Note

Sterilization of Microorganisms by the Supercritical Carbon Dioxide Micro-Bubble Method

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*Lactobacillus brevis* and *Saccharomyces cerevisiae* were completely sterilized by the supercritical (SC) CO₂ micro-bubble method. Gaseous (G) and liquid (LQ) CO₂ were used in a similar manner to compare the sterilizing effect. Among the three treatments, the microorganisms were only effectively sterilized by the SC CO₂ treatment at 25 MPa and 35°C.

Advances in the application of supercritical (SC), gaseous (G), and liquid (LQ) CO₂ have been made regarding the interaction with biological materials or organisms. There are several reports dealing with the influences of SC, G, and LQ CO₂ on proteins, amino acids, enzymes, and microbial cells. Kamihara et al. have described that wet cells (water content of 70–90%) of baker’s yeast, *Escherichia coli*, *Staphylococcus aureus* and conidia of *Aspergillus niger* could be sterilized by treating with SC CO₂ at 35°C and 200 atm for 2 h. Nakamura et al. have reported that wet cells of baker’s yeast were destroyed after being treated with CO₂ at 40°C and 40 atm for more than 3 h. The efficiency of sterilization, however, was insufficient, because it was necessary to prolong the treating time. In our previous paper, some kinds of enzymes in aqueous solutions could be effectively inactivated by the SC CO₂ micro-bubble method in only 30 min. The purpose of this paper is to clarify the effectiveness of the SC CO₂ micro-bubble method for sterilizing microorganisms.

*Lactobacillus brevis* and *Saccharomyces cerevisiae* were used as the test microorganisms. These microorganisms were suspended in physiological saline at the level of 10⁶ CFU/ml and subjected to several CO₂ treatments with Milton Roy X-10 system (Riviera Beach, FL). This system was equipped with a 120-ml treatment vessel incorporating a cylindrical filter (10 μm pore size) made of porous stainless steel for feeding CO₂. In each experiment, 100 ml of a sample solution was loaded into the treatment vessel. Until the pressure had reached the experimental level, CO₂ was fed at 4.0 g/min for about 10 min, and then the flow was stopped. At the end of the treatment, the vessel was depressurized by slowly opening the pressure-regulating valve over a period of about 5 min.

Figure 1 shows the effect of the filter on the sterilization of *L. brevis* and *S. cerevisiae* by the CO₂ treatment for 30 min. The sterilizing effect of SC CO₂ was compared with those of G and LQ CO₂. After the LQ and G CO₂ treatments, the difference in survival ratio between the treatments with and without the filter.

**Fig. 1.** Effect of the Filter on the Sterilization of *L. brevis* and *S. cerevisiae* with Gaseous, Liquid, and Supercritical Carbon Dioxide.
The treatment time was set at 15 min. Symbols: ■, treatment was carried out with the filter (10 μm pore size); ○, treatment was carried out without the filter.

**Fig. 2.** Time-Course for the Sterilization of *L. brevis* and *S. cerevisiae* with Supercritical and Liquid Carbon Dioxide.
The treatment was carried out with the filter (10 μm pore size). Symbols: ▲, 8 MPa; △, 15 MPa; ○, 20 MPa; □, 25 MPa.
were less than one order. On the other hand, after the SC CO₂ treatment, the sterilizing effect with the filter greatly increased with increasing pressure, the difference with and without the filter being more than 4 orders for L. brevis at 35°C and 25 MPa. Similarly, with S. cerevisiae, the difference in survival ratio was more than 3 orders under the same conditions. The micro-bubbles of SC CO₂ could increase the CO₂ concentration from 0.4 to 0.92 mol/liter at 25 MPa and 35°C, and hence could improve the efficiency of sterilization for both microorganisms when compared to treating without the filter. Figure 2 shows the time-course for the survival ratios of L. brevis and S. cerevisiae by the SC CO₂ and LQ CO₂ treatments. The LQ CO₂ treatment for 5 min decreased the survival ratios to 1/10 to 1/10⁶, but further large decreases could not be achieved. The time-course for the survival ratio by the G CO₂ treatment at 4 MPa was similar to that of LQ CO₂ (data not shown). In contrast, the SC CO₂ treatment decreased the survival ratio with increasing treatment time. In particular, the initial sterilizing rate was maintained while increasing the pressure, and treatment at 25 MPa and 35°C for 30 min achieved complete sterilization of L. brevis. Complete sterilization of S. cerevisiae needed more than 30 min under the same conditions. Figure 3 shows the effect of the CO₂ density on sterilization. By treating with micro-bubbles of high density SC CO₂ at 35°C for 30 min, both microorganisms effectively decreased and the survival ratios became 1/10⁶ at 0.9 g/cm³. Thus, high density SC CO₂ above 0.9 g/cm³ would be needed for sufficient sterilization in only 30 min. Nakamura et al. have described that the death of microorganisms by high pressure CO₂ might be caused by mechanical rupture and/or physiological damage related to gas sorption and desorption by the cells. In their system, decompression to atmospheric pressure was achieved within 50 s. On the other hand, in our system, decompression was performed more slowly, taking about 5 min, and hence mechanical rupture would be unlikely to have occurred in the sterilization process. In this respect, Arreola et al. have also described that a sudden pressure decrease may not play a significant role in microbial reduction. It therefore seems that the SC CO₂ micro-bubble method results in sterilization due to the physiological damage. As described in our previous paper, high-density SC CO₂ would accelerate the sorption of CO₂ into enzymes and result in their inactivation. In addition, survival-CO₂ density profiles for the microorganisms resembled the inactivation-CO₂ density profiles for the enzymes. Thus, sterilization might mainly have been due to the inactivation of intracellular and membrane-bound enzymes. As another possibility, the sorption of CO₂ into a microbial cell might cause damage to the cytoplasmic membranes and result in loss of the metabolic functions. We are investigating the mechanism for sterilization to clarify this point.

References