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A NEW MICROBIAL ELECTRODE FOR BOD ESTIMATION

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A new microbial electrode using immobilized *Clostridium butyricum* was prepared for biochemical oxygen demand (BOD) estimation of wastewaters. The current of the electrode was decreased with time until a steady state was reached. The steady state current was in all cases attained within 30–40 min at 37°C, and the maximum current output was obtained at 37°C and pH between 6.2 and 7.0. A linear relationship was obtained between the steady state current and BOD. The steady state current values were reproducible within ±7% of the relative error. The BOD of industrial wastewaters can be estimated by using the microbial electrode. Relative error of the BOD estimation of industrial wastewaters was within ±10%. The current output of the microbial electrode was almost constant for 30 days.

INTRODUCTION

The biochemical oxygen demand (BOD) test is one of the most widely used and important tests in the measurement of organic pollution. Since the BOD test measures biodegradable organic compounds in wastewaters, it requires a long incubation period (5 days at 20°C). A number of papers concerning methods for rapid estimation of the 5-day BOD have been published in recent years (1–3). However, a simple and reproducible method for estimation of 5-day BOD is still required for pollution control.

A method for immobilization of whole cells of hydrogen-producing bacteria, *Cl. butyricum*, was recently developed in this laboratory (4). The immobilized whole cells were capable of continuously evolving hydrogen from glucose under aerobic conditions. A previous paper by us discusses the application of the immobilized whole cells to a biofuel cell which produced a constant current over a period of 15 days (5). The current generated by the cell was the result of the oxidation of hydrogen and formate produced by *Cl. butyricum*. Since bacteria are known to utilize carbohydrates and proteins, the biofuel cell system using immobilized *Cl. butyricum* could be applied to the estimation of the BOD of wastewaters (6). However, the apparatus used for the biofuel cell system was not suitable for continuous BOD estimation of wastewaters. Therefore, a new microbial electrode for BOD estimation has been developed. In this paper, we describe the properties of the microbial electrode as well as its use in estimating the BOD of industrial wastewaters.

MATERIALS AND METHODS

Materials

Yeast extract was purchased from Difco Laboratories, and peptone was purchased from Kyokuto Pharmaceutical Company. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from Wako Pure Chemicals Ind. Other solvents and reagents were commercially available analytical reagents or laboratory grade materials. Deionized water was used in all procedures.

Culture of Microorganisms

Clostridium butyricum IFO 3847 was used in this study. The bacteria cells were grown under anaerobic conditions at 37°C for 9 h in a medium (pH 7.0) containing glucose 10 g/liter, peptone 4 g/liter, yeast extract 4 g/liter, beef extract 2 g/liter, K_2HPO_4 12.5 g/liter and $FeSO_4$ 0.5 g/liter. The cells were isolated by centrifugation at 5°C and $8000 \times g$. The cells were washed twice with oxygen-free 0.1 M phosphate buffer (pH 7.0, 3°C).

Preparation of Microbial Electrode

A strip of nylon net (20 mesh, 700 μm thickness, 3.4 cm diameter) was attached to a platinum electrode with an adhesive. The immobilization of *Cl. butyricum* on the electrode was accomplished as follows: 0.4 g of intact cells and 0.36 g of a mixture of two monomers (90% acrylamide and 10% *N,N*-methylenebisacrylamide) were suspended in 10 ml of physiological saline solution in an ice bath. The solution was then saturated with nitrogen gas. Then 0.5 ml of the solution was cast on the platinum electrode covered with nylon net (effective surface area 9.1 cm^2). The polymerization reaction was initiated with 0.03 ml of 10% dimethylaminopropionitrile and 0.1 mg of potassium persulfate and was allowed to proceed anaerobically for 30 min at 37°C. The resulting microbial electrode was stored in 0.1 M phosphate buffer (pH 7.0) containing 0.5% of glucose at 5°C.

Apparatus

A schematic diagram of the microbial electrode is shown in Fig. 1. The cathode was silver peroxide (Ag_2O_2) and the anode was a platinum electrode. The electrolyte was 50 ml of 0.1 M phosphate buffer (pH 7.0) and anion exchange membrane (Selemion Type AMV, Asahi Glass Company) was used as a separator.

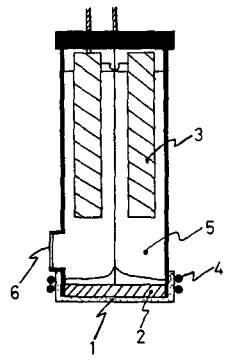

FIG. 1. Schematic diagram of the microbial electrode. 1, Immobilized *Cl. butyricum*; 2, platinum electrode; 3, silver peroxide electrode (Ag_2O_2); 4, O-ring; 5, electrolyte (0.1 M phosphate buffer); 6, anion exchange membrane.

Estimation of BOD by Microbial Electrode

Standard solution (0.1 M phosphate buffer, pH 7.0) containing glucose and glutamate was employed as a model wastewater according to JIS (7). Wastewaters were diluted to 0–220 ppm (BOD) by 0.1 M phosphate buffer (pH 7.0). The microbial electrode was immersed in the sample wastewater and the current of the microbial electrode was measured by a millivoltammeter (Kikusui Electronics Model 114) and the signal obtained was displayed on a recorder (Riken Denshi, Model SP-J5C). The 5-day BOD of the sample wastewater was measured by the "standard method" according to JIS (7).

RESULTS

Response Properties of the Microbial Electrode

Figure 2 shows the relationship between the current obtained by the microbial electrode and the time at various concentrations of the substrate. As hydrogen and formate accumulated at the cathode reacted on the electrode, a high current was initially obtained. The diffusion of hydrogen and formate to the cathode became the rate-determining factor and a steady state current was obtained. As shown in Fig. 2, the response time (time required for the current to reach a steady state) depended on the concentration of glucose and glutamate. Increase of the standard solution concentration shortened the response time. When the microbial electrode was removed from the sample solution and placed in a solution free of organic compounds, the current output of the microbial electrode gradually decreased and returned to zero level within 20 min at 37°C.

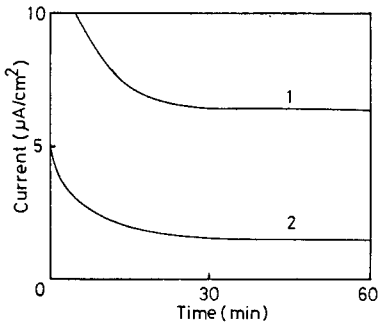

FIG. 2. Relationship between the steady state current and time for a system containing 200 ml of 0.1 M phosphate buffer with (1) 500 mg/liter of glucose and 500 mg/liter of glutamate and (2) 50 mg/liter of glucose and 50 mg/liter of glutamate.

Effect of pH

The effect of pH on the steady state current of the microbial electrode was studied from pH 5.6 to 9.0 for the standard solution containing 50 mg/liter of glucose and 50 mg/liter of glutamate in 0.1 M phosphate or carbonate buffer (Fig. 3). The optimum pH was between 6.2 and 7.0 and the current decreased below pH 6.0 or above pH 7.0. This may be caused by the inactivation of *Cl. butyricum* in gel at lower and higher pH values. Since immobilized *Cl. butyricum* produced organic acids during incubation (2), the pH of the sample was thereafter adjusted to pH 7.0.

Effect of Temperature

The effect of temperature on the steady state current is shown in Fig. 4. The standard solution containing 50 mg/liter of glucose and 50 mg/liter of glutamate in 200 ml of 0.1 M phosphate buffer (pH 7.0) was employed for the experiments. The steady state current increased with increase in temperature. However, no current output was observed at 43°C. This was caused by the inactivation of the bacteria in the polyacrylamide gel by heat. A temperature of 37°C was therefore used in experiments.

Effect of Sodium Chloride

The effect of sodium chloride on the current output of the microbial electrode was examined. The steady state current obtained was constant below 0.4 M of sodium chloride. However, a decrease in current was observed above 0.4 M of sodium chloride. This may be caused by the inactivation of bacteria by sodium chloride.

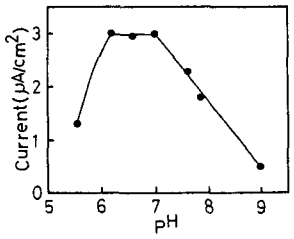

FIG. 3. Effect of pH on the steady state current. Employed were 200 ml of 0.1 M phosphate buffer (pH 5.6–7.8) and 0.1 M carbonate buffer (pH. 9.0), and current was determined 40 min after insertion of the electrode.

Current–BOD Relationship

Figure 5 shows the relationship between the steady state current and the BOD of the standard solution. A linear relationship was obtained between the steady state current and the BOD from 0 to 250 ppm. The steady state current was reproducible within ±7% of the relative error, when the standard solution (50 mg/liter glucose, 50 mg/liter glutamate) was measured repeatedly. The standard deviation was 2 ppm.

The microbial electrode was applied to the estimation of the BOD of wastewaters. Three kinds of industrial untreated wastewater—slaughterhouse (Shibaura, Tokyo), food factory (Kawasaki), and alcohol factory (Inage, Chiba)—were employed in the experiments. Each wastewater was diluted and the 5-day BOD determined by the conventional method (7). Then the current for each diluted wastewater was determined by the microbial electrode. The current–BOD relationship is plotted in Fig. 6. The solid line shows the current–BOD relationship obtained from the various concentrations of the standard solution. As shown in Fig. 6, the current–BOD plots of wastewaters are located near the standard curve.

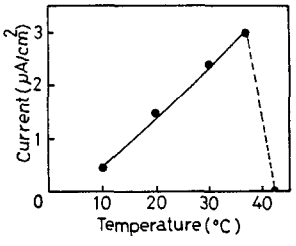

FIG. 4. Effect of temperature on the steady state current. Current was determined 40 min after insertion of the electrode.

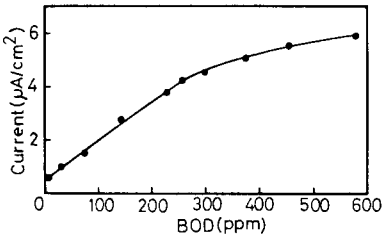

FIG. 5. Relationship between the steady state current and the BOD of the standard solution. A solution of 200 ml of 0.1 M phosphate buffer containing various concentrations of glucose (2.5–400 mg) and glutamate (2.5–400 mg) was employed and current was determined 40 min after insertion of the electrode.

Relative error of BOD estimation of industrial wastewaters was within 10%.

Reusability of the Microbial Electrode

Reusability of the microbial electrode was tested as follows. The current was determined 40 min after it had been immersed in the standard solution (50 mg/liter glucose, 50 mg/liter glutamate). The measurement was repeated every day. No decrease in current output was observed over a 30-day period.

Storage stability of the microbial electrode was examined at 5°C. The steady state current for the diluted standard solution (50 mg/liter glucose, 50 mg/liter glutamate) was measured once per 10 days. The current output was almost constant for 40 days.

DISCUSSION

Many papers have recently been published on methods for the rapid estimation of a 5-day BOD (1-3). However, these methods are not in fact

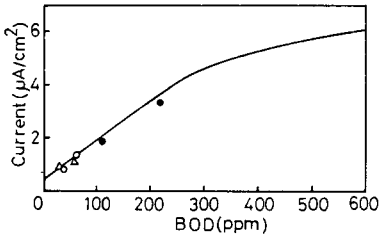

FIG. 6. Current–BOD relationship of industrial wastewaters. Diluted wastewater, 200 ml, was employed and current was determined 40 min after insertion of the electrode. Diluted wastewater of (●) an alcohol factory (1:2000–1:1000), (○) a food factory (1:200–1:100), and (△) a slaughterhouse (1:20–1:10).

rapid or simple. The method described here using a BOD sensor having a microbial electrode can truly be called rapid and simple since it provides an estimated BOD in only 1 h.

There are two major differences in principle between the method described here and the conventional method (7). First, the microbial electrode uses anaerobic bacteria, whereas the conventional method used aerobic microorganisms. The anaerobic bacterium, *Cl. butyricum*, decomposes organic compounds to produce hydrogen or formate (4), and these are oxidized electrochemically on the anode. Furthermore, the stability of *Cl. butyricum* increases against oxygen with immobilization in polyacrylamide gel (4). On the other hand, aerobic microorganisms consume oxygen during decomposition of organic compounds. As shown in Fig. 4, a linear relationship exists between the steady state current of the microbial electrode and the results of the 5-day BOD test. Because of this, the microbial electrode can be used to estimate the BOD. The standard deviation of the microbial electrode was 2 ppm in the case of measurements conducted on the diluted standard solution. This microbial electrode uses a single species of bacteria, whereas the conventional method uses many species of microorganisms obtained from the soil. In spite of using only a single species, the microbial electrode was capable of giving reproducible results in the estimation of BOD. Furthermore, the microbial electrode could be used to estimate the BOD of the wastewaters employed in the experiments described in this paper (Fig. 6). The relative error of measurement was less than 10%. In practice, the error inherent in the 5-day BOD test is more than 10%.

The application of this microbial electrode to the estimation of the BOD is based on the assumption that the organic compounds in the wastewaters can be degraded by *Cl. butyricum*. On the other hand, it will be difficult to use the microbial electrode to estimate the BOD of wastewaters that contain organic compounds which cannot be degraded by *Cl. butyricum*. In such cases, various species of microorganisms isolated from the active sludge of the wastewater to be tested should be used in the microbial electrode. As previously reported (4), immobilized microorganisms remain active for a long time under certain conditions. No decrease in current output was observed over a 30-day period. Therefore, it can be used repeatedly for the estimation of the BOD of wastewaters.

This study provides a method of a rapid estimation of the BOD of certain kinds of wastewaters. Further developmental studies in this laboratory are being directed toward applying the microbial electrode using many species of microorganisms for the estimation of the BOD of other wastewaters.

REFERENCES

1. LEBLANCE, P. J. (1974) J. Water Pollut. Control Fed. 46 : 2202.
2. BUSCH, A. W., and MIRICK, N. (1962) J. Water Pollut. Control Fed. 34 : 354.
3. MULLIS, M. K., and SCHROEDER, E. D. (1971) J. Water Pollut. Control Fed. 34 : 209.
4. KARUBE, I., MATSUNGA, T., TSURU, S., and SUZUKI, S. (1976) Biochim. Biophys. Acta 444 : 338.
5. KARUBE, I., MATSUNGA, T., TSURU S., and SUZUKI, S., (1977) Biotechnol. Bioeng., 19 : 1727.
6. KARUBE, I., MATSUNAGA, T., MITSUDA, S., and SUZUKI, S., (1977) Biotechnol. Bioeng., 19 : 1535.
7. Japanese Industrial Standard Committee (1974) *In* Testing Methods for Industrial Waste Water, JIS K 0102, p. 33.