

# High pressure inactivation of *Escherichia coli* and its giant spheroplast at subzero temperature

M. MOUSSA, J.-M. PERRIER-CORNET<sup>\*</sup>, G. LAWRENCE, and P. GERVAIS

*Laboratoire de Génie des Procédés Alimentaires et Biotechnologiques,*

*ENSBANA, 1 esplanade Erasme, 21000 Dijon, France*

*jperrier@u-bourgogne.fr*

## Abstract

High-pressure treatments necessary to reach high or complete microbial inactivation could be improved through the combination of high pressure with other physical parameters. This work was intended to study coupling effect of high pressure and subzero temperature on microbial inactivation. *Escherichia coli* LCB 320 was treated at different pressure levels (0.1 to 450 MPa, 10 min holding time) at 25°C and -20°C (in liquid conditions). Synergistic and antagonistic interactions between high pressure and subzero temperature were observed with regard to the colony forming ability of *E. coli* bacterium. The membrane integrity of giant spheroplasts ( $\approx 5 \mu\text{m}$  in diameter) generated from this bacterium was assessed. Involvement of water compression was discussed.

## Introduction

Emerging technologies have stimulated considerable interest in the food industry. They have been developed in order to produce high quality foods, including an appropriate microbial decontamination, by minimally processing way. Examples include high hydrostatic pressure (HHP) processing, pulsed electric fields treatment, pulsed UV light technology, cold plasma and use of biopreservatives. Commercial opportunities and research challenge in the HHP processing of foods are particularly interesting (Torres *et al.*, 2005). Such a treatment allows inactivation of pathogenic and spoilage microorganisms (Knorr, 1993). Also, it allows to denature several enzymes responsible for quality deterioration in juices (Polydera *et al.*, 2004). HHP treatment has been found to be less detrimental to low molecular weight food

compounds, due to the stability of covalent bonds to high pressure (Heremans, 1982). Therefore, texture, color, natural flavouring agents and nutritional values are not greatly affected by HHP processing as compared to conventional technologies.

However, to reach high or complete microbial inactivation, a high pressure level and/or long treatment time are required. Therefore, the high cost of HHP treatments is nowadays seriously limiting their industrial applications. For this reason, optimization of HHP processes is very relevant. Low temperature could be a useful parameter to enhance pressure-induced inactivation without modifying food properties (Hashizume *et al.*, 1995; Perrier-Cornet *et al.*, 2004). Nevertheless, HHP processing at low temperature, especially subzero range, has not been studied thoroughly. In addition, there remains a large question about the mechanisms involved in high pressure-low (subzero) temperature interaction with regard to microbial inactivation.

This work was intended to study coupling effect of subzero temperature and high pressure on inactivation of *Escherichia coli* LCB320. This bacterium was submitted to high-pressure treatments at room and subzero temperature (in liquid conditions). Established methods were modified to generate giant spheroplasts of *Escherichia coli* LCB320 in order to assess their membrane integrity and shape modification.

## **Materials and Methods**

### **Growth of bacteria and preparation of giant spheroplasts**

The gram negative bacterium *Escherichia coli* LCB 320 was grown on Luria-Bertani (LB) broth. A single colony was first statically grown for 16 h at 37°C as a subculture. Liquid culture was then prepared by injecting 0.2 ml of subculture into 20 ml of LB broth and this was statically grown to the early stationary phase at 37°C for 24 h.

Giant spheroplasts were prepared from *Escherichia coli* LCB 320 in a manner similar to that described by Ruthe and Adler (1985) and Kubalski (1995). Briefly, 6 ml of cell culture were diluted to 30 ml in LB broth. Cephalixin (Sigma-Aldrich, France), was then added to grow long filamentous cells and the culture was incubated with shaking at 42°C for 3 h. Filaments

were then harvested by centrifugation at 1500 g and the pellet was washed once in 1 ml of 0.8 M sucrose and 10 mM Tris-HCl (pH 7.8) and resuspended in 2.5 ml of the same solution. Addition of 240  $\mu$ l of lysozyme (5 mg/ml) (Sigma-Aldrich, France) and 240  $\mu$ l of Na<sub>2</sub>EDTA (0.125 M) (Sigma-Aldrich, France) gradually converted filaments into giant spheroplasts during incubation (1 h at 25°C).

### **Sample preparation and high pressure treatment**

Samples of less than 1 ml of *Escherichia coli* LCB 320 culture or its giant spheroplast suspension were aseptically transferred to polyethylene bags which were heat-sealed after exclusion of air bubbles.

High pressure treatments were performed using a high pressure vessel (MFM, Montbard, France; inner volume: 4 ml with pressure bear-ability up to 600 MPa). Compression and decompression phases were performed using a hand-type pump (Novaswiss, Switzerland) with a speed ranging from 1 to 3 MPa.s<sup>-1</sup>, depending on target pressure. Pressure inside the sample-chamber was monitored using a pressure-gauge (SEDEME, France). Temperature was measured using a type K thermocouple (NiCr/NiAl, Response Time 70 ms) (Thermocoax, France) passed through the upper plug of the vessel and placed close to the sample. Pressure and temperature data were recorded during the entire pressurization period using a data acquisition device (Instrunet, GW Instruments, USA).

Pressure up to 450 MPa was applied to the samples. Pressurization at 25°C was performed while immersing the high pressure vessel into a thermostated bath. Combined high pressure and subzero temperature treatments were performed as described by Perrier-Cornet *et al.* (2004). In short, temperature was lowered by plunging the high pressure vessel into a first cryostat (F81-HP, Julabo, Germany) maintained at -60°C in order to accelerate thermal transfer. A cooling rate of about 20°C.min<sup>-1</sup> was obtained. Pressure was increased while lowering temperature. The high pressure vessel was then transferred into a second cryostat (RC6CP, Lauda, Germany) maintained at -20°C in order to hold a constant temperature during the combined treatment. The target pressure and subzero temperature were reached at approximately the same time. For treatment at atmospheric pressure, only the temperature

was lowered down to -20°C in the supercooled region as allowed by the experimental design. Freezing phenomena were detected by precise pressure and temperature monitoring. Combined high pressure and subzero conditions were maintained for 10 min. Thereafter, the vessel was reheated by immersion into a thermostated bath maintained at 25°C. Simultaneously, the pressure was released by slowly operating the hand-driven pump. Either come-up and come-down times were about 3 min.

### **Viable cell counts and assessment of membrane integrity**

Viability of the bacterium *Escherichia coli* was estimated by the Colony Forming Unit (CFU) method using LB agar plates. Inactivation results were expressed as the logarithmic decrement of viability.

The integrity of the cytoplasmic membrane of giant spheroplasts was evaluated using the Live/Dead® BacLight™ Bacterial Viability Kit (Molecular Probes, France) according to instructions of the supplier. In brief, to each treated sample of spheroplast suspension, 3 µl of dye solution (20 mM of the red-fluorescent stain Propidium Iodide, PI, and 3.34 mM of the green-fluorescent stain, SYTO® 9). These probes are two nucleic acid stains. SYTO® 9 labels all bacteria, those with damaged membranes and those with intact membranes. However, PI is a membrane-impermeant which could not penetrate cells with intact membranes. Following loss of membrane integrity, PI diffuses into and stains the cells. When mixed with SYTO® 9, PI staining causes a reduction in the SYTO® 9 stain fluorescence in the membrane-damaged cells. Thus, bacteria with damaged membranes stain fluorescent red whereas bacteria with intact membranes stain fluorescent green. Samples were then incubated for 15 min at 25°C. Green fluorescent spheroplasts at 480/500 nm excitation/emission wavelengths and red fluorescence ones at 490/635 nm excitation/emission wavelengths are enumerated on Thoma cell by fluorescence microscope observation (LEICA, France). The red to green fluorescence ratio was used to calculate the percentage of inactivated spheroplasts.

Viability and membrane integrity results were reported as the means of at least three separate experiments.

## Results

### High pressure inactivation of *Escherichia coli* LCB 320 bacterium at 25 and -20°C

Pressure levels ranging from 100 up to 450 MPa were applied for 10 min at two temperatures : 25 and -20°C. Cells were pressurized in their standard culture medium ( $a_w$  0.992). For experiments at subzero temperature, processing was carried out under supercooled conditions. Indeed, thanks to the very small inner volume of the vessel and the static conditions, no crystallization occurred even under theoretical freezing point. In addition, a precise check on pressure and temperature profiles was used to detect possible freezing phenomenon in the samples. If freezing occurred, then a significant increase of pressure (more than 5 MPa) and an exothermic event (about 2°C) were instantaneously recorded.

Figure 1 shows logarithmic decrement of viability of the bacterium *Escherichia coli* LCB 320 as a function of pressure and temperature.

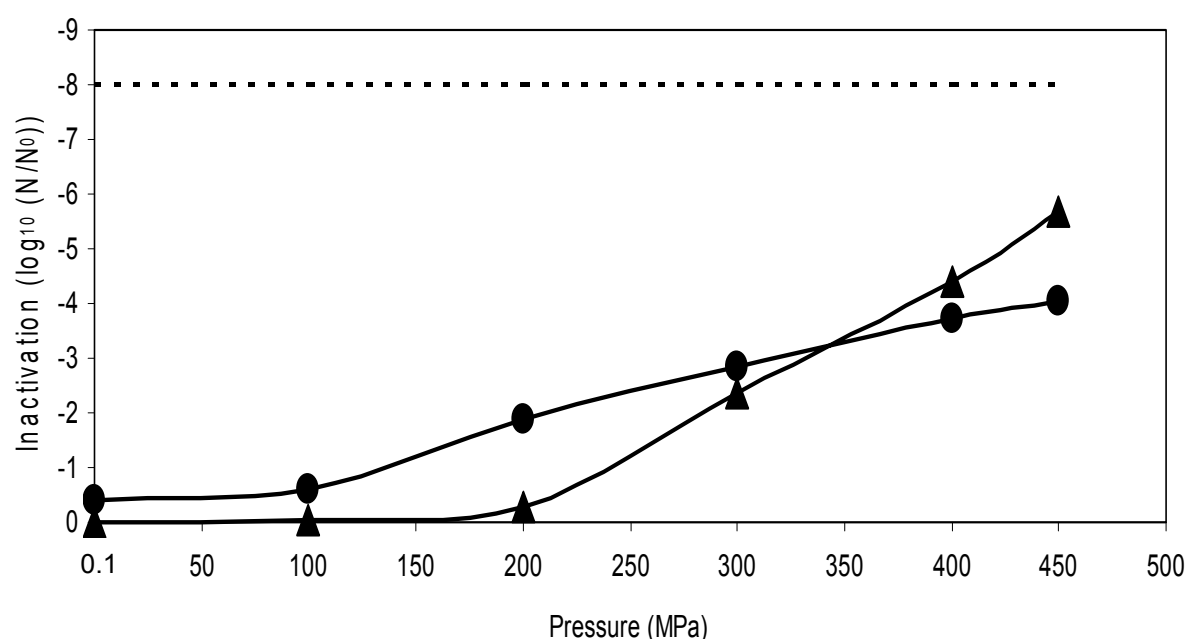


Figure 1. Inactivation of *Escherichia coli* LCB 320 by high pressure treatment for 10 min at 25°C (▲) and -20°C (●). N is the number of CFU.ml<sup>-1</sup> after treatment and N<sub>0</sub> is the number of CFU.ml<sup>-1</sup> of untreated bacteria. The dashed line shows the limit of detection by the CFU method. Standard deviations did not exceed 23%.

At room temperature, pressure up to 200 MPa did not induce appreciable loss of viability. For higher pressure, microbial inactivation increased consistently with pressure and reached a maximum of 5.7 log cycles at 450 MPa. At -20°C, inactivation of *Escherichia coli* was Interestingly more effective than at 25°C for pressure up to 300 MPa. Indeed, at atmospheric pressure, a smooth inactivation was observed (58%) which was induced by the sole effect of subzero temperature in liquid conditions. The onset of pressure-induced inactivation at -20°C began at 100 MPa. A synergistic interaction between high pressure and subzero temperature was observed up to 300 MPa, allowing to improve pressure-efficiency on cell inactivation. However, above 300 MPa, subzero temperature seemed to counteract the inactivating-effect of high pressure. Indeed, at 400 and 450 MPa, pressurization was more effective at 25°C than at -20°C. This unusual trend illustrated an antagonistic interaction between high pressure and subzero temperature.

### **Generating giant spheroplasts of *Escherichia coli* LCB 320**

In the presence of 60 µg/ml of Cephalexin, which blocks septum formation, *Escherichia coli* grew into filaments up to 50 µm long (Figure 2 A). It should be noted that neither higher Cephalexin concentration, nor longer incubation time resulted in a longer filaments.

Viable giant spheroplasts (≈5 µm in diameter, Figure 2 B) resulted from hydrolysis of the peptidoglycan layer of the filaments by treatment with lysozyme and Na<sub>2</sub>EDTA (Martinac *et al.*, 1987). The effectiveness of spheroplast formation was controlled by osmotic shock. Indeed, enzymatic hydrolysis of the cell wall is known to render the cells osmotically sensitive (Birdsell and Cota-Robles, 1967). This osmotic sensitivity was substantiated by adding a few micro-liters of distilled water to a spheroplast sample fixed on microscope glass. However, the resulting ghosts could not be observed by phase contrast microscope.

Giant spheroplasts could be stored at -20°C over months. With respect to their size ( about 3 folds that of bacterium *E. coli*) and their mono-membrane wall, spheroplasts could be considered as a simple cell model suitable for evaluation of morphological changes by microscopic observation and assessment of membrane integrity. In fact, the gram-negative bacterium *E. coli* has a complex cell wall including outer and inner membranes separated by

a peptidoglycan layer. The rigidity of the peptidoglycan layer could prevent or hide the expected shape modifications induced by high pressure treatment.

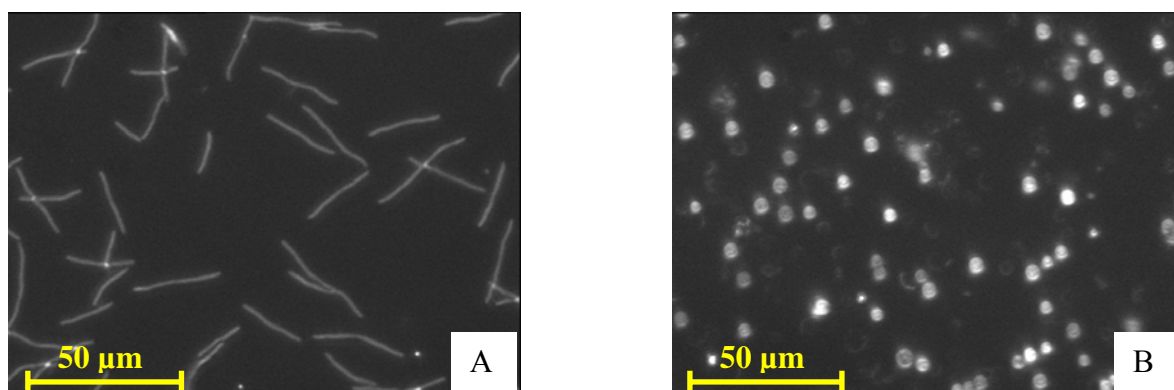


Figure 2. Phase contrast microscopic observation of *Escherichia coli* LCB 320 grown in filaments (A) and converted to giant spheroplasts (B).

#### Effect of high pressure on membrane integrity of giant spheroplasts of *Escherichia coli* LCB 320

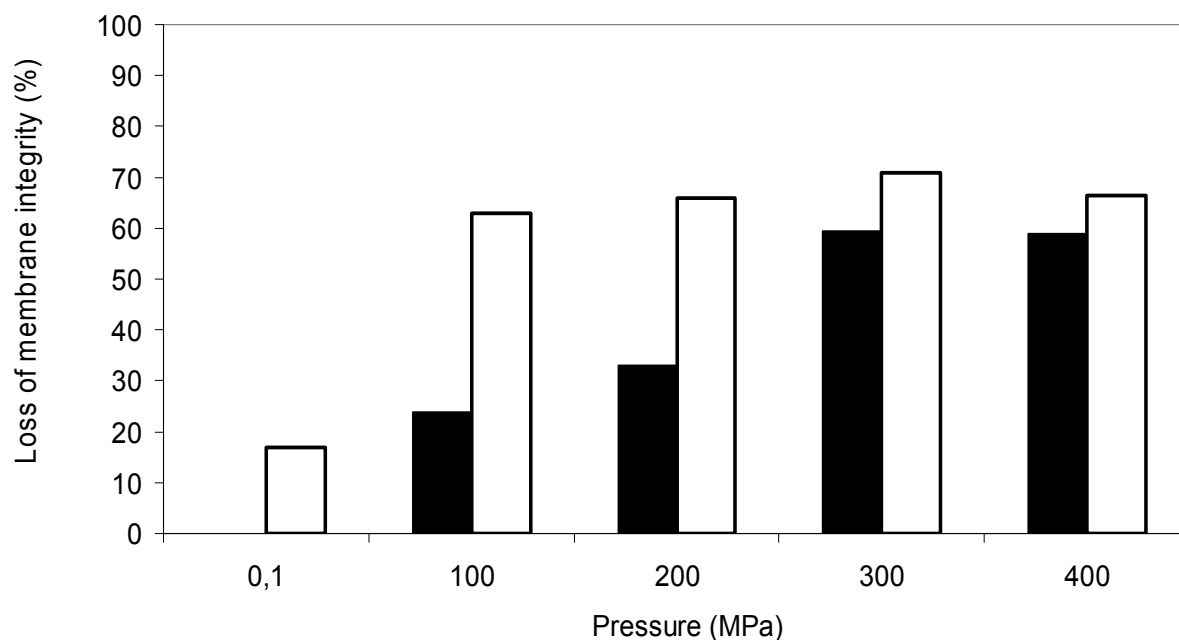


Figure 3. Inactivation of giant spheroplasts of *Escherichia coli* LCB 320 by high pressure treatment for 10 min at 25°C (■) and -20°C (□). Standard deviation did not exceed 25%.

Giant spheroplasts of *Escherichia coli* LCB 320 were submitted to 10 min hyperbaric treatments with a magnitude varying from 100 up to 400 MPa at 25°C and -20°C (in liquid conditions). Figure 3 shows percentage of membrane integrity loss of treated spheroplasts with reference to untreated ones (0.1 MPa, 25°C).

As observed for the bacterium *Escherichia coli*, the sole effect of subzero temperature, in the supercooled range, induced a smooth inactivation of spheroplasts (17%). Pressurization at -20°C was more effective than that performed at 25°C, whatever the pressure level. Indeed, at 100 MPa, inactivation was about 3 folds higher at -20°C than that obtained at 25°C (63% versus 24% respectively). For higher pressure, inactivation increased slightly at -20°C (66% and 71% respectively at 200 MPa and 300 MPa) then decreased at 400 MPa (66.5%). At 25°C, it increased at 200 and 300 MPa (33% and 59.5% respectively) before leveling off at 400 MPa. Accordingly, a synergistic interaction between high pressure and subzero temperature was observed. The magnitude of this interaction did not follow a monotonous pattern (maximum at 100 MPa and minimum at 400 MPa). However, in the contrary of the colony forming ability of *E. coli* bacterium, there was no antagonism observed with regard to the loss of membrane integrity of giant spheroplasts.

## Discussion

In this study, the effect of high pressure and subzero temperature combination was evaluated first with respect to viability of *Escherichia coli* LCB 320. Results showed that subzero temperature could improve pressure efficiency. For example, a 2 log cycles inactivation rate could be obtained through 10 min pressurization at 200 MPa at -20°C and 300 MPa at 25°C. Similar synergistic interactions have been reported by Haschizume *et al.* (1995) and Perrier-Cornet *et al.* (2004) for *saccharomyces cerevisiae* and *Lactobacillus plantarum*. These authors highlighted the similarity between the pressure-temperature dependence of microbial inactivation and the pressure-temperature diagram of protein denaturation. Synergism was observed for pressure up to about 300 MPa. Above 300 MPa, subzero temperature seemed to counteract the inactivating effect of pressure. This unusual pattern of survival was not observed for yeast and gram positive bacteria.



PI/SYTO<sup>®</sup> 9 staining of spheroplasts showed high loss of membrane integrity under combined high pressure subzero temperature treatments. Compared to yeast spheroplasts (Perrier-Cornet *et al.*, 2003), giant spheroplasts of *Escherichia coli* LCB 320 seemed to be more pressure-resistant. In addition, there was no visual morphological damages on *E. coli* spheroplasts as inspected by microscopic observation. Pressure-mediated permeabilization of spheroplasts could be related to the denaturation of transport systems or other protein structures. Such a denaturation could be more marked under the combined action of high pressure and subzero temperature. However, this attractive approach doesn't explain the Baroprotective effect of subzero temperature above 300 MPa.

From a thermodynamical point of view, compression of the cell system resulted in a mechanical energy which could be characterized by the volume change of this system. That means that the compressibility of the cell cytoplasm is the first determining parameter of the amount of transferred energy. With regard to the important quantity of water in the cytoplasm content, water compression could be involved in the pressure-mediated inactivation. In fact, combination of pressure and low temperature resulted in a lower liquid volume and, accordingly, a larger change in the mechanical energy. Differential in compressibility between cell wall and cytoplasm could perturb the membrane integrity and induce cell permeabilization.

## **Conclusion**

Combination of subzero temperature is a potential way to improve pressure efficiency on microbial inactivation. However, possible antagonistic interaction has to be considered. An effective application of such combination need thorough comprehension of the mechanism involved in pressure-induced inactivation. The involvement of water properties is an attractive approach that needs further investigations.

## **References**

1. Birdsell, D. C., and Cota-Robles, E. H., 1967. Production and ultrastructure of lysozyme and ethylenediaminetetraacetate-lysozyme spheroplasts of *Escherichia coli*. *Journal of Bacteriology*, 93 (1), 427-437.

2. Haschizume, C., Kimura, K., and Hayashi, R., 1995. Kinetic analysis of yeast inactivation by high pressure treatment at low temperatures. *Bioscience Biotechnology Biochemistry*, 59, 1455-1458.
3. Heremans, K., 1982. High pressure effects on proteins and other biomolecules. *Annual Review of Biophysics and Bioengineering*, 11, 1-21.
4. Knorr, D., 1993. Effects of high-hydrostatic-pressure processes on food safety and quality. *Food Technology*. 47, 156-161.
5. Kubalski, A., 1995. Generation of giant protoplasts of *Escherichia coli* and an inner-membrane anion selective conductance. *Biochimica et Biophysica Acta*, 1238, 177-182.
6. Perrier-Cornet, J. M., Tapin, S., and Gervais, P., 2003. High-pressure inactivation of yeast and spheroplasts at subzero temperature. In: R. Winter, ed. *Advances in high pressure bioscience and biotechnology II. Proceedings of the 2<sup>nd</sup> International Conference on High Pressure Bioscience and Biotechnology*, 16-19 September, 2002. Heidelberg: Springer, 389-393.
7. Perrier-Cornet, J. M., Tapin, S., Serenella, G., and Gervais, P., 2004. High-pressure inactivation of *Saccharomyces cerevisiae* and *Lactobacillus plantarum* at subzero temperatures. *Journal of Biotechnology*, 115, 405-412.
8. Polydera, A. C., Galanou, E., Stoforos, N.G., and Taoukis, P.S., 2004. Inactivation kinetics of pectin methylesterase of Greek Navel orange juice as a function of high hydrostatic pressure and temperature process conditions. *Journal of Food Engineering*, 62, 291-298.
9. Torres, J. A., and Velazquez, G., 2005. Commercial opportunities and research challenges in the high pressure processing of foods. *Journal of Food Engineering*, 67, 95-112.
10. Ruthe, H.-J., and Adler, J., 1985. Fusion of bacterial spheroplasts by electric field. *Biochimica et Biophysica Acta*, 819, 105-113.