A direct and simple method for rapidly counting viable chains of Leuconostoc in batch cultures

Florence Plihon, Patricia Taillandier, Pierre Strehaiano

A DIRECT AND SIMPLE METHOD FOR RAPIDLY COUNTING VIABLE CHAINS OF *LEUCONOSTOC* IN BATCH CULTURES

F. Plihon*, P. Taillandier, and P. Stréhaiano

Laboratoire de Génie Chimique, CNRS URA 192, ENSIGC
18 Chemin de la Loge, 31078 Toulouse cedex, France
fax : (33) 62 25 23 18

SUMMARY

Plating determines the concentration in viable cells of bacteria but remains time-consuming and inaccurate. A direct and simple method combining two techniques, haemacytometry and epifluorescence, gives both the viability and the concentration in viable cells. The method was evaluated using *Leuconostoc mesenteroides*.

INTRODUCTION

Plating, used to enumerate numbers of viable microorganisms, is both time-consuming and inaccurate (Lange *et al.*, 1993). We present here a simple and direct method that gives the number of viable bacteria by combining two techniques: haemacytometry, which gives the total bacterial concentration (Bourdon and Marchal, 1973) and the epifluorescence, which gives the viability (Mayfield, 1975, 1977; King *et al.*, 1981). We have used *Leuconostoc mesenteroides*, a strain commonly used in cheese product, to evaluate this method. This lactic acid bacterium occurs in chains of various lengths when growing in liquid media (Goel and Marth, 1969a), which perturbs bacterial numbers when plating. Cultures therefore need to be shaken to make the chain length uniform and to lead to reproducible counts (Goel and Marth, 1969b; Martley, 1972).

As viability depends on the acidity of the culture medium (Champagne *et al.*, 1989), viable counts, given both by plating and the direct method, have been determined with and without pH control. The effect of shaking on chain disruption with the strain was also studied to explain the results obtained with the two methods.

MATERIALS AND METHODS

Cultures. Leuconostoc mesenteroides subsp. *mesenteroides* was obtained from an industrial laboratory and was grown on 100 mL liquid MRS medium for 7 h in a 250 mL Erlenmeyer flask at 30°C with magnetic agitation at 250 rpm. For cultures with pH control, a 15-L fermentor vessel was filled with 10 L modified MRS medium (Plihon *et al.*, 1995) and sterilized for 55 min. at 120°C. The pH was maintained at 6.5 with a 5M ammonia solution. The temperature was fixed at 30°C and the stirrer speed at 400 rpm.

Chain length. A drop of a culture was observed under microscopy at 1000x magnification. The number of cells per chain L_i was counted for each chain n_i observed in a microscopic field. A good statistical representation of each chain length L_i in a culture was obtained when counting 200 to 250 chains. An average chain length $((\Sigma n_i L_i)/\Sigma n_i)$ was deduced.

Plate count technique. A 1 mL sample of cellular suspension was spread in each sterile Petri dish and then covered with MRS agar (Champagne and Gardner, 1990) maintained at 45°C. The colonies were counted after 48 h incubation at 30°C. Dilutions were made in sterile water so that an ideal count ranged from 30 to 300 colonies (Lee *et al.*, 1981).

The direct method. It combined haemacytometry, giving the total concentration in chains, and the epifluorescence, giving the viability, both conducted on the same sample. Haemacytometry: the Petit-Salumbeni haemacytometer used to determine the total concentration in chains was adapted to the bacterial numeration. It was composed of 4x4 large squares. The volume of each large square was equal to 1.6 nL. The chains were counted at 400x magnification. Epifluorescence: this method required a microscope coupled with an ultraviolet epilighting system specific for Mg-ANS: the illumination wavelength was 490 nm and the fluorescence wavelength was 515 nm. Mg-ANS (0.3 g) was dissolved in 100 mL Ringer solution (Lee *et al.*, 1981). The staining and the culture sample were mixed in a 1:1 ratio and incubated for 5 minutes before the microscopic observation at 1000x magnification. A chain was considered as being dead when all the cells of this chain fluoresced. The ratio of the nonviable chains to the total chains was determined by first counting the fluorescing chains in a microscopic field under epifluorescence, then the total field in the brightfield mode.

Experimental error. An error E was calculated from n experimental results X_i obtained for each technique and each sample:

$$E\,(\%) = 100 / X. \quad \sqrt{\sum (X_i - X)^2 / (n - 1)}, \text{ with } X = \Sigma X_i / n$$

Three counts were conducted for a same sample and for each method (n=3). The error with plating was found to be less than 10% if the plates contained more than 200 colonies. The error with haemacytometry remained below 5% when the counts ranged between 70 to 100 chains per square. The error fell under 8% when 100 to 150 chains were counted with the epifluorescence.

RESULTS

Comparison between plating and the direct method. When bacteria occurred in chains, each chain was presumed to give rise to a single colony, regardless of its length (Goel and Marth, 1969b). Plating and the direct method, which provided a concentration in viable chains too, could be then compared. Table 1 showed that the acid produced by the bacteria damaged the cells and induced a decrease in the viability when the pH was not controlled. Viability remained total when pH was controlled (Table 2). A 400 rpm agitation with pH control induced a stronger decrease in the average chain length than a 250 rpm agitation with no pH control. The chains disrupted when the average chain length reached 9.5 cells with no pH control and 11 cells with pH control.

The counts with plating remained higher compared to the counts with the direct method with and without pH control. The difference ε between the two methods increased with the

average chain length. However, it remained less high with pH control than with no pH control. The direct method could be compared with plating when the average chain length fell below 5 cells (ε fell below 13 %). The long chains would be more sensitive to shaking than the short ones. As plating required more shaking than the direct method because of the great number of dilutions, this should explain why the viable counts given by plating remained higher than those given by the direct method when the chains were long. To confirm this hypothesis, the effect of shaking was then studied.

Table 1. Comparison between plating and the direct method during a batch of *Ln. mesenteroides* with no pH control.

Time (h)	pH	Viability (%) (epifluo.)	Viable counts		ε† (%)	Average chain length (cells)
			direct method (Log_{10} chains/ml)	plating (Log_{10} CFU/ml)		
0	6.3	100	7.06	/	/	6.0
2	6.1	100	7.62	7.93	68.7	/
3.5	5.7	100	8.13	8.34	49.0	9.5
4.7	5.4	100	8.51	8.76	56.6	9.0
6.25	5.1	100	8.91	9.03	28.6	7.0
7.5	4.7	95.3	9.04	9.09	12.7	6.0
9	4.6	93.2	9.13	9.17	9.7	5.2
10.9	4.5	72.2	9.13	9.15	6.2	5.0
12.25	4.4	55.0	9.09	9.14	10.8	4.8
20	4.3	41.0	8.96	9.00	10.1	4.8

† X1: counts with the direct method. X2: counts on plating. ε (%)=100*(X1 X2)/(X1+X2)*2

Table 2. Comparison between plating and the direct method during a batch of *Ln. mesenteroides* with pH control.

Time (h)	pH	Viability (%) (epifluo.)	Viable counts		ε† (%)	Average chain length (cells)
			direct method (Log_{10} chains/ml)	plating (Log_{10} CFU/ml)		
0	6.5	100	6.48	/	/	6.0
5	6.5	100	8.19	8.22	8.1	7.7
6	6.5	100	8.59	8.52	15.9	11.0
7.5	6.5	100	8.93	9.04	24.7	8.0
8	6.5	100	9.19	9.22	8.9	5.6
9	6.5	100	9.41	9.49	17.2	4.5
9.7	6.5	98.0	9.52	9.56	10.3	3.5
10	6.5	98.0	9.59	9.65	15.1	3.2
11	6.5	98.5	9.72	9.78	12.8	2.6
12	6.5	98.2	9.77	9.81	8.0	2.5
13	6.5	97.9	9.81	9.83	2.5	2.4

† X1: counts with the direct method. X2: counts on plating. ε (%)=100*(X1-X2)/(X1+X2)*2

Effect of shaking on chain disruption

Spontaneous disruption of Ln. mesenteroides chains. The total concentration in chains and the average chain length were determined during a batch culture of *Ln. mesenteroides* without pH control (Fig. 1). The average chain length increased during the first three hours. The cells of each chain duplicated themselves. Each new cell stayed linked to its mother, inducing an increase of the chain length. This result proved that the long chains remained shake-proof when they were shaken at 250 rpm. The chains became unstable when reaching a maximum average chain length equal to 9.5 cells. They began to disrupt spontaneously and a strong disruption occurred during the first half of the growth phase. This characteristic phase was called the "unstable phase". The average chain length decreased slightly in the second half of the growth and kept an average value of 4.8 cells in the stationary phase.

No cell alone and almost no chain with an odd number of cells were observed during growth (data not shown). Long chains of every length, ranging from 2 to 30 cells, grew before the spontaneous disruption. During the unstable phase, the long chains were progressively broken down in order to provide chains with 2, 4, 6 and 8 cells. The chain length became uniform when reaching the stationary phase since more than 60% of the chains consisted of 2 and 4 cells (data not shown).

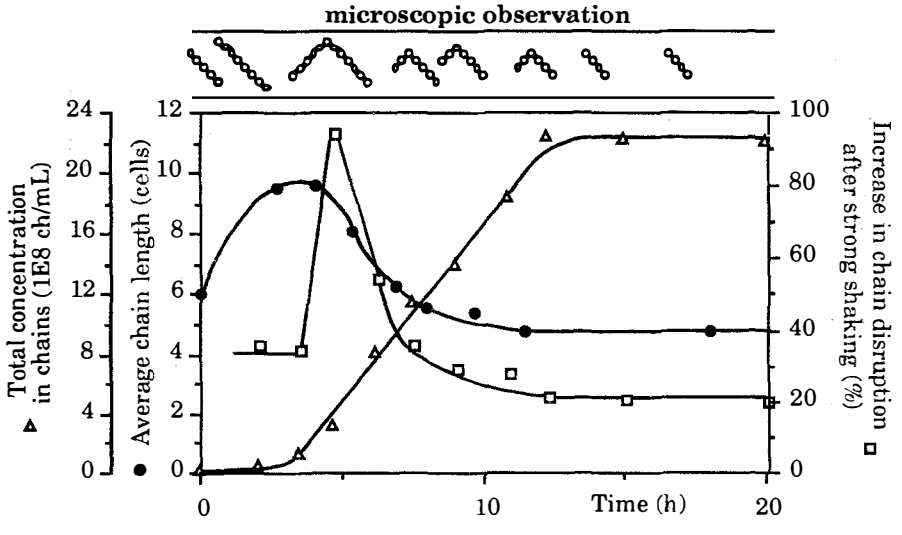

Figure 1. Growth, average chain length and chain disruption after strong shaking of *Ln. mesenteroides* during a batch culture.

Effect of strong shaking on the chain length. Samples were taken at various intervals of the batch culture previously described. Two concentrations in chains were determined for each sample:

- a first concentration C_{bs} was obtained without shaking the sample.
- the sample was then strongly shaken for 30 s by using a vortex mixer (this value was closer to the time of shaking after the dilutions needed for plating). A second concentration C_{as} was obtained.

An instantaneous value $(C_{as}-C_{bs})/C_{bs}$ was deduced (Fig. 1) and represented the increase in chain disruption after shaking. We saw that a rough shaking during the development of the long chains did not induce a strong disruption of these chains. Only 30% of the long chains disrupted according to Fig. 1. Every chain was broken into two parts as $(C_{as}-C_{bs})/C_{bs}$ reached 100 % as soon as the spontaneous disruption occurred. The percentage of chain disruption decreased strongly from 100 % to 30 % during the unstable phase and even fell to 20 % when the average chain length became equal to 5 cells.

DISCUSSION

This investigation shows that the count plate technique and the direct method give similar results during the growth of *Ln. mesenteroides* except during a phase called the "unstable phase", corresponding to a strong decrease of the average chain length. This result is not surprising since the study concerning the shaking effect on chain disruption shows that the chains are relatively shake-proof, no matter their length, when avoiding the unstable phase. But the chains become very sensitive to a strong shaking during the unstable phase. The spontaneous disruption was observed by Martley (1972) when working with three *Streptococcus* strains in unshaken cultures and by Goel and Marth (1969a) when working with eight strains of *Leuconostoc citrovorum* in sterile skim milk. Besides, these two authors showed that this phenomenon was independant of the chain length. This is why the viable counts are higher with plating than with the direct method during the unstable phase. Indeed, plating needs more shaking than the direct method because of the great number of dilutions.

The two methods give similar results especially when reaching the second half of growth in batch cultures of the strain, because the chain length becomes uniform and decreases slightly. This conclusion is very important since the searchers or the manufacturers are essentially interested in the concentration in viable cells at the end of the growth when producing chain forming bacteria. A decrease in the viability indicates at which moment they can stop the culture to stock it. The direct method is cheap, rapid and simple to conduct. It provides two types of information: the viability and the concentration in viable chains of chain-forming bacteria. Besides, by detecting a decrease in the

viability, this method can indicate an abnormal development in growth during the fermentation. The searchers or the manufacturers may therefore react immediately on their production of bacteria and avoid waste of money.

REFERENCES

Bourdon, J.L. and Marchal, N. (1973). Techniques de numération des bactéries. In: *Techniques Bactériologiques*, pp. 203-217, Doin (ed.), Paris.

Champagne, C.P. and Gardner, N. (1990). *Sci. Aliments* 10, 899-905.

Champagne, C.P., Gardner, N. and Doyon, G.(1989). *Appl. Env. Microbiol.* 55:2488-2492.

Goel, M.C. and Marth, E.H. (1969a). *J. Dairy Sc.* 52, 1207 1213

Goel, M.C. and Marth, E.H. (1969b). *J. Dairy Sc.* 52, 1941-1947.

King, L.M., Schisler, D.O. and Ruocco, J.J. (1981). *J. Amer. Soc. Brew. Chem.* 39, 52-55.

Lange, H., Bavouzet, J.M., Taillandier, P., and Delorme, C. (1993). *Biotechnol. Tech.* 7, 223-228.

Lee, S., Robinson, F.M. and Wang, H.Y. (1981). *Biotechnol. Bioeng. Symp.* 11, 641-649.

Martley, F.G. (1972). *N. Z. J. Dairy Sci. Tech.* 7, 7 11.

Mayfield, C.I. (1975). *Can. J. Microbiol.* 21, 727-729.

Mayfield, C.I. (1977). *Can. J. Microbiol.* 23, 75-83.

Plihon, F., Taillandier, P., and Stréhaiano, P. (1995). *Appl. Microbiol. Biotechnol.* 43, 117-122.