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Title	Evaluation of Biochemical Oxygen Demand for Wastewater Based on Kinetic Analysis with Flow-Injection Microbial Biosensor
Author(s)	Mitsuyasu Kawakami
Citation	福岡工業大学研究論集 第49巻 第1号（通巻75号） P1-P7
Issue Date	2016-9
URI	<a href="http://hdl.handle.net/11478/545">http://hdl.handle.net/11478/545</a>
Right	
Type	Research Paper
Textversion	publisher

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## Evaluation of Biochemical Oxygen Demand for Wastewater Based on Kinetic Analysis with Flow-Injection Microbial Biosensor

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### Abstract

A mediated biochemical oxygen demand (BOD) sensor system of flow injection mode was constructed by employing carrier solution containing potassium hexacyanoferrate (III) as a redox mediator. An electrochemical flow cell of three electrodes configuration was developed, in which a gelatin layer containing wastewater-assimilating microbes was deposited on the working electrode. The output current was confirmed to yield a peak with a sample injection, and to result from reoxidation of reduced mediator at the electrode. By employing the peak area as the sensor response, the initial assimilation rate was estimated from the dependence of sensor response on the flow rate of carrier solution. It was demonstrated that reasonable BOD could be obtained for the distillate of a Japanese liquor *shochu* used as a high BOD wastewater sample.

Key words: *BOD, mediated biosensor, microbial biosensor, mediator*

### 1. Introduction

BOD is a major and widely used index to estimate the organic pollution of water environment, which means the amount of oxygen consumed when organic compounds are decomposed by microorganisms in the water. The most widely used assay for BOD measurement is the 5-day biochemical oxygen demand (BOD<sub>5</sub>) [1,2], where the consumption of dissolved oxygen by microbial assimilation during a 5-day incubation period is determined. However, this method suffers serious disadvantage that it is a time-consuming procedure and requires experience and skills to obtain reproducible results. Thus, biosensor-based methods have attracted a lot of attention as an alternative method for BOD estimation [3-5].

Microbial sensor with redox mediator is one of the representative methods for BOD assessment. The mediator acts as an electron acceptor instead of oxygen in the microbial metabolism of organic substrates and shuttles electrons from microbes to the electrode surface. The resulting current produced by re-oxidation of the reduced mediator at the electrode is proportional to the concentration of organic materials. Potassium hexacyanoferrate (HCF) (III) has been known as an efficient mediator and HCF (III)-mediat-

ed BOD determination has been extensively studied [6-17]. The most significant advantage of using ferricyanide as an alternative electron acceptor is its high solubility compared to oxygen. This makes it possible to use much higher microbial populations without rapid depletion of the electron acceptor, whereby affords a rapid BOD measurement. However, most of the researches so far made concerning mediated BOD biosensors are based on the batch systems, and little attempts have been made to apply the mediated BOD biosensors to the flow injection analysis system.

There are two measurement approaches available for BOD biosensor systems, namely the batch and the flow injection techniques. The flow system is advantageous for rapid and repeated measurements compared with the batch mode. In addition, there are two distinct methods for measuring microbial respiration rate, that is, the steady-state method and the initial rate method. In the steady-state method, the current difference between the two steady states is used for the BOD estimation. In the initial-rate method, on the other hand, the initial current change after sample addition is employed as the sensor response. This transient state measurement has been employed not only for the batch system [18-21], but for the flow system [22-26]. Although the transient state method is faster than the steady state method, it usually requires more complicated data processing.

In the flow-type microbial biosensor method only a part

of organic compounds biodegradable for the microbe can be assimilated since the residential time is considerably short. Microorganism has an inherent biodegradation activity as the recognition element and the sensor activity is limited by the microbe content. Then, if excessive substrate is loaded, an erroneous sensor response which is not proportional to the substrate concentration might be obtained. Therefore, use of high biodegradation activity is recommended to obtain sufficient sensor response, because there is usually a limitation of the amount of microbes in biosensor system. In our previous work, mediated BOD biosensor system of flow injection mode employing HCF (III) solution as the carrier solution was demonstrated to be applicable to BOD assessment of shochu distillery wastewater (SDW), and the system utilizing microbe of higher assimilating ability was shown to enable more reliable assessment [27].

In the initial-rate method employing a flow-type microbial biosensor method the rate of assimilation has been calculated from the slope of the tangent to a sensor output – time curve. However, such a manner usually requires more complicated data processing. Then, in the present study, it was attempted to determine the initial assimilation rate from the dependence of the sensor response on the residence time. Further, the apparent pseudo-first order rate constant was determined from the relation between the initial-rate and the substrate concentration in order to minimize the effect of flow rate. Finally, BOD of samples was evaluated utilizing these kinetic parameters. Sensor response measurements were made employing two kinds of SDW as real wastewater samples. Availability of the present assessment has been checked by comparing BOD with that determined by 5-day method

## 2. EXPERIMENTAL

### 2.1 Chemicals and Materials

Potassium hexacyanoferrate (III), potassium hexacyanoferrate (II) trihydrate, polypepton, and other chemicals of the highest grade available were purchased from Wako Pure Chemical Industries (Osaka, Japan). Peptone, yeast extract, and malt extract were supplied by Difco Laboratories (Detroit, MI, USA). Beef extract was supplied by MP Biomedicals (Solon, OH, USA). Gelatin from bovine skin was purchased from Sigma-Aldrich. Two kinds of shochu distillery wastes yielded in shochu production using barley (SDW(B)) and rice (SDW(R)) were kindly provided by Hikari Shuzo (Fukuoka, Japan). These wastes were centrifuged at 5,000 rpm for 10 min and the supernatants were employed as SDW stock solutions, which were stored

in a freezer when not in use. BOD<sub>5</sub> values of these stock solutions were 120,000, and 93,000 mg•L<sup>-1</sup>, respectively.

A graphite rod of 5 mm in diameter (spectroscopic grade; Hitachi Chemical, Tokyo, Japan) was used for the working electrode. The graphite rod was polished with 0.1 μm alumina powder, and rinsed thoroughly with deionized water. Then, the electrode was sonicated in acetone and deionized water successively, and allowed to dry at room temperature. The cleanliness of electrode surface was confirmed by conventional cyclic voltammetry with CH Instruments electrochemical analyzer (model 812A, Austin, TX). An Ag/AgCl electrode was prepared by bulk electrolysis of an Ag wire (1 mmϕ) in 0.1 M KCl and employed as the reference electrode.

### 2.2 Microorganism and Culture

*Burkholderia cepacia* strain M-1 isolated as a SDW-assimilating microorganism from a soil collected at farmland in northern Kyushu was used for the BOD sensor. The strain was cultured aerobically on a reciprocating shaker at 30 °C for 24 h in NBRC No.802 medium (10 g polypepton, 2 g yeast extract, 1 g MgSO<sub>4</sub>•7H<sub>2</sub>O per liter). After the growth, the cells were harvested by centrifugation at 4000 rpm for 10 min at room temperature and washed twice with sterilized water. They were then resuspended in the 802 broth containing 50 % glycerol and stored at -80 °C.

### 2.3 Immobilized Cell Electrode Preparation

After the cells were cultivated, they were harvested at their late exponential phase by centrifugation at 4000 rpm for 10 min. The cells were washed three times with sterilized sodium phosphate buffer solution (0.1 M, pH 7.0). Then the cell suspension was prepared using the same buffer solution so as to its optical density at 660 nm was ca. 0.9. Gelatin (2.0 %) was added separately to the buffer solution and autoclaved at 120 °C for 20 min, followed by incubation at around 40 °C. The calculated amount of cell suspension was added to 10 mL of gelatin solution so that a given amount (dry weight) of the cell would be contained in the cell-gelatin mixture. 20 μL of the cell-gelatin mixture was deposited on the surface of electrode, and left for 1 h at room temperature. Finally 20 μL of glutaraldehyde solution (2.5 %) was dropped onto the cell-gelatin layer, and allowed to stand for 2 min. The immobilized cell electrode was then washed thoroughly with sterilized water and ultimately with the buffer solution.

### 2.4 Sample Preparation

The OECD synthetic wastewater was employed as a

standard solution of BOD estimation. The stock solution of synthetic wastewater was prepared by dissolving 3.2 g peptone, 2.2 g meat extract, 0.6 g urea, 0.56 g  $K_2HPO_4$ , 0.14 g NaCl, 0.08 g  $CaCl_2 \cdot 2H_2O$ , and 0.04 g  $MgSO_4 \cdot 7H_2O$  in 1 liter of the sodium phosphate buffer (0.1 M, pH 7.0). The solution was filtered through a membrane filter (0.25  $\mu m$ ) to remove insoluble particles. The  $BOD_5$  of this solution was 2,600  $mg \cdot L^{-1}$ . The stock solution of SDW was also filtered through a membrane filter. Sample solutions of both the synthetic wastewater and SDW were prepared by diluting the stock solution with the carrier solution just before the response measurement.

## 2.5 Instrumentation

A schematic diagram of the flow injection system is illustrated in Fig. 1 (a). The system consists of a piston pump (model 325DI, Chemco Scientific, Osaka, Japan), a sample injector with a 500  $\mu L$  loop, and a flow cell. PTFE tubes of 1.0 mm in i.d. were employed to connect these parts.

An enlarged view of the flow cell is illustrated in Fig. 1 (b). The flow cell was made of a PTFE sheet and a 3 mm-thick silicone rubber gasket. The inner cavity of the cell was estimated to be about 150  $\mu L$ . A platinum wire (1 mm  $\phi \times 10$  mm) and an Ag/AgCl electrode (1 mm  $\phi$ ) were used as the auxiliary and the reference electrode, respectively. The microbial reactor and the flow cell were kept at 30 °C in an incubator.

## 2.6 Measurement of Sensor Response

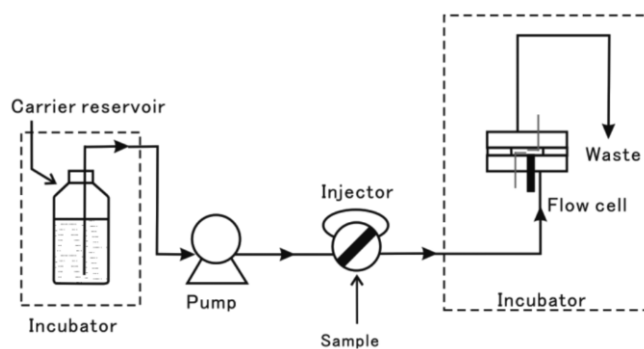
Sodium phosphate buffer (0.1 M, pH 7.0) containing 0.1 M NaCl and 10 mM HCF (III) was used as the carrier. The carrier reservoir was held in an incubator at 30°C, and the flow rate was set at 0.5 ~ 2.5  $mL \cdot min^{-1}$ . Amperometric measurements were made by applying a given potential (250 mV) on the working electrode with a potentiostat (model HECS 318C; Huso, Kawasaki, Japan) bearing the accuracy for current detection of  $\pm 0.5 \sim 1 \%$  of the measured value, which was connected to a personal computer. After the current output has reached a steady state, the sample solution was injected through the injector. The inside of the sensor system was filled with sterilized 0.9 % NaCl when not in use.

## 3. Results and Discussion

### 3.1 Amperometric sensor response

Examples of amperometric response obtained for successive injections of SDW(B) of different dilution factors are shown in Fig. 2. It can be seen that an anodic peak is

(a)



(b)

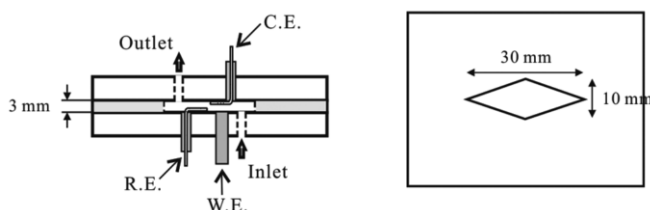


Fig. 1 Schemes of the FIA sensor system (a) and enlarged diagram of the flow cell (b).

obtained with an injection of the sample. Both the peak height and the peak area increased with the concentration of wastewater. In the present work, the electricity obtained from the average peak area determined for triplicate measurements was used as the sensor response. The output response was also determined using the electrode prepared with either gelatin layer alone or autoclaved (120 °C for 20 min) cell-gelatin (6.7  $\mu g$ ). Consequently the outputs observed for both electrodes were found to be almost the same and less than 20 % of that obtained with living cell-gelatin layer for OECD synthetic wastewater. This demonstrates that the sensor output arises predominantly from reoxidation of HCF (II) produced by microbial assimilation, while small amounts of electrochemically active species are contained in the wastewater. In this report the sensor response was not corrected for the contributions of electrochemically active species.

### 3.2 Effect of flow rate

The flow rate of carrier solution affects seriously the sensitivity of the present sensor system, since the progress of microbial assimilation would depend on the residence time in the reactor. Then, the time required for a sample solution to pass through the inner cavity of the measuring cell was employed as the residence time. Dependencies of the sensor responses for SDW(B) on the residence time are illustrated in Fig. 3. It is apparent that the sensor response

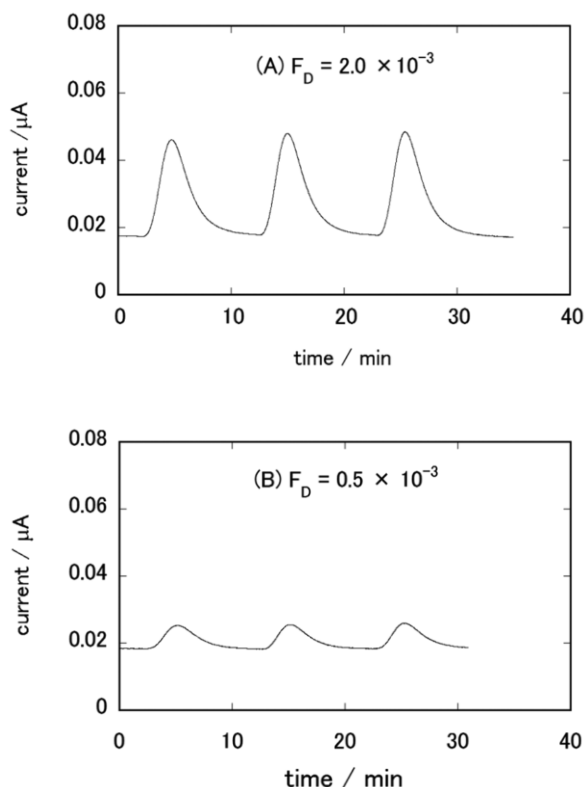


Fig. 2 Diagram of amperometric response obtained for successive injections of SDW(B) of different dilution factors with *Burkholderia cepacia* strain M-1 based sensor system (flow rate =  $0.5 \text{ mL} \cdot \text{min}^{-1}$ ).

increases linearly with increasing residence time for either sample of different concentration examined. Similar dependencies have been also found for the synthetic wastewater and SDW(R) employed as the substrate. This implies that larger quantities of substrate in a sample could be metabolized when the residence time is lengthened. Further the amount of HCF (II) reoxidized at the sensor electrode was shown to increase linearly with the residence time.

### 3.3 Kinetic analysis

The variation of sensor response with residence time makes it possible to analyze the rate of HCF (II) formation. Thus the initial rate of HCF (II) formation ( $v_i$ ) was determined from the slope of the sensor response-residence time plot (Fig. 3). This initial rate would be primarily governed by the rate of microbial assimilation, since it can be considered that the rate of HCF(II) reoxidation on the electrode surface is much faster than that of microbial reaction. Relationships between the initial rate and the dilution factor of substrate are shown in Fig. 4(A) for SDW solutions. A similar relation is also shown for OECD synthetic waste-

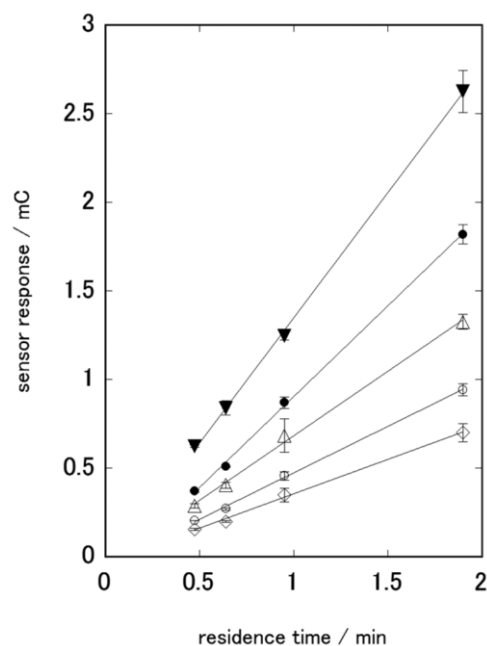


Fig. 3 Relations between sensor response and residence time for SDW(R) of different dilution factor; ( $\blacktriangledown$ )  $2.00 \times 10^{-3}$ , ( $\bullet$ )  $1.33 \times 10^{-3}$ , ( $\triangle$ )  $1.00 \times 10^{-3}$ , ( $\circ$ )  $0.67 \times 10^{-3}$ , and ( $\diamond$ )  $0.50 \times 10^{-3}$ ; microbes content =  $16.7 \mu\text{g}$ .

water in Fig. 4(B) using calculated BOD values instead of the dilution factor. It is seen that the initial rates observed for both SDW solutions display linear dependencies on the substrate concentration, whereas that for the synthetic wastewater deviates from a linear relationship with an increase in BOD. For the former case, the assimilation reactions were demonstrated to be regarded roughly as pseudo-first order reactions. In the latter case, on the other hand, the reaction rate cannot be dealt with in a similar manner as SDW in the concentration range applied here. In relatively low concentration range, however, the metabolic reaction rate for the synthetic wastewater can be considered to vary linearly with substrate concentration as in the case of SDW.

### 3.4 BOD estimation

In the previous report we have estimated BOD values using the slope of calibration plot for SDW and that for the synthetic wastewater employed as the standard. The sensitivity is, however, affected seriously by the flow rate of carrier solution. In this regards, it was demonstrated that the respiration reaction rate obtained by varying the residence time could be apparently treated as pseudo-first order reaction. Then, the slopes of the initial rate — substrate concentration plots, which would refer to apparent rate

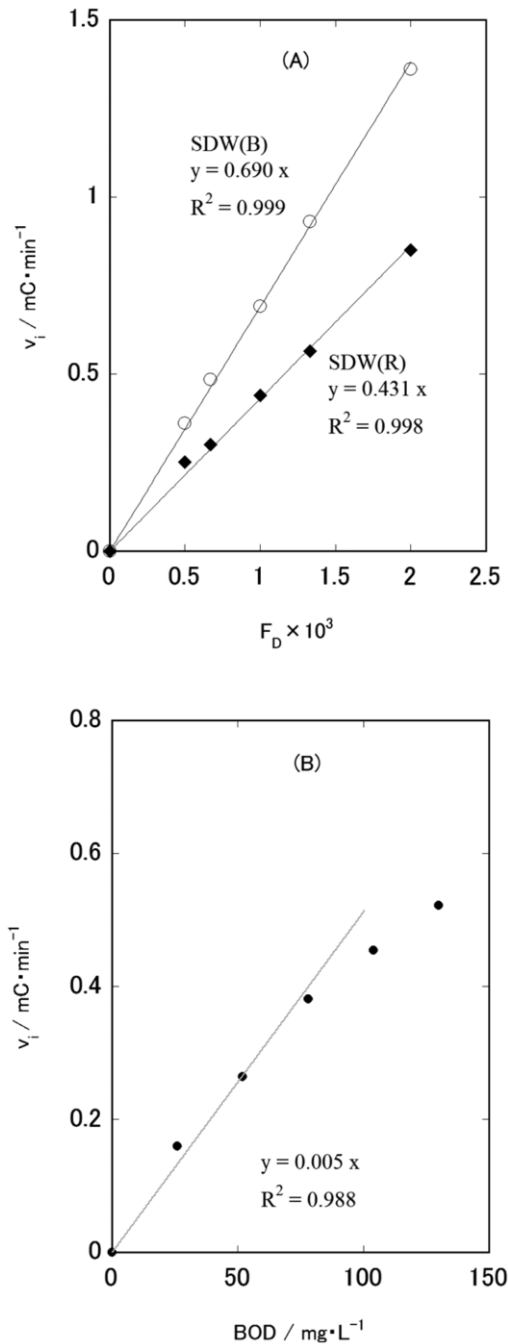


Fig. 4 Relationships between the initial assimilation rate and the substrate concentration for (A) SDW and (B) OECD synthetic wastewater; microbes content = 16.7  $\mu$ g.

constants, have been determined. In the case of synthetic wastewater a straight line was depicted using data obtained for relatively low concentration range (BOD lower than 80 mg·L<sup>-1</sup> (Fig. 4 (B)). The apparent pseudo-first order rate constant  $k_1^{app}$  for the synthetic wastewater was obtained as  $5.0 \times 10^{-3}$  mC·L·mg<sup>-1</sup>·min<sup>-1</sup>. The biochemical oxygen demand, BOD<sub>FIA</sub> for a substrate solution, can be determined by dividing its rate constant by that for the standard solu-

tion. BOD values estimated by using this rate constant are shown in Table 1 compared with BOD<sub>5</sub>. It can be seen that BOD<sub>FIA</sub> values are almost comparable to BOD<sub>5</sub> values for SDW stock solutions.

Table 1 Comparison of BOD obtained by the present sensor system, BOD<sub>FIA</sub> and BOD<sub>5</sub> for SDW.

	BOD <sub>FIA</sub> (mg·L <sup>-1</sup> )	BOD <sub>5</sub> (mg·L <sup>-1</sup> )
SDW(B)	138,000	120,000
SDW(R)	86,200	93,000
OECD synthetic wastewater	130	

In a microbial biosensor system it is fundamental that the measurements should be made under the condition where the microbial assimilation proceeds as a pseudo-first order reaction. The sensor response measurement is therefore needed to be carried out in appropriate ranges of flow rate and substrate concentration. For SDW solutions this requisite was found to be satisfied in the substrate concentration applied. For the synthetic wastewater, on the other hand, the initial rate was found to deviate downward from a linear relationship with an increase in BOD, while a pseudo-first order reaction could be assumed in relatively lower BOD range. If the measurements were made only in relatively high BOD range, it is probable that overestimated or underestimated BOD<sub>FIA</sub> would be obtained. This implies that microbes of high assimilating activities for both substrate and standard solutions should be preferably employed.

#### 4. Conclusion

In the flow injection biosensor system constructed in this work, the initial assimilation rate was determined from the slope of the sensor response-residence time plot. This method is considerably simple as compared to that utilized in the transient state measurement in earlier works [22-26]. The apparent pseudo-first order rate constant could be obtained from the linear dependence of the initial rate on the substrate concentration. BOD<sub>FIA</sub> values evaluated with these rate constants are found to be almost comparable to BOD<sub>5</sub> values for sample wastewaters, demonstrating the present kinetic method to be effective for BOD assessment of SDW.

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