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EFFECT OF HIGH PRESSURE AND SUB-ZERO TEMPERATURE ON SOME GRAM-NEGATIVE BACTERIA

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The objective of the investigations was to determine the viability of selected gram-negative bacteria exposed to high pressure and sub-zero temperature, in the range of 59÷193 MPa and -5÷-20°C, respectively, without the freezing of water. After pressurization for 24 h, at 193 MPa and -20°C, no living cells of gram-negative bacteria *T. thermophilus*, *P. fluorescens* and *E. coli* of the initial population number of 10^8 CFU/mL were detected. After pressure treatment for a shorter time than 24 h, differences in sensitivity appeared between these bacteria. The most resistant was the tested strain of *E. coli*. Reduction in the population of *E. coli* was only about 3.5 log cycles after pressurization for 30 min at -20°C, while *T. thermophilus* and *P. fluorescens* were completely inactivated under these conditions. No living cells of *E. coli* were found after 7 h of pressure treatment at 193 MPa and -20°C. The viability of all tested bacteria was not reduced significantly at 59 MPa, but decreased with the pressure increase. The most sensitive was *P. fluorescens* - a drastic loss of viability in these bacteria occurred during the time of generation of the pressure of 110 MPa at -10°C. In this period, the reduction in viable numbers of *E. coli* did not exceed 1 log cycle in all studied ranges of pressure and temperature. Freezing the samples under atmospheric pressure at -5°C÷-20°C did not exert any influence on the viability of *E. coli* and *T. thermophilus*, while the number of viable cells of *P. fluorescens* decreased by 0.5÷1 log cycle.

INTRODUCTION

In recent years, a great interest has been shown in non-thermal methods of processing and preservation of food. Among them, high hydrostatic pressure seems to be one of the more promising methods for inactivation of undesirable microorganisms and extending the shelf life of food sensitive to changes in sensory properties and nutritive value when preserved by traditional thermal processing. The high pressure technique is used commercially, mainly for preservation of acidic food such as fruit juices and jams [Hoover, 1993; Cheftel, 1995; Cheftel & Culioli, 1997; Hendrickx *et al.*, 1998; Smelt, 1998].

The effectiveness of high pressure in inducing death of microorganisms depends on microbial types and their growth phase, pH and composition of media, as well as on the parameters of the process – the magnitude of pressure, pressurization time and temperature [Patterson *et al.*, 1995; Benito *et al.*, 1999; Alpas *et al.*, 2000]. Similar to other methods of inactivation of microorganisms, gram-negative bacteria and cells in the exponential growth phase are more sensitive to pressure treatment than gram-positive bacteria and cells in the stationary phase of growth [Pagan & Mackey, 2000]. Bacterial spores are very resistant to high pressure. The process of their inactivation proceeds in two stages; first, high pressure induces germination of spores and then destroys vegetative cells [Mills *et al.*, 1998; Raso *et al.*, 1998a,b].

The inactivation of microorganisms depends on the temperature at which cells are treated by high pressure. Usually, above 0°C, they are more resistant to high pressure at 20÷35°C than above and below this range of temperature [Hashizume *et al.*, 1995; Kalchayanand *et al.*, 1998a,b; Ponce *et al.*, 1998].

A new possibility for processing and preservation of food has been created by using high pressure below 0°C. The effects of pressure on the solid-liquid phase transition of water can be used for pressure-assisted freezing and pressure-assisted thawing of food or for storage of the material without the freezing of water [Kalichevsky *et al.*, 1995; Fuchigami *et al.*, 1997]. The limited available data showed that such low-temperature, high pressure conditions also enable inactivation of microorganisms and even more effectively than pressure treatment in a certain range of temperature above 0°C [Kalichevsky *et al.*, 1995; Hashizume *et al.*, 1995; Hayakawa *et al.*, 1998].

The objective of the study was to determine the viability of selected gram-negative bacteria exposed to high pressure and sub-zero temperature without the freezing of water.

MATERIAL AND METHODS

Cultures and growth conditions. The cultures used in this study: *Escherichia coli* K-12 PCM 2560 (NCTC 10538) from Polish Collection of Microorganisms, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences, Wrocław; *Pseudomonas fluo-*

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rescens from Culture Collection of Department of Food Chemistry and Technology, Gdansk University of Technology; and *Thermus thermophilus* HB-8 (ATCC 27634) from German Collection of Microorganisms and Cell Cultures.

All experiments were carried out with cultures in the stationary phase, obtained by growing *E. coli* and *P. fluorescens*, respectively, at 37°C and 26°C in Tryptone Soya Broth supplemented with yeast extract – 6 g/L and *T. thermophilus* at 70°C in the broth containing yeast extract – 4 g/L, peptone – 8 g/L, and NaCl – 2 g/L. The same media, but with the addition of agar, and the same temperature of incubation were used for enumeration of viable CFU in pressure–treated samples and controls serially diluted in phosphate-buffered saline. The media were purchased from BTL Sp. z o.o., Łódź, Poland.

Pressure treatment. The cells were centrifuged at 1500xg for 20 min at 5°C, and the pellets were re-suspended in phosphate-buffered saline (pH 7.0) to give viable counts of about $10^8 \div 10^9$ CFU/mL. Cell suspensions (3 mL of each) were placed in sterile glass test tubes, which were sealed with a moving cork, and kept at 0°C before pressurization.

Pressure was generated as described by Hayakawa *et al.* [1998]. This technique is based on the phenomenon that at sub-zero temperatures, increasing the volume of frozen water generates high pressure in a sealed vessel (Table 1). High pressure lowers the freezing point of water, thus the material is in the unfrozen state up to about -20°C.

TABLE 1. Relationship between temperature and pressure generated in the pressure vessel [Kalichevsky *et al*., 1995].

Temperature (°C)	Pressure (MPa)
-5	59.8
-10	110.9
-15	156.0
-20	193.3

In the experiments, equipment designed and constructed by the Department was used for the generation of pressure. The glass tubes containing cell suspension and a metal spring were placed in a cylindrical, metal vessel filled with water. The vessel was closed without leaving any air bubbles inside. It was gradually immersed during 40 min or 90 min, with the closed side down, in a temperature-controlled bath containing a mixture of ethanol, propylene glycol and distilled water (1:1:1, v/v) as the coolant. The times of pressurization are given in the tables and figures. After pressure treatment at -5, -10, -15, and -20°C, half of the vessel was raised and warmed in the upper part to 15°C measured with a thermocouple. Then the vessel was taken out and placed for a few minutes in a water bath at 20°C. The total time of decompression did not exceed 10 min. In parallel experiments, the suspensions of bacteria were kept at -5÷-20°C under atmospheric pressure.

The cell suspensions were stored in an ice bath before viable counts were determined. Unpressurized cell suspensions were enumerated as controls.

The data presented in the tables and figures are mean values obtained from three independent experiments. The bars on the figures indicate the mean standard deviations for the data points.

RESULTS AND DISCUSSION

Living cells of the three tested species of gram-negative bacteria were not detected after prolonged pressurization, for 24 h at 193 MPa and -20°C (Table 2). In a similar experiment, Hayakawa *et al.* [1998] showed the complete inactivation of some bacteria (*E. coli* and *Lactobacillus brevis*), yeasts (*Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii*) and moulds (*Aspergillus niger* and *Aspergillus oryzae*). However, *Staphylococcus aureus* even after 24 h of pressurization at -20°C was only partially inactivated with viable numbers decreasing from 9.0×10^6 to 7.2×10^2 [Hayakawa *et al.*, 1998].

TABLE 2. Viability of *E. coli*, *P. fluorescens* and *T. thermophilus* following pressurization at 193 MPa at -20°C (generating time of the pressure – 90 min).

Bacterial species	Log CFU/mL		
	Control (unpressurized)	Samples pressurized for	
		30 min	24 h
Escherichia coli	8.8	5.3	Nd[1]
Pseudomonas fluorescens	8.2	Nd[1]	Nd[1]
Thermus thermophilus	8.6	Nd[1]	Nd[1]

[1] not detected

After pressure treatment for a shorter time than 24 h, differences in sensitivity appeared between *T. thermophilus*, *P. fluorescens* and *E. coli* (Table 2). The most resistant was *E. coli*. The reduction in the number of *E. coli* was only about 3.5 log cycles after pressurization for 30 min at -20°C, while *T. thermophilus* and *P. fluorescens* were completely inactivated under these conditions. As shown in Figure 1, these bacteria were even totally destroyed at 156 MPa and -15°C. In the case of *E. coli*, surviving cells were not found after 7 h of

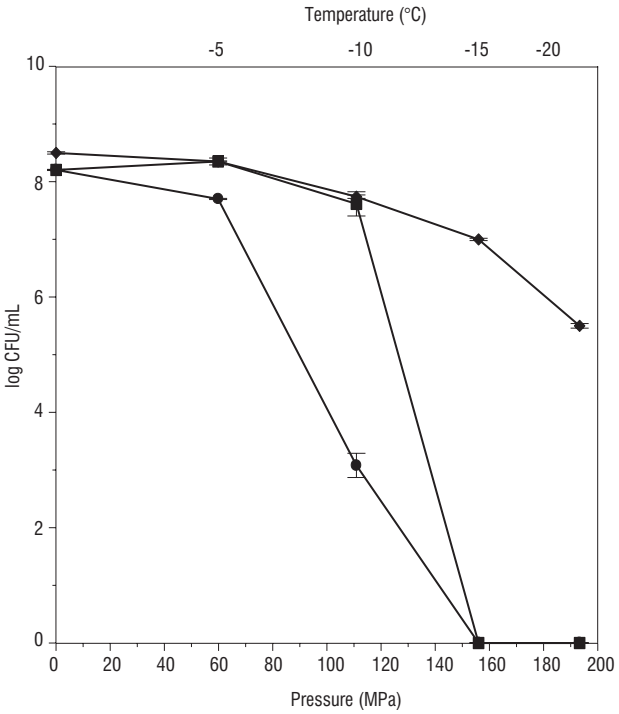

FIGURE 1. The effect of high pressure treatment for 30 min at subzero temperature on viability of *E. coli* (◆), *P. fluorescens* (●) and *T. thermophilus* (■) (generating time of the pressure – 90 min).

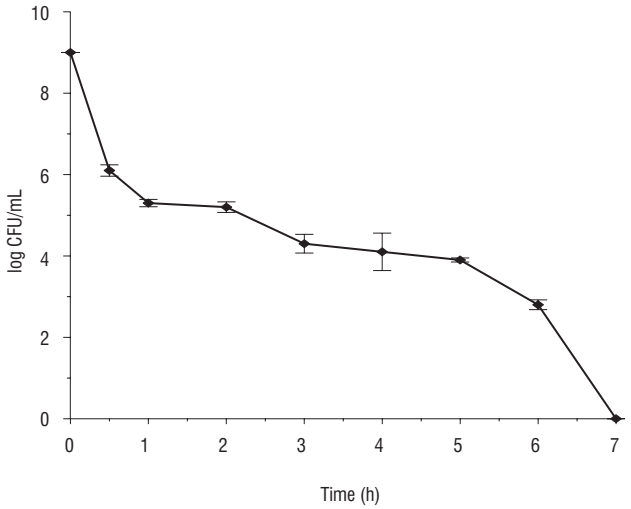

FIGURE 2. The effect of pressurization time at 193 MPa and -20°C on viability of *E. coli* (generating time of the pressure – 90 min).

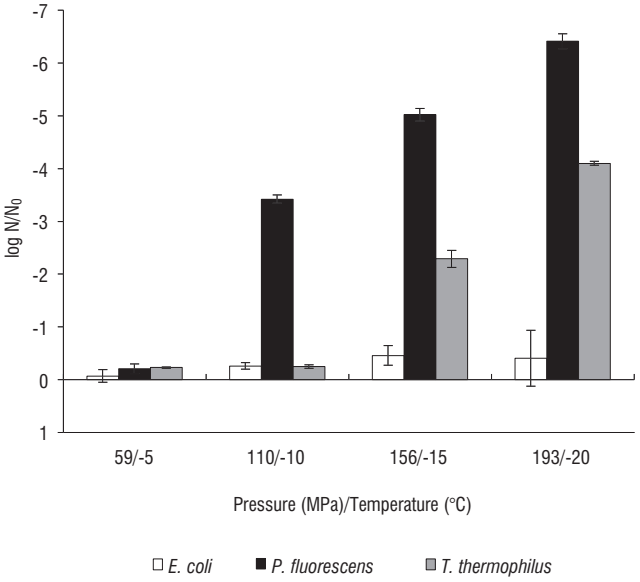

FIGURE 3. The effect of high pressure at subzero temperature on viability loss of *E. coli*, *P. fluorescens* and *T. thermophilus* (during 40 min generating of the pressure). [1)] N – the number of cells detected after pressurization; N_0 – the number of cells in the control.

pressure treatment at 193 MPa and -20°C (Figure 2). In all these experiments, the pressure vessel was gradually immersed in a cooling bath for 90 min and the pressure generated during that time was sufficient to destroy the populations of *T. thermophilus* and *P. fluorescens* at -20°C. If the time was shortened to 40 min, the populations of these two species were only partially inactivated and differences in sensitivity to high pressure between them were observed (Table 3).

TABLE 3. Viability of *E. coli*, *P. fluorescens* and *T. thermophilus* by pressure generated during cooling from 0 to -20°C.

Bacterial species	Log CFU/mL		
	Control (unpressurized)	Pressurized samples	
		Time (min) to generate the pressure of 193 MPa	
		40	90
Escherichia coli	8.5	8.4	6.1
Pseudomonas fluorescens	8.7	1.7	Nd[1]
Thermus thermophilus	8.1	4.0	Nd[1]

[1)] not detected

The effect of pressure in the range of 59÷193 MPa and temperature of -5÷-20°C, on the tested bacteria is given in Figure 3. The number of viable cells of all tested bacteria was not reduced significantly at 59 MPa and then decreased with the pressure increase. *P. fluorescens* was the most sensitive species. The drastic loss of viability of these bacteria occurred at 110 MPa and -10°C. Under the same conditions most cells of *T. thermophilus* population were able to survive, but after treatment at 193 MPa the number of viable bacteria decreased by about 6 log cycles. In the case of *E. coli*, the reduction in the population did not exceed 1 log cycle in the studied range of pressure.

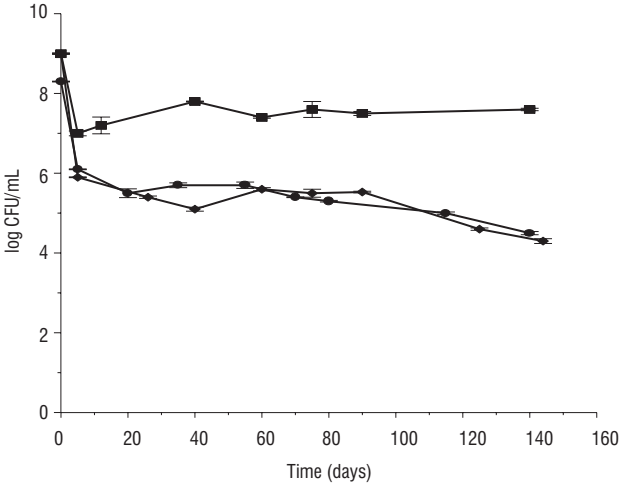

FIGURE 4. The effect of storage at -20°C under atmospheric pressure on viability loss of *E. coli* (◆), *P. fluorescens* (●) and *T. thermophilus* (■).

Freezing the samples under an atmospheric pressure at -5°C÷-20°C did not exert any influence on the viability of the cells of *E. coli* and *T. thermophilus*. The number of viable organisms of *P. fluorescens* decreased by 0.5÷1 log cycle at each temperature (Table 4). During storage of the samples at -20°C at atmospheric pressure, the greatest reduction of the population of all tested bacteria was observed during the first 7 days. However, the viability loss of *P. fluorescens*

TABLE 4. Viability of *E. coli*, *P. fluorescens* and *T. thermophilus* after freezing the samples under atmospheric pressure.

Bacterial species	Log CFU/mL				
	Control (unfrozen)	Samples frozen at temperaure (°C)			
		-5	-10	-15	-20
Escherichia coli	8.2	7.8	7.8	7.7	7.1
Pseudomonas fluorescens	8.7	8.6	8.6	8.4	8.4
Thermus thermophilus	8.2	8.1	8.3	8.4	8.5

and *E. coli* was higher than that of *T. thermophilus* (Figure 4). The number of *T. thermophilus* cells did not change during storage over 7 days at -20°C, while that of *E. coli* gradually decreased under these conditions. On the other hand, the reduction in the population during freeze storage of the most pressure–sensitive *P. fluorescens* was similar to that of the more pressure–resistant *E. coli* (Figure 4).

CONCLUSIONS

The high pressure treatment of bacteria in unfrozen state at a temperature range of -5÷-20°C exerts a greater bactericidal effect than freezing at the same temperature at atmospheric pressure. This experiment demonstrates that, similar to above 0°C, differences in sensitivity to high pressure appeared between different species of gram-negative bacteria. Variations in pressure resistance also probably exist among strains of the same species. Although the pressure achieved in the sealed vessel at -20°C is very effective in reducing the viable numbers of tested bacteria, it may be not sufficient to completely inactivate the more pressure--resistant gram-positive bacteria and some gram-negative bacteria. However, high pressure, in combination with other factors limiting bacterial growth, can be effective in ensuring the microbiological safety of food.

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WPŁYW WYSOKIEGO CIŚNIENIA I UJEMNYCH TEMPERATUR NA NIEKTÓRE BAKTERIE GRAMUJEMNE

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Celem badań było określenie przeżywalności wybranych bakterii gramujemnych pod ciśnieniem 59÷193 MPa w temperaturze -5÷-20°C, bez wymrożenia wody. Działanie ciśnienia 193 MPa przez 24 h w temperaturze -20°C spowodowało całkowitą inaktywację *T. thermophilus*, *P. fluorescens* i *E. coli* pochodzących z populacji o liczebności 10^8 komórek/mL. Różnice wrażliwości pomiędzy bakteriami tych gatunków pojawiły się, gdy traktowano je ciśnieniem w czasie krótszym niż 24 h. Najbardziej odporny okazał się badany szczep *E. coli*. Po 30 minutach działania ciśnienia 193 MPa w -20°C zmniejszenie liczebności tych bakterii wynosiło tylko około 3.5 rzędu logarytmicznego, podczas gdy *T. thermophilus* i *P. fluorescens* uległy całkowitej inaktywacji w tych warunkach (tab. 2). W przypadku *E. coli,* żywych komórek nie wykryto po 7 h działania ciśnienia 193 MPa w -20°C (rys. 2). Ciśnienie do 59 MPa nie wpływało znacząco na liczbę bakterii wszystkich trzech badanych gatunków. Dalsze zwiększanie ciśnienia powodowało zmniejszenie ich przeżywalności. Najbardziej wrażliwy był *P. fluorescens*. Drastyczna utrata przeżywalności tych bakterii następowała już przy dochodzeniu do 110 MPa i temperatury -10°C (rys. 3). W przypadku *E. coli* zmniejszenie liczby komórek nie przekroczyło 1 rzędu logarytmicznego podczas osiągania ciśnienia i temperatury z całego badanego zakresu. Zamrożenie prób pod ciśnieniem atmosferycznym w temperaturze -5°C÷-20°C nie wpływało na przeżywalność komórek *E. coli* i *T. thermophilus*. W przypadku *P. fluorescens* zmniejszenie liczby komórek wynosiło od 0.5 do 1 rzędu logarytmicznego w każdej z tych temperatur (tab. 4).