Sterilization of Microorganisms with Supercritical Carbon Dioxide

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The sterilizing effect of supercritical carbon dioxide (SC-CO_2) is described as compared with gaseous CO_2(G-CO_2) and liquid CO_2(L-CO_2). Baker's yeast, *Escherichia coli*, *Staphylococcus aureus* and conidia of *Aspergillus niger* were sterilized by treating with SC-CO_2 at 200 atm and 35°C when the water content of each microorganism was 70～90%. However, dry cells with a water content of 2～10% could not be sterilized under the same conditions. No sterilizing effect from SC-CO_2 on endospores of *Bacillus subtilis* and *B. stearothermophilus* was observed. G-CO_2 and L-CO_2 produced no sterilizing effect against both wet and dry baker's yeast cells, while wet *E. coli* cells were sterilized with G-CO_2. Although dry conidia of *A. niger* could not be sterilized with SC-CO_2 alone, the addition of ethanol or acetic acid to SC-CO_2 made it possible to sterilize these. Without any decrease in their enzymatic activities, *E. coli* and baker's yeast in α-amylase and lipase preparations were sterilized with SC-CO_2.

The extraction of useful substances from natural products using a supercritical fluid is an interesting separation method and has been applied in various fields.[1,2] Especially in the food industry, SC-CO_2 is considered to be the most favorable solvent because CO_2 is physiologically safe, inexpensive, and easily available in high purity and in large quantity. Moreover, since CO_2 has a relatively low critical pressure (72.8 atm) and critical temperature (31.1°C), and its solvent power near the critical point is easily varied over the wide range through slight changes in temperature and pressure, SC-CO_2 extraction offers greater advantages than the conventional separation methods. We have reported previously on the application of SC-CO_2 extraction to oils from vegetable seeds such as wheat germ[3] and rice bran,[4] and on the removal of organic solvents in the final step of antibiotic purification by SC-CO_2 treatment.[5] Apart from the direct exploitation of the dissolving capacity of SC-CO_2, we found that the number of living microorganisms in the Koji residue decreased remarkably after SC-CO_2 extraction.[6] With sterilization, the use of gases such as ethylene oxide,[7] ultraviolet rays,[7] microwaves,[8] radial rays,[7] superheated steam[9] and extrusion cooking[10] have been studied for many kinds of substances. However, there is no safe method that can sterilize bioactive products such as medicines and food without lowering their quality. Sterilization methods using ethylene oxide or radial rays are promising, but are limited to applicable objects by the law in Japan. This paper describes the sterilizing effects of SC-CO_2 as compared with L-CO_2 and G-CO_2. The feasibility of sterilizing thermally unstable compounds with SC-CO_2 was also investigated.

MATERIALS AND METHODS

Microorganisms. Six strains, *Escherichia coli* B, baker's yeast, *Staphylococcus aureus* 209P, *Aspergillus niger* IFO 6341, *Bacillus subtilis* PCI 129, and *Bacillus stearothermophilus* IAM 11002, were used as test organisms for estimating the sterilizing effect of CO_2.

Enzymes. Lipase OF (Meito Sangyo Co.) and α-amylase AD-C (Amano Pharmaceutical Co.) were used as a

model for heat-sensitive substances.

Preparation of microbial cells. Baker's yeast cells were purchased from Oriental Yeast Co. *E. coli* and *S. aureus* were cultivated in a medium containing 1% polypepton, 0.5% yeast extract, 0.5% sodium chloride and 0.1% glucose (pH 7.2). After incubating for 15~20 hr at 37°C, the cells were harvested by centrifuging at 12,000 rpm for 10 min. *A. niger* was grown on a petri-dish with a malt extract agar medium containing 2% malt extract, 0.1% polypepton and 2% glucose. After incubating for 5~7 days at 30°C, the conidia formed were collected and suspended in distilled water containing 0.1% Tween 80. The conidia suspension was filtered through three layers of gauze to remove hyphae. The conidia in the filtrate were harvested by centrifuging at 5,000 rpm for 10 min. Endospores of the bacilli were prepared according to the standard methods[7] as follows: Both bacillus strains were cultivated on petri-dishes with a nutrient agar medium containing 0.3% beef extract, 0.5% polypepton and 0.005% $MnSO_4$ (pH 6.8). After incubating for 2 days at 37°C for *B. subtilis* and for 2 days at 50°C for *B. stearothermophilus*, the cells were collected and suspended in distilled water and then centrifuged at 12,000 rpm for 10 min. The harvested cells were again suspended in a minimum amount of distilled water, and temperature-treated at 70°C for 10 min for *B. subtilis*, or at 4°C overnight followed by 80°C for 10 min for *B. stearothermophilus*, in order to kill completely vegetative cells. Each microorganism was used as a wet cell after washing with a 0.85% NaCl solution or distilled water and then centrifuging at 12,000 rpm for 10 min, or a dry cell after drying the wet cell in vacuo. The water content of the wet and dry cells was determined by drying them at 105°C for more than 20 hr.

Procedures for sterilization. Sterilization with CO_2 was carried out using the SC-CO_2 extraction apparatus made by Mitsubishi Kakoki Co. and described previously.[3~6] A sample of the microorganism was placed in a cup made of aluminum. This aluminum cup was put on a sample holder in the extraction vessel. Treatment with CO_2 for sterilization was operated in a similar manner as that described in the vegetable oil extraction experiments.[3] In some experiments, ethanol or acetic acid was added as the entrainer to SC-CO_2 with a high-pressure pump at a weight ratio of 2% or 0.5%, respectively.

Measurement of sterilizing effect. The sterilizing effect of CO_2 was evaluated by determining the ratio of the number of living cells after the treatment with CO_2 to those initially added. The number of living cells was determined by measuring the number of colonies grown on each agar medium. As media, Eosin-Methylene Blue, Mannitol-NaCl, Czapek-Dox and Spizizen were used for *E. coli*, *S. aureus*, *A. niger* and *B. subtilis*, respectively.[7] Streptomycin sulfate and penicillin G potassium were added to an agar medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% polypepton and 1% glucose (pH 5.0) for baker's yeast to prevent any bacterial contamination. *B. stearothermophilus* was cultivated at 50°C on the nutrient agar medium to prevent the growth of mesophilic microorganisms. The colony formed on each agar medium was microscopically observed to confirm no contamination with other microorganisms whenever necessary.

RESULTS

Effect of conditions for sterilization

Table I shows the effect of the treatment conditions on *E. coli* and baker's yeast. CO_2 is in a gaseous state in runs 1 and 4, a liquid state in runs 2 and 3, and a SC state in runs 5 and 6. CO_2 exhibited a sterilizing effect against wet *E. coli* cells in all the experiments, and the effect was enhanced with increasing temperature and pressure. Contrary to this, the ratios of living cells were reduced to the order of $10^{-1} \sim 10^{-2}$ in the case of dry *E. coli* cells. In the case of baker's yeast, the sterilizing effect was only observed on wet baker's yeast cells with SC-CO_2. Other states of CO_2 than the SC condition had no sterilizing effect on either wet or dry baker's yeast cells. There was a great difference in the sensitivity toward CO_2 between wet *E. coli* and wet baker's yeast, particularly in the experiments on sterilization with gaseous and liquid CO_2.

Figure 1 shows time courses for the sterilization with SC-CO_2 at 200 atm and 35°C. Here, zero time of the treatment means that the sample was exposed with CO_2 until the establishment of an initial condition at 200 atm (about 20 min) and then the pressure was decreased to 1 atm (about 20 min). During the increase and decrease in pressure, some cells were sterilized and the ratio of living cells became less than unity at zero time of the treament. Wet cells of *E. coli* and baker's yeast were rapidly sterilized in the early stage of the treatment, and then the sterilization effect decreased. In the case of dry cells of both microorganisms, the ratio of living cells was not much decreased regardless of the time for sterilization.

TABLE I. STERILIZING EFFECT OF CO_2 UNDER VARIOUS CONDITIONS

Run	Treatment conditions[a]		Ratio of living cells (−)			
			E. coli		Baker's yeast	
	Temperature (°C)	Pressure (atm)	Wet cells[b]	Dry cells[b]	Wet cells[b]	Dry cells[b]
1	20	40	1.3×10^{-4}	0.30	0.84	1.09
2	20	100	3.1×10^{-5}	0.10	0.47	0.89
3	20	200	3.6×10^{-5}	6.6×10^{-2}	0.13	0.75
4	35	40	9.9×10^{-5}	0.11	0.73	1.08
5	35	100	6.0×10^{-5}	0.12	1.2×10^{-4}	0.65
6	35	200	7.2×10^{-6}	4.7×10^{-2}	5.4×10^{-7}	0.50

[a] Treatment time: 2 hr.
[b] Water content: wet cells, 65~75%; dry cells, 5~15%.

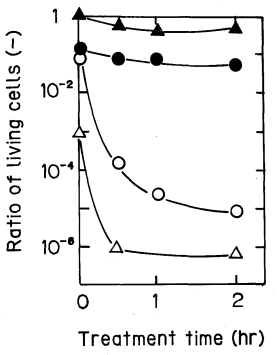

FIG. 1. Time Courses for Sterilization with Supercritical CO_2 at 200 atm and 35°C.
○, wet *E. coli*; ●, dry *E. coli*; △, wet baker's yeast; ▲, dry baker's yeast.

TABLE II. STERILIZING EFFECT OF SUPERCRITICAL CO_2 AT 200 atm AND 35°C

Microorganism	Ratio of living cells (−)	
	Wet cells[a]	Dry cells[a]
Baker's yeast	5.4×10^{-7}	0.50
E. coli	7.2×10^{-6}	0.047
S. aureus	1.5×10^{-5}	0.037
A. niger (conidia)	1.2×10^{-5}	0.88
B. subtilis (endospore)	0.47	0.99
B. stearothermophilus (endospore)	1.07	0.80

[a] Water content: wet cells, 70~90%; dry cells, 2~10%.

Sterilization of different kinds of microorganisms

Table II shows the sterilizing effect of SC-CO_2 at 200 atm and 35°C on other microorganisms. Four kinds of wet microorganisms (baker's yeast, *E. coli, S. aureus* and *A. niger*) were sterilized by being exposed to SC-CO_2 at 200 atm and 35°C for 2 hr. However, dry cells of baker's yeast and *A. niger* remained almost unsterilized under the same conditions. On the other hand, in the case of dry cells of *E. coli* and *S. aureus,* the ratio of living cells was reduced to below 5% of those initially added. The endospore of both bacillus strains was not sterilized.

Effect of an entrainer

An entrainer has been used to modify the properties of CO_2. Brunner and Peter[11] reported that the addition of 10% ethanol to SC-CO_2 increased the solubility of palm oils twenty times as much as that under the same conditions with CO_2 alone. Table III shows the effects of adding ethanol and acetic acid to SC-CO_2. Ethanol and acetic acid were selected because they are used as sterilizing reagents and are harmless even if they remain in such sample as the starting materials for foods. With wet conidia of *A. niger*, the sterilizing effect of CO_2 was strengthened by adding ethanol or acetic acid at a weight ratio of 2% or 0.5%, respectively. The dry conidia were not sterilized with SC-CO_2 alone, but the addition

TABLE III. EFFECT OF AN ENTRAINER ON SUPERCRITICAL CO_2 TREATMENT[a]

Microorganism	Ratio of living cells (−)					
	Wet cells			Dry cells		
	CO_2	CO_2+EtOH[b]	CO_2+AcOH[b]	CO_2	CO_2+EtOH[b]	CO_2+AcOH[b]
A. niger (conidia)	1.2×10^{-5}	$<2.3\times10^{-6}$	$<7.9\times10^{-6}$	0.88	1.2×10^{-6}	$<1.6\times10^{-5}$
B. stearothermophilus (endospore)	1.07	0.62	0.43	0.80	0.49	0.43

[a] Treatment conditions: 200 atm, 35°C, 2 hr.
[b] Ethanol (EtOH) or acetic acid (AcOH) was added to CO_2 at a weight ratio of 2% or 0.5%, respectively.

TABLE IV. STERILIZATION OF AN ENZYME PREPARATION WITH SUPERCRITICAL CO_2

Enzyme	Microorganism	Enzyme activity (%)	Ratio of living cells (−)
α-Amylase	*E. coli*	121	5.2×10^{-5}
	Baker's yeast	135	3.6×10^{-3}
Lipase	*E. coli*	88	8.9×10^{-5}
	Baker's yeast	78	4.7×10^{-3}

Treatment conditions: 200 atm, 35°C, 2 hr.

of ethanol or acetic acid to SC-CO_2 made it possible to sterilize even the dry ones. The ratio of living cells of dry conidia were reduced to 10^{-6} orders of magnitude, which corresponds to that in the experiments on wet conidia with SC-CO_2 alone. In both wet and dry endospores of *B. stearothermophilus*, a sterilizing effect was observed with the addition of ethanol or acetic acid as an entrainer, and about 50% of endospores were inactivated.

Sterilization of enzyme preparations with SC-CO_2

Table IV shows the results for sterilizing enzyme preparations with SC-CO_2 at 200 atm and 35°C for 2 hr. After wet *E. coli* (water content: 74%) or baker's yeast (water content: 68%) was mixed with crude, dry α-amylase or lipase at a weight ratio of 9:1, the microorganisms in the enzyme preparations were sterilized with SC-CO_2. Here, the initial water content of the enzyme preparation with *E. coli* or baker's yeast was 68% or 62%, respectively. *E. coli* cells in the two enzyme preparations were sterilized with SC-CO_2 to a low level of 10^{-5} of the initially added living cells. This ratio almost corresponded to that with the wet *E. coli* cells alone as shown in Table I. In the case of baker's yeast, the ratio of living cells was reduced to 10^{-3} orders of magnitude. This may be due to a little lower water content (62%) compared with that (68%) in the experiment shown in Table I. The remaining enzyme activity after sterilization with SC-CO_2 was not decreased for α-amylase but slightly decreased for lipase.

DISCUSSION

Medicines and foods have to be free from contamination by microorganisms. However, since natural products as starting materials contain a large number of microorganisms, sterilization is essential. Many powdery and granulated products such as Chinese medicines and spices are difficult to sterilize by heat treatment because this causes undesirable

changes in the quality of the products. New sterilization methods including sterilization with superheated steam,[9] and extrusion cooking[10] have been recently investigated. Especially, superheated steam is considered to be safe and is used to sterilize powdery and granulated products such as spices, buckwheat flour, cacao beans and grains on a commercial scale. However, deterioration in the quality of the products is still inevitable by the superheated steam treatment. The inhibitory effect of CO_2 on the growth of some food-related bacteria has been studied,[12] and a modified atmosphere enriched with CO_2 is often commercially used to control microbial spoilage in fresh meat.[13] Enfors and Molin[14] have reported that at atmospheric pressure, 100% CO_2 inhibited the germination of spores of *Bacillus cereus*, and that the same conditions stimulated the germination of spores of *Clostridium sporogenes* and *Clostridium perfringens*. There are few reports[15] on the effect of high pressures of CO_2, except for toxin production with *Clostridium botulinum*.

As already described, the number of living cells after sterilizing with compressed CO_2 was affected by (1) the kind of microorganism, (2) the water content, (3) the state of CO_2, and (4) the addition of ethanol or acetic acid as an entrainer to CO_2. Morphological changes of microbial cells caused by the treatment for sterilizing with SC-CO_2 alone were observed by a scanning electron microscope to evaluate the action of SC-CO_2 on wet cells. Some wet *E. coli* cells were found to have burst, but on the other hand, wet baker's yeast cells did not burst during sterilization with SC-CO_2. The experimental results at zero time of the treatment in Fig. 1 do not coincide with the microscopic observations, because the ratio of living cells at zero time was almost the same for wet and dry *E. coli* cells, while it was greatly different between the wet and dry baker's yeast cells. The death of microbial cells with compressed CO_2 is presumed to have been caused by the inactivation of some enzymes by a pH decrease and/or by the extraction of intracellular substances such as phospholipids. Time courses for the sterilization did not result in straight lines on a semi-logarithmic scale, as shown in Fig. 1, which suggests that a complex mechanism is involved in the sterilization. On the basis of the results just described, further studies to elucidate the mechanism for sterilization with high pressures of CO_2 and on the conditions for sterilizing endospores of Bacillus species are still necessary.

Consequently, SC-CO_2 is expected to be used as a safe sterilizing reagent as well as being an alternative to a solvent for extracting such lipophilic compounds as vegetables and essential oils. As sterilization proceeds during the extraction of oils with SC-CO_2, both oil and a defatted residue free from microorganisms can be obtained. We obtained the sterilized defatted Koji for producing sake by SC-CO_2 extraction without any decrease in the enzymatic activities as previously reported.[6] In the present study, two kinds of enzyme preparations were used as typical models for thermally unstable compounds to determine the effect of SC-CO_2 on enzymatic activities during sterilization. Without any decrease in enzymatic activities, the enzyme preparations seemed to be sterilized with SC-CO_2 to a considerable extent. The results of sterilization for thermally unstable substances other than the enzymes by the SC-CO_2 treatment will be described elsewhere. Thus, sterilization with SC-CO_2 is applicable to heat-sensitive substances as a newly promising alternative to the current methods.

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