MEASUREMENT OF INTRACELLULAR PH DURING SUPERCRITICAL PASTEURIZATION EVALUATED BY CELL FLUORESCENT STAINING

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The study addresses cell inactivation phenomena over bacteria induced by high pressure CO_2, particularly intracellular pH decrease. A common bacteria, *L. monocytogenes* was used as test-microorganism; cells were loaded with cFDASE, a specific fluorescent probe. Different sets of experiments were carried out in batch mode at different treatment conditions of temperature and pressure. The change of the ratio of two fluorescence signals obtained at 490 nm and 440 nm was collected by a spectrofluorometer at emission of 525 nm. The data analysis indicates a progressive intracellular depletion inside the cell caused by the action of CO_2.
This new analytical method provides an innovative and promising technique for indications of cell behaviour during high pressure processes.

INTRODUCTION

In food, microbial inactivation is a crucial parameter in the safety and shelf-life of the product. Acid tolerance of *Listeria* has important implications for their survival in different environments such as food products. One of the most promising treatments to inactivate microorganisms is supercritical CO_2 that increased membrane permeability, easily penetrate through the bacterial cell membrane and accumulate in the cytoplasmic interior of cells. If too much dissolved CO_2 enters the cytoplasm, the cells may be unable to expel all the resulting protons and intracellular pH (pH_i) must start decreasing [1]. Spilimbergo et al. determined [2] extracellular and pH_i of *Bacillus subtilis* after CO_2 treatment. The in situ determination of pH_i is critical and very interesting because of regulation of the cytoplasmatic pH is a requirement for the survival and viability of microorganisms.
In this study, the fluorescent probe cFDASE was considered for determination of pH_i of *Listeria monocytogenes*.

MATERIALS AND METHODS

2.1 Bacterial strain and media

Listeria monocytogenes LMG 13305 a gram-positive bacteria, was used as test microorganism. Starter cultures of the strain were initiated by inoculating a single colony in Brain Heart Infusion Broth (BHIB), at 37°C for 24h. 1 ml of the culture was inoculated into 50 ml of fresh BHIB, at 37°C for 16h. Cells were centrifugated at 8000 rpm for 5 minutes and resuspended in treatment medium (TM), a slightly buffered phosphate solution (10 mM, KH_2PO_4 and $HNa_2O_4P.2H_2O$, Fluka) adjusted to pH 7.0.

2.2 Loading of cells with fluorescent probe

5 (and 6-)-carboxyfluorescein diacetate succinimidyl ester (cFDASE) (C195, Invitrogen) was used to the stain cells for pH_i measurements. For cell staining a modified Breeuwer method [3] was followed: 60 ml of *L. monocytogenes* culture was incubated at 37°C for 30 min in the presence of 1.2 ml of cFDASE (3mM) and 0.6 ml of glucose (1M) (Sigma-Aldrich); the cells were washed, centrifugated at 8000 rpm for 15 minutes, washed again and resuspended in TM for each experiment and calibration curve.

2.3 Counting

Before and after each treatment the viable cell counts were determined by standard plating technique. Every sample was diluted in ringer solution, Oxoid, (dilution 1:10), plated onto Brain Heart Infusion Agar, BHIA (PBI international) and incubated at their optimal condition (48h at 37°C) before counting. The results are expressed as $\log(N/N_0)$ versus time, where N_0 is the initial number of cells in the control sample and N is the number of cells in the sample after treatment.

2.4 Pasteurization equipment

The experiments were performed in a multi-batch apparatus showed in Fig.1. The gas (Messer, carbon dioxide, purity 99.990%) was pumped by means of a volumetric pump (LEWA, mod.LCD1/M910s) with a maximum rate of 11 l/h into 10 stainless steel reactors previously filled with 7 ml of the sample.
Before the compression, the gas is cooled down to $T \approx 4$°C. A thermocontroller, connected with a temperature probe (Pt 100Ω) inside the water bath, maintains the temperature at a constant value (25°C). Each reactor is provided with a magnetic stirrer system (VETROTECNICA, micro-stirrer Velp 10.0161), which assures a sufficient degree of mixing to improve the dissolution of supercritical gas inside the liquid phase. The operating parameters (temperature and pressure) are continuously recorded by a real time acquisition data system (NATIONAL INSTRUMENTS, field point FP-1000 RS 232/RS 485) and monitored by specific software (LabVIEW™ 5.0).
The depressurization step was performed by widely opening the on–off valve, V3 (Fig. 1), and partially opening the micrometric one V4 (Fig. 1) in such a way that the depressurization rate was constant at about 30 bar/min in all the experimental runs. More details of the procedure for pasteurization has been described in previous works [4].

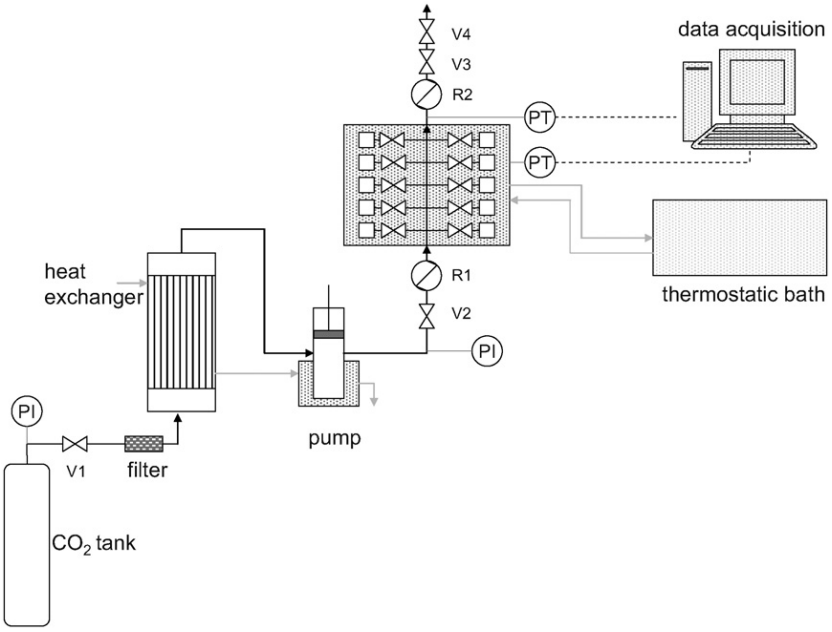

Figure 1: Schematic representation of the high-pressure CO_2 multi-batch apparatus.

2.5 Calibration of pH_i

Calibration curves with cFDASE-loaded *L.monocytogenes* cells were determined to enable calculation of pH_i values from fluorescence ratios obtained by a spectrofluorometer (JASCO FP-770) at emission of 525 nm. The ratios of the pH sensitive wavelength (490 nm) and the insensitive wavelength (440 nm) vs pH_i were determined using 5 ml of TM, containing coloured cells, varying pH from 3.5 to 8. The extracellular and the intracellular pH were equilibrated by addition of valinomycin (1 mM, Fluka) and nigericin (1 mM, Fluka). Each point represents the mean of three different runs. For each experiment a new calibration curve was made.

RESULTS

Several runs of experiments were performed at different operative conditions of temperature, pressure and treatment time. pH_i value was calculated using linear equation (1) obtained from the calibration curve, showed in Fig.2.

$$y = 1.4928x - 7.3579 \quad (1)$$

Figure 3 shows the results obtained at100 bar at 25°C at different treatment time (0, 2, 5, 10 minutes).

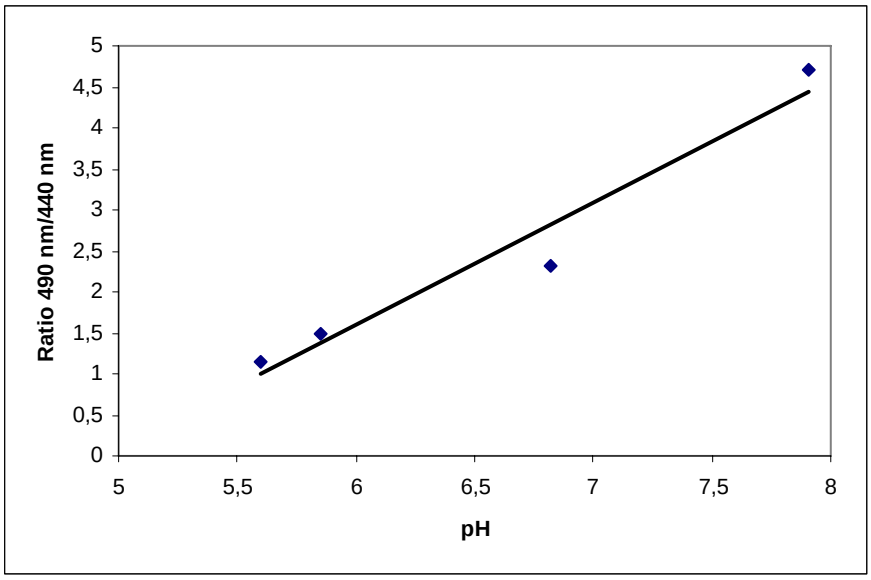

Figure 2: calibration curve for experiment at 100 bar and 25°C.

Table 1 reports the value of the ratio of fluorescence 490/440nm of total and filtrate (after filtration) treated sample; by subtracting the singular value (at 490 and 440 nm, respectively), the ratio of fluorescence of intracellular treated sample was calculated; finally by means of the calibration curve (1) the pH_i value obtained.

	Time (min)	**Total**	**Filtrate**	**Intracellular**	**Intracellular pH**
		Ratio 490/440nm	**Ratio 490/440nm**	**Ratio 490/440nm**	
Untreated	0	4,52	5	2,96	7,14
Treated	0	2,13	1,70	1,94	6,53
	2	1,77	2,13	1,58	5,17
	5	1,8	2,42	-0,05	4,48
	10	1,67	2,06	3,21	7,08

Table 1: value of ratio 490/440 of total, filtrate and intracellular fluorescence

From Table 1 and Figure 3 it is easy to note a pH_i decreased below the detection limit has been reached within 2 minutes at 100 bar and 25°C. Furthermore, after 10 minutes of treatment the pH_i value is equal to the extracellular pH one.

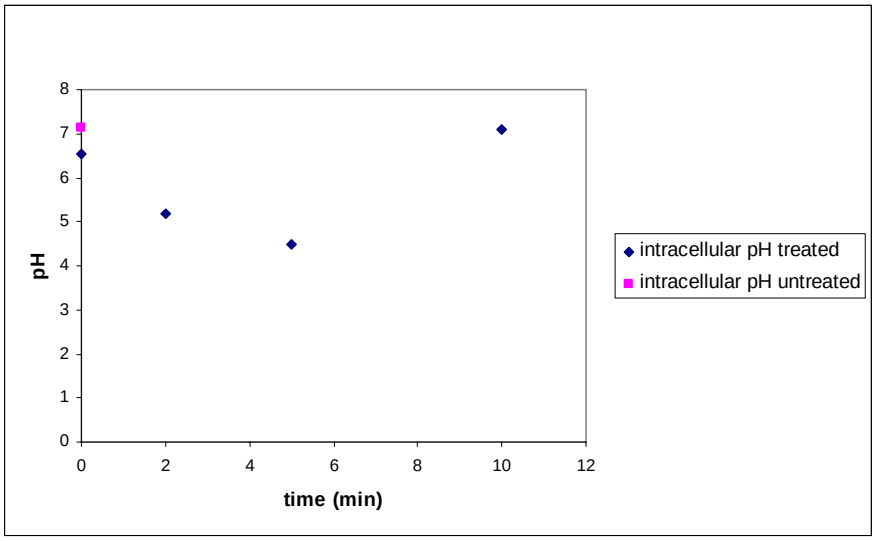

Figure 3: trend of pH_i of *Listeria monocytogenes* at 100 bar and 25°C

Before performing the microbiological analyses, we have verified that the dye was not toxic to the cells (data not reported). Certain of this, we plated each treated samples at different treated times to obtain the inactivation curve showed in Figure 4.

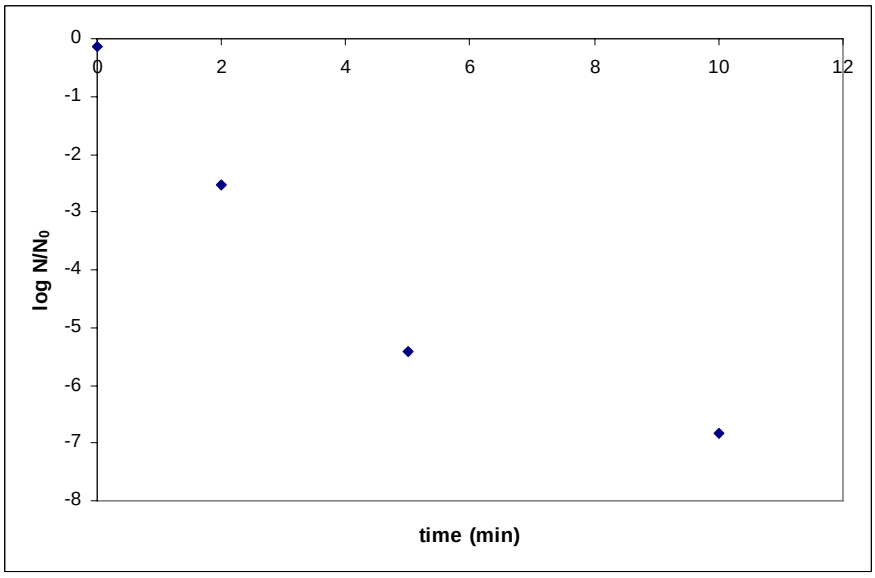

Figure 4: microbial inactivation with SC-CO_2 at 100 bar and 25°C; N_0 was $3.4*10^8$

CONCLUSION

The results obtained lead to the following conclusions:

- Total microbial inactivation was obtained after a treatment of 5 minutes, pressure 100 bar and temperature 25°C.
- After 5 minutes of treatment the bacteria cells are completely permeabilized: we demonstrate that after a sudden decrease down to 4.5, the intracellular pH equals to the extracellular pH probably because the cytoplasmatic content completely leaks out.

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