Sterilization of Microorganisms with Supercritical Carbon Dioxide

Masamichi KAMIHIRA, Masayuki TANIGUCHI and Takeshi KOBAYASHI

Department of Chemical Engineering, Faculty of Engineering,
Nagoya University, Chikusa-ku, Nagoya 464, Japan

Received August 8, 1986

The sterilizing effect of supercritical carbon dioxide (SC-CO₂) is described as compared with gaseous CO₂ (G-CO₂) and liquid CO₂ (L-CO₂). Baker’s yeast, Escherichia coli, Staphylococcus aureus and conidia of Aspergillus niger were sterilized by treating with SC-CO₂ 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-CO₂ on endospores of Bacillus subtilis and B. stearothermophilus was observed. G-CO₂ and L-CO₂ produced no sterilizing effect against both wet and dry baker’s yeast cells, while wet E. coli cells were sterilized with G-CO₂. Although dry conidia of A. niger could not be sterilized with SC-CO₂ alone, the addition of ethanol or acetic acid to SC-CO₂ 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-CO₂.

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-CO₂ is considered to be the most favorable solvent because CO₂ is physiologically safe, inexpensive, and easily available in high purity and in large quantity. Moreover, since CO₂ 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-CO₂ extraction offers greater advantages than the conventional separation methods. We have reported previously on the application of SC-CO₂ extraction to oils from vegetable seeds such as wheat germ³ and rice bran,⁴ and on the removal of organic solvents in the final step of antibiotic purification by SC-CO₂ treatment.⁵ Apart from the direct exploitation of the dissolving capacity of SC-CO₂, we found that the number of living microorganisms in the Koji residue decreased remarkably after SC-CO₂ extraction.⁶ With sterilization, the use of gases such as ethylene oxide,⁷ ultraviolet rays,⁷ microwaves,⁸ radial rays,⁷ superheated steam⁹ and extrusion cooking¹⁰ 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-CO₂ as compared with L-CO₂ and G-CO₂. The feasibility of sterilizing thermally unstable compounds with SC-CO₂ 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 CO₂.

Enzymes. Lipase OF (Meito Sangyo Co.) and α-amylase AD-C (Amano Pharmaceutical Co.) were used as a
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 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% MnSO₄ (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.

Measurement of sterilizing effect. The sterilizing effect of CO₂ was evaluated by determining the ratio of the number of living cells after the treatment with CO₂ 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. 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 1 shows the effect of the treatment conditions on E. coli and baker’s yeast. CO₂ 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. CO₂ 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 ~ 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-CO₂. Other states of CO₂ 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 CO₂ between wet E. coli and wet baker’s yeast, particularly in the experiments on sterilization with gaseous and liquid CO₂.

Figure 1 shows time courses for the sterilization with SC-CO₂ at 200 atm and 35°C. Here, zero time of the treatment means that the sample was exposed with CO₂ 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 treatment. 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.
Sterilization of different kinds of microorganisms

Table II shows the sterilizing effect of SC-CO₂ 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-CO₂ 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 CO₂. Brunner and Peter reported that the addition of 10% ethanol to SC-CO₂ increased the solubility of palm oils twenty times as much as that under the same conditions with CO₂ alone. Table III shows the effects of adding ethanol and acetic acid to SC-CO₂. 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 CO₂ 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-CO₂ alone, but the addition
Table III. Effect of an Entrainer on Supercritical CO₂ Treatment

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Wet cells CO₂</th>
<th>Wet cells CO₂ + EtOH₈</th>
<th>Wet cells CO₂ + AcOH₈</th>
<th>Dry cells CO₂</th>
<th>Dry cells CO₂ + EtOH₈</th>
<th>Dry cells CO₂ + AcOH₈</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. niger (conidia)</td>
<td>1.2 x 10⁻⁵</td>
<td>&lt;2.3 x 10⁻⁶</td>
<td>&lt;7.9 x 10⁻⁶</td>
<td>0.88</td>
<td>1.2 x 10⁻⁶</td>
<td>&lt;1.6 x 10⁻⁵</td>
</tr>
<tr>
<td>B. stearothermophilus (endospore)</td>
<td>1.07</td>
<td>0.62</td>
<td>0.43</td>
<td>0.80</td>
<td>0.49</td>
<td>0.43</td>
</tr>
</tbody>
</table>

a. Treatment conditions: 200 atm, 35°C, 2 hr.
b. Ethanol (EtOH) or acetic acid (AcOH) was added to CO₂ at a weight ratio of 2% or 0.5%, respectively.

Table IV. Sterilization of an Enzyme Preparation with Supercritical CO₂

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Microorganism</th>
<th>Enzyme activity (%)</th>
<th>Ratio of living cells (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-Amylase</td>
<td>E. coli</td>
<td>121</td>
<td>5.2 x 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>Baker's yeast</td>
<td>135</td>
<td>3.6 x 10⁻³</td>
</tr>
<tr>
<td>Lipase</td>
<td>E. coli</td>
<td>88</td>
<td>8.9 x 10⁻⁵</td>
</tr>
<tr>
<td></td>
<td>Baker's yeast</td>
<td>78</td>
<td>4.7 x 10⁻³</td>
</tr>
</tbody>
</table>

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

Sterilization of enzyme preparations with SC- CO₂:
Table IV shows the results for sterilizing enzyme preparations with SC-CO₂ 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-CO₂. 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-CO₂ 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-CO₂ 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
Sterilization with Supercritical CO₂

changes in the quality of the products. New sterilization methods including sterilization with superheated steam,⁹ and extrusion cooking¹⁰ 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 CO₂ on the growth of some food-related bacteria has been studied,¹² and a modified atmosphere enriched with CO₂ is often commercially used to control microbial spoilage in fresh meat.¹³ Enfors and Molin¹⁴ have reported that at atmospheric pressure, 100% CO₂ 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 reports¹⁵ on the effect of high pressures of CO₂, except for toxin production with Clostridium botulinum.

As already described, the number of living cells after sterilizing with compressed CO₂ was affected by (1) the kind of microorganism, (2) the water content, (3) the state of CO₂, and (4) the addition of ethanol or acetic acid as an entrainer to CO₂. Morphological changes of microbial cells caused by the treatment for sterilizing with SC-CO₂ alone were observed by a scanning electron microscope to evaluate the action of SC-CO₂ 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-CO₂. 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 CO₂ 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 CO₂ and on the conditions for sterilizing endospores of Bacillus species are still necessary.

Consequently, SC-CO₂ 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-CO₂, both oil and a defatted residue free from microorganisms can be obtained. We obtained the sterilized defatted Koji for producing sake by SC-CO₂ extraction without any decrease in the enzymatic activities as previously reported.⁵ In the present study, two kinds of enzyme preparations were used as typical models for thermally unstable compounds to determine the effect of SC-CO₂ on enzymatic activities during sterilization. Without any decrease in enzymatic activities, the enzyme preparations seemed to be sterilized with SC-CO₂ to a considerable extent. The results of sterilization for thermally unstable substances other than the enzymes by the SC-CO₂ treatment will be described elsewhere. Thus, sterilization with SC-CO₂ is applicable to heat-sensitive substances as a newly promising alternative to the current methods.

Acknowledgments. The authors wish to thank Messrs. T. Aki, T. Murakami and H. Tashiro of Mitsubishi Kakoki Co. for their helpful advice and kind suggestions. This work was supported in part by a Grant-in-Aid for Special Project Research (No. 59124003) from the Ministry of Education, Science and Culture of Japan.

REFERENCES

4) M. Taniguchi, T. Tsuji, M. Shibata and T.


