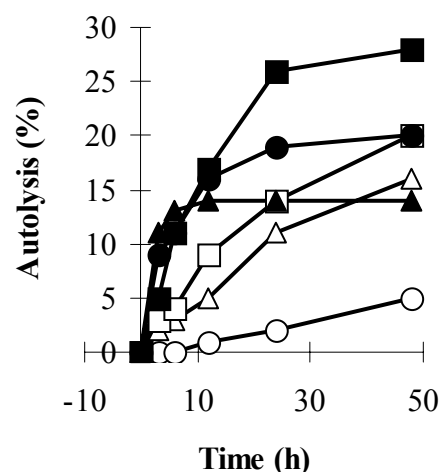


Figure 3.
Influence of the temperature on the autolysis of strain CNRZ 1772. Exponential phase harvested cells resuspended in potassium phosphate buffer (50 mM pH 6.5) for 24 h at 7°C (\circ), 12°C (Δ), 20°C (\square), 30°C (\blacksquare), 42°C (\bullet) and 50°C (\blacktriangle).

Table 1. Influence of mono and divalent cations on the autolysis^a

Cations	Concn.	Autolysis (%)	
		CNRZ 1772	CNRZ 1470
Non		27	35
NaCl	100 mM	39	44
	200 mM	38	49
	300 mM	42	56
KCl	100 Mm	25	36
	200 mM	28	39
	300 mM	31	49
MnCl ₂	10 μ M	9	21
	100 μ M	12	16
	1000 μ M	5	9
MgCl ₂	10 M	19	26
	100 μ M	16	27
	1000 μ M	13	11
CaCl ₂	10 μ M	18	37
	100 μ M	15	27
	1000 μ M	19	25

^a All the experiments were repeated three times and variability was < 10%.

Discussion

Transferring the bacteria from culture medium to a buffer system in which the bacteria will be in a starvation condition is a widely used technique for estimating potential autolytic properties of the bacteria^{6,13,12}. By using this method factors influencing autolysis of leuconostocs were investigated for the first time. The strains showed a spontaneous autolysis

when they were transferred from culture medium to buffer. Obtaining of different level of autolytic extent from both strains confirm that autolysis is a strain dependent phenomenon. Similar results were observed for *Lactococcus lactis*^{22,17} and *Propionibacteria*¹³. Chapot-Chartier et al² and Wilkinson et al^{23,24} visualized the same phenomenon during the maturation process of cheeses while the *Lc. lactis* strains were used as starter cultures.

The extent of the autolysis was closely dependent on the growth phase. In this report, tested strains showed the most efficient autolysis at the early exponential phase. Similar results were reported for *Lc. lactis*^{14,15}, *Propionibacteria*¹⁶ and for *Lactobacillus acidophilus*¹⁰. Exponential phase cells that express the highest level of lysis support of the idea of the involvement of autolysins in the cell growth⁷. By contrast, Buist et al.¹ postulated a major autolytic enzyme from lactococcal strains, which is involved in stationary phase. Meanwhile, maximal autolysis for *Streptococcus thermophilus* was observed in the transitional phase between exponential and stationary phase¹⁸. Valence et al.²⁰ reported two optimal harvesting points for *Lb. helveticus*. The first was at transitional phase and second one was at early the exponential phase.

pH and temperature are the important factors in cheese ripening. In our experiments for both strains the optimum pH for autolysis was detected between 5.5 and 7 values. These values are in good relation with pH values required for the ripening process of cheese. In a study performed on *Lb. acidophilus*⁹ authors reported higher level of autolysis extent at 6.5 pH value.

Monovalent cations notably NaCl induced the extent of the autolysis of both strains. Wilkinson et al.²³ studied the factors influencing autolysis of starter bacteria during Cheddar cheese ripening and postulated that increasing amount of NaCl resulted in an increase in activities of autolytic marker enzymes. The results obtained in the present study can be used in the selection of proper leuconostocs bacteria for ripening of several cheeses varieties.

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