APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1988, p. 1330–1333
0099-2240/88/061330-04$02.00/0

Vol. 54, No. 6

Continuous-Sterilization System That Uses Photosemiconductor Powders

TADASHI MATSUNAGA,* RYUZO TOMODA, TOSHIAKI NAKAJIMA, NORIYUKI NAKAMURA, AND TAMOTSU KOMINE

Department of Applied Chemistry for Resources, Tokyo University of Agriculture and Technology, Koganei Tokyo, 184, Japan

Received 18 November 1987/Accepted 2 March 1988

We report a novel photochemical sterilization system in which *Escherichia coli* cells were sterilized with photosemiconductor powders (titanium oxide). For sterilization that could be used in practice, it was necessary to separate the TiO_2 powders from the cell suspension. Therefore, semiconductor powders were immobilized on acetylcellulose membranes. We constructed a continuous-sterilization system consisting of a TiO_2-immobilized acetylcellulose membrane reactor, a mercury lamp, and a masterflex pump. As a result, under the various sterilization conditions examined, *E. coli* (10^2 cells per ml) was sterilized to <1% survival when the cell suspension flowed in this system at a mean residence time of 16.0 min under irradiation (1,800 microeinsteins/m^2 per s). We found that this system was reusable.

The sterilization of microbial cells is important in the medical and bioindustrial fields. Antibiotics and bactericides have been used for this purpose. Microbial cells are also physically sterilized by heat treatment and UV irradiation. Usually, the effective methods induce many problems, for instance, toxicity or mutagenesis by the reagent. Therefore, a new and effective sterilization method is still required. Various electrochemical methods have been developed for the sterilization of microbial cells. The passage of an alternating current through cell suspensions causes inhibition of cell division or results in bacterial death. Toxic substances such as free chlorine (9), Pt complex (10), and H_2O_2 (12) generated by electrolysis have been shown to be responsible for the sterilization effect. Recently, we have reported (4, 5) that microbial cells are electrochemically oxidized on a graphite electrode and that the electron transfer between microbial cells and the electrode is mediated by intracellular coenzyme A (CoA). Electrochemical oxidation of microbial cells resulted in a decrease in respiratory activity and cell death (6) when the constant potential (0.74 V versus the saturated calomel electrode) was applied to *Saccharomyces cerevisiae* cells attached to the electrode surface. Irradiation of semiconductors with light causes the formation of an electron hole (e^-, h^+) pair, and the reaction of these photogenerated species with species in solution leads to the products of redox reactions (2). *n*-Type semiconductor powders such as TiO_2 have been shown to be applicable to the photocatalytic oxidation of water (11), cyanide (1), and acetic acid (3). Light-sensitized photochemical reactions involving TiO_2 on the surface have also been used for the degradation of contaminants in water (8). Recently, the semiconductor powders were applied to the sterilization of microbial cells. *S. cerevisiae* cells were sterilized photoelectrochemically in the presence of photosensitive semiconductor powders (TiO_2) suspended in the water as a catalyst, since CoA in the microbial cells was oxidized with the photosemiconductor powders (7). However, in a practical sterilization system the semiconductor particles must be separated from the microbial cell suspension. Therefore, we immobilized TiO_2 particles on an acetylcellulose membrane, and a continuous sterilization system was developed with a TiO_2-immobilized membrane.

* Corresponding author.

MATERIALS AND METHODS

Materials. Titanium oxide (Aerosil P-25; 99.99% anatase type) was obtained from Japan Aerosil. Beef extract and peptone were purchased from Kyokuto Pharmaceutical Co. Other reagents were commercially available or laboratory-grade materials. Deionized water was used in all procedures.

Culture of microorganism. Cells of *Escherichia coli* were grown under aerobic conditions at 37°C for 12 h in 100 ml of nutrient broth (pH 7.2) containing 1 g of beef extract, 1 g of peptone, and 0.5 g of sodium chloride. The cells were centrifuged at 8,000 × *g* (4°C) for 10 min, washed, and suspended in sterilized water. The cell concentrations of the different preparations were determined with a hemacytometer.

Preparation of TiO_2-immobilized membrane. TiO_2 particles were washed twice and suspended in water. This suspension was centrifuged at 8,000 × *g*, and the supernatant was removed. TiO_2 particles were dried and then ground in a mortar to arrange the size of particles. Acetylcellulose was dissolved in acetone, and this solution was spread onto plates and permitted to stand under semidry conditions. TiO_2 particles were dispersed at 4.0 mg/cm^2 on the semidry acetylcellulose membrane and then were completely dried. The TiO_2-immobilized membrane (60 cm^2, 240 mg of TiO_2 per membrane) was inserted spirally into the glass tube (diameter, 6.5 mm; length, 30 cm).

Sterilization by TiO_2 powders and TiO_2-immobilized membrane. For sterilization by TiO_2 powders, 10 mg of TiO_2 powders was suspended in sterilized water. The suspended cells were added to the solution containing the TiO_2 powders. The reaction vessel was illuminated with a mercury lamp (HF 100x; Iwasaki Electric Co.) at a light intensity of 1,100 microeinsteins/m^2 per s. The light intensity was measured in the center of the vessel surface with a radiometer (model 65A; Yellow Springs Instrument Co., Yellow Springs, Ohio) and corrected for the adsorption of UV radiation by the glass.

The continuous-sterilization system is depicted in Fig. 1.

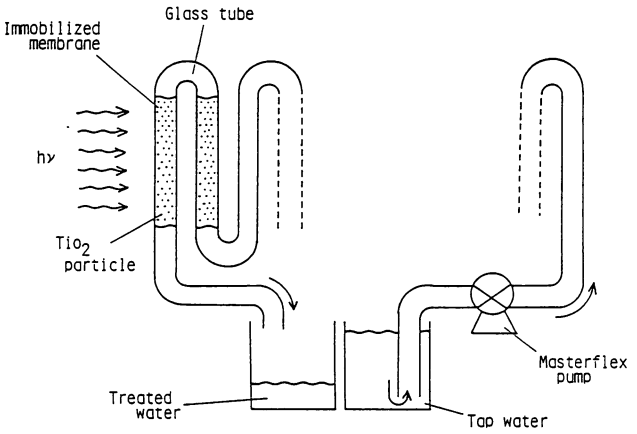

FIG. 1. Schematic diagram of the continuous-sterilization reactor.

The continuous-sterilization system consisted of 26 glass tubes (the effective volume for sterilization was 260 ml), a mercury lamp, and a masterflex pump (Cole-Parmer Instrument Co.). The cell suspension (10^2 cells per ml) was passed through the glass tubes containing the TiO_2-immobilized membrane under light irradiation. The light intensity was measured in the center of the glass tube as described above.

Determination of viable cell numbers. The number of viable cells in the solution was determined by plating suitably diluted samples and then counting the colonies which appeared on the nutrient broth agar plate after 24 h of incubation at 37°C.

Determination of CoA and a dimer of CoA. The CoA concentration was determined by the phosphotransacetylase method of Stadtman et al. (13). The dimer of CoA was determined by high-pressure liquid chromatography (LC-6A; Shimadzu, Kyoto, Japan) with a column (7.6 by 100 mm; 9-μm particle size; Asahipak). Samples were injected into the liquid chromatograph after the column was equilibrated with a mobile phase (200 mM $NaH_2PO_4 \cdot 2H_2O$, 75%; CH_3CN, 25%) at a flow rate of 1 ml/min.

RESULTS AND DISCUSSION

Sterilization of *E. coli* by TiO_2 powders. The time course of viable *E. coli* cells when cell suspensions (10^3 cells per ml) were incubated with TiO_2 powders under mercury light irradiation is shown in Fig. 2. The number of viable cells decreased gradually, and sterilization was complete after 60 min. On the other hand, a decrease in viable cell numbers was not observed under light irradiation when semiconductor powders were absent.

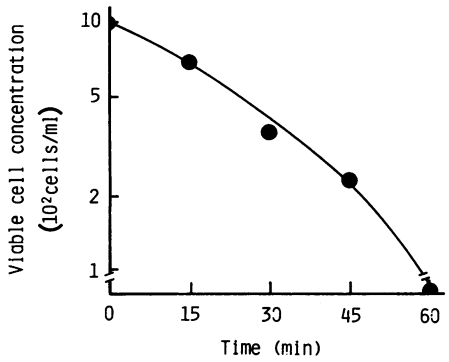

FIG. 2. Time course of viable cell when cell suspensions of *E. coli* (10^3 cells per ml) were incubated with TiO_2 powders under light irradiation (4,300 microeinsteins/m^2 per s).

TABLE 1. Sterilization of *E. coli* cells at various cell concentrations by TiO_2 powders[a]

Cell concn (cells/ml)	Surviving ratio (%) for TiO_2 powders at concn (mg/ml) of:	
	0	1
10^2	100	0
10^3	94	0
10^4	92	32
10^5	100	84

[a] The reaction was carried out for 30 min under irradiation with 1,100 microeinsteins/m^2 per s.

Sterilization of *E. coli* cells was carried out at various concentrations (10^2, 10^3, 10^4, and 10^5 cells per ml). Table 1 shows the surviving ratio of cells when a mixture of TiO_2 powders and cell suspension was illuminated for 30 min with a mercury lamp at a light intensity of 1,100 microeinsteins/m^2 per s. The cells were completely sterilized when 10^2 to 10^3 cells per ml were employed. On the other hand, at concentrations above 10^4 cells per ml, the surviving ratio of cells increased with an increase in the initial cell concentration. The sterilization system with photosemiconductor particles is suitable for samples containing a low concentration of bacteria (below 10^3 cells per ml). For further experiments, water samples containing 10^2 cells of *E. coli* per ml were employed.

Optimum conditions for continuous sterilization by TiO_2-immobilized membrane. In a practical situation, it is necessary to separate TiO_2 powders from the cell suspension. Therefore, TiO_2 powders were immobilized on an acetylcellulose membrane. The effect of the light intensity on the surviving ratio of *E. coli* cells when suspensions containing 10^2 cells per ml were passed through a TiO_2-immobilized membrane reactor system at a mean residence time of 16.0 min is shown in Fig. 3. The surviving ratio decreased with increasing light intensity. The cells were completely sterilized at 1,800 microeinsteins/m^2 per s. On the other hand, about 90% of the cells flowed out under dark conditions.

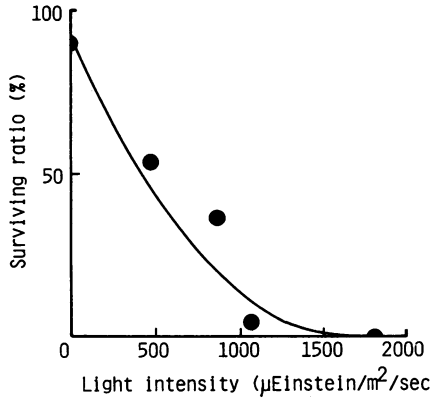

FIG. 3. Effect of light intensity on the surviving ratio of *E. coli* cells. The cell suspensions containing 10^2 cells per ml were passed through the TiO_2-immobilized membrane (containing 6.24 g of TiO_2) reactor system at a mean residence time of 16.0 min.

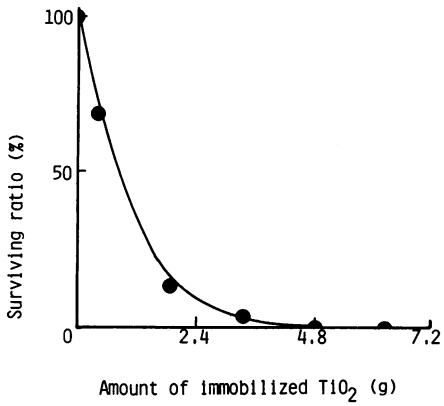

FIG. 4. Effect of the amount of immobilized TiO_2 powders on the surviving ratio of *E. coli* cells. The cell suspension, containing 10^2 cells per ml, was passed through the system at a mean residence time of 16.0 min under irradiation with 1,800 microeinsteins/m^2 per s.

Figure 4 shows the effect of the amount of immobilized TiO_2 powders on the surviving ratio when suspensions containing 10^2 cells per ml were passed through the system at a mean residence time of 16.0 min under irradiation with 1,800 microeinsteins/m^2 per s. The surviving ratio decreased with an increase in the amount of immobilized TiO_2 powders. The cells were completely sterilized when more than 4.8 g of TiO_2 powders were used for the system. Figure 5 shows the effect of the mean residence time on the surviving ratio when 10^2 cells of an *E. coli* suspension were passed through the system under an irradiation of 1,800 microeinsteins/m^2 per s. The cells were sterilized at mean residence times of 16.0 min, and the surviving bacteria decreased with an increase in the mean residence time.

Continuous sterilization of *E. coli* cells by TiO_2-immobilized membrane reactor. Continuous sterilization of *E. coli* cells was carried out with the TiO_2-immobilized membrane reactor (Table 2). Cell suspensions were passed through the system at a mean residence time of 16.0 min under irradiation with 1,800 microeinsteins/m^2 per s. Sterilization was completed when less than 10^3 cells per ml were used. The efficiency of sterilization decreased with an increase in the cell concentration above 10^4 cells per ml.

Stability of the TiO_2-immobilized membrane reactor. To investigate the stability of the TiO_2-immobilized membrane

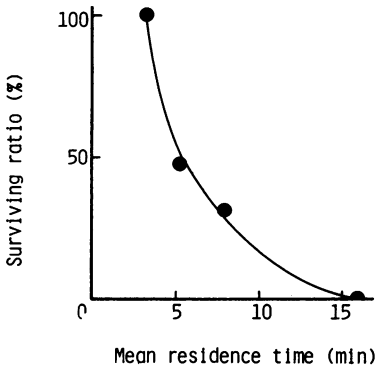

FIG. 5. Effect of the mean residence time on the surviving ratio when 10^2 cells of an *E. coli* suspension were passed through the system under irradiation with 1,800 microeinsteins/m^2 per s.

TABLE 2. Continuous sterilization of *E. coli* cells at various cell concentrations by a TiO_2-immobilized membrane reactor system[a]

Cell concn (cells/ml)	Surviving ratio (%)
10^2	0
10^3	0
10^4	6
10^5	36

[a] The flow rate was 16.3 ml/s. The effective volume for sterilization was 260 ml; the amount of TiO_2 used was 6.24 g. The light intensity was 1,800 microeinsteins/m^2 per s.

reactor, the system was operated continuously for a week. The surviving ratio of *E. coli* cells was measured each day, when the cell suspension containing 10^2 cells per ml was passed through the reactor at a mean residence time of 16.0 min at a light intensity of 1,800 microeinsteins/m^2 per s. The *E. coli* cells were sterilized to <1% survival. The surviving ratio of cells did not increase for 1 week. This system was very stable.

Mechanism of sterilization by TiO_2 powders. We have shown in recent years that CoA mediates an electron transfer between the cell and an electrode or a semiconductor (4, 5, 7). When whole cells of *E. coli* were photoelectrochemically sterilized with TiO_2 powders, the CoA content decreased in the cell (T. Matsunaga, unpublished data). The time course of the CoA and dimeric CoA concentration when CoA solution (0.36 μmol/ml) was incubated with TiO_2 powders under light irradiation is shown in Fig. 6. CoA decreased with an increase in the irradiation time and with an increase in the dimeric CoA concentration. CoA was not detected after 40 min of irradiation. On the other hand, the dimeric CoA concentration was about 0.2 μmol/ml at the same time.

The light irradiation of TiO_2 powders caused the formation of an electron hole pair in particles. Under light irradiation TiO_2 powders were excited and, as a result, generated electrons in the conduction band that were transferred to oxygen as the acceptor, and the hole in the valence band of TiO_2 received an electron from CoA as the donor. Then, the formation of dimeric CoA was induced.

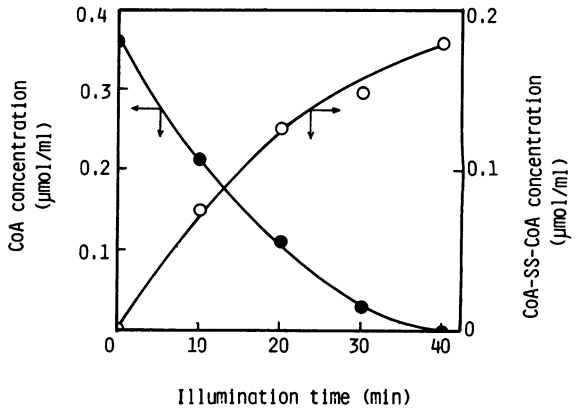

FIG. 6. Time course of the CoA and dimeric CoA (CoA-ss-CoA) concentrations. The CoA solution (0.36 μmol/liter) was incubated with TiO_2 powders (10 mg/ml) under light irradiation with 7,000 microeinsteins/m^2 per s. CoA bind to each other with disulfide bonds.

LITERATURE CITED

1. **Frank, S. N., and A. J. Bard.** 1977. Heterogeneous photocatalytic oxidation of cyanide and sulfite in aqueous solutions at semiconductor powders. J. Phys. Chem. **81**:1484–1488.
2. **Fujishima, A., and K. Honda.** 1972. Electrochemical photolysis of water at a semiconductor electrode. Nature (London) **238**:37–38.
3. **Kraeutler, B., and A. J. Bard.** 1978. Heterogeneous photocatalytic synthesis of methane from acetic acid—new Kolbe reaction pathway. J. Am. Chem. Soc. **100**:2239–2240.
4. **Matsunaga, T., and Y. Namba.** 1984. Selective determination of microbial cells by graphite electrode modified with adsorbed 4,4′-bipyridine. Anal. Chim. Acta **159**:87–94.
5. **Matsunaga, T., and Y. Namba.** 1984. Detection of microbial cells by cyclic voltammetry. Anal. Chem. **56**:798–801.
6. **Matsunaga, T., Y. Namba, and T. Nakajima.** 1984. Electrochemical sterilization of microbial cells. Bioelectrochem. Bioenergetics **13**:393–400.
7. **Matsunaga, T., R. Tomoda, T. Nakajima, and H. Wake.** 1985. Photoelectrochemical sterilization of microbial cells by semiconductor powders. FEMS Microbiol. Lett. **29**:211-214.
8. **Ollis, D. F.** 1985. Contaminant degradation in water. Environ. Sci. Technol. **19**:480–484.
9. **Pareilleux, A., and N. Sicard.** 1970. Lethal effects of electric current on *Escherichia coli*. Appl. Microbiol. **19**:421–424.
10. **Rosenberg, B., L. Van Camp, and T. Krigas.** 1965. Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. Nature (London) **205**:698–699.
11. **Schrauzer, G. N., and T. K. Guth.** 1977. Photolysis of water and photoreduction of nitrogen on titanium dioxide. J. Am. Chem. Soc. **99**:7189–7193.
12. **Shimada, K., and K. Shimahara.** 1982. Responsibility of hydrogen peroxide for the lethality of resting *Escherichia coli* B cells exposed to alternating current in phosphate buffer solution. Agric. Biol. Chem. **46**:1329–1337.
13. **Stadtman, E. R., G. D. Novelli, and F. Lipman.** 1951. Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. J. Biol. Chem. **191**:365–376.