Hydrostatic Pressure Resistance of 14 Genera of Microbes under Controlled Room Temperature

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The pressure resistance of microbes (14 genera) was investigated at pressures ranging from 300 MPa to 500 MPa at 20°C for 20 min using a compact pressure vessel. *Acinetobacter* sp., *Pseudomonas fluorescens* and *Vibrio parahaemolyticus*, all of which are gram negative bacteria, and *Saccharomyces cerevisiae* were dead at 300 MPa, although gram positive bacteria were all alive at that pressure. At 400 MPa, *Salmonella Typhimurium*, *Morganella morganii*, *Leuconostoc mesenteroides*, and *Candida parapsilosis* were dead. At 500 MPa, *Escherichia coli* and *Lactobacillus plantarum* were dead. However, *Staphylococcus aureus*, *Enterococcus faecalis*, *Corynebacterium* sp., and *Neosartorya hiratsukae* (ascospores) survived even at 500 MPa, and the organisms except for *N. hiratsukae* were all gram positive bacteria.

Keywords: high pressure, sterilization, pressure resistance, *E. coli*

High hydrostatic pressure was applied to sterilization of microbes for the first time by Hite (1899). Nearly a century later, high hydrostatic pressure as one of the food processing techniques was investigated widely by Hayashi (1989). Especially, the pressure treatment has been recognized as a sterilization technique without heating, and many reports have been presented on it. Some of the reports indicated that the temperature during pressurizing significantly affects the inactivation of organisms even at the room temperatures (Horie *et al.*, 1992; Okazaki *et al.*, 1996). In addition, it is known that the pressurizing temperature rises as a result of adiabatic compression, which probably affects the inactivation of organisms by high pressure. In spite of the presence of this problem, there have rarely been such reports in which the temperature histories during the pressure treatment were indicated.

Thus, the object of this study is to clarify the pressure resistance of various microbes under the controlled and monitored temperature. A compact pressure vessel capable of easily controlling the sample temperature and of recording the change in the temperature during the pressure treatment was used for this purpose. Using this vessel, the pressure resistance of 14 genera of microorganisms was measured.

Materials and Methods

Test microbes *Escherichia coli* (IAM 1253), *Staphylococcus aureus* (209P), *Lactobacillus plantarum* (IAM 1216), *Pseudomonas fluorescens* (IAM 12022), *Leuconostoc mesenteroides* (IAM 1046), *Enterococcus faecalis* (IFO 8033), *Saccharomyces cerevisiae* (IAM 4954) and *Candida parapsilosis* (IFO 10564) were used. Furthermore, *Salmonella typhimurium*, *Morganella morganii*, *Vibrio parahaemolyticus*, and *Acinetobacter* sp., all of which were obtained from the Department of Food Science and Technology, Tokyo

University of Fisheries, *Corynebacterium* sp. derived from the Tokyo Metropolitan Food Technology Research Center, and *Neosartorya hiratsukae* (isolated from spoiled drink) were also used.

Pressurizing unit The unit previously described was used (Okazaki *et al.*, 1994).

Preparation of microbial suspension A certain number of the strain cells (*E. coli*, *S. typhimurium*, *S. aureus*, *M. morganii*, *P. fluorescens*, *Acinetobacter* sp., and *Corynebacterium* sp.) was inoculated into glucose broth, which consists of meat extract 3 g (Oxoid, England), yeast extract 3 g (Nihon-seiyaku, Tokyo), bacto peptone 10 g (Difco Laboratories, Detroit, Mich.) and glucose 5 g/l, pH 7.0. *V. parahaemolyticus* was inoculated into glucose broth + 3% NaCl, and *L. plantarum*, *L. mesenteroides* and *E. faecalis* were inoculated into Man Rogosa Sharpe media (MRS, Oxoid, England). *S. cerevisiae* and *C. parapsilosis* were inoculated into malt extract broth (Difco Laboratories). Incubation temperatures were the following: *E. coli*, *S. typhimurium*, *S. aureus* and *E. faecalis* at 35°C, *Acinetobacter* sp., *M. morganii*, *L. plantarum*, *L. mesenteroides*, *S. cerevisiae* and *Corynebacterium* sp. at 30°C, and *P. fluorescens* and *C. parapsilosis* at 25°C for 1-4 days until these microbes grow sufficiently. After *N. hiratsukae* was incubated on a potato dextrose agar (PDA, Nissui-seiyaku, Tokyo) at 30°C for 100 days, the plates were homogenized with sterile distilled water for a few minutes, and then the homogenized suspension containing ascospores was gained. These microbial cultures had been stored at 5°C. Each microbial culture was diluted with 10 volumes of the same medium used for the incubation, just before usage to produce a microbial suspension.

Procedure for pressurizing microbe A 1.2-ml of the microbial suspension was poured in a sample container (a 4×100-mm sterile silicone rubber tube sealed at one end)

without air bubbles, and the open end of the container was closed with a silicone rubber plug. The sample container filled with the suspension was inserted in a pressure vessel, which was immersed in a water bath (Thermo minder H-100, TAITEC Ltd., Koshigaya) controlled accurately at temperatures ranging from 10°C to 50°C for 5 min to make sure the initial temperature of each sample suspension was regulated to the same level. After that, the vessel was pressurized at 100–500 MPa for 10 min to 40 min. It took about 30 s to reach 400 MPa and about 15 s to return to atmospheric pressure.

Measurement of temperature A thermocouple (CA, 1.5 mm ϕ) was fixed in the center of a sample container to measure the temperature changes in the microbial suspensions, which were recorded as the digital data per 5 s during the pressure treatments.

Survival microbial cell count The pressurized tubes were aseptically opened, and the contents were withdrawn from each for plating. After cultivation at 35°C for 2 days, the colony growing on agar plates was enumerated. The plates used were the following: standard agar +3% NaCl for *V. parahaemolyticus*, MRS for *L. plantarum*, *L. mesenteroides* and *E. faecalis*, DPA for fungi, and standard agar for other microbes.

Assessment of pressurized sterilization The pressurized content (0.2 ml) in each tube was aseptically poured into 4 ml of broth medium in 5 test tubes, respectively. When the organisms did not grow in all of the 5 test tubes after 7 days, they were judged to be sterilized.

Results and Discussion

The effect of incubation time on the growth of *E. coli* cells pressurized at 30°C is shown in Table 1. The organism pressurized at both 100 MPa and 200 MPa grew during every treatment time after incubation for only a day. The organism treated at 300 MPa for 5 min and 10 min also grew for a day, although pressurization for longer than 15 min resulted in no growth of the organism. However, when the incubation time

was prolonged for 4 days, the organism grew even during the treatment for 40 min. Furthermore, the organism pressurized at 400 MPa for 5 min grew only after incubation for 4 days. After incubation for 7 days and 15 days, the number of the growth tubes no longer increased. Based on these results, an incubation time of at least 7 days was necessary to determine the sterilization of the organism.

When the pressure became high or the pressurized time became long, the time necessary for determining the growth indicated the tendency to become long. Miyao *et al.* (1993) showed that the pressurization prolonged the starting time of the growth for the gram positive bacteria due to injury to the cells by high pressure. This result was similar to our result although the organism used in this study was the gram negative bacteria.

The effect of the storage period of the microbial culture on the pressure resistance of *E. coli* was investigated after the microbial culture was stored for 0–30 day at 5°C. The result is shown in Table 2. As the storage period of the microbial culture became longer, the pressure resistance increased. Especially, although the organism did not grow with the exception of the pressurizing time for 5 min when the microbial culture stored for 0 day was pressurized at 400 MPa, the number of the growth tubes rose with the increase in the storage period. This result indicated that the pressure resistance of the organism became high during the storage. Thus, the storage period of the microbial culture (*E. coli*) affected the pressure resistance. The number of the growth tubes between the storage period for 15 days and that for 30 days was not different, and therefore, the microbial cultures used in this study were preserved for more than 15 days at 5°C.

Satomi *et al.* (1995) indicated that the organisms incubated at the relatively high temperatures (37°C or 44°C) had low pressure resistance. In our study, the shift of the temperature from 35°C (incubation temperature) to 5°C (store temperature) caused an increase in the pressure resistance. Although it is known that a change in incubation temperature affects the fluidity of the bacterium cytoplasmic membrane (Morris

Table 1. Effect of incubation time on growth of pressurized *E. coli* cells.

Inc. time ^{a)} (days)	Pressure (MPa)	Time (min)					
		5	10	15	20	30	40
1	100	5 ^{b)}	5	5	5	5	5
	200	5	5	5	5	5	5
	300	5	1	0	0	0	0
	400	0	0	0	0	— ^{c)}	—
2	300	5	5	5	5	5	0
	400	0	0	0	0	—	—
4	300	5	5	5	5	5	1
	400	1	0	0	0	—	—
7	300	5	5	5	5	5	1
	400	2	0	0	0	—	—
15	300	5	5	5	5	5	1
	400	2	0	0	0	—	—

E. coli cells, 4.6×10^7 CFU/ml, suspended in glucose broth were pressurized at 30°C and were subcultured at 35°C. The number of sample tubes subcultured was 5 per set.

^{a)}Incubation time.

^{b)}Number of positive growth tubes.

^{c)}Not examined.

Table 2. Effect of storage time of microbial culture of *E. coli* at 5°C on pressure resistance.

St. time ^{a)} (days)	Pressure (MPa)	Time (min)					
		5	10	15	20	30	40
0	300	5 ^{b)}	5	5	5	5	1
	400	2	0	0	0	0	— ^{c)}
3	300	—	5	—	5	2	1
	400	5	5	1	0	0	—
6	300	—	5	—	5	5	4
	400	3	3	1	0	0	—
15	300	—	5	—	5	5	5
	400	5	5	5	2	0	—
30	300	—	5	—	5	5	5
	400	5	5	5	2	0	—

E. coli cells, 4.8×10^7 CFU/ml at 0 day and 4.0×10^7 CFU/ml at 30 days storage, suspended in glucose broth were pressurized at 30°C and were subcultured for 7 days at 35°C. The number of sample tubes subcultured was 5 per set.

^{a)}Storage time at 5°C.

^{b)}and ^{c)} See Table 1.

& Clarke, 1981), the situation of the membrane of *E. coli* might change even during the storage period at low temperature. As the result, the pressure resistance of *E. coli* might become high.

The effects of temperature during the pressure treatment on the pressure resistance of *E. coli* is shown in Table 3. Even though it was pressurized at 20°C for 40 min, the organism was alive. However, the organism was dead under the conditions at 30°C for 40 min and at 40°C for 30 min. This result showed that the pressurizing temperature influences the pressure resistance of *E. coli* in the room temperature ranges from 20°C to 40°C, at which the organism can grow sufficiently. Some studies have previously been reported on the effects of temperature on pressure sterilization. For instance, when *Candida parapsilosis* was treated at 400 MPa for 10 min, the survival ratio was 100% at 24°C and 1% at 34°C (Horie *et al.*, 1992). In the case of spores of *Bacillus subtilis*, when the spore suspension was pressurized at 400 MPa, the death rate at 35°C became faster than that at 25°C (Okazaki *et al.*, 1996). Furthermore, when a kind of yeast, *Rhodotorula rubra*, suspended in the solution of Aw 0.94 (50% sucrose) was treated at 400 MPa for 15 min, less than a 2-log cycle reduction occurred at 30°C and a 7-log cycle was inactivated at 45°C, though the number of cells was not decreased at 25°C (Oxen & Knorr, 1993). These results indicate that, even if the species of organisms are different, room temperatures between 20°C and 40°C affect pressure resistance sufficiently.

It is known that the sample temperature increases as a result of the adiabatic compression when the sample is pressurized within a relatively short time. This phenomenon is very important to understand the pressure resistance of organisms, because the pressure resistance is affected even by room temperature as indicated in Table 3. The temperature and pressure history curves of the sample directly measured during the pressure treatments are shown in Fig. 1. The sample temperature rose about 8°C from the initial temperature for each case pressurized up to 400 MPa at 20°C, 30°C and 40°C, and a period of more than 60 s was necessary for it to return to the initial temperature. After the pressure treatments, the sample temperature dropped about 13°C as a result of the adiabatic expansion during the release of the pressure. So, all samples shown in Table 3 definitely trace the temperature history curves such as Fig. 1 during the pressure treatments. Thus, the maximum and minimum temperatures of the sample treated at 20°C are predicted to be 28°C and to be 7°C, respectively.

The temperature history curve of the sample measured directly during pressurizing had rarely been indicated in the

previous reports, even though the treatment temperature was mentioned in the reports. Therefore, the following experiment was arranged to clarify the effect of the increase in the sample temperature as a result of adiabatic compression. The microbial suspension of *E. coli* cells is pressurized for only 30 s at 400 MPa, and then the sample temperature rises to about 8°C from the initial level. This rising temperature is almost maintained during the 30 s as shown in Fig. 1, and therefore the rising temperature was utilized as the real pressurized temperature instead of the initial value. *E. coli* suspensions were pressurized at 400 MPa at the initial temperatures ranging from 10°C to 50°C; the measured maximum sample temperatures and the numbers of survival cells are indicated in Table 4.

Although the survival number at 20°C hardly decreased, the survival number at 10°C, 30°C and 40°C decreased to one-twenty, 3 log cycles and 6 log cycles, respectively. At 50°C, the surviving organism was not detected. Each real pressurized temperature was the initial one+about 8–9°C as shown in Table 4, and the temperature expressing the highest pressure resistance was 28°C. When the treatment temperatures become higher or lower than 28°C, the pressure resistance became lower than the result at 28°C. Based on this result, the temperature region showing the highest pressure resistance is thought to exist at around 28°C. Furthermore, if the pressurized treatments are carried out at below 18°C or

Fig. 1. Temperature history curves of sample suspension during the pressure treatments at 400 MPa for 120 s. Symbols (▼ 20°C, ● 30°C, ▲ 40°C). The up-arrow is the starting point of the pressure treatments and the down-arrow is the end point.

Table 3. Effect of temperature during pressurizing at 400 MPa on pressure resistance of *E. coli*.

Pressure (MPa)	Temperature (°C)	Time (min)				
		10	15	20	30	40
400	20	5 ^{a)}	5	5	5	5
	30	5	5	5	2	0
	40	5	5	5	0	0

Initial population of *E. coli* was 9.1×10^6 CFU/ml.

^{a)}Number of positive growth tubes.

Table 4. Effect of temperature increase during pressure treatment on *E. coli* cells at 400 MPa.

Initial temperature (°C)	Sample temperature (°C)	Survivors (CFU/ml)
10.2	18.1	1.7×10^5
20.2	28.4	1.9×10^6
30.2	38.5	3.5×10^3
40.1	49.0	5.0×10
50.1	59.1	no detection

The times increasing, holding and decreasing pressure were 30 s, 10 s, and 10 s, respectively. Initial population of *E. coli* was 2.6×10^6 CFU/ml.

above 38°C as the real pressurized temperatures, the pressure resistance of organisms is probably revealed to be lower. This result is in agreement with those of Horie *et al.* (1992) and Okazaki *et al.* (1996) as mentioned above.

Microbes (14 genera), 6 of which are gram negative bacteria, 5 of which are gram positive ones and 3 of which are yeasts and fungi, were pressurized under the following conditions; the microbe suspensions preserved for more than 15 days at 5°C were used as the samples. The incubation period to determine the growth after pressurizing was 7 days. The pressurizing conditions were at 300 MPa, 400 MPa and 500 MPa for 20 min at 20°C (initial temperature), and the maximum rising temperature of the sample is then measured to be about 28°C.

Table 5 shows the results. *Acinetobacter* sp., *P. fluorescens* and *V. parahaemolyticus* (all gram negative), and *Saccharomyces cerevisiae* were dead at 300 MPa, although gram positive bacteria were all alive at that pressure. At 400 MPa, *S. Typhimurium*, *M. morganii*, *L. mesenteroides* and *C. parapsilosis* were dead. At 500 MPa, *E. coli* and *L. plantarum* were dead. However, *S. aureus*, *E. faecalis* and *Corynebacterium* sp. and *N. hiratsukae* (ascospores) survived even at 500 MPa and these organisms except for *N. hiratsukae* were all gram positive bacteria.

Among these results, the bacteria possessing the highest pressure resistance were *S. aureus*, *E. faecalis* and *Corynebacterium* sp., all of which were cocci as well as gram positive bacteria. It is known that the cell walls of gram positive bacteria are thicker and firmer than those of the gram negative ones (Roger *et al.*, 1976), and therefore the difference in cell wall structure may directly affect the pressure resistance. Furthermore, the fact that the shape of the bacteria is spherical may also increase the structural strength against high pressure. In the previous studies, it was indicated that *S. aureus*, *E. faecalis*, and *Corynebacterium* sp. have the

pressure resistance even at 600 MPa (Ohmori & Shigehisa, 1990; Miyao *et al.*, 1993). Also in our study, these bacteria were alive at 500 MPa•20°C•20 min. Consequently, it will be necessary to pressurize at higher than 500 MPa in order to sterilize them at 20°C.

E. coli was dead under the conditions of 400 MPa•20°C•10 min (Kajiya *et al.*, 1993) and decreased to 10² CFU/ml from 10⁸ CFU/ml under the same conditions (Arroyo *et al.*, 1997). Our results indicate that *E. coli* was alive at 400 MPa•20°C•20 min and dead at 500 MPa•20°C•20 min and so had higher pressure resistance than that shown in the results of the previous reports.

V. parahaemolyticus was rapidly killed by the pressure condition at 1700 atm (172 MPa) •23°C (Styles *et al.*, 1991). In our results, the microbe was inactivated by the pressurization at 300 MPa•20°C•20 min.

S. typhimurium decreased to less than 3 log cycles at 3400 atm (344 MPa) for 20 min (the pressurizing temperature is not shown, Metrick *et al.*, 1989) and to 10² CFU/ml from 10⁸ CFU/ml at 400 MPa•20°C•10 min (Arroyo *et al.*, 1997). Our results revealed that the organism was living at 300 MPa•20°C•20 min but was dead at 400 MPa•20°C•20 min.

The ascospores (spores in a ascus produced during a teleomorph) of *N. hiratsukae* survived at 500 MPa•20°C•20 min, and our results indicated a relatively higher value than the pressure resistance of other fungi. The generation of ascospores might possess a higher pressure resistance than others such as conidia and mycelia.

In conclusion, some factors affecting pressure resistance existed in the pressurizing procedure. That is, an incubation time of the pressurized microbe of 7 days was needed to determine the sterilization. The microbial culture stored for 15 days at 5°C had a higher pressure resistance than the culture just after cultivation. The increase of sample temperature as a result of adiabatic compression affected the pressure resistance of *E. coli* considerably, and the pressure treatment at 20°C showed the highest pressure resistance. Under these conditions, the pressure resistance of microbes (14 genera) was investigated at pressures ranging from 300 MPa to 500 MPa at 20°C for 20 min. Three genera (all gram negative) of them were dead at below 400 MPa, and 3 genera (all gram positive) of them survived even at 500 MPa, indicating that it is probable that gram positive organisms have higher pressure resistance than gram negative ones.

References

- Arroyo, G., Sanz, P.D. and Prestamo, G. (1997). Effect of high pressure on the reduction of microbial populations in vegetables. *J. Appl. Microbiol.* **82**, 735–742.
- Hayashi, R. (1989). Application of high pressure to food processing and preservation, Philosophy and development. In "Engineering and Food," ed. by W.E.L. Spiess and H. Schubert, Vol. 2, 815–826.
- Hite, B.H. (1889). The effect of pressure in the preservation of milk. *Bull.* **58**, 15–35. w. Va. Univ. Agric. Exp. Sta., Morgantown.
- Horie, Y., Kimura, K., Ida, M., Yoshida, Y. and Ohki, K. (1992). Identification and pressure-sterilization of a candida strain resistant to high pressure. *Nippon Nogeikagaku Kaishi*, **65**, 975–980 (in Japanese).
- Kajiya, N., Akizumi, K., Abei, K., Nagata, M., Egashira, T. and Miyake, Y. (1993). Sterilization of *Escherichia coli* by high pressure. *Nippon Shokuhin Kogyo Gakkaishi*, **40**, 406–413 (in Japanese).

Table 5. Pressure resistance of 14 genera of bacteria and fungi.

Genera	Initial populations	Pressure (MPa) ^{a)}		
	(CFU/ml)	300	400	500
Gram negative bacteria				
<i>Escherichia coli</i>	1.3×10 ⁷	5 ^{b)}	5	0
<i>Salmonella typhimurium</i>	3.3×10 ⁷	5	0	0
<i>Morganella morganii</i>	2.2×10 ⁷	5	0	0
<i>Pseudomonas fluorescens</i>	3.2×10 ⁷	0	0	0
<i>Vibrio parahaemolyticus</i>	3.5×10 ⁶	0	0	0
<i>Acinetobacter</i> sp.	4.8×10 ⁷	0	0	0
Gram positive bacteria				
<i>Staphylococcus aureus</i>	8.2×10 ⁶	5	5	5
<i>Enterococcus faecalis</i>	5.9×10 ⁷	5	5	5
<i>Lactobacillus plantarum</i>	2.4×10 ⁷	5	5	5
<i>Leuconostoc mesenteroides</i>	9.4×10 ⁷	4	0	0
<i>Corynebacterium</i> sp.	1.1×10 ⁷	5	5	5
Fungi				
<i>Saccharomyces cerevisiae</i>	— ^{c)}	0	0	0
<i>Candida parapsilosis</i>	1.0×10 ⁶	5	0	0
<i>Neosartorya hiratsukae</i> ^{d)}	2.4×10 ⁸	5	5	5

^{a)}Pressurized at 20°C for 20 min.

^{b)}Number of positive growth tubes.

^{c)}Not counted.

^{d)}Ascospores.

- Metrick, C., Hoover, D.G. and Farkas, D.F. (1989). Effects of high hydrostatic pressure on heat-resistant and heat-sensitive strains of *Salmonella*. *J. Food Sic.*, **54**, 1547-1549.
- Miyao, S., Shindoh, T., Miyamori, K. and Arita, T. (1993). Effects of high pressurization on the growth of bacteria derived from *surimi*. *Nippon Shokuhin Kogyo Gakkaishi*, **40**, 478-484 (in Japanese).
- Morris, G.J. and Clarke, A. (1981). "Effect of low temperature on biological membranes" ed. by Academic Press, New York, p. 41-53.
- Ohmori, T. and Shigehisa, T. (1990). Application of high hydrostatic pressure to the processing of meat and meat products. *Pressure-Processed Food*, Kyoto, Japan, 131-139 (in Japanese).
- Okazaki, T., Yoneda, T. and Suzuki, K. (1994). Combined effects of temperature and pressure on sterilization of *Bacillus subtilis* spores. *Nippon Shokuhin Kogyo Gakkaishi*, **41**, 536-541 (in Japanese).
- Okazaki, T., Maeshige, S. and Suzuki, K. (1996). Combined effects of temperature and pressure on inactivation of heat-resistant bacteria. *High Pressure Bioscience and Biotechnology*, Kyoto, Japan, pp. 415-418.
- Oxen, P. and Knoor, D. (1993). Baroprotective effects of high sokute concentrations against inactivation of *Rhodotorula rubra*. *Food Sci. Technol.*, **45**, 153-160.
- Roger, S., Edward, A. and John, I. (1976). "The microbial world (4th edition)," Prentice-Hall Inc., New Jersey.
- Satomi, M., Yamaguchi, T., Okuzumi, M. and Fujii, T. (1995). Effect of several conditions on the recovery of pressure-injured bacteria. *Shokuhin Eiseigaku Zasshi*, **36**, 344-351 (in Japanese).
- Styles, M.F., Hoover, D.G. and Farkas, D.F. (1991). Response of *Listeria monocytogenes* and *Vibrio parahaemolyticus* to high hydrostatic pressure. *J. Food Sic.*, **56**, 1404-1407.