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Growth Inhibition of Microorganisms by Hydrostatic Pressure

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The pressure range that prevents growth of microorganisms (two yeast, three lactic acid bacteria, *E. coli*, three bacilli and one clostridium) was investigated in order to apply it to food processing. The growth of the microorganisms could be restrained in a pressure range of 40–70 MPa depending on the species of microorganism. Growth of *Lactobacillus plantarum* was inhibited at 70 MPa, and two kinds of yeast at 40 MPa. The pressurization treatment is presumed to induce the germination of the spores of *Bacillus* and *Clostridium*, and the germination of *B. subtilis* and *B. stearothermophilus* were especially remarkable. Most of the germinated spores were killed at the optimum growth temperature of each microorganism under pressurized conditions. Practical usefulness of these results was verified in the autolysis process of fish meat without decomposition. Growth inhibition and inactivation of spores by pressurization treatment at less than 100 MPa can be utilized as a new technique for killing microorganisms and for producing food.

Keywords: pressurization, growth inhibition, microorganisms, autolysis.

Many researchers have investigated the effect of high hydrostatic pressure (HHP) on inactivation of microorganisms since the HHP treatment for milk preservation was first reported more than 100 years ago by Hite (1899). Hayashi (1989) suggested the availability of HHP in food processing, though he proposed the exclusive utilization of pressure ranges higher than 200 MPa. On the other hand, Okami (1990) also suggested utilization of relatively low pressure of less than 100 MPa for food-preservation and storage of food materials.

ZoBell and Johnson (1949) studied the effect of pressure on the growth of many kinds of microorganisms, and showed that hydrostatic pressure from 50 MPa to 60 MPa retarded their growth. Clouston and Wills (1969) showed that germination of *Bacillus pumilus* spores was accelerated at 500 atm (c.a. 50 MPa). Recently, Furukawa and Hayakawa (2001) reported that *B. stearothermophilus* spores can be sterilized at 95°C, 60 MPa. However, such relatively low pressures have not yet been applied in practice.

Recently, Okazaki *et al.* (2003) applied the growth inhibition behavior of microorganisms under pressurized conditions to autolysis of fish meat. The report showed that the fish meat could be autolyzed in an extremely short period without decomposition at 60 MPa. This result also indicates the growth inhibition of microorganisms by pressurization treatment can be applied to other enzymatic processings without decomposition. To apply the HHP to food processing, it is necessary to clarify the growth behavior of microorganisms under pressurized conditions.

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The purpose of this study was to determine the pressure range capable of inhibiting growth of microorganisms, and growth inhibition by pressurization treatment was also confirmed in autolysis of fresh fish meat as a viable application.

Materials and Methods

Test microorganisms Two kinds of yeast (*Saccharomyces cerevisiae* IAM 4178, *Zygosaccharomyces rouxii* IFO 1877), three kinds of lactic acid bacteria (*Lactococcus lactis* IAM 1198, *Lactobacillus plantarum* IAM1041, *Tetragenococcus halophilus* IAM 1673), *Escherichia coli* IFO 3301, three kinds of *Bacillus* (*B. subtilis* No.1403 Research Laboratory, Japan Canners Association, *B. coagulans* No. 1101, *B. stearothermophilus* No. 1001), and *Clostridium sporogenes* PA 3679 were used.

Pressurizing apparatus The pressurizing apparatus (Hikarikouatsu-kiki Co., Ltd., Hiroshima), capable of pressurizing up to 400 MPa and heating up to 80°C simultaneously, was used in this study. The pressure chamber of the apparatus is 100 mm × 300 mm in size and is heated by an outside ribbon heater (600 w). The water in the pressure chamber was used as a pressurizing medium. The pressure was confirmed by watching a pressure gauge (Heise, Model-CC, USA).

Preparation of microorganisms Yeast: *S. cerevisiae* and *Z. rouxii* were inoculated into the YPD medium, which consisted of yeast extract 10 g (Nihon-seiyaku, Tokyo), polypepton 20 g (Nihon-seiyaku), glucose 20 g and agar 15 g/L, pH6.0. They were cultured at 30°C for a day. Lactic acid bacteria: *L. lactis*, *L. plantarum*, and *T. halophilus* were inoculated into the GAM broth (Nissui Seiyaku, Ltd., Tokyo)

and also cultured at 30°C for a day. In the case of *T. halophilus*, 5% NaCl was also added to the GAM broth. *E. coli* was inoculated into glucose broth (GB) and cultured at 35°C for a day. *Bacillus* and *Clostridium*: the spores previously prepared were used for this test (Okazaki *et al.*, 2000).

Procedure for pressurization treatment A few milliliters of each growth culture or spore suspension were aseptically injected into 100 ml of the media, which had been packed in a polyester pouch. The medium was YPD in yeast, GAM in lactic acid bacteria and *C. sporogenes* spore, and GB in *E. coli* and *Bacillus*, respectively. The initial population of each microorganism inoculated in the pouch was in a range of 10^5–10^6 CFU/ml. When fish meat was substituted for media, fresh anchovies were roughly homogenized with a hand mincer, and the microorganisms prepared as above were inoculated to the minced anchovies so as to be 10^4–10^7 CFU/g, then they were packed in a polyester pouch.

The inoculated pouches were set in the chamber of the pressurizing apparatus to grow, and then pressurized at 0.1–70 MPa for 0–48 h at the appropriate temperatures. In the case of *C. sporogenes*, the pressurized period was prolonged to 100 h. It took about 90 s to reach 70 MPa and about 10 s to return to atmospheric pressure. The sample was taken out at the given time, and the viable counts and spores were measured.

Population of microorganisms. The pouches after pressurization treatment were aseptically opened and the contents were withdrawn, and then serially diluted in sterile saline (0.85% NaCl). Each diluted sample was poured into duplicate plates. YPD agar was used for yeast, GAM agar for lactic acid bacteria and *C. sporogenes*, Standard agar (Nissui Seiyaku Ltd.) for *Bacillus* and *E. coli*. Only *C. sporogenes* was cultivated under anaerobic condition by using an Anaero Pack (Mitsubishi Gas Chemical Co., Inc.). After being cultivated on the agar plates for 2–4 days at the appropriate temperatures, the colonies on the plates were counted. For measurement of the spore number, the colonies were counted after being heat-treated at 80°C for 10 min.

Microscopy Microorganisms treated under pressurized conditions were observed with an optical microscope (OLYMPUS, BX-51).

Results and Discussion

Figure 1 shows the growth behavior of two kinds of yeast (*S. cerevisiae* and *Z. rouxii*) under pressurized conditions. At atmospheric pressure (0.1 MPa), the population of their viable counts increased soon after cultivation, and they generated much gas. At 40 MPa and 50 MPa, the viable counts decreased gradually, and had reduced to about one-tenth after 24 hours. Thus, pressurization treatment at higher than 40 MPa was able to prevent these yeasts from growing. It was reported that the growth of *S. cerevisiae* was inhibited at 40 MPa (ZoBell and Johnson, 1949), and our results were in agreement with these. In addition, the growth of other microorganisms used in the present study was also delayed or inhibited under a pressurized condition compared to the atmospheric pressure condition as shown in the following figures.

Figure 2 shows the growth behavior of three kinds of lactic acid bacteria (*L. lactice*, *L. plantarum*, *T. halophilus*) under pressurized conditions. At 50 MPa, the viable counts of *L. lactice* decreased gradually, but those of *L. plantarum* and *T. halophilus* increased. The growth of *T. halophilus* stopped completely at 60 MPa, and the viable counts decreased gradually at 70 MPa. In the case of *L. plantarum*, at 60 MPa the lag period became longer and then afterwards it grew slightly, but at 70 MPa the growth stopped completely and viable counts decreased gradually. In this

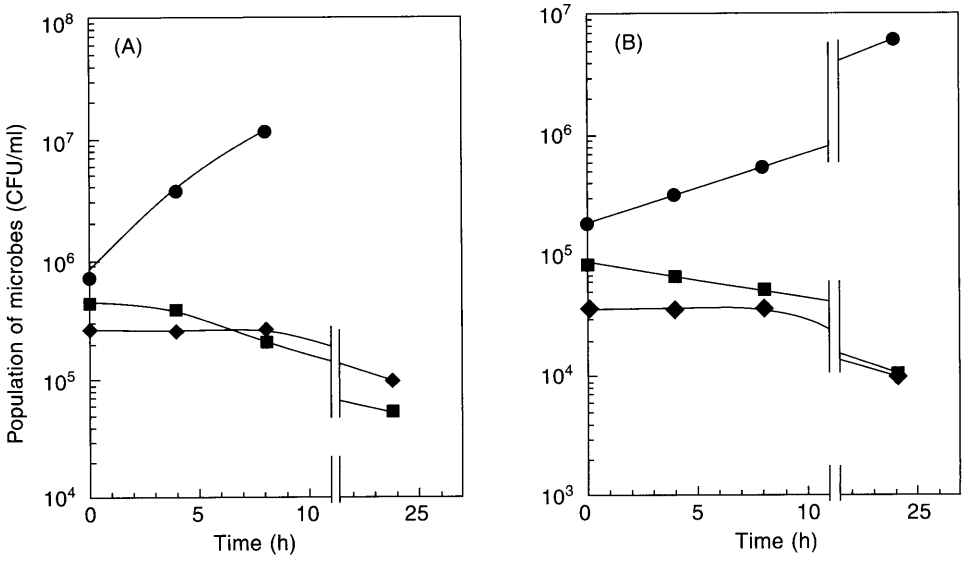

Fig. 1. Changes in viable counts of yeast, *S. cerevisiae* (A) and *Z. rouxii* (B) in culture during pressurization treatment at 30°C. ●, 0.1 MPa; ■, 40 MPa; ◆, 50 MPa.

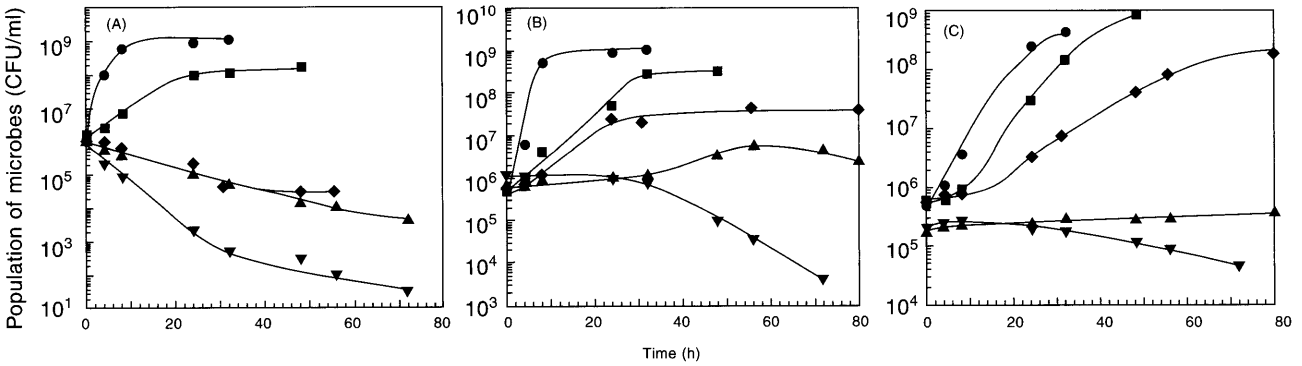

Fig. 2. Viable counts of lactic acid bacteria, *L. lactice* (A), *L. plantarum* (B), and *T. halophilus* (C) in culture under pressurized conditions at 30°C. ●, 0.1 MPa; ■, 40 MPa; ◆, 50 MPa; ▲, 60 MPa; ▼, 70 MPa.

study *L. lactice* did not grow under pressurized conditions of 50–60 MPa, although it was reported that it could grow at that pressure (ZoBell and Johnson, 1949). These results suggested that pressure treatment at over 70 MPa is needed to prevent the growth of lactic acid bacteria.

Figure 3 shows the growth behavior of *E. coli* and vegetative cells of *B. subtilis* under pressurized conditions. Both of them almost stopped growing at 50 MPa, and their viable counts gradually decreased at 60 MPa. ZoBell and Johnson (1949), on the other hand, reported that *E. coli* and *B. subtilis* were able to grow even at 50 MPa and 60 MPa, respectively. The present result (Fig. 3) did not coincide with theirs. This difference in results might have been because the growth of microorganisms in the present study was judged from viable counts, while ZoBell and Johnson measured the increase in turbidity of liquid medium to judge the growth behavior. Then, we also measured the turbidity of liquid medium during the cultivation process of *E. coli* under pressurized conditions (Fig. 4). The turbidity increased even at 50 MPa, however, the viable counts were unchanged at the same pressure as shown in Fig. 3. The same differences between turbidity and viable counts

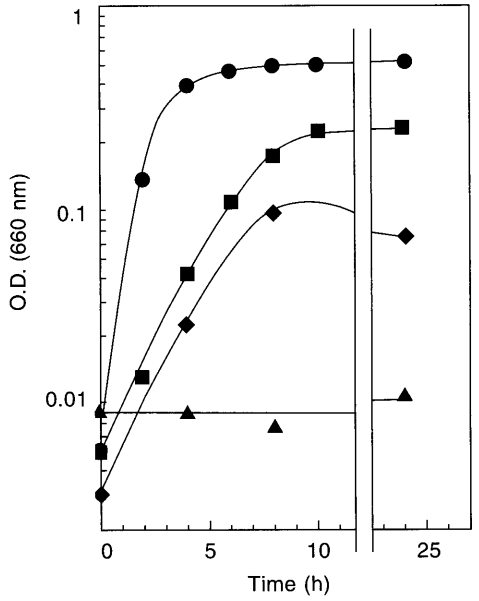

Fig. 4 Changes in relative turbidity of liquid medium for *E. coli* during pressurization treatment at 40°C. ●, 0.1 MPa; ■, 40 MPa; ◆, 50 MPa; ▲, 60 MPa. each plot corresponds to those of Fig. 3(A).

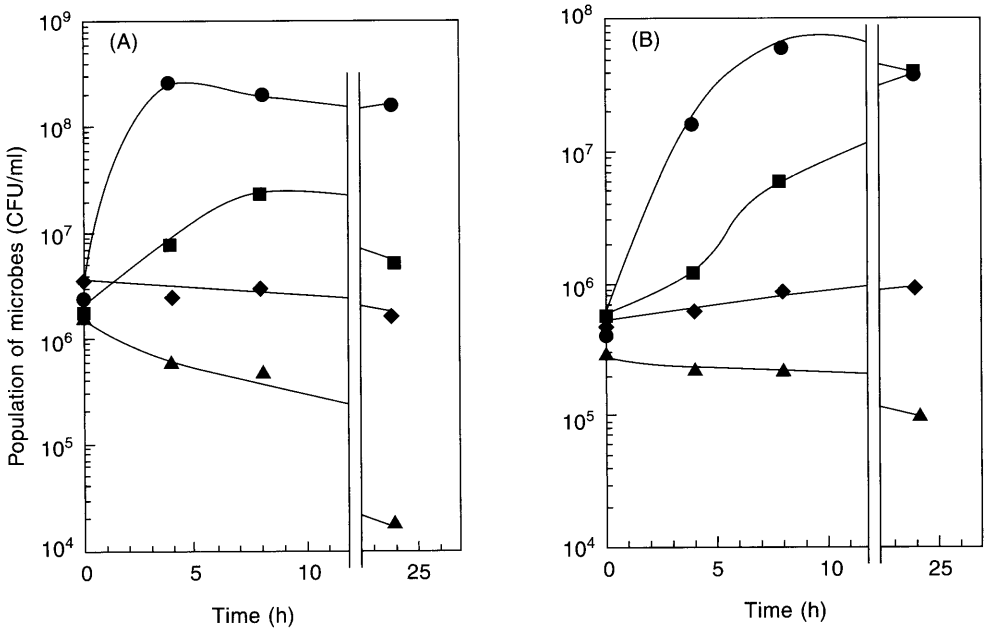

Fig. 3 Viable counts of *E. coli* (A) and vegetative cells of *B. subtilis* (B) in culture under pressurized conditions at 40°C. ●, 0.1 MPa; ■, 40 MPa; ◆, 50 MPa; ▲, 60 MPa.

were also observed in the other microorganisms used in this study (data not shown). Therefore, an increase in turbidity does not always mean that microorganisms grow, if the judgment of growth is confined to the condition under pressure. On the other hand, it was observed by microscopy that the shape of vegetative cells remarkably lengthens at 50 MPa (photo not shown) as demonstrated before (ZoBell and Cobet, 1962). This phenomenon might be one of the reasons that both the judgment of growth from turbidity and viable counts were in disagreement.

Figure 5 shows the growth behavior of three kinds of *Bacillus* and *Clostridium* spores under pressurized conditions. At 50 MPa, the growth of each *Bacillus* was inhibited entirely. Furthermore, the population of each *Bacillus* decreased, especially, those of *B. subtilis* and *B. stearothermophilus* diminished remarkably after 24 h. The viable counts of *C. sporogenes* increased at 40 MPa up to that at 0.1 MPa after 140 h, though the lag period lengthened to 72 h from 48 h. At 50 MPa, the viable counts gradually decreased and the growth was completely restrained. These results showed that the spore growth of the four kinds of spore-forming-bacteria was inhibited at 50 MPa.

However, the question still remained why the viable counts of spores decreased under the pressurized condition at 50 MPa, because it has been reported that *Bacillus* spores are not inactivated even at 12,000 atm (c.a. 1,200 MPa) (Larson *et al.*, 1918). The magnitudes of the pressure applied in the present study (40–60 MPa) were much lower than the reported value. On the other hand, it is known that the pressurization treatment induces germination of spores (Clouston and Wills, 1969; Sale *et al.*, 1970; Furukawa and

Table 1 Number of spores in culture after pressurization treatment for 4 h.

Microorganisms	Pressure (MPa)	Initial spores	Spores after 4-hour treatment
C. sporogenes	0.1	8.1×10^4	8.5×10^4
	40	7.7×10^4	2.6×10^4
	50	7.2×10^4	1.3×10^4
B. subtilis	0.1	4.2×10^6	5.3×10^6
	40	4.8×10^6	7.4×10^3
	50	5.3×10^6	9.7×10^2
	60	4.4×10^6	8.2×10^2
B. coagulans	0.1	3.1×10^5	2.6×10^5
	40	3.2×10^5	—*
	50	3.0×10^5	2.9×10^4
B. stearothermophilus	0.1	9.0×10^4	1.8×10^5
	40	1.0×10^5	1.9×10^3
	50	1.3×10^5	1.0×10^3

*No data

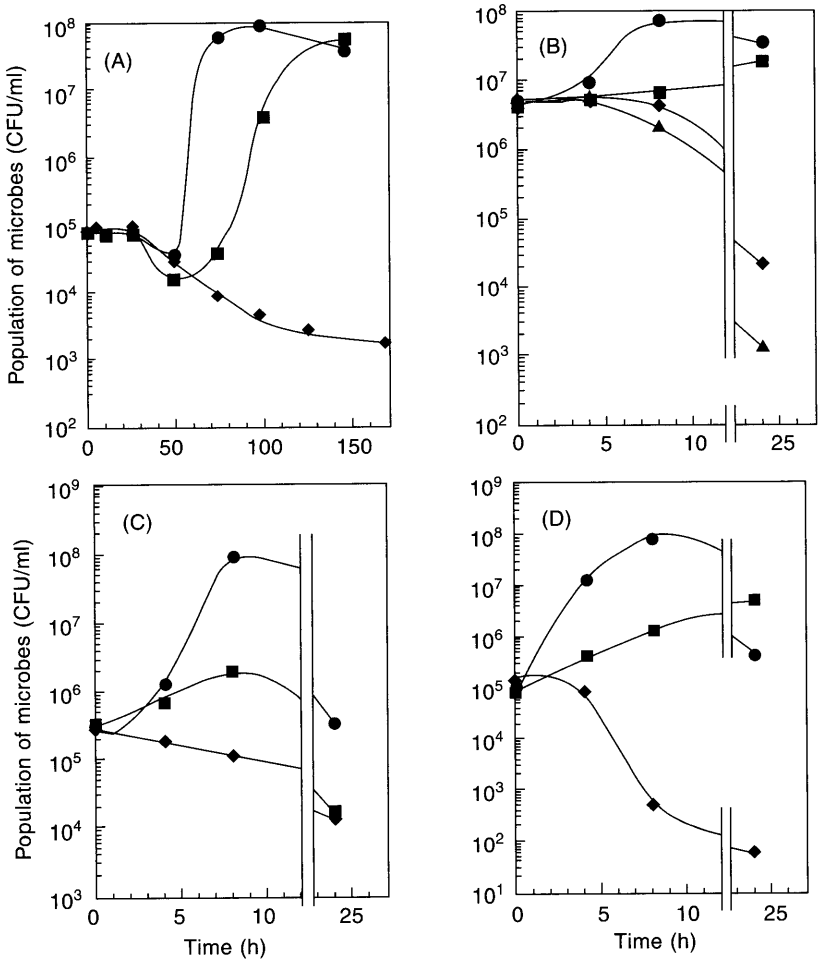

Fig. 5 Viable counts of *C. sporogenes* spores (A) at 30°C, *B. subtilis* spores (B) at 40°C, *B. coagulans* spores (C) at 55°C and *B. stearothermophilus* spores (D) at 55°C in culture under pressurized conditions. ●, 0.1 MPa; ■, 40 MPa; ◆, 50 MPa; ▲, 60 MPa.

Hayakawa, 2001). Table 1 shows the change in number of spores after pressurization treatment for 4 hours. The spore population of each bacterium decreased. Especially, in the case of *B. subtilis,* the spore population was reduced to 10^3 from 10^6 CFU/ml. The reduction of spore number indicated that the heat resistance of spores was lost within 4 hours under pressurized conditions, because the spore population was measured after being heated at 80°C for 10 min. Furthermore, the appearance of spores changed to a dark color from a bright one (photo not shown). These phenomena are recognized as proof of spore germination. It is believed that some of the spores germinated under a pressurized condition within 4 h. Therefore, the reason why the viable counts decreased under the pressurized conditions for 24 h (Fig. 5) was presumed to be that the spores were inactivated after germination. In addition, considering that the vegetative cells of *B. subtilis* were not killed by pressurization treatment at 50–60 MPa for 24 h as shown in Fig. 3, it is thought that inactivation of spores occurred before they changed to vegetative cells.

A new technique, autolysis of unsalted fish protein under pressurized condition was proposed by Okazaki *et al.* (2003). However, they did not check the growth inhibition of each microorganism by pressure holding. We therefore applied the pressure conditions capable of inhibiting the growth of each microorganism to the autolysis of fish meat from a practical standpoint. Each microorganism was added to the minced anchovies, and they were incubated under pressurized condition for 48 h at 30–55°C. The change in population of the microorganisms is shown in Table 2. The minced anchovies were decomposed under atmospheric pressure in all runs of the tests, except for those at 55°C, while all of the pressurized anchovies retained a good smell. At 30°C, the viable counts of *S. cerevisiae* and *Z. rouxii* decreased at 50 MPa for 48 h. The viable counts of *L. lactice*, *L. plantarum* and *T. halophilus* did not increase under a pressurized condition at 60–70 MPa, while those of *C. sporogenes* decreased in one-order at 50 MPa. At 40°C, the viable counts of *E. coli* somewhat decreased at 60 MPa as compared with the initial viable counts. On the contrary, the viable counts of *B. coagulans* and *B. subtilis* decreased in the extent of two-order and five-order, respectively at 60 MPa. At 55°C, the viable counts of *B. coagulans* and *B. stearothermophilus* spores decreased in the extent of approximately one-order and two-order, respectively at 50 MPa. The pressure conditions determined above to inhibit the growth of microorganisms were able to restrain their growth even in fresh fish meat. Furthermore, reduction of the spore population under the pressurized conditions was also confirmed even in fresh fish meat.

Table 2. Changes in population of microorganisms added to minced anchovies after pressurization treatment for 48 h.

Temp. (°C)	Microorganisms	Viable counts (CFU/ml)			
		Pressure (MPa)	Initial	0.1 MPa	Pressurization
30	No addition	50	1.1×10^3	d*	1.0×10^3
	S. cerevisiae	50	1.2×10^6	d	1.2×10^5
	Z. rouxii	50	1.5×10^4	d	< 300
	C. sporogenes spores	50	2.6×10^5	d	6.5×10^4
	L. lactice	60	1.0×10^6	d	2.7×10^5
	L. plantarum	70	2.4×10^6	d	1.8×10^6
	T. halophilus	60	3.4×10^6	d	1.1×10^6
40	No addition	60	6.1×10^4	d	< 300
	E. coli	60	5.8×10^6	d	3.9×10^5
	B. subtilis spores	60	1.6×10^7	d	< 300
	B. coagulans spores	60	2.4×10^5	d	1.6×10^3
55	No addition	50	1.3×10^3	4.5×10^3	< 300
	B. coagulans spores	50	7.4×10^5	1.3×10^6	7.0×10^4
	B. stearothermophylus spores	50	4.5×10^5	8.9×10^5	1.0×10^3

*d, decomposition

In conclusion, the growth of each microorganism used in the present study could be controlled by pressurization treatment (40–70 MPa) though the magnitude of pressure to inhibit the growth was dependent on the species of microorganism. Practical usefulness of these results was also verified in the autolysis process of fresh fish meat. In addition, it was presumed that some of the *Bacillus* and *Clostridium* spores were induced to germinate by pressurization, and these germinated spores were then killed during the pressurization. These results support Okami's suggestion (1990) that it is possible to preserve foods without decomposition under a pressurized condition below 100 MPa.

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