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# Development of mediated BOD biosensor system of flow injection mode for *shochu* distillery wastewater

Shinichi Oota [a], Yuta Hatae [b], Kei Amada [c], Hidekazu Koya [c], Mitsuyasu Kawakami [c,*]

[a] *Materials Science and Production Engineering, Graduate School of Engineering, Fukuoka Institute of Technology, Higashi-ku, Fukuoka 811-0295, Japan*
[b] *Functional Materials Engineering, Graduate School of Engineering, Fukuoka Institute of Technology, Higashi-ku, Fukuoka 811-0295, Japan*
[c] *Department of Life, Environment and Materials Science, Faculty of Engineering, Fukuoka Institute of Technology, Higashi-ku, Fukuoka 811-0295, Japan*

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ABSTRACT

Although microbial biochemical oxygen demand (BOD) sensors utilizing redox mediators have attracted much attention as a rapid BOD measurement method, little attempts have been made to apply the mediated BOD biosensors to the flow injection analysis system. In this work, a mediated BOD sensor system of flow injection mode, constructed by combining an immobilized microbial reactor with an electrochemical flow cell of three electrodes configuration, has been developed to estimate BOD of *shochu* distillery wastewater (SDW). It was demonstrated consequently that the mediated sensing was realized by employing phosphate buffer containing potassium hexacyanoferrate as the carrier. The output current was found 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 effects of flow rate and pH of the carrier on the sensitivity were investigated. The sensor system using a microorganism of high SDW-assimilation capacity showed good performance and proved to be available for estimation of BOD of SDW.



## 1. Introduction

BOD (biochemical oxygen demand) 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 ($BOD_5$) (APHA, 2005; JISC, 2008), where the consumption of dissolved oxygen by microbial assimilation during a 5-day incubation period is determined. Thus, the 5-day method is a time-consuming procedure. Further, it also requires experience and skills to obtain reproducible results.

Many efforts have been made to develop an alternative method for BOD estimation, and consequently, a whole cell biosensor assay has been proposed as a rapid and reliable method. The first microbial biosensor for BOD estimation has been constructed by coupling an immobilized microorganisms membrane and a dissolved oxygen electrode (Karube et al., 1977). The change in the output current of oxygen electrode is measured as the sensor response, which arises from oxygen consumption caused by microbial oxidation of organic compounds. A flow-through BOD sensor has been also developed using living yeast *Hansenula anomala* (Kulys and Kadziauskiene, 1980). The microbial electrode sensor employing yeast *Trichosporon cutaneum* has been authorized as a Japanese Industrial Standard (JISC, 1990).

There have been various BOD sensors reported to date for which the kind of microorganisms used and/or the measurement method are different among them (Baronian, 2004; Kale and Mehrotra, 2009). It is usually said that microbes of low specificity and high oxidation activity for a wide range of organic compounds is desirable as the biological recognition element of BOD sensor. However, separate species of microorganisms has different selectivities and oxidation activities for a given solute. It seems likely that BOD values obtained by the rapid estimation method tend to be lower than a practical value when organics of hard to be bio-oxidized are contained in a water sample. Then, utilization of a mixed culture (Jia et al., 2003; Tan et al., 1993; Suriyawattanakul et al., 2002) or a microbial consortium (Liu et al., 2000; Rastogi et al., 2003) has been also attempted. The reproducibility of the sensor with pure culture is usually superior to that of the sensor using mixed culture or consortium.

There are two measurement approaches available for BOD biosensor systems, viz. 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, namely 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

* Corresponding author. Tel.: +81 92 606 3779; fax: +81 92 606 0728.
*E-mail address:* kawakami@fit.ac.jp (M. Kawakami).

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employed as the sensor response. This transient state measurement has been employed not only for the batch system (Riedel et al., 1988; Tan et al., 1993; Velling and Tenno, 2009), but for the flow system (Sohn et al., 1995; Sangeetha et al., 1996; Yang et al., 1997; Riedel et al., 1998; Liu et al., 2000, 2004a,b; Chan et al., 2000).

Recently, microbial BOD sensors utilizing redox mediators have received much attention. 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 reoxidation 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)-mediated BOD determination has been extensively studied (Pasco et al., 2000, 2004; Yoshida et al., 2000, 2001; Trosok et al., 2001, 2002; Catterall et al., 2001, 2003; Nakamura et al., 2007; Chen et al., 2008). 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.

*Shochu* is a Japanese distilled alcoholic beverage produced from rice, barley, buckwheat, sweet potato, and so on. The amount of distillery waste yielded during its production has increased markedly with a recent increase in the amount of production. The supernatant of distillery waste contains various organic compounds such as proteins and amino acids, and is an acidic solution of considerably high BOD. Then, many attempts have been made concerning the treatment procedure to lower BOD and the effective utilization as an organic resource of the wastewater. In such investigations determination of the actual BOD is indispensable and the development of reliable approach for BOD estimation is required. In the present study, *shochu* distillery wastewater (SDW) was employed as a real wastewater sample.

The flow-type biosensor method usually tends to yield a lower BOD value in comparison with the conventional $BOD_5$ method, because only a part of organic compounds biodegradable for the microbes used can be assimilated in the residential time. Microorganisms of high biodegradation activity are required to obtain sufficient sensor response. Then, it would be inevitable to use appropriate microorganisms in order to determine BOD of SDW, since it is specific wastewater of complicated composition.

In this study, a yeast strain, which has been isolated from an activated sludge as a microbe proliferating rapidly in diluted SDW, was employed as the biological recognition element. The yeast strain was immobilized on silica gel particles and packed into a fixed bed reactor. A flow cell of three electrode configuration was assembled and used as a transducer. A flow injection sensor system was constructed by arranging the reactor and flow cell separately. It was attempted to detect electrochemically the reduced mediator generated by microbial metabolism by employing HCF(III) solution as the carrier. The characteristics of the sensor system were investigated by using the peak area as the sensor response. Further, availability of the present system was checked by comparing BOD of SDW obtained by the present sensor system with that determined by 5-day method.

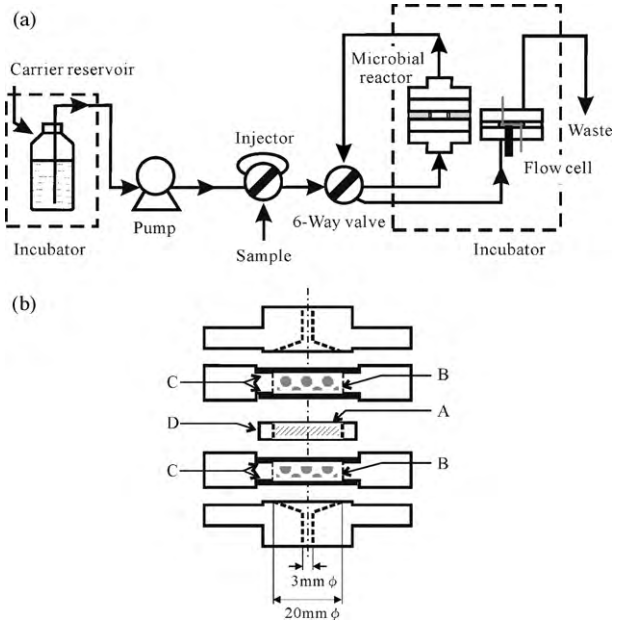


**Fig. 1.** Scheme of the BOD sensor system (a) and enlarged diagram of the microbial reactor (b). A: immobilized microbe, B: glass beads, C: stainless steel mesh screen, D: PTFE immobilized cell holder.

## 2. Experimental

### 2.1. Chemicals and materials

Pottasium hexacyanoferrate(III), pottasium hexacyanoferrate(II) trihydrate, 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). Silica gel (Wakogel G, 30–50 mesh) was used as the support for immobilization of microbes. *Shochu* distillery waste yielded during *shochu* production using barley was kindly provided by Hikari Shuzo (Fukuoka, Japan). The waste was centrifuged at 5000 rpm for 10 min and the supernatant was employed as SDW stock solution, which was stored in a freezer when not in use. $BOD_5$ and pH of the stock solution were 120,000 mg $L^{-1}$ and 3.9, respectively.

A graphite rod of 3 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. An Ag/AgCl electrode was prepared by bulk electrolysis of an Ag wire (1 mm $\phi$) in 0.1 M KCl and employed as the reference electrode.

### 2.2. Microorganism and culture

A SDW-assimilating microorganism was isolated from activated sludge sampled at a municipal sewage treatment plant (Kitakyushu, Japan). The sludge was dispersed into distilled water, and the supernatant was inoculated into appropriately diluted SDW. After incubation at 30 °C for a few days, the yeast strain SH-3 was isolated by streak-purification on SDW Gellan Gum plates. The strain was identified as *Candida krusei* (*Issatchenkia orientalis*) sp. by MMID (Mitsui Norin Microorganism Identification Service) based on 28S rDNA sequence analysis. *C. krusei* strain 1664 obtained from NBRC was also employed for comparison since it was observed to grow fairly well in diluted SDW. These strains were cultured aerobically on a reciprocating shaker at 30 °C for 24 h in YM broth (3 g $L^{-1}$ yeast extract, 3 g $L^{-1}$ malt extract, 5 g $L^{-1}$ peptone, 10 g $L^{-1}$ glucose). 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 YM broth containing 50% glycerol and stored at −80 °C.

### 2.3. Cell immobilization

Yeast cultures were grown aerobically on a rotary shaker at 30 °C for 24 h in YM broth added with silica gel particles. The culture was decanted and the residual particles were washed three times with sterilized phosphate buffer (0.1 M, pH 7.0). The resulting immobilized microbes particles were then resuspended in sterilized 0.9% NaCl solution and stored in a refrigerator for more than 24 h.

### 2.4. Sample preparation

OECD synthetic wastewater was used as a standard solution to calibrate the sensor system. 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 $MgSO_4\,7H_2O$ in 1 L of the phosphate buffer. The solution was filtered through a membrane filter (0.25 μm) to remove insoluble particles. The $BOD_5$ of this solution was 2600 mg $L^{-1}$. 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 biosensor 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 μL loop, a six-port switching valve, a microbial reactor, and a flow cell. PTFE tubes of 1.0 mm in i.d. were employed to connect these parts. Two Rheodyne Teflon rotary valves were used as the injector and the switching valve. The 6-way valve was switched to the reactor port when the response to BOD sample was measured. On the other hand, the valve was switched to the flow cell port when the sensitivity of the electrode to HCF(II) was checked.

An enlarged diagram of the microbial reactor is illustrated in Fig. 1(b). The reactor consists of an immobilized cell holder part (D), a set of supporting part, and a pair of connecting parts. The cell holder is a cylindrical PTFE pipe of 20 mm in i.d., 3 mm in thickness, and 12 mm in height, into which immobilized microorganism particles (A) have been packed. The supporting part was made of a PTFE rod (60 mm $\phi$). Its cylindrical hole (20 mm in diameter) was packed with glass beads (B) and both sides were supported with two pieces of SUS mesh screen (C).

The flow cell was made of a PTFE sheet and a 3 mm-thick silicone gasket. The inner cavity of the cell was estimated to be about 0.7 mL. A platinum wire ($1\phi \times 10$ 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 procedure

After the immobilized microbes particles were packed into the cell holder, the packed bed was thoroughly washed by passing overnight sterilized 0.9% NaCl solution at 0.5 mL $min^{-1}$. 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 1.0–2.5 mL $min^{-1}$. Amperometric measurements were made by applying a given potential on the working electrode with a potentiostat (model HECS 318C; Huso, Kawasaki, Japan) bearing the accuracy for current detection of ±0.5–1% 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 with a sampling tube (500 μL). The inside of the sensor system was filled with sterilized 0.9% NaCl when not in use.

## 3. Results and discussion

### 3.1. Measurement methods

The applied potential of the working electrode is an important factor affecting the responsibility and sensitivity of the sensor system. Ferricyanide was found to have the oxidation and the reduction peak at 290 and 130 mV (*vs.* Ag/AgCl), respectively, in the phosphate buffer by cyclic voltammometry. Then, the response to 1 mM HCF(II) was measured by applying a potential of 250, 300, and 400 mV (*vs.* Ag/AgCl). In this case, the microbial reactor was bypassed, and the substrate was sent directly to the flow cell. It was observed consequently that the sensitivity was almost independent on the electrode potential in the range applied here and the base line stability was most excellent at 250 mV. Accordingly, the applied potential was fixed at 250 mV in further experiments.

In order to determine BOD of real wastewater with biosensor method, it is needed to measure for a calibration solution at the same conditions. Then, the sensor responses for glucose–glutamic acid solution (GGA) and OECD synthetic wastewater, both of which have been used widely as the calibration solutions, were determined. It was consequently found that the response for the standard GGA solution ($BOD_5$ = 220 mg $L^{-1}$) was considerably lower than that for the synthetic wastewater ($BOD_5$ = 130 mg $L^{-1}$). The reproducibility of the response measurements for GGA was comparatively poor. Further, SDW could be presumed to contain a variety of proteins as the constituents. Then, the synthetic wastewater having analogous composition to that of SDW was employed as the calibration solution in the present study.

The sensor output signals arising from electrochemically active species contained in sample were examined by injecting sample solutions into the system without passing through the microbial reactor. Consequently, it was observed that the signals observed for OECD synthetic wastewater and SDW sample could be negligible when the working electrode was covered with a cellulose dialysis membrane, whereas the signals for ferrocyanide solution was reduced to about 10% of that obtained with a bare electrode. Then, subsequent measurements were made with a membrane-covered electrode. The sensitivity of the working electrode was checked periodically by determining the magnitude of output signals for 1 mM HCF(II).

### 3.2. Amperometric sensor response

Examples of amperometric response obtained for successive injections of OECD synthetic wastewater of different concentration with strain SH-3 based sensor system are shown in Fig. 2. The concentration of organic materials in the wastewater was calculated on the basis of $BOD_5$ = 2600 mg $L^{-1}$ for the stock solution. It can be seen that an anodic peak is 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. On the other hand, it was confirmed that the sensor responses obtained by using immobilized cell particles which was autoclaved at 120 °C for 20 min before use were only 1–2% of those obtained with living cells. This demonstrates the sensor output to arise from reoxidation of HCF(II) which is produced by microbial assimilation in the reactor.

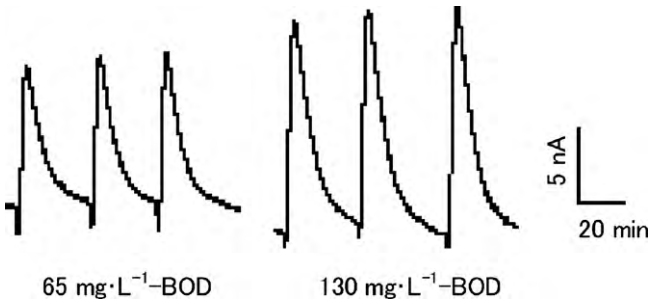


**Fig. 2.** Diagram of amperometric response obtained for successive injections of OECD synthetic wastewater of different concentration with the strain SH-3 based sensor system (flow rate = 1.5 mL min$^{-1}$).

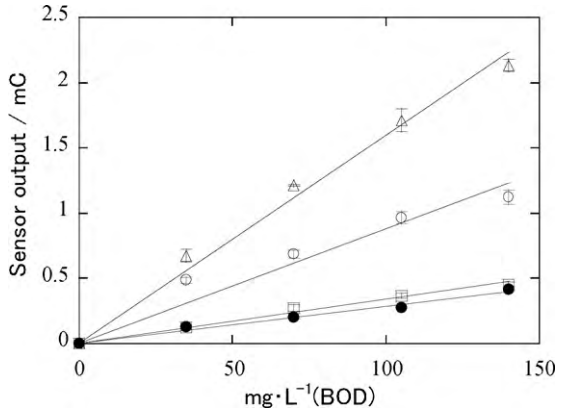


**Fig. 3.** Dependence of calibration on flow rate of carrier solution. OECD synthetic wastewater was analyzed with the strain SH-3 based sensor system at the flow rate of (△) 1.0 mL min$^{-1}$, (○) 1.5 mL min$^{-1}$, (□) 2.0 mL min$^{-1}$, and (●) 2.5 mL min$^{-1}$.

### 3.3. Effect of flow rate

The flow rate could be expected to affect seriously the sensitivities of the present sensor system, since the extent of microbial assimilation would depend on the residence time in the reactor. Dependence of the calibration curves for synthetic wastewater on the flow rate of carrier solution is illustrated in Fig. 3. Almost linear correlations were obtained between the sensor response and the calculated organics concentration in the range applied here. Variation of the sensitivities evaluated by the slope of calibration plots with flow rates is depicted in Fig. 4. As expected the sensitivity of the system increased with decreasing flow rate. This implies

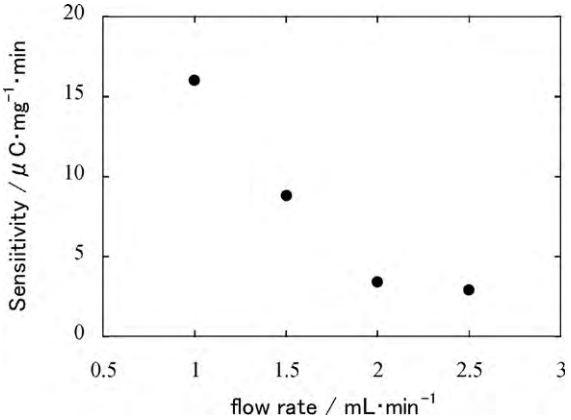


**Fig. 4.** Variation of sensitivity with flow rate of carrier solution. OECD synthetic wastewater of 140 mg L$^{-1}$-BOD was analyzed with the strain SH-3 based sensor system.

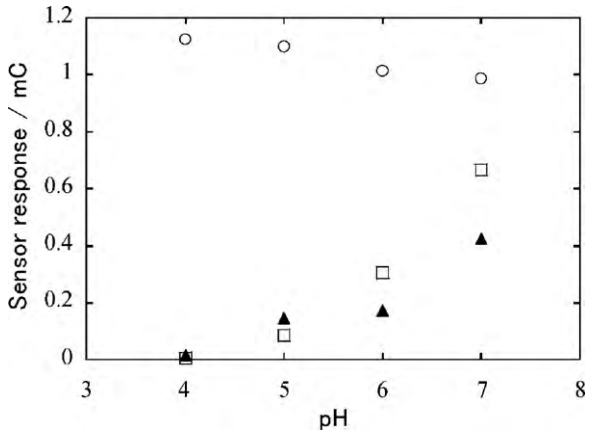


**Fig. 5.** Effects of carrier solution pH on the sensor response. OECD synthetic wastewater of 70 mg L$^{-1}$-BOD (▲), SDW of DR = $10^{-3}$ (□) and 1 mM potassium ferrocyanide (○) were analyzed independently with the strain SH-3 based sensor system.

that larger quantities of organic compounds in a sample could be metabolized when the residence time is lengthened.

The time required for a measurement is, on the other hand, an important factor for practical use. The time taken to recover the base line increased with decreasing the flow rate. Then, by considering the sensitivity and the measuring time, further measurements were made at a flow rate of 1.5 mL min$^{-1}$, where the time required for one measurement from sample injection to baseline recovery was about 30 min.

### 3.4. Effects of carrier solution pH

Effects of the carrier solution pH on the sensor response were investigated for synthetic wastewater (65 mg L$^{-1}$) and SDW (dilution rate = $1 \times 10^{-3}$) using the strain SH-3 based sensor system. The response for 1 mM HCF(II) was also examined without passing through the microbial reactor. It is seen from Fig. 5 that the sensitivity for ferrocyanide is slightly affected by the pH. On the other hand, the carrier solution pH has found to exert remarkable effects on the sensor responses for synthetic wastewater and SDW. The highest response for either synthetic wastewater or SDW was obtained at pH 7.0, and the responses decreased with lowering pH down to pH 4.0. These results may indicate that the metabolism of the microbe becomes more active in neutral pH range.

### 3.5. Effects of microbial strain

Fig. 6 shows the effects of the kind of microbial strain on the sensor calibration. The concentration of SDW was expressed by dilution rate. For both OECD synthetic wastewater (a) and SDW (b) sample, almost linear relationships were obtained with two different strain-based systems. On the other hand, in the case of *T. cutaneum* (NBRC 10466), which has been most frequently employed as the microorganism for BOD sensor, peaks of unusual shapes were sometimes observed and reproducible results could not be obtained. This implies that *T. cutaneum* is not appropriate to determine BOD of SDW with the present sensor system. It is seen from Fig. 6 that the sensitivities of strain SH-3 based system for both samples are higher than those of the NBRC 1664 based system. This may suggest the sensitivity to depend on the inherent assimilating ability of microorganism used, if there is not a marked difference in the amounts of immobilized microbes used for the both system.

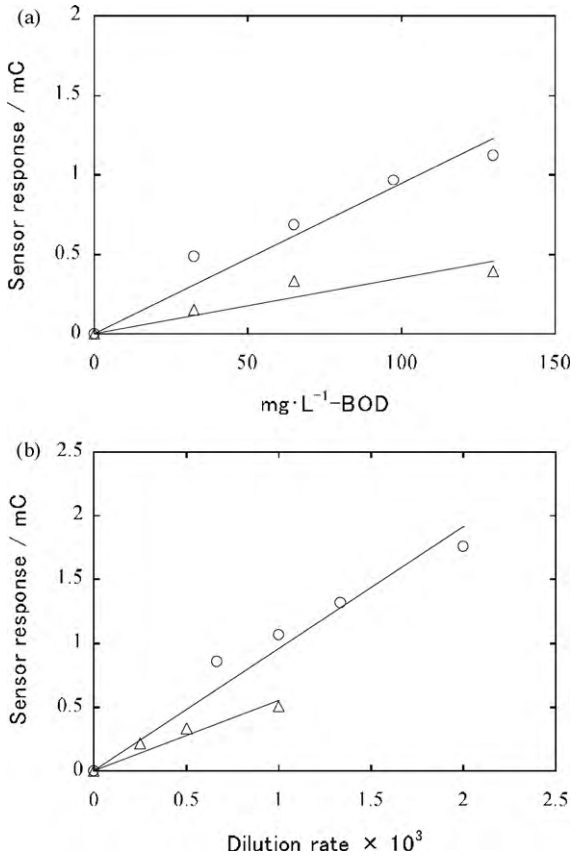


**Fig. 6.** Effects of the kind of microbial strain on calibration for (a) OECD synthetic wastewater and (b) SDW (○) SH-3, (△) 1664.

### 3.6. BOD estimation and sensor stability

BOD values estimated from the slopes of calibration plots in Fig. 6 were 102,000 and 156,000 mg $L^{-1}$ for the strain SH-3 and NBRC 1664 based sensor system, respectively. The former result is relatively close to $BOD_5$ (120,000 mg $L^{-1}$). It seems likely that the sensor system of higher sensitivity affords more reliable results. For the NBRC 1664 based system, BOD value may be somewhat overestimated due to its relatively poor bio-oxidative capacity. These results demonstrate the present sensor system to be applicable to the estimation of BOD of SDW.

The response stability of the flow cell could be remarkably improved by covering the working electrode with a dialysis membrane, which could prevent or reduce the adsorption of organics in sample solution. The microbial reactor in the present sensor system has primarily high operational stability compared to that employed in the steady-state method since a relatively small amount of sample is injected. Further, it was found that a stable baseline could be established more quickly by filling the system with 0.9% NaCl when not in use as compared to the carrier solution. Consequently, the system was found to show good reproducibility for at least 1 week, while further prolonged stability test has not yet been made.

## 4. Conclusion

The mediated BOD biosensor has attracted much attention as a rapid measuring method and an alternative to the conventional 5-day method. In the flow injection biosensor system constructed in this work, it was demonstrated that the mediated sensing was realized by employing phosphate buffer containing HCF(III) as the carrier. The output current was found to yield a peak after sample injection, and to result from reoxidation of reduced mediator, HCF(II), at the electrode. The peak area could be used as the sensor response, and the sensitivity was affected by the flow rate and pH of the carrier solution. The sensor system using a microorganism of high SDW-assimilation capacity showed good performance and proved to be available for estimation of BOD of SDW. In order to demonstrate the applicability of the present sensor system for BOD determination to wastewater samples other than SDW, further experiments are currently under way.

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