Disruption of Microbial Cells by the Flash Discharge of High-pressure Carbon Dioxide

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In order to develop a novel sterilization method for heat-sensitive materials, the disruption of microbial cells by the rapid release of gas pressure was examined under various conditions of pressure, temperature, treatment time and water content of the cells. Wet cells of baker's yeast were completely destroyed, after the microorganisms had been saturated with CO₂ gas at 40°C and 40 atm for more than 3 h when the pressure was suddenly discharged. On the other hand, dry cells were poorly killed even under the same experimental conditions. In particular, N₂ gas with low solubility in water had no effect on the survival ratio of the yeast. From these results, the death of microorganisms may be caused by mechanical breakage and/or physiological damage related to gas sorption and desorption by the cells.

Heat sterilization is the traditional and most popular method to prevent foods from microbial spoilage. This process, however, may often cause undesirable changes in the quality of the products such as decomposition of the nutrients, production of off-flavors, and deterioration of the color and texture because of its high temperature. For example, orange juice flavor is heat-sensitive; therefore, its pasteurization process must minimize the flavor loss. In particular, spices and herbs are very difficult to sterilize by heat treatment without lowering their quality.

To resolve these problems, a variety of novel sterilization methods, including no or a minimal heating process, have been widely studied. Among these methods, the use of ethylene oxide or radial rays is indeed effective, but its application is limited in scope to special materials by law in Japan. A new method with superheated steam has been used to sterilize powdery and granulated food materials, but its intensive localized heating effects sometimes result in a deterioration in their quality. Although antimicrobial agents are also utilized in the food industry for food preservation, they do not always exert the optimal bactericidal effects. The lethal effects of (ultra) high hydrostatic pressure have recently received a great deal of attention, and are applied in a few food processes such as the preparation of strawberry jam. The extremely high pressures in excess of 1000 atm, nevertheless, result in expensive facilities for batch operation, and some undesirable changes in the sensory properties are still inevitable.

In the present paper, we describe the disruption of microbial cells by a rapid release of gas pressure under moderate conditions (less than 40 atm). This technique, which was first reported by Fraser in 1951 and then developed by several workers for unit operation to recover intracellular enzymes and recombinant-DNA proteins from microbial cell cultures, consists of sequential pressurization and explosive decompression. This method for the disruption of microorganisms is considered to proceed as follows. With pressurization, the microbial cells are gradually penetrated and filled with gas, usually carbon dioxide. After saturation by the gas, the applied pressure is suddenly released. In this step, the absorbed gas will rapidly expand within the cells; therefore, a part of the cells may be mechanically ruptured like a popped balloon.

By using baker's yeast as the test organism, we examined and compared in detail the sterilizing effects by flash discharge of high pressure CO₂ or N₂ gas under various conditions of pressure, temperature, treatment time and moisture content of the cells. Under each experimental condition, the initial rate for gas desorption was estimated by assuming the desorption to proceed with quasi-equilibrium, and the relationship between the sterilization efficiency and the desorption rate was evaluated to elucidate the sterilization mechanism. Our results may be useful for the establishment of a simple, safe and inexpensive sterilization method for heat-sensitive materials.

Materials and Methods

Microorganism, Saccharomyces cerevisiae (baker's yeast), a Gram-positive eukaryote with a two-layer structure for the cell wall including mannanprotein units and structural glucon units, was purchased from Oriental Yeast Co., Tokyo, Japan. The initial water content of the cells was adjusted by adding sterile distilled water to nominally dry cells with about 8% moisture content, the microbe being used in this study without precultivation.

Equipment. Pressurization and 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 illustrated in Fig. 1. A chromel-alumel needle-type thermocouple (Kobayashi Rikakikai Co., Tokyo, Japan) and a pressure transducer (pressure range of 0–500 kg/cm²; G; less than 1 ms minimum response time, Ohkura Electric Co., Tokyo, Japan) were connected through Union-type fittings to the vessel to monitor the time-course of temperature and pressure, respectively, and 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. If a greater rate of decompression was desired, the line filter was removed in some experiments. The vessel and all other parts exposed to high pressure were constructed in stainless steel. Between each sterilization run, the system was cleaned with sterile distilled water and 70% ethanol.

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Procedure for sterilization. A glass cup containing about 1.0 × 10⁶ cells of baker’s yeast moistened with sterile distilled water was placed in the slurry reservoir. The vessel was then enclosed and put into a thermostatically controlled water bath to maintain a designated temperature in the range from 0°C to 40°C. After the temperature had become stable, commercially available CO₂ or N₂ gas was gradually (to minimize the effect of heating due to compression of the gas) forced into the vessel through a three-way valve until the desired pressure of 10–40 atm was reached. The bacterial sample was then kept for a certain period up to 5 h 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 gas from the vessel. In general, it took approximately 2.5 min for our system with the line filter to attain 40 atm, decompression to atmospheric pressure was achieved within 50 s, and the maximum initial discharge rate being estimated to be about 5 atm/s (see Fig. 7). All experiments were performed in duplicate or triplicate.

Determination of the sterilization efficiency. To evaluate the sterilization efficiency of the procedure, the survival ratio for each sample was determined by an agar-plate (viable) count. All samples, experimental groups treated with high-pressure gas, and control groups incubated at the same temperature for the same period under an air atmosphere were serially diluted with sterile distilled water and plated on a malt agar medium to enumerate the colonies (living cells) after incubating the plates at 30°C for 2 days. Each survival ratio is expressed as the mean viable cell for the experimental groups divided by the mean viable count for the control groups. Morphological changes in the microbial cells were assessed by direct observation with a scanning electron microscope (model JSM-5300LV, JEOL, Tokyo, Japan).

Results and Discussion

Bactericidal effects on baker’s yeast

As shown in Figs. 2–4, the death of baker’s yeast by the flash discharge of high-pressure CO₂ gas was found to be markedly affected by the pressurization conditions. Within the limits of this study, the bactericidal effect of the CO₂ treatment was dramatically increased with increasing pressure ranging from 20 atm to 40 atm (Fig. 2), by the temperature in the range of 20°C to 40°C (Fig. 3), and by the length of treatment time ranging from 0.5 h to 3 h (Fig. 4).

Under the most appropriate conditions (40 atm pressure; 40°C temperature; and more than 3 h treatment time), the survival ratio of wet cells with approximately 80% moisture content could be reduced to 1/10⁶.

The initial water content of the cells and the solubility of the gas in water were also primary factors which influenced the bactericidal effects (Figs. 2 and 5). As shown in Fig. 5, wet cells with more than 60% moisture content were entirely destroyed when the organisms were saturated with CO₂ gas, which is highly soluble in water, at 40 atm and 40°C for 5 h, before the pressure was suddenly released.

On the other hand, dry cells with less than 40% moisture content were poorly killed, even under the same experimental conditions. In contrast to the CO₂ treatment, N₂ gas with limited solubility in water had no effects on the survival ratio of the yeast; 91.2% of the wet cells remained unsterilized after N₂ saturation at 40 atm and 40°C for 4 h.

The initial or average decompression rate with high-
pressure CO₂ gas also seemed to influence the degree of sterilization efficiency. A comparison of the experimental data in the presence or absence of the line filter (Fig. 2) indicates that more rapid decompression without the filter generally resulted in a higher reduction of the survival ratio. This may also be the case for Fig. 4, where the sterilization efficiency of the CO₂ treatment with the filter at 40 atm and 30°C is lower than that without the filter at 20 atm or 30 atm. Further studies will be required to precisely elucidate the bactericidal effect of the decompression rate.

From these results, the reduction in the survival ratio is thought to be highly correlated with the gas absorption and desorption by the cells. A similar conclusion has also been independently demonstrated by other workers, using several kinds of gases (CO₂, N₂, N₂O, C₂H₄, and Ar) and microorganisms (S. cerevisiae, E. coli, S. marcescens, S. aureus, B. abortus, E. tenella, B. subtilis, etc.), but this report is the first to systematically analyze in detail the sterilizing effects by a rapid release of gas pressure under relatively mild conditions (less than 40 atm).

**Morphological changes in baker's yeast**

A scanning electron microscope was then utilized to examine the morphological changes in the baker's yeast. Figure 6 shows micrographs of the wet cells before and after the CO₂ treatment at 40 atm and 40°C for 5 h. After the treatment, the appearance of “holes” and “wrinkles” was recognized in the cell surfaces (lower panel), and some cells were found to have completely burst (middle panel), while normal cells appeared as “spheres” with a particularly smooth surface (upper panel). Although we have not confirmed the extent of the cells encountering such morphological changes, these observations suggest that yeast cells, at the least some of them, will be mechanically ruptured by the CO₂ treatment.

**Effects of initial gas desorption rate**

As described in the introduction, at the moment of flash discharge, the overfilled gas is rapidly desorbed in the microbial cells to introduce an unbalanced pressure between the inside and outside of the cells. If this pressure difference exceeds a certain limit, the cell walls cannot endure the stress and, as a result, the cells will be mechanically ruptured. According to this sterilization mechanism, the initial rate of gas desorption is considered to be the most important factor, and was tentatively calculated by a simple method to be correlated with the experimental sterilization efficiency. This simple method does not take into consideration rate processes such as the nucleation of gas bubbles in the cells, and the desorption of gas is assumed to proceed in a quasi-equilibrium state. The initial gas desorption rate can then be expressed by the following equation:

\[ (-dq/d\theta)_0 = [(dq/dp) + (dq/dT) \cdot (dT/dp)]_0 \cdot (dp/d\theta)_0 \]  

(1)

where \( q \) is the amount of gas sorbed in a unit volume of the cells, \( p \) and \( T \) are the pressure in the vessel and the absolute temperature in the microbial sample, respectively, and suffix 0 expresses the initial state. Equation (1) can be rewritten as Eq. (2) below, if the temperature of the sample changes similarly to that of an ideal gas in the reversible
Fig. 7. Time-Course for the Pressure Change Corresponding to Rapid Decompression from 40 atm to Atmospheric Pressure in the Presence of the Line Filter.

adiabatic expansion, and when the pressure decreases exponentially as expressed by Eq. (3).

$$(- \frac{dq}{dT})_0 = -a \cdot (p_0 - p_a) \cdot \left\{ \frac{dq}{dp} + \left[ (\gamma - 1)/\gamma \right] \cdot \left( T/\rho \right) \cdot \left( \frac{dq}{dT} \right)_0 \right\}$$

$$p/(p_0 - p_a) = \exp(-aT)$$

where symbol $a$ is the time constant, suffixes 0 and $a$ express the initial and the atmospheric states, respectively, and $\gamma$ is the ratio of the constant-pressure specific heat to constant-volume specific heat.

Water, proteins and polysaccharides in the microbial cells are assumed to be the main components of the CO$_2$-absorber or adsorbent, and the quantity of $q$ is given as a function of the pressure and temperature, which can be evaluated by using the data for CO$_2$ absorption into water$^{12}$ and CO$_2$ adsorption to several kinds of proteins$^{13}$ and polysaccharides.$^{14}$ Carbon dioxide absorbed into water is the greater part of $q$, unless the moisture content of the cells is extremely low.

Figures 7 and 8 show the observed time-course for pressure, and the relationship between the temperature and pressure when the microbial samples were quickly decompressed from 40 atm to atmospheric pressure. The time-course for the pressure decrease with the line filter could be correlated with Eq. (3), especially at an initial temperature ranging from 30°C to 40°C, and the initial discharge rate was calculated to be approximately 5 atm/s (time constant $a=0.13$ s$^{-1}$) and independent of the temperature. The temperature decrease was less significant than that for the adiabatic expansion of an ideal gas, at least in the presence of the filter. The initial slopes of $d\rho/dt$ and $(dT/dt)$ were determined from the data in Figs. 7 and 8, and then used to evaluate the initial gas desorption rate by Eq. (1).

Figure 9 illustrates the relationship between the calculated gas desorption rate and the observed sterilization efficiency, corresponding to changes in the applied pressure from 10 atm to 40 atm (● in Fig. 2) and to the initial water content of the cells from 40% to 80% (● in Fig. 5). The survival ratio of the yeast was greatly decreased with increasing gas desorption rate, and became quite low as the initial rate of gas desorption approached 0.8–1.0 cm$^3$/cm$^2$-cell$^{-1}$·s. In the case of a temperature change (● in Fig. 3), the relationship between the survival ratio and the initial gas desorption rate was counter to our expectation. The estimated gas desorption rate, however, changed a little with the temperature changing from 20°C to 40°C, and this contradictory result may be compromised if the rate process, which depends on temperature, is taken into consideration to estimate the initial gas desorption rate. Another explanation for the foregoing contradiction may be possible, because the temperature influences not only the gas desorption rate but also the physical properties of the cell membranes and walls. As indicated by Lin and his coworkers,$^{9,10}$ high process temperatures tend to enhance the transfer rate of CO$_2$ and also to relax the cell walls to facilitate the permeability of CO$_2$. It will also be necessary to consider the effects of temperature on the elasticity of the cell walls and cytoplasmic membranes.

Sterilization mechanism

An explosive decompression system such as that with our apparatus involves a pressurization process that is responsible for penetration of the applied gas into microbial cells, and subsequent rapid decompression resulting in gas expansion within the cells. In this system, the death of
microorganisms is considered to have occurred mainly due to mechanical rupture by the desorbed gas at the moment of flash discharge.\textsuperscript{6-10} In fact, the effects of sudden decompression have been confirmed to be beneficial to the lysis of microbial cells such as yeast, \textit{E. coli} and \textit{B. subtilis} to recover intracellular proteins (enzymes) and nucleic acids.\textsuperscript{8-10} and Fraser had shown that \textit{E. coli} cells remained nearly intact when the pressure of a culture was slowly released.\textsuperscript{6} Our microscopic observations in Fig. 6 also support this hypothesis, part of the yeast cells being found to be mechanically ruptured.

In this explanation, the initial rate of gas desorption is thought to play an important role with high influence on the survival ratio of microorganisms. We, therefore, have estimated the initial desorption rate under each experimental condition, and then discuss the relationship between the observed sterilization efficiency and the desorption rate, as described earlier. The death of baker's yeast, however, could not always be explained by our simple method. Although a more exact evaluation of the gas desorption rate should be conducted, in addition to the effects of sudden decompression, another distinct mechanism may provide a lethal action on the microorganism in the pressurization treatment.

The inhibitory effects of CO\textsubscript{2} under pressure on microbial cells have been extensively studied.\textsuperscript{1-5,15,16} The overall mechanism underlying the death of microorganisms is still not well understood, but one possibility exists from the gas sorption of the cells. When microbial cells were pressurized with CO\textsubscript{2}, the gas gradually penetrated into the cells. This sorbed gas may cause the inactivation of key enzymes related to the essential metabolite process due to the decrease of pH inside the cells and/or to the solubilization of intracellular substances such as phospholipids and hydrophobic compounds in the cell walls and cytoplasmic membranes, as several workers\textsuperscript{1-3,15} have already pointed out. A similar mechanism may play an important role, even in the explosive decompression system.

In preliminary experiments, we found that wet cells of baker's yeast were killed to a considerable extent, even if the decompression rate was extremely small. This is in conflict with Fraser's observation,\textsuperscript{6} and more work along this line is now in progress. In addition, Arreola \textit{et al.} have revealed high microbial numbers from samples collected early during their explosive decompression experiments, and argued that a sudden pressure decrease may not play a significant role in microbial reduction, at least with their system.\textsuperscript{11} From these results, the death of microorganisms in our system may also be partially due to physiological damage by the CO\textsubscript{2} gas used in the pressurization process.

In conclusion, the sterilization mechanism is considered to result from complex effects involving physiological damage as well as mechanical rupture, even with the explosive decompression system. The lethal effects of CO\textsubscript{2} gas under high pressure seem to have resulted in the early death of baker's yeast, and the rapid expansion of the absorbed gas at the moment of flash discharge may predominantly have resulted in the disruption of both the sterilized and remaining cells. If this is true, the initial rate for gas desorption should be correlated with the magnitude of cell disruption, and will be the most important factor to influence the degree of recovery for useful intracellular substances.

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