EFFECT OF CERAMIC POWDER SLURRY ON SPORES OF BACILLUS SUBTILIS

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The effects of magnesium oxide (MgO), calcium oxide (CaO), and zinc oxide (ZnO) powder slurries on spores of Bacillus subtilis were examined. The CaO and MgO powder slurries were able to kill the spores of B. subtilis in physiological saline. The efficacy of the CaO powder slurry was much higher than that of the MgO powder slurry. It was considered that the efficacy of the CaO powder slurry against the spores depended on effects of Ca²⁺ and some factors due to contact between spores and the CaO powders. When the spores and nutrients such as growth medium existed together, it was observed that the MgO, CaO and ZnO powder slurries exhibited antibacterial activity against spores of B. subtilis. There was no difference in sensitivity to the ceramic powder slurry between the vegetative cells and the spores of B. subtilis. Further, the supernatant of the ceramic powder slurries promoted the germination of the spores. Therefore, it was suggested that the ceramic powder slurries weakened a high dormancy of spores, and acted on the germinated spores.

Introduction

Recently, consumption of processed foods has increased markedly, and a diet containing less salt or less sugar is desirable. These circumstances have enhanced the importance of microbial control in food processing. And, biodegradation and microbial contamination have become a serious problem in various fields.²⁻², ⁸⁻⁻², ¹⁷⁻⁻², ²²⁻⁻². The use of antibacterial ceramics for these problems holds considerable attention as a new antibacterial technique.¹⁴⁻⁻², ²⁰⁻⁻², ²¹⁻⁻². However, the antibacterial mechanisms of ceramics have not been elucidated, and the evaluation method for the growth inhibitory effect (antibacterial activity) of ceramics has also not been established yet.

We have proposed application of the conductance method to evaluate the antibacterial activity of the materials which are slightly soluble or insoluble to water, such as ceramics. The conductance method could provide quantitative and quick evaluation of the antibacterial activity of ceramic powder slurries.

We examined the antibacterial activity of 26 ceramic powders, and ten ceramic powders exhibited the antibacterial activity. For example, calcium oxide (CaO) and magnesium oxide (MgO) powders inhibited bacterial growth and exhibited a bactericidal action. Zinc oxide (ZnO) powder acted stronger on gram-positive bacteria than gram-negative bacteria and exhibited a bacteriostatic action.²⁴⁻⁻²

Some species of bacteria, including Bacillus and Clostridium, produce spores in their stationary phase of growth. The spores have high dormancy and resistivity to heat and various chemicals. Therefore, pasteurization of bacterial spores, which are difficult to kill, is an important step in food processing. Hence, bacterial spores have been used for many years as biological indicators to monitor and validate sterilization processes in food and health.¹⁹⁻⁻²

However, the effect of ceramic powders on bacterial spores has not been studied. In this study, MgO, CaO and ZnO powders are employed, and the efficacy of these powder slurries on spores of B. subtilis was examined. Moreover, the antibacterial mechanisms of the ceramic powder slurries on spores were studied.

1. Materials and Methods

1.1 Test bacteria

Bacillus subtilis ATCC 6633 was used as a test bacterium. The test bacteria were incubated in Brain Heart Infusion (BHI) broth (Difco) at 310 K for 24 h.

1.2 Preparation of spore suspension

A 0.5 ml portion of the BHI broth culture of B. subtilis was inoculated in 100 ml of a sporulation medium (Beef extract 5 g, Bacto-peaton 1 g, NaCl 0.5 g, MnSO₄ 0.05 g, H₂O 100 ml, pH 7.0). The medium was incubated

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at 310 K for a week. The spores were washed three times with physiological saline by centrifugation at 5,000 x g for 10 min, and resuspended in saline. The final spore suspensions were stored at 277 K. The spores were confirmed by staining with methylene blue. Activation of spores by heat treatment was not performed throughout this study.

1.3 Preparation of ceramics powder

MgO, CaO, and ZnO (Kishida Chemicals) were used as test ceramic powders. The slurries of ceramic powder were prepared with physiological saline with the same method as described in the previous study. The mean particle size of the MgO, CaO, and ZnO powders were 3.6, 2.7, and 2.6 μm, respectively.

1.4 Shaking with ceramic powder slurry in saline

0.1 ml of the spore suspension (10^8 CFU/ml) was added to a vial (φ 35 mm) containing 5 ml of the ceramic powder slurry, and the mixture was shaken in a reciprocal water bath (110 strokes/min) at 310 K. From time to time, samples were drawn from the slurry. Each 0.1-ml sample was diluted 10^3 to 10^5 fold with physiological saline and overspread on Trypticase Soy Agar plate (Eiken Chemical). After incubation at 310 K for 24 h, the number of colonies was enumerated. Ethylenediaminetetraacetic acid (EDTA: Wako Chemicals) was used as a chelating agent of Ca^{2+}. NaOH and KOH were used to adjust pH.

1.5 Monitoring of spore germination

MgO, CaO, and ZnO powder slurries of 100 mg/ml were prepared. The supernatant solutions of the slurries were obtained by centrifugation at 15,000 x g for 20 min. Spores were inoculated into 0.15 ml of BH broth and mixed with 0.15 ml of the supernatant of the slurry. Spore germination was monitored by measurement of the changes in optical density of the mixture at 660 nm during incubation at 310 K. Tosoh Micro Plate Reader A4i was used for monitoring of the optical density. Initial spore concentration was approximately 3 × 10^7 CFU/ml.

1.6 Conductance method

The changes in conductance of the growth medium were measured by the Bactometer Model 64 (bioMérieux) with the same procedure in the previous study, and the antibacterial activity of the ceramic powder was evaluated. Initial concentrations of vegetative cells and spore suspensions of B. subtilis were 5.7 × 10^3 and 1.3 × 10^3 CFU/ml, respectively. After incubation, a small quantity of the slurry was cultured in BH broth at 308 K for 24 h as described in the previous study. Whether or not the antibacterial activity of the ceramic powder is due to a bactericidal action or a bacteriostatic action was examined.

2. Results and Discussion

2.1 Efficacy of ceramic powder slurry on spores in physiological saline

We examined whether or not ceramic powder (MgO, CaO, and ZnO) slurries were able to kill bacterial spores. Figure 1 illustrated the effect of the MgO powder slurry on the spores of B. subtilis. N/N_0 represents the survival ratio of the spores. N_0 is the concentration of viable spores in the slurry at the initial state (approximately 10^7 CFU/ml). The survival ratio did not decrease until 2 h had passed. Although a temporal reduction of the survival ratio was observed after incubation for 150 min, the efficacy of the MgO powder slurry on the spores was slight.

Figure 2 illustrated the results of the CaO powder slurry. The survival ratio of the spores in the CaO powder slurry decreased with increasing incubation time and powder concentration. Therefore, it was found that the MgO and CaO powders were able to kill the spores of B. subtilis in physiological saline. Especially, the CaO powder slurry had a significant efficacy against the spores. Since these ceramic powder slurries were
Fig. 3 Survival ratio of spores of *B. subtilis* in ZnO powder slurry

Fig. 4 Effect of EDTA of efficacy of CaO powder slurry on spores of *B. subtilis* and effect of supernatant of CaO powder slurry completely saturated, the contact between the spores and the powders would contribute to the killing of the spores.

The survival ratio of the spores of *B. subtilis* in the ZnO powder slurry did not decrease, even after 3 h (Fig. 3). The ZnO powder slurry did not show efficacy on the spores in physiological saline.

2.2 Effects of pH of slurry and of Ca$^{2+}$ in the slurry

The CaO and MgO powder slurries have high pH values -12.6 and 10.4, respectively. The effect of alkaline treatment on the spores was thus examined using KOH (pH 13.0) and NaOH (pH 10.8 and 13.0). The CaO powder slurry reduced the survival ratio of the spores to $10^{-2}$ after 30 min. However, alkaline treatment did not reduce the survival ratio of the spores (Figs. 1 and 2).

Ca$^{2+}$ is particularly important in the resistance of spores to heat, some antibacterial agents and ultraviolet radiation. Figure 4 illustrated the effect of EDTA on the efficacy of the CaO powder slurry on the spores. The concentration of the CaO powder was 6.3 mg/ml. The surface of the CaO powder is almost covered by Ca(OH)$_2$. Therefore, the concentration of Ca$^{2+}$ in the slurry would be nearly equal to the solubility of Ca(OH)$_2$. $1.72 \times 10^{-2}$ M$^{12}$. The pH value of the CaO powder slurry calculated from the solubility of Ca(OH)$_2$ almost agrees with that measured by a pH meter. The efficacy of the CaO powder slurry on the spores decreased with addition of EDTA. Therefore, the efficacy of the CaO powder slurry on the spores depends on the Ca$^{2+}$ concentration and some factors due to contact between spores and the CaO powders.

In addition, as shown in Fig. 4, the spores were also killed only by the supernatant not including the CaO powders. Since the CaO powder slurry was completely saturated, the pH value of the supernatant is 12.6, which is the same as that of the slurry. The effect of the supernatant on the spores was maintained even after washing the CaO powders with physiological saline a few times (data not shown). The components of the supernatant are Ca$^{2+}$ and OH$^-$. Therefore, the Ca$^{2+}$ in the slurry will weaken the dormancy of the spores.

2.3 Antibacterial activity of ceramic powder slurry including nutrients

Considering application of these ceramic powders to food processing, the efficacy of the ceramic powder slurries must be examined in the medium including nutrients. In the previous study$^{24}$, we used the conductance method to evaluate the antibacterial activity of ceramic powder slurries. The conductance method depends on the detection of
a conductance change of the agar medium caused by bacterial metabolism\textsuperscript{3}. The module used for the measurement of the conductance change contains nutrient components of agar medium. And so, the efficacy of the MgO, CaO and ZnO powder slurries on the sponges in the system including medium component, such as nutrients was examined by the conductance method.

A conductance change is detected when the concentration of the test bacteria reaches approximately 10\textsuperscript{7} CFU/ml. The time required to reach this concentration is called “detection time” (DT). From the change in the value of DT, the antibacterial activity of the ceramics powder slurry can be evaluated.

Figures 5(A) and 5(B) illustrate the antibacterial activity of the CaO powder slurry on the vegetative cells and the spores of \textit{B. subtilis}, respectively. Percent conductance change is plotted against incubation time. For the vegetative cells, the DT of the control was approximately 6 h. The value of DT was delayed with an increase in powder concentration. The DT at concentrations of 3.1 and 6.3 mg/ml were about 7 and 8 h, respectively. The DT was not detected over 12.5 mg/ml for the measurement of 48 h. From the incubation in BHI broth after the conductance measurement, it was found that the CaO powder slurry exhibited a bactericidal action on the vegetative cells of the \textit{B. subtilis} over 12.5 mg/ml.

As shown in Fig.5 (B), CaO powder slurry exhibited efficacy on the bacterial spores too. For the spores, the DT of the control was approximately 8.5 h. The difference in the DT values between the vegetative cells and the spores of \textit{B. subtilis} is due to the time required to propagate from spores to vegetative cells. The antibacterial activity of the CaO powder slurry against the spores was enhanced with increasing powder concentration. The DT at 6.3 mg/ml was approximately 12.7 h. DT was not observed over 12.5 mg/ml. At higher than this concentration, the CaO powder slurry also exhibited a bactericidal action on the spores.

The results of the antibacterial activity of the MgO, CaO and ZnO powders against the vegetative cells and the spores of \textit{B. subtilis} are summarized in Table 1. DT/DT\textsubscript{cont} is the ratio of the DT at the specified powder concentration to that of control. As shown in Table 1, the MgO and ZnO powders also exhibit antibacterial activity against the spores of \textit{B. subtilis}. The minimal inhibitory concentrations of MgO and CaO powders against the spores (DT was not detected) were 12.5 mg/ml, and were equal to those against the vegetative cells of \textit{B. subtilis}. Moreover, based on the result with BHI broth incubation, the MgO and CaO powders exhibited a bactericidal action on both the spores and the vegetative cells.

The ZnO powder was also effective for the spores of \textit{B. subtilis}. For both the spores and the vegetative cells, the DT values were delayed by the addition of the ZnO powder, and not detected over 1.6 mg/ml. Based on incubation with BHI broth, it was found that the ZnO powder exhibited a bacteriostatic action against both the spores and the vegetative cells over 1.6 mg/ml.

### Table 1. Evaluation of antibacterial activity of the MgO, CaO and ZnO powder slurries on the vegetative cells and the spores of \textit{B. subtilis} by conductance method

<table>
<thead>
<tr>
<th>Powder conc.[mg/ml]</th>
<th>DT/DT\textsubscript{cont}.</th>
<th>Vegetative cells</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgO 25.0</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>12.5</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>6.3</td>
<td>1.48</td>
<td>1.61</td>
<td>1.10</td>
</tr>
<tr>
<td>3.1</td>
<td>1.11</td>
<td>1.06</td>
<td>0.94</td>
</tr>
<tr>
<td>1.6</td>
<td>0.93</td>
<td>0.87</td>
<td>0.90</td>
</tr>
<tr>
<td>0.8</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>CaO 25.0</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>12.5</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>6.3</td>
<td>1.30</td>
<td>1.42</td>
<td>0.93</td>
</tr>
<tr>
<td>3.1</td>
<td>1.12</td>
<td>0.93</td>
<td>0.93</td>
</tr>
<tr>
<td>1.6</td>
<td>1.01</td>
<td>0.87</td>
<td>0.90</td>
</tr>
<tr>
<td>0.8</td>
<td>0.93</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>ZnO 25.0</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
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<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>6.3</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
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<tr>
<td>3.1</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>1.6</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
</tr>
<tr>
<td>0.8</td>
<td>4.30</td>
<td>4.47</td>
<td>4.47</td>
</tr>
<tr>
<td>0.4</td>
<td>2.47</td>
<td>2.37</td>
<td>2.37</td>
</tr>
<tr>
<td>0.2</td>
<td>2.21</td>
<td>2.20</td>
<td>2.20</td>
</tr>
<tr>
<td>0.1</td>
<td>1.11</td>
<td>1.41</td>
<td>1.41</td>
</tr>
<tr>
<td>0.05</td>
<td>1.01</td>
<td>1.17</td>
<td>1.17</td>
</tr>
</tbody>
</table>

N.D: DT was not detected during incubation for 48 h with underline: bacteriostatic effect, without underline: bactericidal effect.

From the results, the MgO, CaO and ZnO powder slurries exhibited an efficacy for pasteurization of the spores of \textit{B. subtilis} under the condition that the spores and nutrients coexisted. Therefore, in the system including various nutrients, such as food processing, the use of these powder slurries will be effective for preventing bacterial contamination.

#### 2.4 Effect of ceramic powder slurries on germination of spores

In order to clarify the efficacy of the MgO, CaO and ZnO powder slurries on the spores, we examined the effect of the supernatant of the slurries on the germination of the spores of \textit{B. subtilis}. The growth from bacterial spores is roughly divided into three stages, that is, germination, outgrowth and vegetative growth\textsuperscript{13}. There are numerous reports on physiological and other changes which take place in a spore at the point of germination. These changes include losses in dipicolinic acid and calcium content of the cell, losses of refractivity and optical density, an increase in permeability to dyes and loss of heat resistance. They can also include losses in resistance to chemicals which exhibit antibacterial activity\textsuperscript{27}.

Hachisuka \textit{et al.} found that the optical density of a spore suspension of \textit{B. subtilis} markedly decreased with the progress of spore germination, and that the decrease in optical density correlated with the loss of heat resistance as a standard for spore germination\textsuperscript{6}. Many workers have evaluated spore germination by measurement of the decrease in optical density of the spore suspension at 660 nm\textsuperscript{7, 10, 15}. We also observed spore germination by this method.

**Figure 6** illustrated the effects of the supernatant of
the MgO, CaO and ZnO powder slurries on the germination of the spores of B. subtilis. BHI broth was used as a germination medium. In the case of the control, the optical density decreased to 90% of the initial state after 1 h. The decrease in the optical density shows the progress of spore germination. It was reported by many workers that heat treatment (preincubation at the range from 333 to 363 K) weakened the high dormancy of spores and acted as a trigger of spore germination. This heat activation is carried out to cause the initiation of germination simultaneously and enhance percent germination. Under favorable conditions, spores activated by heat treatment become easy to generate together and lose their high resistance to various stresses. In this study, however, heat activation was not carried out. Therefore, a slight decrease in the optical density of the control indicated that, even in BHI broth, few spores that were not activated by heat treatment germinated.

On the other hand, for the supernatant of slurry, the values of the optical density decreased more than 25% in 1 h. This remarkable decrease means that spore germination is promoted by the addition of the supernatant. Therefore, metallic ions in the supernatant of the slurries might contribute much to weaken the dormancy of the spores and act as triggers of spore germination. Similar results were obtained in the cases of MgCl₂, CaCl₂, and ZnCl₂ solutions (data not shown). Many workers also reported that inorganic ions took part in spore germination.

7, 16, 21 In those studies, it was reported that the spore germination of B. megaterium, which were activated at 337 K for 10 min, was promoted by the addition of MgCl₂, CaCl₂, and ZnCl₂.

Under the condition that the spores and nutrients existed together, there was no difference in the sensitivity to the ceramic powder slurry between the vegetative cells and the spores of B. subtilis. Therefore, it was considered that the ceramic powder slurry acted on the germinated spores. In the case of MgO and CaO powder slurries, the high pH would generate severe stress on the germinate spores, vegetative cells and the spores which lost their high dormancy. However, as described in the previous study, it was suggested that there were factors other than the pH effect on growth inhibition by the MgO and CaO powder slurries. It was considered that the antibacterial activity of the ZnO powder slurry originates from the surface. The action mechanisms of these ceramic powder slurries and damaged parts in bacteria are now being examined in further detail.

In the previous study, we examined the mutagenicity of the MgO, CaO and ZnO powders. Most mutagens are proved to be carcinogens. Screening tests for mutagenicity of these ceramic powders are needed before their application. These powders were found not to be mutagenic, and the MgO powder showed antimutagenicity. Calcium preparations including MgO, CaO, and ZnO have been used as food additives for a long time. Issiki et al. reported that a calcium preparation, of which the major component was Ca(OH)₂ and CaO, suppressed bacterial growth. Furthermore, Mine et al. applied a calcium preparation to meat processing and reported an improvement of storage quality and tenderness, and an increase in moisture content of products were observed. Our results in this study have proved that the MgO, CaO and ZnO powder slurries exhibit antibacterial activity not only against vegetative cells, but also bacterial spores. Therefore, it will be possible to apply these powder slurries to food processing.

We have already used the acid range, such as acetic acid and various organic acids, as a method for keeping the quality of food safe for a long time. On the other hand, techniques for keeping quality of food in the alkaline range have not been developed yet. The MgO and CaO powder slurries have high pH values. An application of the alkaline range to food processing would also be effective and attractive.

Concluding Remarks

In this study, the effects of MgO, CaO and ZnO powder slurries on spores of B. subtilis were examined. The following results and conclusions were obtained.

1) The CaO and MgO powder slurries were able to kill spores of B. subtilis in physiological saline. The CaO powder slurry had stronger efficacy compared with the MgO powder slurry.

2) The efficacy of the CaO powder slurry on spores of B. subtilis could depend on the effects of Ca²⁺ and some factors due to contact between the spores and the CaO powders.

3) Under the condition that the spores of B. subtilis and nutrients existed together, it was observed that the MgO, CaO and ZnO powder slurries exhibited antibacterial activity against the spores. There was no difference in sensitivity to the ceramic powder between the vegetative cells and the spores of B. subtilis. The MgO and CaO powder slurries exhibited a bactericidal action and the ZnO powder slurry exhibited a bacteriostatic action on the spores.
4) The supernatant of the MgO, CaO and ZnO powder slurries promoted spore germination of *B. subtilis*. Therefore, it was considered that ceramic powder slurries did not inhibit the spore germination, and acted on the germinated spores and the spores which lost their high dormancy.

**Nomenclature**

N = concentration of viable cells in the suspension of spores or vegetative cells [CFU/ml]

N<sub>0</sub> = initial state

**Literature cited**


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