Journal of Industrial Microbiology & Biotechnology (2002) 29, 296–298

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SHORT COMMUNICATION
Indirect conductimetric assay of antibacterial activities

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The applicability of indirect conductimetric assays for evaluation of antibacterial activity was examined. The minimal inhibitory concentration (MIC) obtained by the indirect method was consistent with that by the direct conductimetric assay and the turbidity method. The indirect assay allows use of growth media, which cannot be used in the direct conductimetric assay, making it possible to evaluate the antibacterial activity of insoluble or slightly soluble materials with high turbidity, such as antibacterial ceramic powders.
Journal of Industrial Microbiology & Biotechnology (2002) **29,** 296–298 doi:10.1038/sj.jim.7000314

Keywords: conductimetric assay; antibacterial activity; antibiotic; CaO; MgO; ZnO

Introduction

The conductimetric assay detects bacterial growth and metabolism *via* the electrical conductivity obtained by contacting a pair of electrodes directly with growth medium and supplying the electrodes with an alternating current (direct conductimetric assay). The number of microorganisms can then be estimated rapidly based on the correlation between microbial concentration and the time required to detect a conductivity change [4]. This method is widely applied for rapid microbial detection in foodstuffs such as fish [3], meat [5], milk [6], and soft drinks [7], and in clinical trials [2]. The key advantage of the conductimetric assay is its applicability to highly turbid samples, such as foods and insoluble materials [11]. However, because bacterial growth is detected as a conductivity change of the growth medium, the range of media suitable for these measurements is limited. For example, selective media such as mannitol salt agar cannot be used because of the high salt concentration. The method is also unsuitable for detecting filamentous fungi and yeast, which do not produce sufficient electrolytes [4].

To overcome these disadvantages, an indirect conductimetric assay was proposed, in which the CO_2 produced by microorganisms is detected as an electrical change [10]. In the indirect conductimetric assay, a pair of electrodes contacts a CO_2-absorbent material rather than contacting the bacterial suspension, sample, or growth medium directly. This is the ''indirect'' concept of this methodology. Bolton [1] reported that indirect conductimetry is a powerful tool with which to detect bacteria and yeast. We have shown that media unsuitable for direct assay can be applied to the indirect assay to detect bacteria [12]. Moreover, we have successfully analyzed the kinetic bacterial growth rate by using an indirect assay [13].

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Received 2 April 2002; accepted 30 July 2002

Inorganic antimicrobial materials are being used increasingly for decontamination and the prevention of biodeterioration in many fields [8,14]. MgO, CaO, and ZnO are inexpensive materials with strong antibacterial activity [11], but a method for evaluation of the antimicrobial activity of such inorganic antimicrobial materials has yet to be established. In this study, we examined the applicability of the indirect conductimetric assay for evaluating the antibacterial activity of inorganic materials for the first time. Specifically, we examined several kinds of antibiotics and metallic oxides (MgO, CaO, and ZnO) and compared the results obtained with the indirect assay, the direct assay, and the turbidity method.

Materials and methods

Microorganisms

Escherichia coli 745 and *Staphylococcus aureus* 9779 were obtained from the Tokyo Metropolitan Research Laboratory of Public Health. The bacteria were stored at $-80^{\circ}C$, then thawed and incubated in brain heart infusion (BHI) broth (Eiken Chemicals, Tokyo, Japan) at 37°C for 20 h. The cells were washed once with sterile saline (0.85 wt/vol.% NaCl) and resuspended in saline to yield a specified density.

Antibacterial agents

The antibiotics penicillin G (Sigma, St. Louis, MO, USA), chloramphenicol (Sigma), nalidixic acid (Daiichi Pharmaceutical, Tokyo, Japan), and rifampicin (Daiichi Pharmaceutical) were dissolved in sterile distilled water, absolute ethanol, 1 M NaOH, and methanol, respectively. Several twofold dilutions of the antibiotic solutions were then prepared with sterile distilled water ($100-0.1\ \mu g\ ml^{-1}$).

MgO, CaO, and ZnO powders (Kishida Chemicals, Osaka, Japan) were heated at 180°C for 20 min and stored in a desiccator. The powders were suspended in sterile saline to yield powder concentrations of $100-0.1\ mg\ ml^{-1}$ by twofold dilution.

Indirect conductimetric assay

Conductivity was measured using a Bactometer Microbial Monitoring System model 64 (BioMérieux, Marcy-l'Etoile, France),

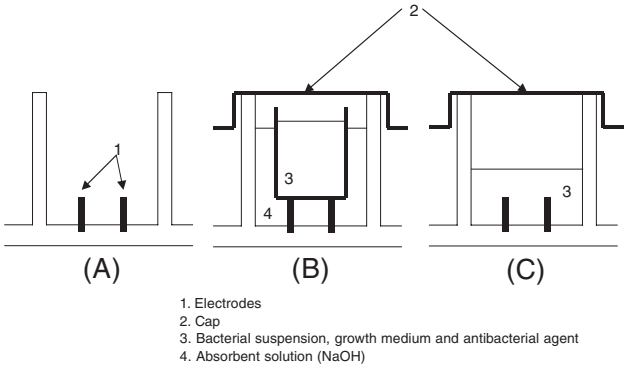

Figure 1 (A) Well, (B) indirect conductimetric assay, (C) direct conductimetric assay.

which includes a display, computer, printer, and bactometer processing unit (BPU) and which can simultaneously monitor up to 64 separate samples [12]. We measured the conductivity in a capped module with 16 individual wells, each containing paired electrodes (Figure 1A). To apply this system for an indirect assay, inner wells of Teflon were placed in each well (Figure 1B). The test strains, *E. coli* 745 and *S. aureus* 9779, were suspended in BHI broth or soybean casein digest (SCD; Eiken Chemicals) broth at approximately 10^3 CFU ml^{-1}. The number of viable CFU was determined by pour-plating on nutrient agar (Eiken Chemicals).

The antibiotic solution (0.15 ml) and bacterial suspension (0.15 ml) were poured into the inner well, and the NaOH solution at pH 12.0 (1.0 ml) was dispensed into the volume outside the inner well. The module was covered with the cap and set in the BPU, and conductivity was monitored for 48 h at 37°C.

In the case of the ceramic powders, modified plate count agar (BioMérieux) or SCD agar was used as the growth medium for the test bacteria. Sterilized hot agar (0.2 ml) was poured into the well until it covered the electrodes. After the agar solidified, the bacterial suspension (0.05 ml) and the powder slurry (0.05 ml) were pipetted into the inner well.

Direct conductimetric assay

The antibiotic solution (0.25 ml) and the bacterial suspension (0.25 ml) were poured directly into the well.

In the case of ceramic powders, sterilized hot agar (0.5 ml) was poured into the well until it covered the electrodes (Figure 1C). After the agar solidified, the bacterial suspension (0.1 ml) and the powder slurry (0.1 ml) were pipetted into the well [11,12]. The module was set in the BPU and conductivity was monitored for 48 h at 37°C.

Turbidity method

The antibiotic solution (0.5 ml) and the bacterial suspension (0.5 ml) were poured into the test tube. The tube was incubated at 37°C, samples were withdrawn from time to time, and their optical density at 660 nm (OD_{660}) was measured.

Results and discussion

Figure 1B shows the configuration of the indirect conductimetric assay. The alkaline solution (NaOH) absorbs the CO_2 produced by

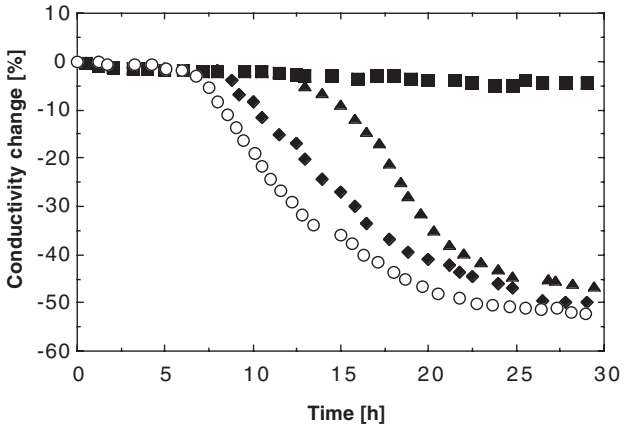

Figure 2 Conductivity curves for *E. coli* 745 after addition of chloramphenicol by indirect conductimetric assay. (○) 0 $\mu g\ ml^{-1}$; (◆) 0.8 $\mu g\ ml^{-1}$; (▲) 1.6 $\mu g\ ml^{-1}$; (■) 3.1 $\mu g\ ml^{-1}$.

bacteria in the inner well due to metabolism and growth according to the following reaction:

$$2OH^- + CO_2 \rightarrow CO_3^{2-} + H_2O \qquad (1)$$

Consequently, the molecular conductivity of the NaOH solution ($\Delta\Lambda_0$) reduces by:

$$\begin{aligned}\Delta\Lambda_0 &= \Lambda_0^{CO_3^{2-}} - 2\Lambda_0^{OH^-} \\ &= 118.6 - 2\times 198.6 \\ &= -278.6\ \text{S cm}^2/\text{mol CO}_2\ \text{absorbed} \end{aligned} \qquad (2)$$

The electrical conductivity of the absorbent decreases due to the decrease in the concentration of OH^-, which increases electric conduction [10].

Figure 2 shows the conductivity changes in the NaOH solution (pH 12.0) when chloramphenicol was added to *E. coli* 745. The time required to detect a change in electrical conductivity is called the detection time (t_D), and is used as the criterion for bacterial growth. For *E. coli* 745, the bacterial concentration at t_D was approximately 6×10^7 CFU ml^{-1} [13]. The t_D of the control

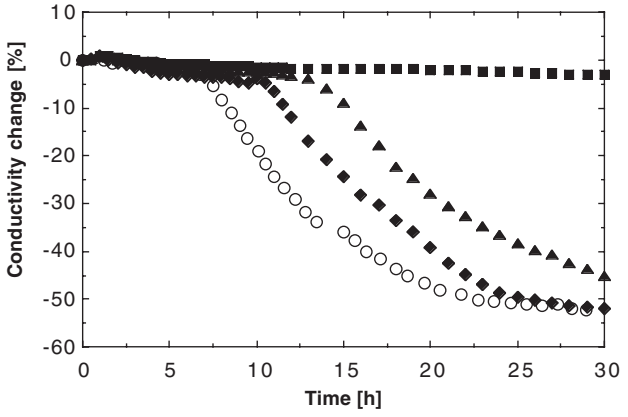

Figure 3 Conductivity curves for *E. coli* 745 after addition of CaO by indirect conductimetric assay. (○) 0 mg ml^{-1}; (◆) 0.4 mg ml^{-1}; (▲) 0.8 mg ml^{-1}; (■) 1.6 mg ml^{-1}.

Table 1 Minimal inhibitory concentration of antibacterial agents against *E. coli* 745 and *S. aureus* 9779

Growth media	Antibacterial agent	Minimal inhibitory concentration [μg ml^{-1}]					
		E. coli 745			*S. aureus* 9779		
		Indirect assay	Direct assay	Turbidity method	Indirect assay	Direct assay	Turbidity method
BHI	Penicillin G	25.0	25.0	25.0	1.6	1.6	1.6
	Chloramphenicol	3.1	3.1	3.1	3.1	3.1	3.1
	Nalidixic acid	3.1	6.3	3.1	50.0	50.0	50.0
	Rifampicin	12.5	12.5	12.5	1.6	1.6	1.6
MPCA	CaO	1.6×10^3	3.1×10^3	–	3.1×10^3	3.1×10^3	–
	MgO	6.3×10^3	6.3×10^3	–	6.3×10^3	6.3×10^3	–
	ZnO	5.0×10^4	5.0×10^4	–	1.6×10^3	1.6×10^3	–
SCD broth	Penicillin G	25.0	N.D.	25.0	3.1	N.D.	3.1
	Chloramphenicol	6.3	N.D.	6.3	3.1	N.D.	3.1
	Nalidixic acid	6.3	N.D.	6.3	50.0	N.D.	50.0
	Rifampicin	12.5	N.D.	12.5	1.6	N.D.	1.6
SCD agar	CaO	1.6×10^3	N.D.	–	1.6×10^3	N.D.	–
	MgO	1.6×10^3	N.D.	–	1.6×10^3	N.D.	–
	ZnO	1.6×10^3	N.D.	–	1.6×10^3	N.D.	–

N.D.=not detected; (–) no trial.

(0 μg ml^{-1}) was approximately 6 h, which was necessary for growth from 10^3 to 10^7 CFU ml^{-1}. Thus, the delay seen in the figure represents inhibited bacterial growth as a result of addition of the antibacterial agent. An increase in the concentration of CP increased the t_D values, and t_D at concentrations of 0.8 and 1.6 μg ml^{-1} were approximately 9 and 12 h, respectively. A t_D could not be measured at concentrations greater than 3.1 μg ml^{-1}.

In recent years, the use of inorganic antimicrobial agents has attracted interest for the control of microbes [9,14]. The key advantages of inorganic antimicrobial agents are improved safety and stability over organic antimicrobial agents. It has been reported that some metallic oxides, such as MgO and CaO, have antimicrobial activity [11]. However, it is difficult to evaluate the activity of these materials by conventional methods such as halo tests and turbidometry due to their insolubility.

Figure 3 shows the conductivity curves when CaO powder was added to *E. coli.* The addition of the CaO powder also delayed the conductivity change of NaOH, indicating that the CaO powder inhibited bacterial growth. No changes in the conductivity were observed over 1.6 mg ml^{-1}. Because the indirect assay is independent of the turbidity of samples, the antimicrobial activities of many agents, including insoluble and slightly soluble materials such as ceramic powder, can be evaluated.

The minimal inhibitory concentrations (MICs) of the antibacterial agents measured by the indirect assay are summarized in Table 1. The MIC represents the minimum concentration of agent at which the t_D is not measurable. In the turbidity method, MIC is defined as the minimum concentration at which no change in OD_{660} could be observed in the medium. Table 1 shows that the values of MIC for BHI broth and MPCA obtained by the indirect assay are almost equal to those obtained by the direct assay. Furthermore, whereas no clear conductivity change was observed for SCD broth and SCD agar in the direct assay, the indirect assay produced accurate measurement of the antibacterial activity for both, with MIC almost identical to that obtained by the turbidity method. These results demonstrate that the indirect assay can be used to evaluate antimicrobial efficacy under a variety conditions irrespective of turbidity or the kind of growth media. In recent years, many goods and materials treated or finished with antimicrobial agents have been introduced into the market. The indirect assay may, therefore, prove to be an important tool for evaluation of the performance of antimicrobial products.

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