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Note

Effect of Food Additives on the Inactivation of Bacterial Spores by Reciprocal Pressurization Treatment

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The effects of food additives on the inactivation of spores of *Bacillus subtilis*, *Bacillus coagulans* and *Bacillus stearothermophilus* by reciprocal pressurization (six reciprocal 5-min pressurization cycles) and continuous pressurization (30-min pressurization) were investigated at 400MPa. As food additives, glucose (12%, w/v), sodium chloride (6%, w/v) and ethanol (20%, w/v) were selected. RP treatment increased the inactivation ratio of three strains. Addition of food additives decreased the inactivation ratio in continuous pressurization and reciprocal pressurization treatment except in the case of the addition of sodium chloride in reciprocal pressurization treatment.

Key words : Hydrostatic pressure/Reciprocal pressurization/Bacterial spore/Inactivation/ Food additives.

Heat sterilization is the most commonly used bacterial inactivation method in the food industry (Walker and LaGrange, 1991). However, heat sterilization to inactivate bacterial spores usually results in detrimental changes in the nutritive value, color and flavor of foods (Joslyn, 1991). On the other hand, high hydrostatic pressure can inactivate microorganisms without altering the flavor and nutrient components in foods (Cheftel, 1992). Hence, major investigations are currently focused on the potential of hydrostatic pressure treatments as alternatives to heat treatments (Hoover et al., 1989).

In hydrostatic pressure treatments, bacterial spores were found to be more resistant than vegetative bacteria (Cheftel, 1992; Sonoike 1997; Timson and Short,1965), surviving up to 1200 MPa (Johnson and ZoBell, 1949; Larson et al., 1918; Sale et al., 1970; Timson and Short, 1965). Hence, it has been suggested that bacterial spores are unlikely to be killed by the hydrostatic pressure treatment at room temperature (Sale et al. 1970; Sonoike 1997). Therefore, the inactivation effects of hydrostatic pressure on bacterial spores in a combination with heat (Gould, 1973; Mallidis and Drizou, 1991; Okazaki et al., 1994; Roberts and Hoover, 1996), irradiation (Crawford et al., 1996), low pH (Roberts and Hoover, 1996) and bacteriocins such as nisin (Roberts and Hoover, 1996) have been studied.

We have investigated the effect of the impulsive force generated by quick decompression on the inactivation of bacterial spores (Hayakawa et al., 1998). From this work, we considered that the reciprocal compression and decompression with hydrostatic pressure could increase the inactivation of and injury to bacterial spores compaed to continuous pressurization. It was indicated that reciprocal pressurization (six reciprocal 5-min pressurization cycles, RP treatment) was more effective in inactivating the bacterial spores than continuous pressurization (30-min pressurization, CP treatment) (Furukawa et al., 2000a and 2000b).

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There were many studies on the effects of food additives on the inactivation of bacterial spores (Alderton et al., 1980; Briggs and Yazdany, 1970; Cook and Gilbert, 1969; Hänulv et al., 1977; Lóez et al., 1996; Mazas et al., 1999; Murrell and Scott, 1966; Periago et al., 1998). In some of these studies, saccharides (Hänulv et al., 1977; Mazas et al., 1999) and salts (Briggs and Yazdany, 1970; Cook and Gilbert, 1969; Hänulv et al., 1977; Lóez et al., 1996; Mazas et al., 1999; Periago et al., 1998) were used as food additives.

In this study, the effects of the food additives, glucose, sodium chloride and ethanol, on the inactivation of bacterial spores by RP and CP treatment were investigated.

The bacteria used were *Bacillus subtilis* IFO13722, *Bacillus coagulans* IFO12583 and *Bacillus stearothermophilus* IFO12550, obtained from the Institute for Fermentation (Osaka). Log-phase-cultures of *B. subtilis*, *B. coagulans* and *B. stearothermophilus* grown in nutrient broth (Eiken Chemical Co., Ltd., Tokyo) were transferred to soil-infusion-agar-plates (Berry and Brandshaw 1980), which consisted of nutrient agar (Eiken Chemical Co., Ltd., Tokyo) plus a soil extract. The plates were incubated at 37℃ (*B. subtilis* and *B. coagulans*) or 55℃ (*B. stearothermophilus*) for 10 d.

Spores were collected by flooding the surface of the culture with sterile distilled water followed by scraping the surface with a sterile microscope glass slide. After collection, spores were washed three times by centrifugation at 4,000 x g for 30min, resuspended in sterile distilled water and stored at 4 ℃ until use. Spore suspensions were heated at 70℃ for 30min to inactivate vegetative cells. Suspensions were diluted to give approximately 10^6 colony forming units (CFU) ml^{-1}.

Spore suspensions were sealed in sterile screw-capped plastic tubes (1.5ml capacity; Greiner Labortechnik Co., Ltd., Germany), and then pressurized. The equipment used was a prototype pressurization apparatus (Hayakawa et al. 1994). The time needed to achieve the treatment pressure was approximately 30s. The decompression time was approximately 0.1s. Temperature of the pressure cell was regulated by a thermocontrolled water bath (Haake GH, Germany). Spores were treated at 400MPa. *B. subtilis* spores were more heat sensitive in RP treatment than *B. coagulans* and *B. stearothermophilus* spores (Furukawa et al., 2000a and 2000b). Because of this, *B. subtilis* spores were treated at 45 ℃, while *B. coagulans* and *B. stearothermophilus* spores were treated at 75℃. A total holding period of 30min and the reciprocal number of 1 and 6 were used in this study. Here, RP treatment consists of six cycles of compression (5min) and quick decompression.

The number of survivors was determined by the colony count method using nutrient agar. The plates were incubated at 37℃ (*B. subtilis* and *B. coagulans*) or 55℃ (*B. stearothermophilus*) for 24h and then enumerated. Water activity of the solution was measured by the rotronic Hygroskop DT water activity meter (Gunze Sangyo Co., Tokyo). Spores were observed by a phase-contrast microscopy (Horikoshi et al. 1993).

In a previous study, addition of 12% (w/v) glucose more decreased the inactivation ratio of *B. stearothermophilus* spores more than 6% (w/v) glucose in the hydrostatic pressure treatment (Furukawa et al. 2000). Similarly, 6% (w/v) sodium chloride decreased the inactivation ratio more than the 3% (w/v) sodium chloride, and 20% (v/v) ethanol decreased the inactivation ratio more than the 10% ethanol (Furukawa et al. 2000). Because of this results, glucose (Nakalai tesque Inc., Kyoto) was added to the spore suspension at 12% (w/v). Sodium chloride (Nakalai tesque Inc., Kyoto) was added to the spore suspension at 6% (w/v), ethanol (Nakalai tesque Inc., Kyoto), which is known as an antiseptic agent (Larson, 1991), was added to the spore suspension at 20% (v/v).

All experiments were done in triplicate. The data presented are the means of three replicate experiments. Significant differences were determined to be those with 5% level of significance ($p<0.05$) by Student's *t* test.

The effects of food additives on the inactivation of spores of *B. subtilis* (45℃, 400MPa), *B. coagulans* (75℃, 400MPa) and *B. stearothermophilus* (75℃, 400MPa) by RP and CP treatments were investigated. These temperature and pressure conditions had significantly increased the inactivation ratio by RP treatment in comparison with that of CP treatment as shown in our previous studies on the inactivation and germination of spores by RP and CP treatment (Furukawa et al., 2000a and 2000b).

Concentrations of food additives (glucose; 12%, w/v, sodium chloride; 6%, w/v, ethanol; 20%, v/v) were determined from our previous study (Furukawa and Hayakawa, 2000). In that study, these food additives decreased the inactivation rate of the bacterial spores by low hydrostatic pressure (below 100MPa) treatments.

RP treatment was significantly more effective than CP treatment in inactivating the spores of *B. subtilis* (Fig. 1), *B. coagulans* (Fig. 2) and *B. stearothermophilus* (Fig. 3) in the suspensions containing glucose,

sodium chloride and ethanol ($p<0.05$). We observed the spores by phase-contrast microscopy. Almost all spores were darkened after the treatments (data not shown).

In CP treatments, surviving spores of *B. subtilis* (Fig. 1), *B. coagulans* (Fig. 2) and *B. stearothermophilus* (Fig. 3) significantly increased more in the suspensions which containing glucose, sodium chloride and ethanol than in the distilled water suspension ($p<0.05$). In RP treatments, surviving spores of *B. subtilis* (Fig. 1), *B. coagulans* (Fig. 2) and *B. stearothermophilus* (Fig. 3) significantly increased more in the suspensions containing glucose and ethanol than in the distilled water suspension ($p<0.05$). However, in RP treatments, surviving spores of *B. coagulans* (Fig. 2) and *B. stearothermophilus* (Fig. 3) were significantly smaller in number the suspensions containing sodium chloride than in the distilled water suspension ($p<0.05$). With *B. subtilis* (Fig. 1), surviving spores after RP treatment increased more in the sodium chloride suspension than in the distilled water suspension. However, against *B. subtilis*, RP treatments were much more effective than CP treatments in the sodium chloride suspension.

From these results, it was indicated that the addition of glucose and ethanol to the spore suspensions did not increase the effects of RP treatments to inactivate of bacterial spores. On the other hand, the addition of sodium chloride was effective in spore inactivation. Several works have suggested that the addition of saccharides and salts increased the heat resistance of microorganisms (Hänulv et al., 1977; Mazas et al., 1999). Only in CP treatments did present results correspond to previous results regarding heat sterilization. However, the effect of sodium chloride in RP treatments was different from that in heat

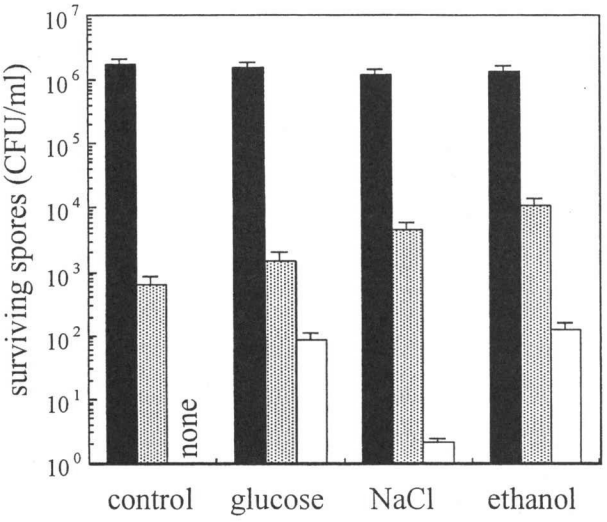

FIG. 1. Effect of food additives on surviving spores of *B. subtilis* treated by continuous and reciprocal pressurization at 45℃, 400 MPa. Black, no treatment; gray, continuous pressurization; white, reciprocal pressurization. "Control" treatment in distilled water. Additives tested are 12% glucose solution, 6% sodium chloride solution, and 20% ethanol solution.

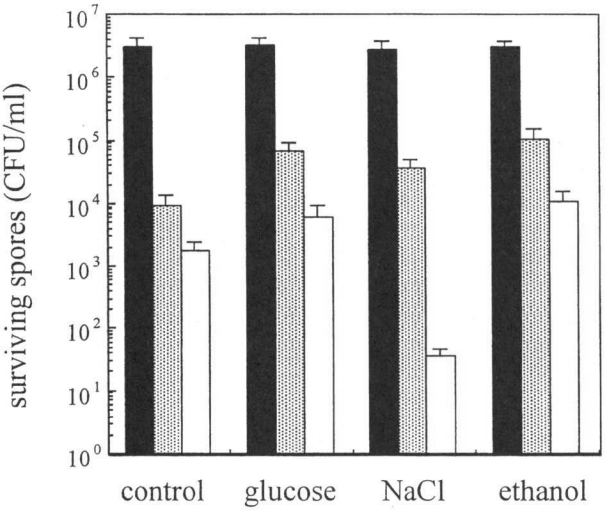

FIG. 2. Effect of food additives on surviving spores of *B. coagulans* treated by continuous and reciprocal pressurization at 75℃, 400 MPa. Black, no treatment; gray, continuous pressurization; white, reciprocal pressurization. "Control" treatment in distilled water. Additives tested are 12% glucose solution, 6% sodium chloride solution, and 20% ethanol solution.

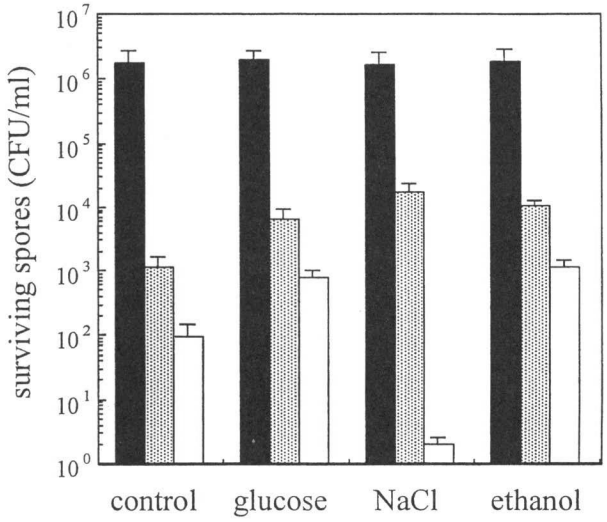

FIG. 3. Effect of food additives on surviving spores of *B. stearothermophilus* treated by continuous and reciprocal pressurization at 75℃, 400 MPa. Black, no treatment; gray, continuous pressurization; white, reciprocal pressurization. "Control" treatment in distilled water. Additives tested are 12% glucose solution, 6% sodium chloride solution, and 20% ethanol solution.

sterilization. It is known that the heat sensitivity of spores decreases in proportion to the decrease of water activity of the spore suspension (Alderton et al., 1980; Hänulv et al., 1977; Mazas et al., 1999; Murrell and Scott, 1966). Addition of food additives decrease the water activity. The water activities were 0.98 for the glucose, 0.97 for the sodium chloride and 0.94 for the ethanol added solution, respectively. Thus, it was considered that the decrease in the water activity decreased the inactivation in CP and RP treatment. However, sodium chloride in RP treatment increased the inactivation ratio. We considered there was another mechanism at work.

Hydrostatic pressure treatment can initiate the germination of dormant bacterial spores in a germinator free suspension (Clouston and Wills, 1969; Gould and Sale, 1970), and sensitive germinated spores are inactivated (Sale et al., 1970). Decrease in the water activity decreases the germination of bacterial spores under high hydrostatic pressure (Raso et al., 1998). Therefore, it was considered that the decrease in the water activity by the addition of glucose and ethanol decreased the germination rate, and, as a result, gave a large number of survivors. Ethanol can kill vegetative cells but had little effect on killing bacterial spores (Larson, 1991). It was considered that the addition of ethanol did not inactivate the spores in the present study.

In our previous study, the addition of sodium chloride decreased the inactivation of bacterial spores by low hydrostatic pressure treatments (Furukawa and Hayakawa, 2000). However, in the present study, the addition of sodium chloride increased the effectiveness of RP treatments in the inactivation of the bacterial spores. Some researchers reported that the addition of sodium chloride increased the inactivation ratio of bacterial spores by heat treatments (Briggs and Yazdany; 1970; Cook and Gilbert, 1969; Lóez, et al., 1996). It could be considered that our results for the RP treatment corresponded to these reports. In general, the medium to which sodium chloride has been added is used in the estimation of injured spores (Feeferry et al., 1987; Gonzáez et al., 1997; Iandolo and Ordal, 1966; Morichi, 1989; Tsuchido, 1999); therefore, injured spores are sensitive to sodium chloride. It was indicated that the spores were significantly inactivated more by RP treatments than by CP treatments at the pressure above 200MPa (Furukawa et al., 2000a; Furukawa et al., 2000b). The inactivation ratio of spores in RP treatment exceeded the ratio in CP treatment at the second decompression in *B. subtilis* (Furukawa et al., 2001). The injury of spores increased depending on the number of RP cycles, and this injury was estimated by using the medium with sodium chloride for *B. subtilis* spores (Furukawa et al., 2001). Therefore, in the present study, it was considered that the lower inactivation ratio by CP treatments and the higher that ratio by RP treatments in the sodium chloride solution could be attributed to the increase of the injured and sodium chloride sensitive spores brought about by RP treatments.

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