Applied and Environmental Microbiology, Aug. 2002, p. 4162–4167 Vol. 68, No. 8
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.8.4162–4167.2002
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Accelerated Death Kinetics of *Aspergillus niger* Spores under High-Pressure Carbonation

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Received 17 December 2001/Accepted 30 May 2002

The death kinetics of *Aspergillus niger* spores under high-pressure carbonation were investigated with respect to the concentration of dissolved CO_2 (dCO_2) and treatment temperature. All of the inactivation followed first-order death kinetics. The *D* value (decimal reduction time, or the time required for a 1-log-cycle reduction in the microbial population) in the saline carbonated at 10 MPa was 0.16 min at 52°C. The log *D* values were linearly related to the treatment temperature and the concentration of dCO_2, but a significant interaction was observed between them.

Heat treatment in food processing is a broadly effective method for inactivating human pathogens and spoilage microorganisms. However, the high temperature involved can cause undesirable changes in nutritional and sensory properties.

The toxicity of CO_2 has been demonstrated for a wide range of microorganisms (5, 6, 12, 17). Carbon dioxide, while exerting antimicrobial activity, causes little harm in foods, additives, etc.; therefore, it is a suitable agent for controlling food spoilage microorganisms (22, 23, 29). In recent years, the influence of high-pressure CO_2 on the vegetative cells of various species has been demonstrated (1, 3, 4, 7, 8, 9, 10, 11, 13, 14, 18, 20, 28).

We succeeded in dissolving CO_2 in an aqueous medium to a nearly saturated level by supplying high-pressure CO_2 in microbubbles (high-pressure carbonation). As a consequence, the treatment time and temperature required for microbial inactivation could be substantially reduced (15, 16, 26). Recently, it was shown that the antimicrobial effect was not related to the pressure of CO_2 but to the concentration of dissolved CO_2 (dCO_2) (27). The experiments were carried out with saturated dCO_2 concentrations under various combinations of pressure and temperature conditions.

Most spoilages of fruit juice are caused by yeast and mold, because the germination of bacterial spores is inhibited at the pH of fruit juice (30). Shimoda et al. (27) have reported that the inactivation temperature of *Saccharomyces cerevisiae* was reduced by about 30°C under high-pressure carbonation. As mold spores have only a moderate thermal resistance, the heat treatment in fruit juice production is intended primarily to inactivate mold spores. The effects of pressurized CO_2 on the inactivation of mold spores, however, have not been investigated. A mesophilic mold, *Aspergillus niger,* was used as a test strain because it is a common contaminant of foods and other products (24).

The purpose of the present paper was to investigate separately the effects of dCO_2 concentration, treatment pressure, and temperature on the death kinetics of *Aspergillus niger* spores.

Fungal strain. Spores of *Aspergillus niger* (ATCC 16888) were obtained from the Japan Collection of Microorganisms (Wako, Saitama, Japan).

Preparation of the spore suspension of *A. niger*. The strain was cultured on potato dextrose agar plates (Eiken Chemical Co., Ltd., Tokyo, Japan) at 25°C over 10 days. Spores were collected by washing the surface of the culture. Finally, a spore suspension (about 10^8 to 10^9 CFU/ml) was prepared in physiological saline with 0.005% Tween 20.

High-pressure carbonation treatment. To simplify controlling the treatment temperature and dCO_2 concentration, an original apparatus (Fig. 1) was constructed. At first, 200 ml of physiological saline was introduced into a vessel for carbonation. Microbubbles of pressurized CO_2 were dispersed into the saline solution from a stainless steel filter (pore size, 10 μm) attached to the bottom of the carbonation vessel. After the carbonation, the solution was left to stand for about 10 min under pressure and then pressed out by introducing pressurized CO_2 into the headspace. A suspension of *A. niger* spores (~10^8 to ~10^9 CFU/ml) was introduced into the flow of carbonated saline at a flow rate of 0.33 to 2.8 ml/min. The total flow rate of the carbonated saline and spore suspension ranged from 3.3 to 28 ml/min. After the carbonated saline containing the spores flowed through a residence tube (4-mm inside diameter, 56 cm in depth), it was decompressed via a pressure control valve, with the pressure being kept within ±0.1 MPa. It took only a few milliseconds to decompress the stream of carbonated spore suspension, since the entire stream flowed through the pressure-regulating port (about 1 mm^2) under propulsion by a spring. The apparatus was placed in a water bath to keep the treatment temperature within ±0.2°C. The volume of gaseous CO_2 generated from the carbonated spore suspension was measured in the outlet of the pressure control valve. Treatment conditions were as follows: pressure was at 5, 8, 10, 15, and 19 MPa; temperatures were at 44, 46, 48, 50, and 52°C; inactivation times were 0.25 to 2.1 min; and dCO_2 concentrations wre 0 to 24.2 γ. The concentration of dCO_2 was

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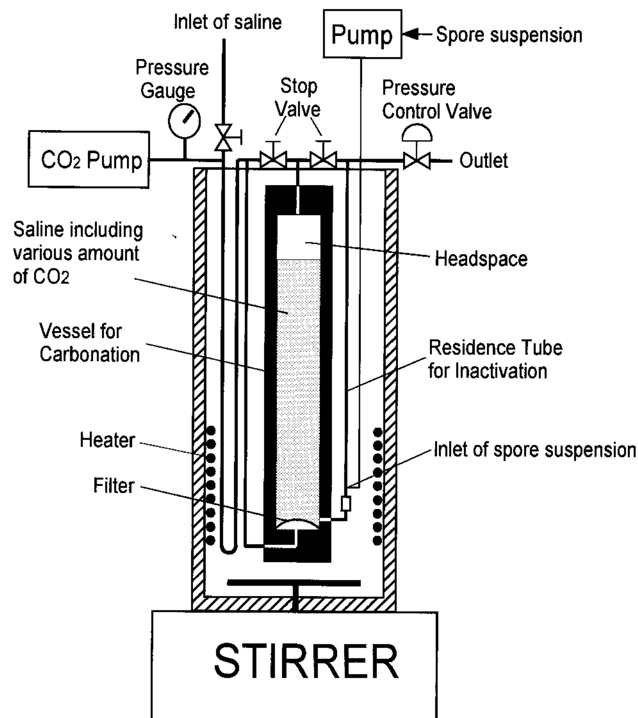

FIG. 1. Schematic diagram of the apparatus for microbial inactivation under high-pressure carbonation.

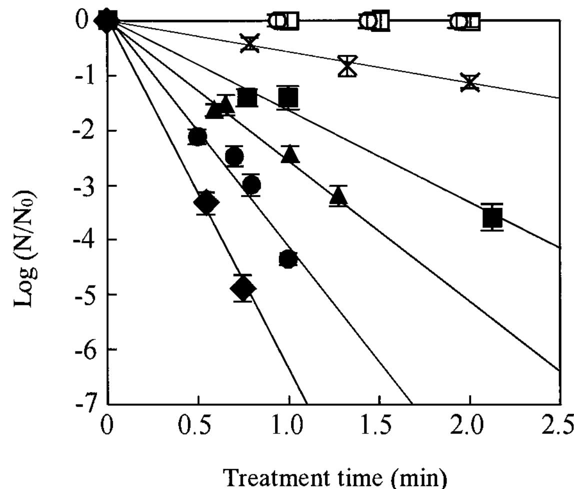

FIG. 2. Inactivation behaviors of *A. niger* spores in carbonated saline. The saline was carbonated at 10 MPa, and the concentrations of dCO_2 were ~21.7 to ~24.2 γ. Symbols indicate results of experiments in carbonated saline at 44°C (×), 46°C (■), 48°C (▲), 50°C (●), and 52°C (◆); in physiological saline at 52°C (○); and in McIlvaine buffer (pH 3.0) at 52°C (□)

estimated as a Kuenen gas absorption coefficient (γ); e.g., a γ value of 24 means that there were 24 volumes of CO_2 (in a normal state) in 1 volume of liquid.

Enumeration of spore counts. Samples were serially diluted with sterile physiological saline. Initial spore counts and counts of survivors were determined by plating 0.1-ml diluted or nondiluted samples on triplicate plates of potato dextrose agar (Eiken Chemical Co.). Colonies were counted after incubation at 30°C for 3 days.

Replication and statistical treatment. All experiments were done in triplicate. The data presented are the means of results of three replicate experiments.

Kinetic parameters for inactivation of microbial cells under high-pressure carbonation. The approach to describing changes in microbial populations as a function of time uses the survivor curve equation $\log [N/N_0] = -t/D$, where N is the microbial population at any time (t), N_0 is the initial microbial population, and D is the decimal reduction time, or time required for a 1-log-cycle reduction in the microbial population.

The influence of temperature on the inactivation rates under high-pressure carbonation was expressed in terms of the thermal resistance constant $[Z_{CO_2}(T)]$ with the model $\log [D/D_{T_0}] = -(T - T_0)/Z_{CO_2}(T)$.

The reference decimal reduction time (D_{T_0}) is the magnitude at a reference temperature (T_0) under a given concentration of dCO_2. The thermal resistance constant $Z_{CO_2}(T)$ is the temperature increase needed to accomplish a 1-log-cycle reduction in the D value at a given dCO_2 concentration.

The influence of the dCO_2 concentration on the inactivation rates was expressed in terms of the CO_2 resistance constant $[Z_{\text{temperature}}(\gamma)]$ using the following model: $\log [D/D_{\gamma_0}] = -(\gamma - \gamma_0)/Z_{\text{temperature}}(\gamma)$.

The reference decimal reduction time (D_{γ_0}) is the magnitude under a reference concentration (γ_0) of dCO_2 at a given temperature. The CO_2 resistance constant $Z_{\text{temperature}}(\gamma)$ is the increase in the concentration of dCO_2 needed to accomplish a 1-log-cycle reduction in the D value at a given temperature.

Figure 2 shows the survival curves of *A. niger* spores in the saline carbonated with microbubbles of CO_2 at 10 MPa. The relationships between the values of $\log [N/N_0]$ and treatment time strongly suggested first-order death kinetics. The concentration of dCO_2 in the treated medium was 24.4 γ for 44°C, 24.2 γ for 46°C, 23.0 γ for 48°C, 21.7 γ for 50°C, and 22.9 γ for 52°C. These values ranged from 87 to 90% of the saturated levels estimated from the data of Seidell and Linke (25) and were reasonable values if we take into account a 10% dilution with spore suspension.

The D values of *A. niger* spores in carbonated saline at 10 MPa were estimated from the slopes of straight lines in Fig. 2. The values were 0.16 min for 52°C, 0.24 min for 50°C, and 0.39 min for 48°C (Table 1). On the other hand, the spores in

TABLE 1. Decimal reduction time, D value, in the inactivation of *A. niger* spores under high-pressure carbonation

Temp (°C)	dCO_2 concn (γ)	D (min)
44	24.4	1.74
46	24.2	0.60
48	23.0	0.39
50	21.7	0.24
52	22.9	0.16
52[a]	0	
52[b]	0	

[a] Physiological saline without CO_2.
[b] McIlvaine buffer (pH 3.0) without CO_2.

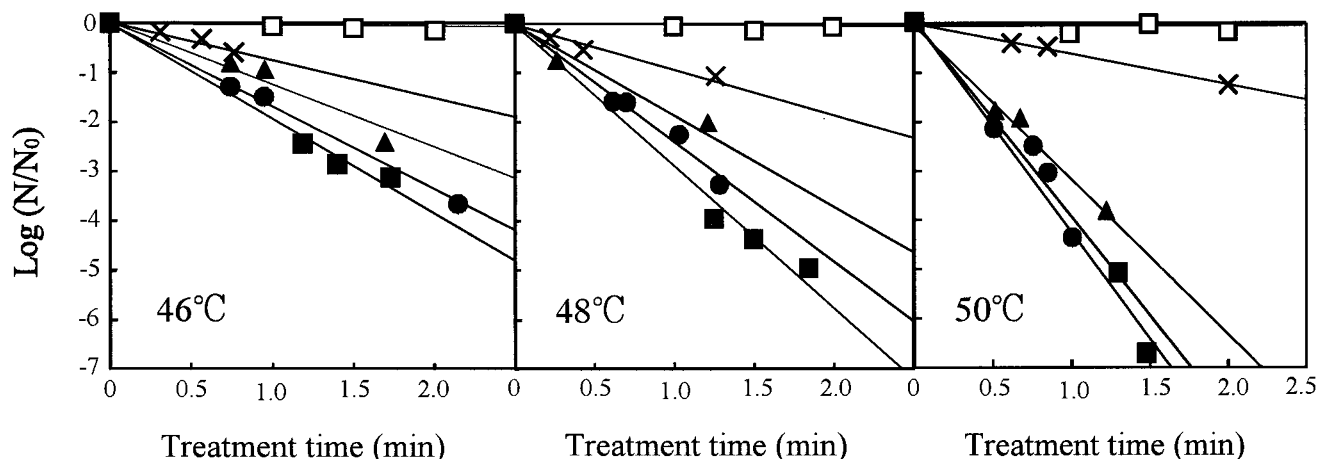

FIG. 3. Inactivation behaviors of *A. niger* spores in carbonated (closed symbols) and noncarbonated (□) saline. Carbonations were carried out to the saturated levels at 5 MPa (×), 8 MPa (▲), 10 MPa (●), and 19 MPa (■).

physiological saline and McIlvaine buffer (pH 3.0) without CO_2 were not killed at 52°C in the limited times.

The effect of treatment pressure on the inactivation of *A. niger* spores is shown in Fig. 3. The spore suspension was introduced into saline carbonated at 5, 8, 10, and 19 MPa. In every treatment, the plots of the values of log $[N/N_0]$ against treatment time showed a linear relationship. The slopes of three survival curves under carbonation at 5 MPa gave almost the same value independently of the treatment temperature, but the slopes became steep as the treatment pressure and temperature increased. On the other hand, *D* values in Fig. 4 show no significant difference at a fixed dCO_2 concentration (γ = 15.5), independently of the treatment pressure. This result indicated that the antimicrobial activity of CO_2 was dependent on the concentration of dCO_2 and not on the treatment pressure. Thus, the increase in the dCO_2 concentration was found to cause the increase in the inactivation rates at a given temperature, as shown in Fig. 3.

To elucidate the effects of the concentration of dCO_2 and treatment temperature on the CO_2 inactivation of *A. niger* spores, treatments were done with various combinations of dCO_2 concentration and treatment temperature. The plots of the values of log $[N/N_0]$ against treatment time suggested that they have a linear relationship (Fig. 5). Comparison of these four plots seemed to suggest the existence of a significant interaction between the effects of treatment temperature and dCO_2 concentration on inactivation.

Figure 6 shows the plots of log *D* values against the concentration of dCO_2 at 46, 48, 50, and 52°C. These plots gave linear relationships, and the slopes increased as the treatment temperature increased. These results indicated that the CO_2 sensitivity of *A. niger* spores was consistent in the wide range of the dCO_2 concentration at a given temperature but that the sensitivity increased as the treatment temperature increased. The CO_2 resistance constants, $Z_{temperature}(\gamma)$, estimated from the slopes of straight lines in Fig. 6 are listed in Table 2. The values were significantly influenced by the treatment temperature and decreased from 21.8 γ at 46°C to 6.4 γ at 52°C. This demonstrated that the CO_2 sensitivity increased as the treatment temperature increased.

The CO_2 resistance constant $[Z_{temperature}(\gamma) = 7.2\ \gamma]$ for the inactivation of *S. cerevisiae* under high-pressure carbonation was independent of treatment temperature (27). On the other hand, the CO_2 resistance constant for *A. niger* spores was significantly dependent on the treatment temperature (Table 2). This might be attributed to structural and physiological differences between *S. cerevisiae* cells, which are living cells, and *A. niger* spores, which are resting cells. To make clear the interaction between CO_2 and thermal resistance constants, the log *D* values were plotted against the treatment temperature, with the concentration of dCO_2 being taken as a parameter. Figure 7 was constituted from the values on the straight lines in Fig. 6 and shows a very interesting profile of the inactivation of *A. niger* spores under high-pressure carbonation. That is, in the low dCO_2 concentration (γ < 10), the log *D* values linearly increased with the increase in the treatment temperature. This

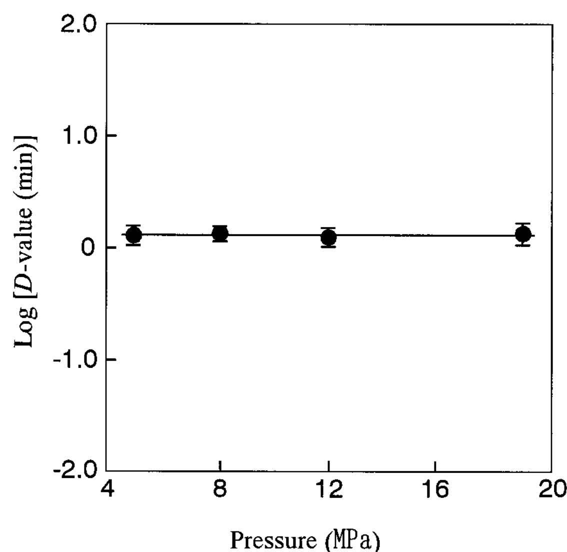

FIG. 4. Effect of the treatment pressure on the inactivation of *A. niger* spores at a fixed concentration (15.5 γ) of dCO_2. The treatment temperature was 50°C.

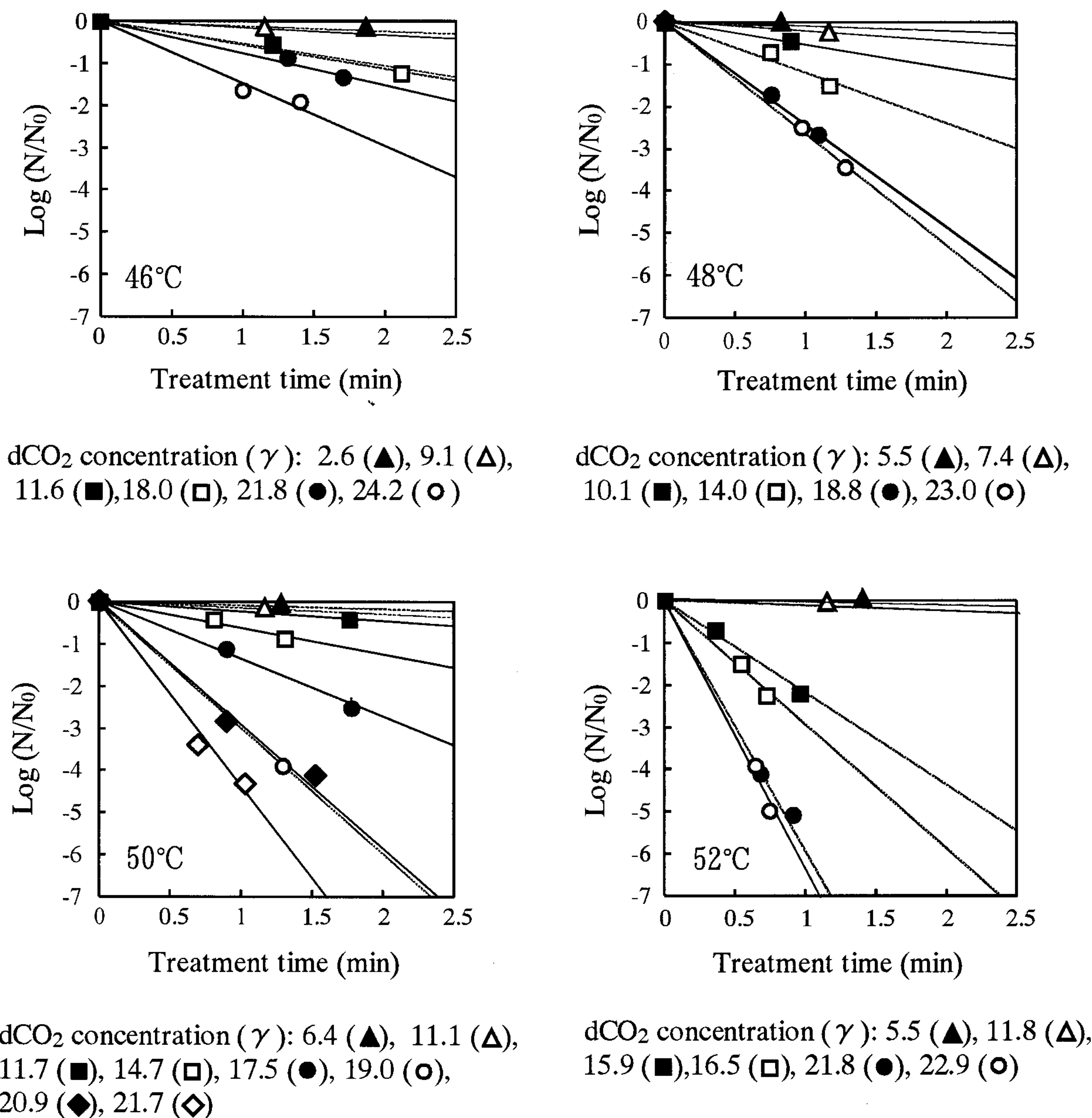

FIG. 5. Effect of dCO_2 concentration on the inactivation of *A. niger* spores under carbonation at 10 MPa.

phenomenon is contradictory to what is commonly known about pasteurization. The treatments under the conditions of lower pressure and lower temperature might have some advantages with respect to food quality as well as equipment cost, although the antimicrobial activity was quite limited and thus a prolonged treatment time was needed. At the medium concentration of dCO_2 (γ value of 10 to 20), the *D* values were hardly influenced by the treatment temperature. At the high concentration ($\gamma > 20$), the log *D* values linearly decreased with the increase in the treatment temperature. In the carbonated saline at 52°C and 25 γ, the *D* value of *A. niger* spores was reduced by about four cycles in a log scale compared with the value in noncarbonated medium. The values of the thermal resistance constant (Z_{CO_2}) estimated from the slopes in Fig. 7 are listed in Table 3. These values indicated that the thermal sensitivity became larger as the concentration of dCO_2 increased. As shown in Fig. 7, there was a significant interaction between the treatment temperature and dCO_2 concentration. To explain the interaction is very difficult, but it might be caused by the difference in the levels of temperature dependence on the distribution of CO_2 between the medium and spores and the thermal sensitivity of the spores under high-pressure carbonation.

The antimicrobial effects of compressed CO_2 have been extensively studied to elucidate the mechanism. In such studies, the death of target cells has been variously explained as follows: the acidification by dCO_2 may cause the inactivation of key enzymes related to the essential metabolic process (1, 5, 6, 7, 13, 18), the extraction of intracellular substances such as hydrophobic compounds in the cell wall and cytoplasmic mem-

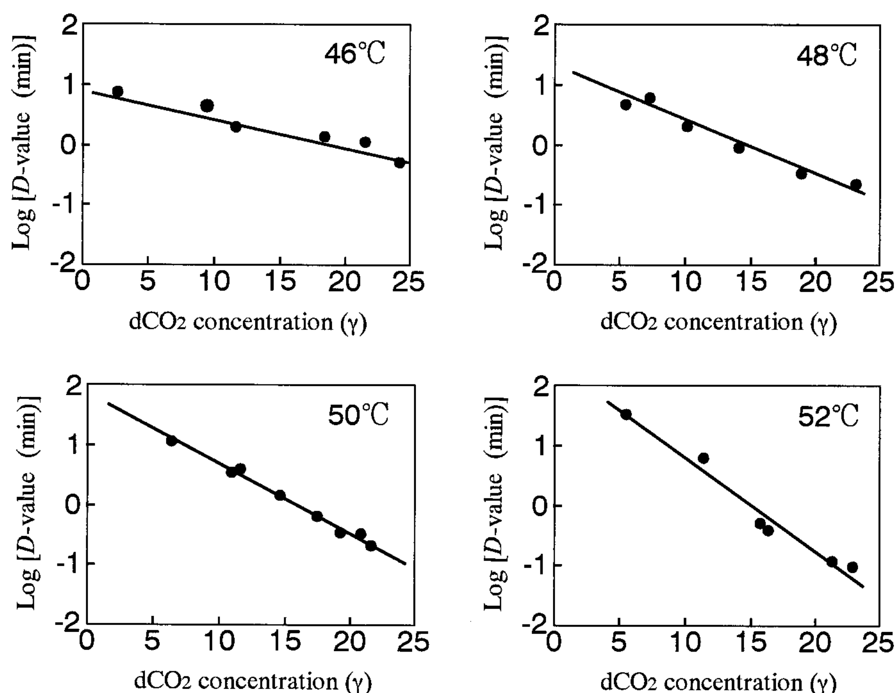

FIG. 6. Relationship between the log D value and dCO_2 concentration.

brane may result in microbial death (18), cell rupture due to the expansion of CO_2 within the cells may induce a loss of viability (3, 26), the damage to the cell membrane due to swelling with compressed CO_2 may kill the cells, or an "anesthesia effect" may induce the inhibition of metabolic systems (13, 14, 27). Authors have demonstrated that the inactivation power of CO_2 was much greater in a continuous treatment (sudden decompression) under high-pressure carbonation than in a batch treatment (slow decompression) (26). In the continuous treatments, the enhancement of the inactivation power could be attributed to cell bursting due to sudden expansion of compressed CO_2 in the cells. Debs-Louka et al. (3) observed no significant difference in viability after a subcritical CO_2 treatment with three different decompression times, but they described how rapid decompression under supercritical conditions could provoke cell rupture.

As alternative explanations for the antimicrobial effects of CO_2, it will be worthwhile to consider the decrease of intracellular pH which is induced by ready penetration of CO_2 into the cells and by its dissociation within the cells, as well as the anesthesia effect due to the accumulation of CO_2 in the cytoplasmic membrane. These inactivation mechanisms could be attributed to the specific effects of CO_2 compared to the effects of other organic acids used as an acidulants (2, 9, 19, 21). They may also affect microbial inactivation during high-pressure carbonation.

To elucidate the inactivation mechanism, it will be important to differentiate the inactivation caused by sudden decompression from that which occurs during high-pressure carbonation.

In conclusion, the inactivation of *A. niger* spores under high-pressure carbonation followed first-order death kinetics. The separate and combined effects of temperature and dCO_2 concentration on inactivation were elucidated. These results per-

TABLE 2. CO_2 resistance constant, $Z_{temperature}(\gamma)$, in the inactivation of *A. niger* spores under high-pressure carbonation

Temp (°C)	$Z_{temperature}(\gamma)$
46	21.8
48	11.5
50	8.4
52	6.4

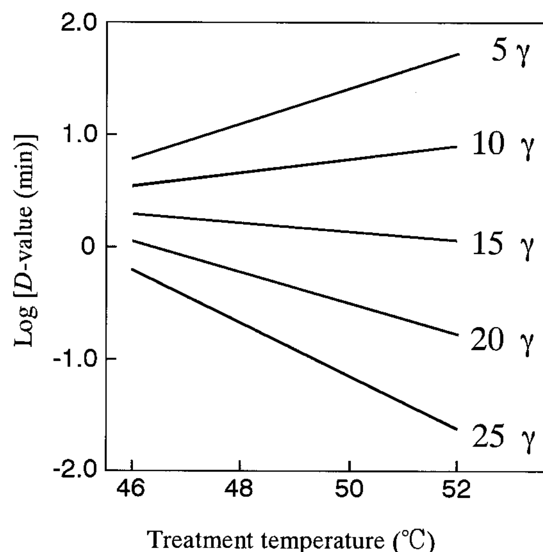

FIG. 7. Effect of the interaction between the treatment temperature and dCO_2 concentration on the log D values of *A. niger* spores under carbonation.

TABLE 3. Thermal resistance constant, $Z_{CO_2}(T)$, in the inactivation of *A. niger* spores under high-pressure carbonation

dCO_2 concn (γ)	$Z_{CO_2}(T)$
5	−6.1
10	−14.7
15	31.0
20	7.2
25	4.2

mit an estimation of the D value for the inactivation of *A. niger* spores at any temperature and any dCO_2 concentration. The high-pressure carbonation treatment could be realized in fruit juice processing, because there is no problem in incorporating such a treatment into a commercial process.

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