Inactivation of Food Microorganisms by High-pressure Carbon Dioxide Treatment with or without Explosive Decomposition

Atsushi Enomoto,‡ Kozo Nakamura,* Kiyotaka Nagai, Takeki Hamamoto, and Masaru Hikoda

Department of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, 1–5–1 Tenjin, Kiryu, Gunma 376, Japan

*Department of Applied Biological Chemistry, Graduate School of Agriculture and Agricultural Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113, Japan

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In order to elucidate the sterilization mechanism underlying the explosive decompression system, baker’s yeast was pressurized with CO₂, N₂O, N₂, or Ar gas at 40 atm and 40°C for 4 h, and then explosively discharged. The survival ratio was markedly decreased only by the treatments with CO₂ and N₂O, which are relatively soluble gases in water, suggesting that the microorganisms’ death may be highly correlated with gas absorption by the cells. Lower decompression rates to atmospheric pressure, however, led to neither any lower reduction of remaining cells nor any smaller release of total cellular proteins. Furthermore, operating with a longer treatment time and smaller number of repetitions was usually more lethal than with a shorter time and more frequent repetition. From these results, most of the yeast cells appear to have been sterilized during the pressurization process. The spore cells of B. megaterium are considered to have been killed in a somewhat different manner, because of their distinct sensitivity to the applied gases.

Key words: sterilization; high-pressure carbon dioxide; food microorganism; survival ratio; decompression rate

Although heat sterilization is the traditional and most popular method for protecting foods from microbial spoilage, it may often cause hostile changes in the nutritional and sensory properties of the products because of the high temperature involved. To develop and establish a novel and effective alternative to heat treatment, the lethal action of high-pressure CO₂ on microorganisms, with no or only a minimal heating process, has recently received a great deal of attention.

Among the methods, the explosive decompression system consists of a pressurization stage to induce penetration of the applied gas into the microbial cells and a subsequent explosive discharge that results in rapid gas expansion within the cells. With this system, the greater part of the microbial cells are believed to be mechanically ruptured like a popped balloon at the moment of flash depressurization.1–6 This technique was first reported by Fraser in 1951, and he found that E. coli cells were significantly disrupted by the quick release of CO₂ gas from 500 lb/in² (about 34.0 atm) to atmospheric pressure.1 This method has subsequently been developed mainly for unit operation to recover intracellular enzymes, recombinant-DNA proteins and nucleic acids from microbial cell cultures.1–5 We have recently demonstrated with a similar system that 10⁸ cells/ml of baker’s yeast could be thoroughly killed after CO₂ saturation at 40 atm and 40°C for 3 h, and proposed this as a new method for sterilizing food microorganisms.5,8

Other approaches involving no flash pressure drop, using high-pressure CO₂ treatment alone, have also been widely studied to provide the required lethal action on various kinds of microbes.9–17 For example, Kamihira et al. have confirmed that four kinds of microorganisms, baker’s yeast, E. coli, S. aureus, and conidia of A. niger, were highly sterilized by contact with supercritical CO₂ at 200 atm and 35°C for 2 h.11 In addition, Wei et al. have examined the ability of high-pressure CO₂ treatment for controlling pathogenic bacteria in model food systems, and found that their treatment at 13.7 MPa (136.1 atm) and 35°C for 2-h was capable of effectively killing S. typhimurium or L. monocytogenes in spiked chicken meat, egg yolk, shrimp, orange juice, etc.15 From these investigations, most microbial cells appear to be sterilized mainly due to the inactivating effect of CO₂ under pressure; this may be in conflict with the foregoing explanation of the sterilization mechanism for explosive decompression.

In this present study, baker’s yeast (Saccharomyces cerevisiae) and the spore cells of Bacillus megaterium were selected as the test organisms. Using the explosive decompression system, we examined and compared in detail the sterilizing effect of pressurization and subsequent flash decompression treatments with CO₂, N₂O, N₂, or Ar on these microbes under various conditions of pressure, temperature, and treatment time. In particular, such important factors as the magnitude of the discharge rate and the number of repeated releases, which were expected to strongly influence the degree of sterilization in the rapid decompression stage, were carefully investigated to elucidate the sterilization mechanism underlying the explosive decompression system. From these results, we discuss the differences in lethal mechanism by the high-pressure CO₂ treatment with and without the rapid pressure discharge. Our findings may be useful for establishing a simple, safe, inexpensive, and effective sterilization method for heat-sensitive materials.

‡ Corresponding author. Atsushi Enomoto, Department of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, 1–5–1 Tenjin, Kiryu, Gunma 376, Japan (TEL: 0277–30–1464; FAX: 0277–30–1465; E-mail: enomoto@cc.gunma-u.ac.jp).
Materials and Methods

Microorganisms. Saccharomyces cerevisiae (baker’s yeast), one of the Gram-positive eukaryotes with a two-layer structure for the cell wall including mannoprotein units and structural glucan units, was purchased from Oriental Yeast Co. (Tokyo, Japan). Bacillus megaterium ATCC 2872, a spore-forming Gram-positive bacteria, was kindly provided by Ms. N. Amaya, Dr. T. Nagai, and Mr. T. Saeki of Suntory Ltd. (Osaka, Japan). The spore cells possess extremely robust and rigid cell walls comprising the cortex, inner coat and outer layer, and are, as a result, highly resistant to heat, radiation, and most chemical disinfectants.

Apparatus. Pressurization and the subsequent decompression experiments were carried out in the slurry reservoir of a preparative column with an internal volume of approximately 40 ml (GL Sciences, Tokyo, Japan), as described elsewhere. A chromel-alumel needle-type thermocouple (Kobayashi Rikikai Co., Tokyo, Japan) and a pressure transducer (Oshika Electric Co., Tokyo, Japan) were connected through Union-type fittings to the vessel to monitor the time-course of temperature and pressure, respectively. In addition, a line filter with a pore size of 2 μm (CRF-2, GL Sciences) was also mounted to avoid unnecessary dissemination of the microbial slurry. The vessel and all other parts exposed to high pressure were made of stainless steel. Between each sterilization run, the system was cleaned with sterile distilled water and 70% ethanol.

Procedure for sterilization. A glass cup containing about 1 × 10^6 cells of the yeast or 10^6 cells of the spore in 1 ml of sterile distilled water was placed in the slurry reservoir. The vessel was then placed in a thermostatically controlled water bath to maintain the designated temperature in the range from 20°C to 60°C, and commercially available CO₂, N₂, N₂O, N₂, or Ar gas was gradually introduced into the vessel through a three-way valve until the pressure had reached the desired level between 10 atm and 60 atm. The bacterial sample was then kept for a certain period up to 24h at constant temperature and pressure during each experiment. At the end of the experiment, the valve was opened as rapidly as possible to allow a flash discharge of the applied gas from the vessel. In general, the time to thoroughly release the applied pressure was within approximately 50 s. If a slower rate for decompression was desired, the vessel was carefully depressurized at an almost constant rate for 1.5–2 h. All experiments were performed at least in duplicate.

Determination of the sterilization efficiency. To assess the sterilization efficiency of the procedure, the survival ratio for each sample was determined by an agar-plate count. All samples were serially diluted with sterile distilled water (for the yeast) or with 0.1% peptone solution (for the spore) and then plated on a malt (for the yeast) or nutrient-broth (for the spore) agar medium to enumerate the colonies after incubating the plates for 2 days (for the yeast) or at 37°C for 24 h (for the spore). The survival ratio is expressed as the mean viable count for the experimental group (treated with the high-pressure gas) divided by that for the control group (incubated at the same temperature for the same period in an air atmosphere).

Evaluation of the bactericidal effects of explosive decompression. The lethal action on the yeast cells, which was induced by the decompression process, was carefully evaluated by 3 distinct approaches. Firstly, two sets of baker’s yeast samples with an initial number of 10^6 cells/ml were exposed to CO₂ and N₂O at 40 atm and 40°C, and then decompressed to atmospheric pressure at 2 different rates: one was decompressed as rapidly as possible (explosive decompression; an average discharge rate of 48 atm/min), and the other was much more slowly depressurized at an almost constant rate of 0.33 atm/min. The survival ratio of each sample was then determined as just described.

Next, the effect of the repeated release of the applied CO₂ pressure was examined to compare its ability to elevate the sterilization efficiency on baker’s yeast. The vessel containing about 10^6 cells/ml of the yeast was pressurized with CO₂ at 40 atm and 40°C for a designated treatment time (0.25, 0.5, 1.2, or 4 h), and explosively decompressed to ambient pressure. As soon as possible, CO₂ gas was again charged into the vessel under the same pressurization conditions for the same period. This operation with explosive decompression was conducted up to 4 times.

Finally, the amount of total proteins released from 10^6 cells of the yeast was determined and the results compared after treatments at 40 atm and 40°C for 4 h with or without the explosive decompression. After the high-pressure treatment, each sample was centrifuged, and the total protein content of the supernatant was then determined by Lowry’s method with bovine serum albumin (Seikagaku Kogyo Co., Tokyo, Japan) as the protein standard.

Results and Discussion

Bactericidal effects of several kinds of high-pressure gas

In order to develop a novel and effective method for sterilizing harmful microorganisms without adversely affecting the quality of food products, S. cerevisiae and spore cells of B. megaterium as model food organisms were first pressurized with CO₂, N₂O, N₂, or Ar gas at constant pressure and temperature, held at the same conditions for a period of 4 h or 24 h, and then rapidly decompressed. As shown in Table I, 10^6 cells of the baker’s yeast were killed to a considerable extent after the microbe had been exposed to CO₂ or N₂O gas, which is highly soluble in water, at 40 atm and 40°C for 4 h. On the other hand, both N₂ and Ar gases, which have poor solubility in water, had almost no effect on the survival ratio of the yeast even under the same experimental conditions. From these results, the yeast cells did not appear to be killed only due to the high applied pressure up to 40 atm, and their death is considered to be highly correlated with gas absorption by the cells.

In contrast to the baker’s yeast, the spore cells of B. megaterium seemed to be sterilized by the high-pressure gases in a somewhat different manner; the survival ratio was markedly decreased by the CO₂ treatment at 60 atm and 60°C for 24 h, while the other 3 gases, including N₂O, which exerted a significant bactericidal effect on the yeast, had no lethal action on the spore even under the same conditions (Table I). It is noteworthy that the spore cells may not have been killed by the heat treatment alone. Taking into consideration the advantages of CO₂ over many other gases in food applications, for example, its nontoxicity, nonflammability, and inexpensiveness, it is concluded that CO₂ may be the most useful gas for sterilizing food organisms such as bacterial spores and baker’s yeast.

Effects of the applied pressure and temperature

It has been well documented that the pressure and temperature conditions of the applied CO₂ gas are generally dominant factors which strongly affect the degree of sterilization. Figure 1 shows the pressure and temperature dependence of the sterilizing effect under a constant treatment time (24h). The bactericidal effect of the CO₂ treatment on baker’s yeast was found to be dramatically enhanced with increasing pressure ranging from 10 atm to 40 atm and temperature in the range of 20°C

<table>
<thead>
<tr>
<th>Gas</th>
<th>Baker’s yeast</th>
<th>Spores of B. megaterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO₂</td>
<td>1.50 × 10⁻⁷</td>
<td>1.56 × 10⁻⁶</td>
</tr>
<tr>
<td>N₂O</td>
<td>2.00 × 10⁻⁵</td>
<td>0.90</td>
</tr>
<tr>
<td>N₂</td>
<td>0.93</td>
<td>0.93</td>
</tr>
<tr>
<td>Ar</td>
<td>0.98</td>
<td>0.91</td>
</tr>
</tbody>
</table>

a The yeast cells, which had been adjusted to approximately 1 × 10^6 cells/ml at the initial stage, were pressurized with the indicated gas at 40 atm and 40°C for 4 h, and then explosively decompressed.

b The spore cells (about 1 × 10^8 cells/ml) were treated with the indicated gas at 60 atm and 60°C for 24 h, and then explosively decompressed.
Fig. 1. Effects of Pressure and Temperature on the Survival Ratio of Baker's Yeast.
The yeast cells (about 10^6 cells/ml) were subjected to high-pressure CO₂ under the indicated pressure and temperature for 24h, and the pressure was then quickly discharged.

Table II. Effect of Decompression Rate on the Survival Ratio of Baker's Yeast

<table>
<thead>
<tr>
<th>Decompression</th>
<th>Average rate (atm/min)</th>
<th>Survival ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Explosively</td>
<td>48</td>
<td>1.00 × 10^-7</td>
</tr>
<tr>
<td>Slowly</td>
<td>0.33</td>
<td>1.50 × 10^-7</td>
</tr>
</tbody>
</table>

* After 10^6 cells/ml of baker's yeast had been saturated with CO₂ at 40 atm and 40°C for 4h, they were released to ambient pressure explosively or slowly.

to 40°C, and these two factors tended to synergistically act with each other; the survival ratio became nearly the same between 10^-4 and 10^-5 under conditions of 20 atm and 40°C, 30 atm and 35°C, and 40 atm and 30°C. These results are consistent with our previous works^a,b in which the treatment time was designed to be within 4h.

Lethal effects of explosive decompression

To elucidate the sterilization mechanism underlying the explosive decompression system, the lethal action induced in the depressurization stage was carefully evaluated by three different approaches. Firstly, we examined the effects of the average discharge rate on the survival ratio of baker's yeast. The results in Table II show that the slower decompression rates would not always lead to a lower reduction of the remaining cells; the survival ratio for the 2 sets of yeast cells both became quite small, approximately 10^-7, in spite of the large difference in the average decompression rates (48 and 0.33 atm/min). Although it is necessary to take into account that the slower discharge rates result in longer contact time with the high-pressure gas, these results suggest that the sterilizing effect of explosive decompression may be much lower than expected.

The repeated release of the applied CO₂ pressure was then examined and compared for its ability to elevate the sterilization efficiency on baker's yeast. As shown in Fig. 2, when the total time of the pressurization process was the same, operation with a longer treatment time and smaller number of repetitions was usually more effective for killing the yeast cells than that with a shorter treatment time and more frequent repetition. For example, the survival ratio was found to be approximately 10^-3 after the CO₂ treatment for 2h with no repeated pressure release, whereas 4 sets of the similar treatment for 0.5h resulted in about a one-order-lower reduction of the survival ratio. Although Lin et al. have observed an improvement in the disruption and inactivation rates of baker's yeast with a series of pressure release/recompression treatments with CO₂ gas in the range between 1000 psi (about 68.0 atm) and 3000 psi (204 atm),^a,b,d these results suggest that the explosive decompression of CO₂ would influence the survival ratio of the yeast cells to a lesser extent, at least with our system.

Our previous observations with a scanning electron microscope have shown that the yeast cells, at the least some of them, may be mechanically ruptured by the CO₂ treatment with explosive decompression.^c,d To evaluate the extent of the cells subjected to such mechanical breakage, especially that caused by the flash discharge, the amount of total proteins released from 10^8 cells of the yeast was determined and compared after treatments at 40 atm and 40°C for 4h with or without explosive decompression. As shown in Table III, the amount released after both high-pressure treatments was found to be about 3 times greater than that following incubation in an air atmosphere (the control value). According to the assumption that proteins normally comprise 40–50% of the total weight of dry yeast cells,^a, b approximately 30% of the total cellular proteins

Fig. 2. Effect of Repeated Explosive Decompression on the Survival Ratio of Baker's Yeast.
The yeast cells (about 10^6 cells/ml) were exposed to CO₂ gas at 40 atm and 40°C for the indicated length of treatment time, and then explosively decompressed. This operation was conducted up to 4 times. The total pressurization time is expressed as "t₁" in the figure.

Table III. Effect of Decompression Rate on the Release of Total Proteins from Baker's Yeast

<table>
<thead>
<tr>
<th>Decompression</th>
<th>Average rate (atm/min)</th>
<th>Total proteins (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Explosoely</td>
<td>48</td>
<td>6.86</td>
</tr>
<tr>
<td>Slowly</td>
<td>0.44</td>
<td>7.05</td>
</tr>
<tr>
<td>No pressuriz.</td>
<td>—</td>
<td>2.43</td>
</tr>
</tbody>
</table>

* The yeast cells (about 10^6 cells/ml) were subjected to CO₂ gas at 40 atm and 40°C for 4h, and then depressurized to atmospheric pressure explosively or slowly.

* The yeast cells were incubated at the same temperature for the same period in an air atmosphere.
was estimated to have been released by the CO₂ treatment. Table III, however, also shows that more rapid decompression would not always lead to a greater release of total proteins, suggesting that the microbial cells may not be ruptured mainly due to explosive decompression. Taking into consideration Tables II, III, and Fig. 2 together, most of the yeast cells appear to have been sterilized during the pressurization step, and not during the rapid discharge stage in our system.

Sterilization mechanism

An explosive decompression system such as that with our apparatus always involves pressurization with an appropriate gas, usually CO₂, and then rapid decompression of the applied pressure. Under pressurization, the microbial cells are thought to be gradually penetrated and filled with the gas. When the applied pressure is then suddenly released, the absorbed gas will quickly expand within the cells. With this mechanism, the death of the target organisms has been, therefore, believed to have occurred mainly due to mechanical rupture that would be caused by the desorbed gas at the moment of flash discharge.¹⁻⁶ Our present results with the baker's yeast shown in Tables II, III, and Fig. 2, however, suggest that the sudden pressure drop may not play such a significant role in microbial reduction, as Arreola et al. have also pointed out.¹⁴⁻¹⁵ Furthermore, when using a similar experimental system, Kumagai et al. have recently found that the sterilization rate for S. cerevisiae could be described as a first-order reaction, and also argued that the yeast cells were killed largely during the pressurization stage,¹² although their discharge rate (8 MPa/h) is considered to be rather slow. From these results, most of the yeast cells did not appear to have been killed during the explosive decompression stage.

The inhibitory effects of CO₂ under pressure on various kinds of microorganisms have been extensively studied to elucidate the sterilization mechanism.¹⁻³⁻¹²⁻¹⁵ In these reports, the death of the target cells is usually explained as follows: during pressurization with CO₂, the absorbed gas may cause the inactivation of key enzymes related to the essential metabolic process, probably due to the decreased pH value inside the cells and/or the solubilization of intracellular substances such as hydrophobic compounds in the cell wall and cytoplasmic membrane. A similar mechanism also seems to play an important role, even with the explosive decompression system. In addition to such a mechanism, the results of our previous microscopic observations⁵⁻⁸ and of the present study as shown in Table III suggest that some of the yeast cells may also be mechanically broken by the pressurization process, and not in the decompression stage. When pressurized with CO₂ gas, gas absorption may cause swelling of the microbial cells, thereby resulting in their mechanical rupture. In conclusion, microbial cells such as baker's yeast are considered to be predominantly killed due to complex effects of the absorbed gas that involve physiological damage as well as mechanical rupture during the pressurization process. In this context, it may not be worthwhile to clearly distinguish between high-pressure CO₂ treatments with and without explosive decompression, at least when the average discharge rate is less than 48 atm/min.

In contrast to the foregoing explanation, different experimental data have also been reported to support the lethal action of rapid discharge by the explosive decompression system, as already mentioned. For example, Fraser¹⁰ has shown that E. coli cells were recovered nearly intact after the pressure of a culture was slowly released, while rapid discharge under similar experimental conditions resulted in a significant decrease in the viable number of microbial cells. Furthermore, we have just found that higher decompression rates up to 48 atm/min could lead to about a two-order-higher reduction of the remaining cells when 1 x 10⁶ cells/ml of baker's yeast had been treated with CO₂ at 40 atm and 40°C for 4 h (preliminary data). In the case of these experiments, a considerable number of the microbial cells can be assumed to have remained unsterilized at the end of pressurization, and the bactericidal effect due to the explosive decompression may, therefore, become clearly detectable. On the other hand, most of the other experimental procedures are usually thought to be responsible for the extremely small number of cells remaining at the same point, and explosive decompression may not actually exert the lethal effect. If this is true, it should be said that explosive decompression essentially possesses potentially lethal activity toward microorganisms such as E. coli and baker's yeast.

Although the exact mechanism remains obscure at this time, other sterilizing effects appear to act on the bacterial spores; the survival ratio of baker's yeast became quite small by treating with N₂O at 40 atm and 40°C for 4 h, whereas a more severe treatment at 60 atm and 60°C for 24 h had almost no effect on the spore cells of B. megaterium (Table I). In addition, no significant release of total proteins from 10⁹ cells of the bacterial spores was apparent even after the CO₂ treatment at 40, 60, or 100 atm and 60°C for 24 h (data not shown), suggesting that the spore cells may not have been mechanically broken. Further investigations are required to precisely elucidate why the high-pressure CO₂ treatment with our system had a lethal effect on the spore cells of B. megaterium.

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References