EVALUATION OF GROWTH INHIBITORY EFFECT OF CERAMICS POWDER SLURRY ON BACTERIA BY CONDUCTANCE METHOD

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The growth inhibitory effect of 26 ceramic powder slurries on bacteria was evaluated by measurement of the conductance change of the growth medium caused by bacterial metabolism (conductance method). *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis* were used as test bacteria. It was found that the growth of the test bacteria was inhibited by 10 ceramic powders. Magnesium oxide (MgO) and calcium oxide (CaO) powder slurries had a growth inhibitory effect on all test bacteria. In the cases of MgO and CaO powder slurries, there was no difference in sensitivities among the test bacteria. These powder slurries exhibited bactericidal action on the test bacteria. On the other hand, the zinc oxide (ZnO) powder slurry inhibited the growth of Gram-positive bacteria stronger than Gram-negative bacteria. The effect was bacteriostatic action. The conductance method could provide quantitative and simple evaluation of the growth inhibitory effect of ceramic powder slurries on bacteria, and was more applicable than the conventional methods, such as the halo test.

Introduction

Recently, microbial contamination and deteriorations induced by microorganisms are spreading to various fields, such as foodstuffs, plastic materials, building materials[20], petroleum products, electronic equipment, optical instruments, and textile materials[23]. Infection and contamination in medical treatment are always serious problems. In response to these problems, new pasteurization and antibacterial technologies are desired, and the application of ceramics materials which have growth inhibitory effects on bacteria is attracting considerable attention. Antibacterial ceramic is milled into synthetic fibers, for example, to make the fabric antibacterial[16, 17]

However, there have been no fundamental studies on the growth inhibitory effect of the ceramic itself on bacteria, and an evaluation method has not yet been established. Antibacterial activity is usually evaluated by the halo test (Agar Plate method) or AATCC method. These methods are applicable to an antibacterial agent that is able to diffuse or penetrate into agar or broth. But, they are unsuitable in the cases of agents with a slight solubility or insoluble agents[9]. Therefore, it is necessary to develop a method for quantitative, quick, and easy evaluation of the effect of the materials such as ceramics. The conductance method depends on the detection of the conductance change generated by microbial growth and metabolism[2, 6, 19]. In particular, application to the detection of microbial contamination in foodstuffs, such as meat[1, 5, 13] or milk[7], have been reported. The conductance or impedance method has recently been applied to clinical trails, such as a rapid detection of urinary tract infection[3] and a rapid monitoring of microbial contamination in blood[4]. The present objective is to discuss the applicability of the conductance method for the evaluation of the growth inhibitory effect of a ceramic powder slurry on bacteria and the inhibition mechanism of bacterial growth.

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1. Materials and Methods

1.1 Test organism

Test bacteria used in this study are stored at the Tokyo Metropolitan Research Laboratory of Public Health and shown in **Table 1**. *S.aureus* A and B were identified as meticillin-resistance *S.aureus* (MRSA). The test bacteria were cultured in Brain Heart Infusion (BHI) broth (Difco) at 310 K for 24h on a reciprocal shaker. The culture was then suspended in sterile physiological saline to yield a final bacterial concentration of approximately 10^3 CFU/m*l*.

1.2 Preparation of Ceramics powder slurry

We selected 26 ceramic powders including metallic oxides, nitrides, and carbides. The ceramic powders used in this study are listed in **Table 2**. The ceramic powder was heated at 453 K for 20 min. The powder was suspended in sterile physiological saline to yield a specified concentration.

Table 1 Test bacteria

Bacteria	Strain	Origin
Gram-positive		
Staphylococcus aureus	9779	food
	A	clinic
	B	clinic
Bacillus subtilis (vegetative cells)	U20	food
Gram-negative		
Eschricha coli	745	food
Salmonella typhimurium	TSA2121	food
Pseudomonas aeruginosa	46	clinic

Table 2 Ceramics powder

	Ceramics	Grade	
Oxides	BeO	99.9%	1
	MgO	Extra grade	1
	CaO	Extra grade	1
	TiO_2(rutile)	99.5%	1
	TiO_2(anatase)	98%	2
	V_2O_5	Extra grade	2
	MnO_2	Extra grade	2
	NiO	99%+	2
	ZnO	Extra grade	1
	Al_2O_3(α-)	99%, 5μ	1
	Al_2O_3, active neutral	70~230mesh	3
	In_2O_3	99. ~%	2
	SiO_2	Extra grade	1
	pbO_2	95%+	2
	SnO_2	99%	2
	Bi_2O_3	99%	2
Nitrides	TiN	99%	1
	BN	99.5%,1~16μ	1
	Si_3N_4	99%, 0.98μ	1
Carbides	TiC	98%	1
	NbC	99.5%, 325mesh	1
	TaC	99%, 3μ	1
	Mo_2C	99%, 1~2μ	1
	WC	99%	1
	B_4C	99%, 220mesh	1
	SiC	#16	1

[1] from Kisida Chemicals
[2] from Wako Chemicals
[3] from Merck

1.3 Apparatus

For measurement of the conductance change caused by bacterial growth, BACTOMETER® microbial monitoring system model 64 (bio Mérieux VITEK) was used. This system includes a Bactometer processing unit (BPU) equipped with incubator, a microcomputer, a display, and a printer (**Figure 1A**). The BPU contains two independent temperature controlled incubators and is capable of monitoring up to 64 separate samples. For the test, a Bactometer disposable module was used (**Figure 1B**). The module is divided into 16 individual wells, which contain paired electrodes (**Figure 1C**). The disposable modules have caps to prevent evaporation of samples during incubation.

1.4 Measurement of the conductance change of growth medium

Modified plate count agar (DIFCO) was used as the growth medium. Sterilized hot agar was poured into the well of the module until it covered the electrodes. After the agar solidified, both the bacterial suspension and the ceramic powder slurry were pipetted into the well (Figure 1C). The caps were placed back on the modules. The modules were set in the BPU, and the conductance change of the agar was monitored during incubation at 308 K for 48 h.

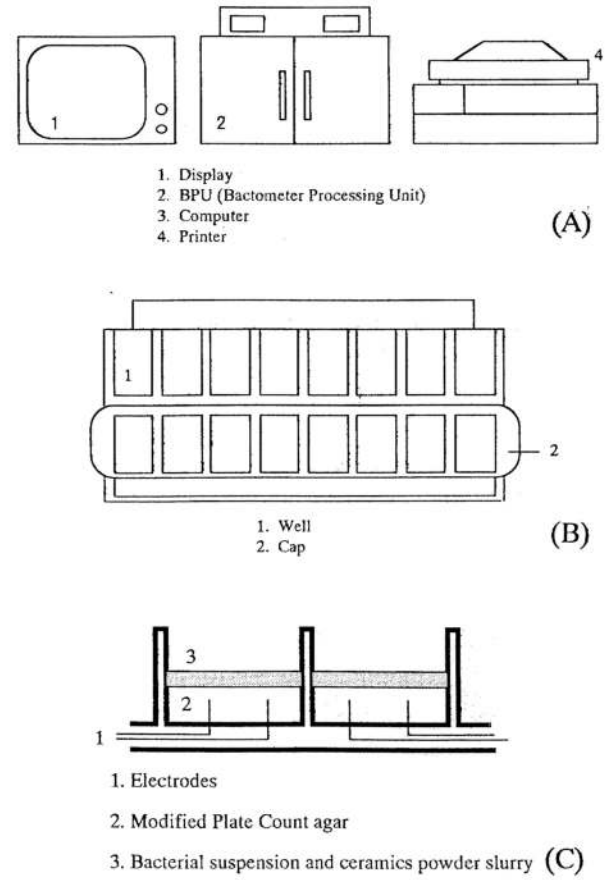

Fig. 1 Scheme of apparatus.
A: Bactometer. B: Scheme of module
C: Scheme of well

1.5 Distinction of bactericidal or bacteriostatic action

After incubation, a small quantity of the slurry from the well was cultured in BHI broth at 308 K for 24 h. At that time, because the sample was diluted with BHI broth about a thousand times, the effect of powder accompaniying the sample on bacterial growth could be neglected. Furthermore, identification of the test bacteria was performed. Eosin Methylene Blue agar was used for *E. coli* and *S. typhimurium*. Nalidixic acid cetrimide agar was used for *P. aeruginosa*. Mannitol salt agar containing 3% egg yolk was used for *S. aureus*. Based on the results, it was determined whether the growth inhibitory effect of ceramic powder slurries was due to bactericidal or bacteriostatic action.

1.6 Measurement of pH value of ceramics powder slurry.

All powder slurries were prepared at 25 mg/m*l*. Each slurry was stirred, and the pH value was confirmed to be steady. Then the pH value of the slurry was measured by pH meter while stirring.

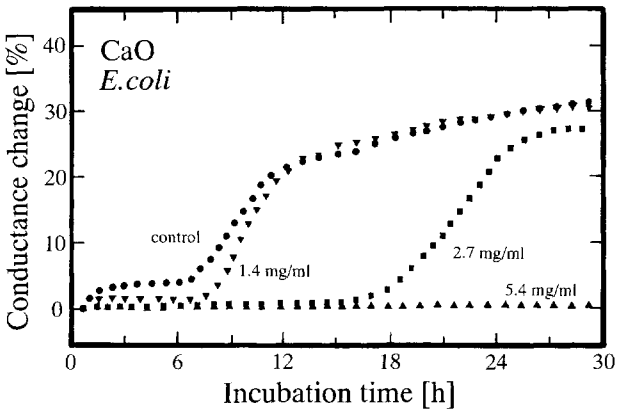

Fig. 2 Effect of CaO powder slurry on growth of *E. coli*

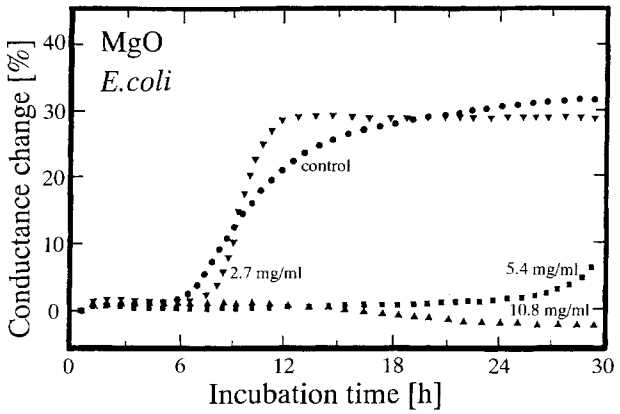

Fig. 3 Effect of MgO powder slurry on growth of *E. coli*

2. RESULTS

2.1 Measurement of growth inhibitory effect by conductance method

The conductance change was detected when the test bacteria reached a threshold concentration of approximately 10^7 viable organisms per m*l* for the Bactometer. The time required to reach this threshold concentration is called the "detection time (*DT*)". We used the *DT* value as a criterion for the evaluation of the growth inhibitory effect of ceramics powder slurry on bacteria.

Figure 2 presents the conductance curves for *E. coli* in the CaO powder slurry. Percent conductance change was plotted against incubation time in hours. The *DT* of the control (the CaO powder concentration is 0 mg/m*l*) was about 6 h, which was necessary for growth from 10^3 to 10^7 CFU/m*l*. Hence, if the *DT* is delayed by adding ceramic powder slurry, it can be distinguished that the ceramic powder slurry has a growth inhibitory effect on the bacteria. On the other hand, if the *DT* decreases, it can be judged that the ceramic powder slurry has a growth promoting effect on the bacteria.

As shown in Fig. 2, the increase in the concentration of the CaO powder slurry increases the *DT*. The *DT* at a concentration of 1.4 mg/m*l* was about 8 h, and the *DT* was delayed to 18 h at a concentration of 2.7 mg/m*l*. The delay of the *DT* indicates that the CaO powder slurry inhibits the growth of *E. coli*. The CaO powder slurry completely inhibited the growth of *E. coli* at a slurry concentration higher than 5.4 mg/m*l* for the incubation time of 48 h. In the case of the MgO powder slurry, the *DT* was not observed at a slurry concentration over 10.8 mg/m*l* within 48 h (**Figure 3**). Based on the results of incubation with BHI broth, it was found that the CaO and MgO powder slurries exhibited bactericidal action on *E. coli* in these regions of the slurry concentration.

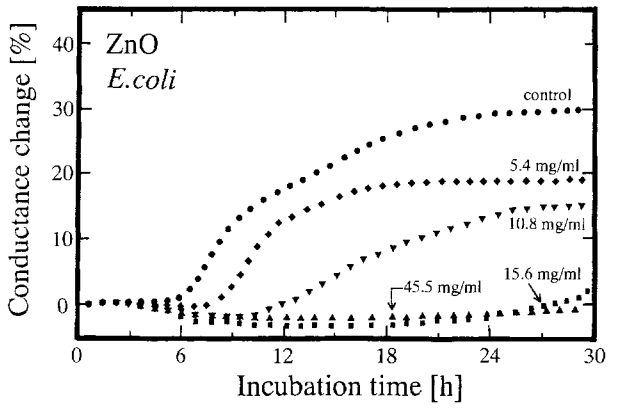

Fig. 4 Effect of ZnO powder slurry on growth of *E. coli*

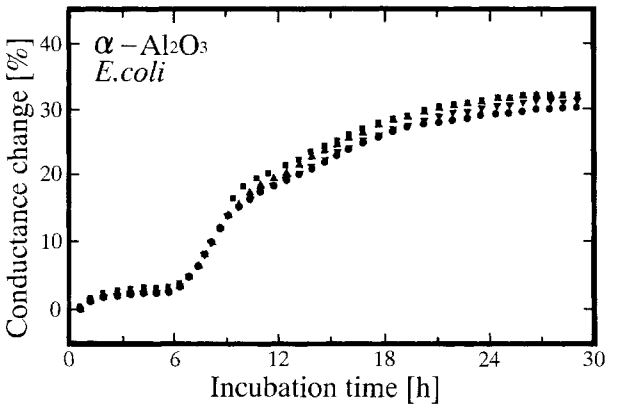

Fig. 5 Effect of α - Al_2O_3 powder slurry on growth of *E. coli* Symbols; ●, control; ▼, 62.5 mg/m*l*; ■, 125 mg/m*l*; ▲, 250mg/m*l*

The growth inhibition of *E. coli* by the ZnO powder slurry is illustrated in **Figure 4**. The increase in the ZnO powder slurry concentration induced a delay of *DT*, which indicated that the ZnO powder slurry had a growth inhibitory effect on *E. coli*. Furthermore, the maximum values of conductance change attained at slurry concentrations of 5.4 or 10.8 mg/m*l* were lower than that of the control. These differences in conductance change indicate that cell division stops earlier than the control. *DT* was not observed at a slurry concentration over 45.5 mg/m*l*. According to the results of the incubation with BHI broth, it was found that the ZnO powder slurry had a bacteriostatic action on *E. coli* at a slurry concentration up to 100 mg/m*l* and showed a bactericidal action over 100 mg/m*l*. The efficacy of these powder slurries did not decrease by washing the powders with physiological saline three times (data not shown).

On the other hand, if the *DT* decreases, it can be

Table 3 Changes in detection time by addition of Al_2O_3-active powder

Bacteria	Concentration [mg/ml]	DT [h]
S. aureus 9779	0	10.5
	12.5	10.4
	25.0	10.2
	50.0	9.8
	100.0	9.0
E. coli 745	0	5.7
	12.5	5.5
	25.0	5.6
	50.0	5.6
	100.0	6.0

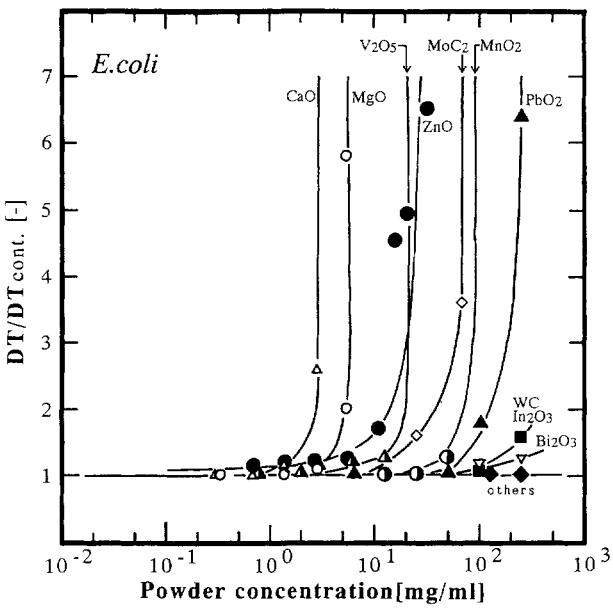

Fig. 6 Comparison of growth inhibitory effects of ceramic powder slurries on *E. coli*

judged that the ceramics powder slurry has a growth promoting effect on the bacteria. The result of the α-Al_2O_3 powder slurry for *E. coli* was shown in **Fig. 5**. There was no delay of the *DT* due to the α-Al_2O_3 powder, and the conductance curves at any powder concentration were the same as that at 0 mg/ml (control). The α-Al_2O_3 powder slurry had no effect on the growth of *E. coli*. The Al_2O_3-active powder slurry actually promoted bacterial growth. **Table 3** shows the changes in *DT* by adding the Al_2O_3-active powder. In the case of *S. aureus* 9779, the *DT* decreased with an increase in powder concentration. However, this effect was not observed for *E. coli*.

2.2 Comparison of growth inhibitory effect of ceramic powder slurries on bacteria

The growth inhibitory effects of 26 ceramic powder slurries on the test bacteria were evaluated by the conductance method. Eight oxide and two carbide ceramic powder slurries inhibited the growth of the test bacteria. **Figure 6** shows a comparison of growth inhibitory effects of eleven ceramic powders on *E. coli*. The abscissa $DT/DT_{cont.}$ represents the ratio of the *DT* at specified concentrations of ceramic powder slurry to that at 0 mg/ml (control). The ceramic powder whose curve showed

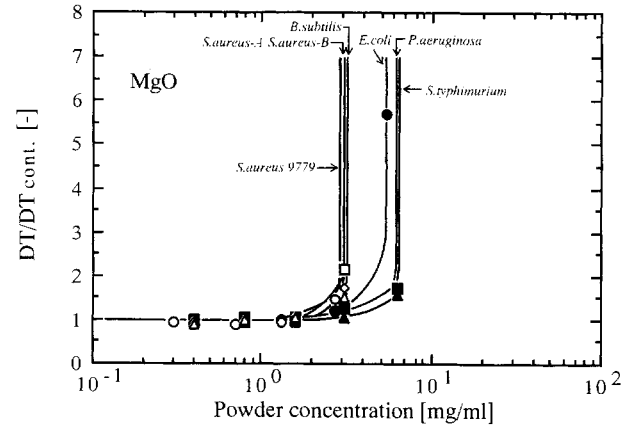

Fig. 7 Growth inhibition of test bacteria by MgO powder slurry

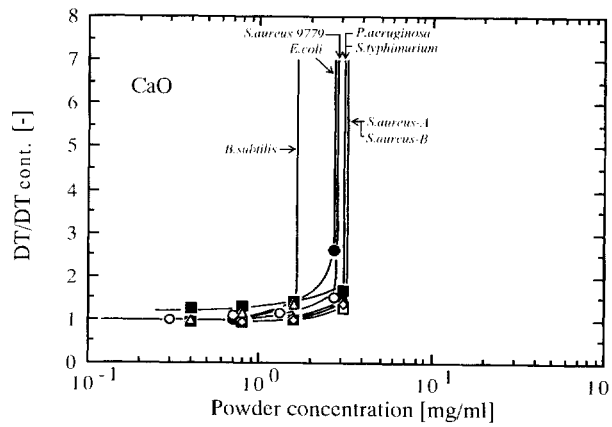

Fig. 8 Growth inhibition of test bacteria by CaO powder slurry

a steep rise at a lower powder concentration shows more growth inhibitory effect on the bacteria. When the ceramic powder does not show the growth inhibitory effect, the value of $DT/DT_{cont.}$ is unity. As shown in Fig. 6, alkaline earth metallic oxides, such as the MgO and CaO powders, were most effective on *E. coli*. In order of degree of the effect, powders were ranked in the order of V_2O_5 , ZnO, MoC_2, MnO_2, PbO_2, WC, In_2O_3, and then Bi_2O_3 powders. The ZnO and PbO_2 powder slurries exhibited bacteriostatic action on *E. coli* within the incubation time of 48 h.

2.3 Antibacterial spectra of ceramics powder slurries.

Two powders, MgO and CaO, and the ZnO powder were selected from among the ceramics powders with bactericidal and bacteriostatic action, respectively. The growth inhibitory effects of these powder slurries on vegetative cells were examined by the conductance method. **Figures 7** and **8** showed the sensitivities of the test bacteria to the MgO and CaO powder slurries. As shown in Figs.7 and 8, both the MgO and CaO powder slurries inhibited bacterial growth and showed bactericidal action on all vegetative cells used in these experiments. Although there appeared to be no large difference in sensitivities among the test bacteria to the MgO powder slurry, the slurry had a tendency to inhibit the growth of Gram-positive bacteria stronger than Gram-negative bacteria. The

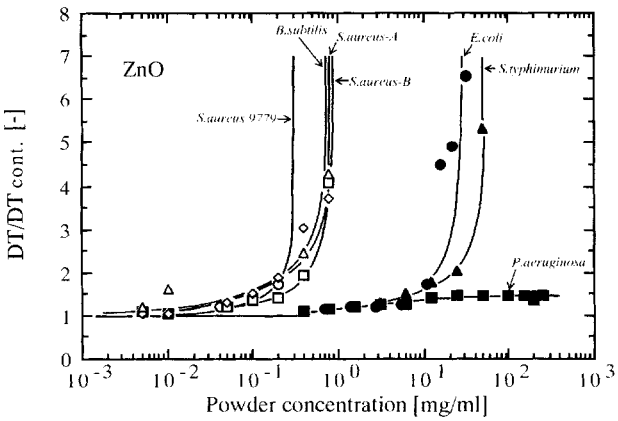

Fig. 9 Growth inhibition of test bacteria by ZnO powder slurry

differences in sensitivity among the test bacteria toward the CaO powder slurry was smaller than those toward the MgO powder slurry. In both powder slurries there appeared to be no differences in sensitivities among three strains of *S. aureus,* including MRSA (*S. aureus* A and B). From the results, the MgO and CaO powder slurries are shown to have a broad antibacterial spectrum.

The character of the growth inhibitory effect of the ZnO powder slurry was very different from those of the MgO and CaO powder slurries (**Figure 9**). There was a large difference in sensitivities among the test bacteria toward the ZnO powder slurry. For the Gram-positive bacteria, the growth inhibitory effect of the ZnO powder slurry was markedly stronger than those of the MgO and CaO powder slurries. The inhibitory effect was due to bacteriostatic action at 25-50 mg/m*l* and to bactericidal action at higher concentrations. For the Gram-negative bacteria, the inhibitory effect of the ZnO powder slurry was weaker than those of the MgO and CaO powder slurries, and did not appear at all for *P. aeruginosa.*

3. Discussions

3.1 Evaluation by conductance method

The growth inhibitory effects of ceramics materials on bacteria are usually determined using the halo test. The halo test was unable to evaluate the effect without specifying both the depth of the agar plate and the spread of bacterial concentration on the plate. Furthermore, it was necessary to conduct experiments in which the slurry concentration was higher than approximately 100 mg/m*l* because the powder slurry ran out on the agar plate at lower concentrations. The halo test could evaluate the growth inhibitory effect of ceramics on bacteria qualitatively, but was unsuitable for the quantitative evaluation of the effect of insoluble powders such as the ceramic powder. In addition, it was very hard to identify whether the bacteria in the halo were alive (bacteriostatic action) or dead (bactericidal action) and to observe continuous bacterial growth. Evaluations by another method were necessary to precisely grasp the effect.

Table 4. pH values of ceramic powder slurries

pH	Ceramic powder
12.6	CaO
11.3	Bi_2O_3
10.4	MgO
9.5	Al_2O_3
8.4	B_4C
8.0	Al_2O_3-active neutral
7.8	ZnO, Mo_2C
7.7	In_2O_3
7.5	TiN BeO, SiC(7.0)
7.0~6.0	BeO, SiC, TiO_2-rutile
	SiN_4, NiO, TiO_2-anatase, BN
	PbO_2, WC, SiO_2
5.9	TiC
5.8	TaC
3.3	SnO_2, NbC
2.6	MnO_2
2.4	V_2O_5

The shake flask method (plate count method) is complex and laborious, and the time required for the microorganisms to grow sufficiently and to form visible colonies is 24 to 48 h. The turbidometry method[14] is unsuitable because of the turbidity of ceramic powder slurries. On the other hand, the conductance method can automatically determine the number of microorganisms, even in turbid samples. Thus, we attempted to evaluate the growth inhibitory effect of ceramic powder slurries on bacteria by using the conductance method.

Based on the foregoing results, the conductance method could provide quantitative, fast, and easy evaluation of the growth inhibitory effect of ceramic powder slurries on bacteria. Furthermore, it could be realized whether the inhibitory effect of ceramics powder slurriy was due to bactericidal action or to bacteriostatic action by incubation with BHI broth. The conductance method was more applicable than the conventional method (ie. halo test) for evaluation of the growth inhibitory effect of ceramic powder slurries.

3.2 Factors in bacterial growth inhibition by ceramic powder slurries

We considered the factors in bacterial growth inhibition by the MgO, CaO, and ZnO powder slurries. Ceramic powders in the air usually adsorb H_2O molecules at ordinary temperature, and hydroxyl groups exist on the surface[24]. In particular, the MgO and CaO absorb H_2O and CO_2 easily. In the slurry, the surface of the MgO and CaO is almost entirely covered by hydroxide. Therefore, for the these powders, it was considered that the growth inhibitory effect of the hydroxide rather than the oxide is examined. **Table 4** lists the pH values of ceramic powder slurries. Among ceramics, alkaline earth metallic oxides, such as MgO and CaO, are relatively soluble in water. Slurries of these powders have high pH values. The pH effects of these ceramic power slurries is considered to be a primary factor of the bactericidal action. However, agar medium in the well of a Bactometer module has a buffer action to some extent. So, the pH value of the slurry decreases. In fact, at a MgO powder concentration of 10.8 mg/m*l* (Fig. 3), and at a CaO powder concentration of 5.4 mg/m*l* (Fig. 2), the bulk pH values of the slurries in the well were confirmed

to decrease below pH 8.5 and 9.0, respectively, within about three minutes after the slurry was pipetted onto the agar in the well. The pH values have a bacteriostatic potential, but do not have a killing effect on the test bacteria. The effect of the supernatant of slurry on bacterial growth was examined using a conductance method. There were no delay of *DT* value and no changes of the shape of conductance curve of *E. coli* and *S. aureus*. The supernatant of the CaO powder slurry showed a slight delay of *DT* value (data not shown). These results suggest that the growth inhibitory effects of MgO and CaO appear very near the surface or on the surface, and depend on the local pH on the surface. However, the growth inhibitory effect of the Bi_2O_3 powder slurry, which also had a high pH value was markedly weak compared to those of the MgO powder slurry as shown in Table 4. There may also be other factors other than the pH effect on growth inhibition by the MgO and CaO powder slurries.

Alkaline earth metal oxides are known as catalysts that exhibit remarkable activity and interesting selectivity for isomerization of olefins, alkylation of phenol with methanol, and dimerization of pyridine derivatives. In these reactions, the electron spin resonance spectra of O_2^- ion is observed on the surfaces of these oxides[10]. The active oxygen generation of these ceramic powders may take part in growth inhibition by these powder slurries, and its examination is now in progress in our laboratory.

In the case of powder slurries with neutral pH, the growth inhibitory effects depend on the metal ions in the ceramics, but not on pH effect. Studies on oligodynamic action (bactericidal action caused by a small amount of metal ion) of various metals to bacteria have been reported by various investigators[21, 22, 25]. The ZnO powder slurry had stronger growth inhibitory effect on Gram-positive bacteria than Gram-negative bacteria and exhibited the bacteriostatic effect. Zinc is known to show antimicrobial activity, and growth inhibition by the ZnO powder slurry can possibly be attributed to zinc. The supernatant of the ZnO powder slurry had no effect on growth of *E. coli* and *S. aureus* in the conductance method. Eluted zinc ions are considered not to inhibit the bacterial growth because of the low solubility of ZnO. Therefore, it is natural to suppose that the growth inhibitory effect of the ZnO powder slurry originates from the powder surface. The action mechanisms of these ceramic powder slurries are now being examined in further detail.

Bacteria in ceramic powder slurries exist and grow in liquid, on agar and on liquid-solid interfaces. Considering that supernatants of MgO, CaO and ZnO had no or slight effect on bacterial growth, lots of bacteria would be adsorbed and/or killed at liquid-solid interfaces. It is reported that the rates of glucose uptake and oxygen consumption of *E. coli* were altered by adsorption on various liquid-solid interfaces[15]. Navarro *et al.* and Marcipar *et al.* also reported that the immobilization of yeast on porous glass and ceramics drastically changed their metabolism, such as ethanol production[12, 18]. There was a case where the α-Al_2O_3 powder did not affect the metabolism and growth rate (Fig.4). However, as shown in Table 3, the conductance method can study the whole metabolism and the mean growth rate of bacteria, but not each metabolism and each growth rate of bacteria under different conditions. In addition, the work of Hattori[8] and Lee *et al.*[11] demonstrated the shift of pH value for maximum oxidative activity and the appearance of resistance to heat and antimicrobial agents. If metabolism and the growth of bacteria on ZnO powder decrease or almost stop, the measurement of changes in metabolism and the growth rate is important. Therefore, it is also necessary to study the growth inhibitory effect of ceramic powders from the viewpoint of interface-effect.

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