

A new BOD estimation method employing a double-mediator system by ferricyanide and menadione using the eukaryote *Saccharomyces cerevisiae*

Hideaki Nakamura^{a,b,*}, Kyota Suzuki^a, Hiroaki Ishikuro^a, Shintaro Kinoshita^a,
Rui Koizumi^a, Seisaku Okuma^a, Masao Gotoh^{a,b}, Isao Karube^{a,b,*}

^a School of Bionics, Tokyo University of Technology, 1404-1 Katakura, Hachioji, Tokyo 192-0982, Japan

^b Research Center of Advanced Bionics (RCAB), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo University of Technology, 1404-1 Katakura, Hachioji, Tokyo 192-0982, Japan

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Abstract

A new biochemical oxygen demand (BOD) sensing method employing a double-mediator (DM) system coupled with ferricyanide and a lipophilic mediator, menadione and the eukaryote *Saccharomyces cerevisiae* has been developed. In this study, a stirred micro-batch-type microbial sensor with a 560 μL volume and a two-electrode system was used. The chronamperometric response of this sensor had a linear response between 1 μM and 10 mM hexacyanoferrate(II) ($r^2 = 0.9995$, 14 points, $n = 3$, average of relative standard deviation and R.S.D._{av} = 1.3%). Next, the optimum conditions for BOD estimation by the DM system (BOD_{DM}) were investigated and the findings revealed that the concentration of ethanol, used to dissolve menadione, influenced the sensor response and a relationship between the sensor output and glucose glutamic acid concentration was obtained over a range of 6.6–220 $\text{mg O}_2 \text{ L}^{-1}$ (five points, $n = 3$, R.S.D._{av} 6.6%) when using a reaction mixture incubated for 15 min. Subsequently, the characterization of this sensor was studied. The sensor responses to 14 pure organic substances were compared with the conventional BOD₅ method and other biosensor methods. Similar results with the BOD biosensor system using *Trichosporon cutaneum* were obtained. In addition, the influence of chloride ion, artificial seawater and heavy metal ions on the sensor response was investigated. A slight influence of 20.0 g L^{-1} chloride ion and artificial seawater (18.4 $\text{g L}^{-1} \text{ Cl}^-$) was observed. Thus, the possibility of BOD determination for seawater was suggested in this study. In addition, no influence of the heavy metal ions (1.0 $\text{mg L}^{-1} \text{ Fe}^{3+}$, Cu^{2+} , Mn^{2+} , Cr^{3+} and Zn^{2+}) was observed. Real sample measurements using both river water and seawater were performed and compared with those obtained from the BOD₅ method. Finally, stable responses were obtained for 14 days when the yeast suspension was stored at 4 °C (response reduction, 93%; R.S.D. for 6 testing days, 9.1%).

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1. Introduction

The biochemical oxygen demand (BOD) has been used widely for several decades as an index of organic pollution in industrial wastewater or natural waters. The conventional 5-day (BOD₅) method is time-consuming and requires 5 days to obtain

results (JIS K 0102) [1]. To solve the problem, the first microbial biosensor for rapid BOD estimation was developed by Karube et al. [2]. Since this method was proposed, many variations of this BOD sensor have been developed. The history of BOD sensor developments was well summarized in two literature by Nakamura and Karube [3,4]. After the first report, a practical sensor system was developed by Hikuma et al. [5]. It employed the eukaryote *Trichosporon cutaneum* as an omnivorous and viable microbe and was utilized as an indication of the dissolved oxygen concentration (BOD_{DO}). This BOD_{DO} method was established as a Japanese Industrial Standard (JIS K 3602) [6], and many variations followed, such as thermophilic bacteria for long-term stability [7], dead *Bacillus subtilis* cells [8],

* Corresponding authors at: Research Center of Advanced Bionics (RCAB), National Institute of Advanced Industrial Science and Technology (AIST), Tokyo University of Technology, 1404-1 Katakura, Hachioji, Tokyo 192-0982, Japan. Tel.: +81 4 26 372149; fax: +81 4 26 374058.

E-mail addresses: nakamura@bs.teu.ac.jp (H. Nakamura), karube@bs.teu.ac.jp (I. Karube).

slime mold [9], *Pseudomonas putida* SG10 for highly sensitive detection [10,11] and multi-microorganisms for seawater [12]. In addition, this principle was applied to soil diagnosis for agricultural fields [13]. However, these methods required a sensitive DO electrode due to the extremely low solubility of oxygen in water ($8.84 \text{ mg O}_2 \text{ L}^{-1}$ at 1 atm, 20°C). Hence, a practical and portable system has not yet been developed. Alternative methods have been created; these sensors indicate bacterial luminescence by *Photobacterium phosphoreum* [14] and surface photovoltage with *T. cutaneum* [15], although neither sensor has been realized for practical use as a portable device.

As a next-generation technique, the single-mediator (SM) system was applied using ferricyanide (FC) and bacteria *Pseudomonas fluorescens* biobar V as a new estimation method of the BOD (BOD_{SM}) by Yoshida et al. [16]. FC proved to be an efficient mediator for shuttle electrons from the redox center of reduced bacterial enzymes to the electrode in the presence of organic compounds [17,18]. The BOD_{SM} sensor could easily be improved as a portable device with bacterial sensor chips because of the use of highly dissolved FC [19]. However, *P. fluorescens*, which is used in the BOD_{SM} sensor developments, is a prokaryote, known as a unicellular organism; therefore, sensor stability was the greatest concern with regard to the sensor's practical applications.

In recent years, a double-mediator (DM) system combining FC and menadione, a lipophilic mediator, was studied using the eukaryote *Saccharomyces cerevisiae* by Baronian et al. [20]. The study revealed that menadione can penetrate the outer cell membrane. Eukaryotes, such as yeasts that function under both aerobic and anaerobic conditions, are easily handled, omnivorous to many kinds of organic substances, and stable in saline solutions. Therefore, the baker's yeast *S. cerevisiae* was selected as the most available and suitable microbe for the new estimation method of BOD.

2. Experimental

2.1. Materials

Menadione (Vitamin K₃, 2-methyl-1,4-naphthoquinone) was purchased from MP Biochemicals, LLC (Germany). Potassium FC, potassium hexacyanoferrate(II), glucose, glutamic acid, and ethanol were purchased from Wako Pure Chemicals (Osaka, Japan). Triton X-100TM (TritonTM) was purchased from Sigma Chemicals (USA). The other chemicals used in this study were of reagent grade. Water was used after reverse osmosis.

Ten-fold-concentrated phosphate-buffered saline (PBS10) contained 2 g KH_2PO_4 , 29 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 80 g NaCl and 2 g KCl and was adjusted to pH 7.0 with HCl; a total volume of 1 L was obtained by mixing with pure water. PBS was prepared by 10-fold dilution of PBS10. To calibrate the amperometric response of the BOD sensor, a standard was prepared by dissolving equimolar amounts of both potassium hexacyanoferrate(II) and potassium FC to a concentration of 400 mM (FF mixture).

For the BOD_{DM} measurement, 480 mM FC was prepared in PBS10. TritonTM was also dissolved in PBS10 to give a 1.5%

(w/v) solution. For experimental use, a 0.05% solution was prepared from the 1.5% solution with PBS10 before use. Menadione was dissolved in 99.5% ethanol to give an 80 mM solution and stored in a light-proof container [20]. The LB medium was prepared with 0.25 g of NaCl, 0.5 g of Bacto Triptone, and 0.25 g of yeast extract in 50 mL at pH 7.0. A BOD standard solution containing 1.5 g L^{-1} glucose and 1.5 g L^{-1} glutamic acid (GGA) was employed as a model wastewater of $2200 \text{ mg O}_2 \text{ L}^{-1}$ according to the Japan Industrial Standard committee (JIS K 0102, 1974) [1]. All of the solutions were stored at 4°C .

2.2. Microorganism and cell cultures

Baker's dry yeast was purchased from Nisshin Foods Inc. (Super Camellia, Tokyo) and grown by two steps. In pre-culture, the cells were grown under aerobic conditions at 180 rpm and 28°C for 9 h in 2 mL of a sterilized YPD medium (40 mg of glucose, 40 mg of PolypeptoneTM and 20 mg of yeast extract in 2 mL, pH 7.0) using a test tube. One microlitre of the pre-cultured medium was inoculated to the fresh YPD medium (200 mL), and the main culture was performed at 120 rpm for 15 h using a Sakaguchi flask. After growth, the yeast cells were harvested by centrifugation for 3 min at 3000 rpm and washed three times with a 0.9% NaCl solution (4°C). The yeast was re-suspended in 25 mL of a NaCl solution and starved by shaking at 120 rpm and 28°C for 2 h. Following starvation, the yeast was washed three times, re-suspended in a NaCl solution to an optical density (OD_{600}) of 45, and stored at 4°C before use.

2.3. Construction of the BOD sensor

The sensor device was built using pilling polyethylene terephthalate (PET) sheets (0.2 mm thickness) and expanded plastic boards (1 mm thickness) (Fig. 1). A screen-printed carbon electrode sheet was placed upside down and sandwiched between one expanded plastic board and three boards. The area of each electrode was determined to be 18 mm^2 ($7.5 \text{ mm} \times 2.4 \text{ mm}$). In addition, a PET sheet was glued to the bottom. The sensor device employing a two-electrode system had a cuvette volume of $562.5 \mu\text{L}$ ($10 \text{ mm} \times 12.5 \text{ mm} \times 4.5 \text{ mm}$), which enabled setting a magnetic micro-stirrer bar ($8.0 \text{ mm} \times 1.5 \text{ mm}$) in the cuvette.

2.4. Experimental procedure

The sensor device was stirred using a micro-stirrer (Cellstar CC-303, AsOne, Japan) and controlled through an electrochemical analyzer (SHV-100, Hokuto Denko or CHI-1202, BAS, USA). Prior to the electrochemical measurement, the stirrer was stopped, and chronoamperometry was performed by poisoning the carbon-working electrode +900 mV relative to the carbon reference/counter electrode for 3 s. The current output obtained at the endpoint of the chronoamperometry was taken as the sensor response. In this study, a sufficiently high potential was applied for the oxidation of hexacyanoferrate(II) to prevent

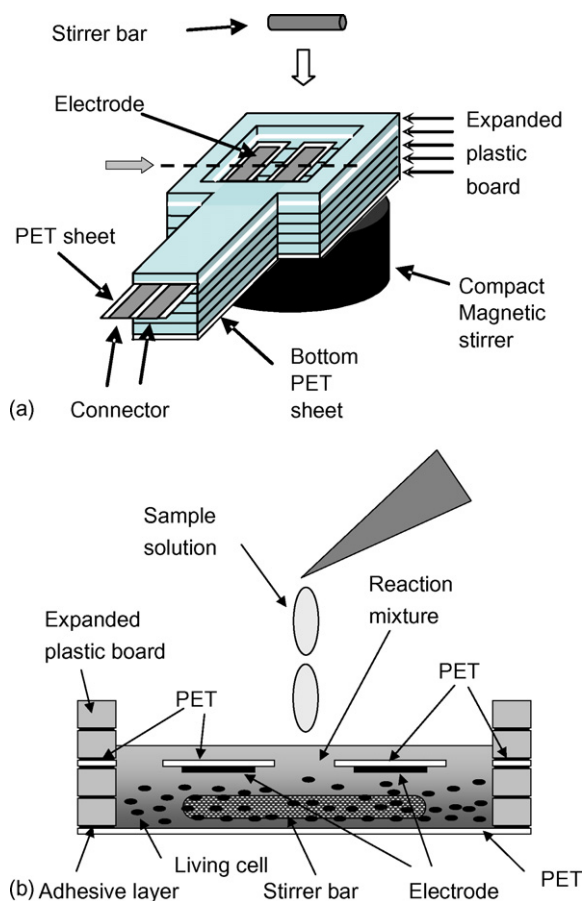


Fig. 1. Schematic diagram of the sensor device. (a) Squint view of the sensor device. (b) Cross-sectional view from a gray arrow in the diagram of the squint view.

the equilibrium potential at the working electrode from having an influence on the drift. In addition, we decided to ignore the influence of electro-active substances dissolved in real samples on the chronoamperometry. All experiments were performed three times at room temperature and with a reaction volume of 500 μL .

The sensor device was preconditioned using diluted household bleach and stirring at 100 rpm for 4 min. The sensor device's response was verified by confirming a 155 μA signal to 10 mM FF.

For the calibration curve to the hexacyanoferrate(II) concentration, the designated concentration of the FF mixture was added to the sensor cuvette for one measurement, and the measurement was performed without reaction time and stirring.

In the experiments using the microbial sensor, 41.5 μL of 480 mM FC, 5 μL of 20 mM or 80 mM menadione, 5 μL of 0.05% (w/v) TritonTM, 50 μL of a yeast suspension ($\text{OD}_{600} = 45$), or 25 μL of a yeast suspension with 25 μL of 0.9% NaCl, 350 μL of sample, and 48.5 μL of water were added to the sensor cuvette to give a final volume of 500 μL . The reaction in the sensor cuvette was started by the final addition of the yeast suspension under stirring at 100 rpm. One electrochemical measurement was performed after a 15 min incubation.

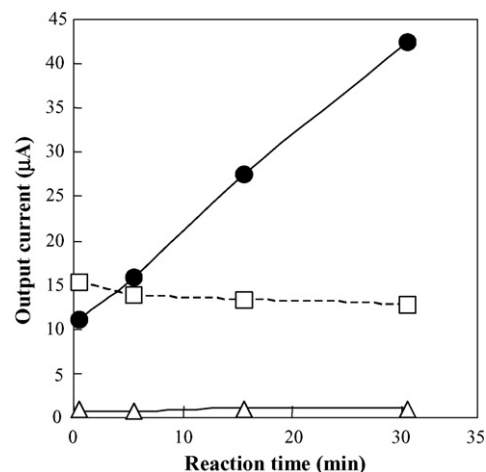


Fig. 2. Sensor responses to the double-mediating reaction. (●) With both FC and menadione, (□) with menadione only and (Δ) with FC only. Fifty microlitres of 240 mM FC in PBS (pH 7.0), 1.5 μL of 20 mM menadione, 30 μL of a yeast suspension ($\text{OD}_{600} = 15$), and 60 μL of an LB medium were added to the sensor cuvette, and the final volume was adjusted to 300 μL by PBS. The electrochemical measurement was performed at 0.5, 5.5, 15.5 and 30.5 min after the addition of the yeast suspension.

2. Results and discussion

2.1. Sensor responses by a DM system

As part of the preliminary investigation, we examined the possibility of obtaining a sensor response from living yeast cells containing a reaction mixture using SHV-100 without stirring. Fig. 2 shows the results obtained under three different conditions. The response from yeast suspensions increased when the reaction mixture contained both FC and menadione. These results show that menadione can penetrate the yeast outer wall and produce a menadione radical, which then transfers the electron to FC. The FC is reduced to hexacyanoferrate(II), which is finally reoxidized to FC at the working electrode.

On the other hand, in the absence of either FC or menadione, no sensor response changes were observed. The results indicate that FC without menadione could not act as an electron acceptor of *S. cerevisiae* during the catabolic process of organic substances, although the menadione response 0.5 min later was greater than the DM response, possibly due to the presence of decomposed menadione. The other side, menadione without FC produced menadione radical; however, the radical could not be detectable to the electrode. On the bases of these facts, the responses were not obtained from these measuring conditions.

2.2. Characterization of a sensor system

Our sensor system was fabricated to evaluate the microbial BOD_{DM} method, and its basic response was examined using CHI-1202 by generating a calibration curve with unstirred 500 μL aliquots of potassium hexacyanoferrate(II). As shown in Fig. 3, a calibration curve was obtained from the 0 to 50 mM FF mixture, a linear range with excellent correlation ($r^2 = 0.9995$,

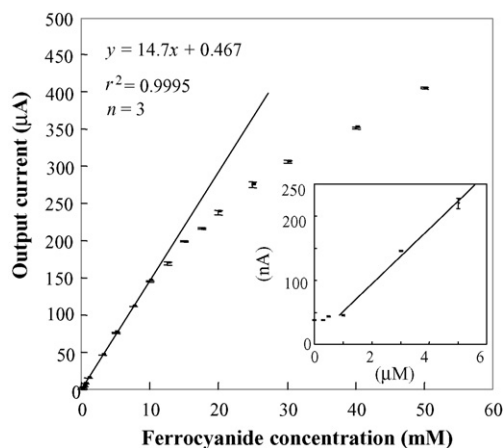


Fig. 3. Calibration curve for potassium hexacyanoferrate(II) dissolved in PBS.

$y = 14.7x + 0.467$, 14 points, $n = 3$, averaged R.S.D. = 1.26%) from the 1 μM to 10 mM FF mixture, and a detection limit from the 0.5 μM FF mixture. Thus, 40 mM FC was adopted as the initial FC concentration for subsequent BOD_{DM} experiments.

2.3. Optimization of the sensor responses

For the optimization of the measuring conditions, a micro-stir system was adopted to improve both the reaction efficiency and measuring reproducibility. For this purpose, the design of the sensor device was slightly changed to create space for rotating a micro-stir bar, and the rotation speed was set to 100 rpm. In addition, the reaction time was set to 15 min for rapid estimation. By repetitive use of the sensor device, the magnitudes of the sensor responses were reduced (data not shown), probably due to adsorption of hydrophobic menadione on the electrode surface. The regeneration of the electrode surface was then examined using a diluted bleaching agent for 4 min, which enabled the repetitive use of the device. Thus, the reproducible measurements by this microbial sensor could be realized, and the relative standard deviation (R.S.D.) taken by three measurements could be less than 10%.

Subsequently, the optimization of the electric potential of the working electrode, the pH in the reaction mixture, and the concentration of TritonTM, phosphate, menadione, and ethanol was investigated.

The working electrode was held at a sufficiently high electric potential to reoxidize the hexacyanoferrate(II) ion produced by the reduction of FC in the menadione radical as a result of the existence of microorganisms. Therefore, the operating potential was held at +900 mV versus a counter electrode serving as a reference electrode in this study.

The effects of the pH on the sensor responses to 110 mg O₂ L⁻¹ GGA were investigated at pH 5.0, 6.0, 7.0, 8.0 and 9.0 by the addition of 5 μL of 80 mM menadione and 50 μL of a yeast suspension (OD₆₀₀ = 45) (data not shown). As a result, almost identical responses between pH 6.0 and pH 9.0 were obtained; however, the responses at pH 5.0 were slightly smaller than the other responses. This is quite likely because the solubility of menadione in an aquatic solution is lowered under acidic

conditions. In general, the pH of natural water and wastewater is nearly neutral; therefore, the potential of the BOD_{MD} sensor could be shown by this experiment. For subsequent experiments, measurements were performed at pH 7.0.

To reduce the adsorption of menadione onto the surface of the carbon electrodes, TritonTM as a surfactant was used in this study. At first, the effects of TritonTM on the responses to a 10 mM FF mixture were investigated using a pure TritonTM solution from 0.0001, 0.0001, 0.001, 0.005, 0.01, 0.1 and 1% (w/v). Then, the responses were dramatically reduced from 0.001% TritonTM due to the formation of a membrane on the electrode surface (data not shown). Next, we examined the effects of TritonTM at 0.00001, 0.00025, 0.0005, 0.001, 0.001, 0.01 and 0.1% on the response of the BOD_{DM} sensor to 110 mg O₂ L⁻¹ GGA. The responses gently decreased with an increase in the concentration of TritonTM. In general, the existence of a surfactant in a cell suspension is fatal to microorganisms. However, it was insignificant when using yeast because yeast has a thick outer-cell wall. Thus, the concentration of TritonTM was set at 0.0005% as a final concentration in subsequent experiments.

Microbial sensor responses are known to decrease when the phosphate concentration drops below 10 mM [5]. Thus, the effects of phosphate on the responses to 110 mg O₂ L⁻¹ GGA were examined at 2.0, 10, and 40 mM phosphate as the final concentration in the sensor cuvette. The response decreased with a decrease in the phosphate concentration from 2 mM (data not shown). Here, we adopted the phosphate concentration of 8.9 mM, which is close to 10 mM, as a final concentration, which is possibly enough to obtain a sufficient response.

Ethanol is used as a solvent of menadione and is an assimilable organic substance. Hence, the effects of ethanol on the responses to 110 mg O₂ L⁻¹ GGA were investigated at 1.0, 1.5 and 2.0% (v/v) as the final concentration in the sensor cuvette. As we expected, the responses increased with a linear increase in the ethanol concentration (data not shown). These results suggested that the oxidation rate of ethanol was faster than that of some substrates, such as glucose and glutamic acid. This result indicated that the amount of ethanol used as a solvent of menadione has to be reduced as much as possible; thus, 1.0% (v/v) ethanol was adopted as a final concentration in the sensor cuvette.

Subsequently, the effects of the menadione concentration on the sensor responses to 110 mg O₂ L⁻¹ GGA were examined from 0.1 to 0.8 mM at the final concentration in the sensor cuvette. As shown in Fig. 4, the responses drastically and efficiently increased with an increase in the menadione concentration up to 0.2 mM.

Hence, the optimum conditions obtained by these experiments were used for all further studies.

2.4. Calibration curve for GGA

Various concentrations of the standard BOD solution were applied to the system to obtain calibration curves under three different conditions, as shown in Fig. 5. The response increased with increasing the BOD concentration of the standard GGA solution, and the best relationship could be obtained under the conditions using 5 μL of 80 mM menadione and 50 μL of

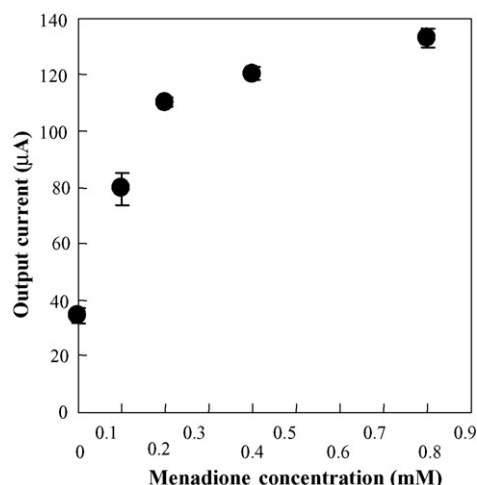


Fig. 4. Effects of menadione.

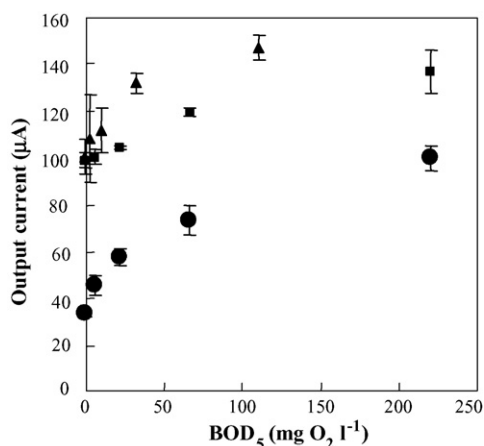


Fig. 5. Calibration curve for GGA. (▲) Five microlitres of 80 mM menadione and 50 μL of a yeast suspension ($\text{OD}_{600} = 45$), (■) 5 μL of 20 mM menadione and 50 μL of a yeast suspension, (●) 5 μL of 20 mM menadione and 25 μL of a yeast suspension with 25 μL of a 0.9% NaCl solution.

yeast suspension ($\text{OD}_{600} = 45$). Then, a relationship between the increase in the output current (BOD response) and the BOD concentration from 6.6 to 220 $\text{mg O}_2 \text{L}^{-1}$ with the GGA solution was obtained in the BOD_{DM} sensor. The reproducibility of the sensor responses in the calibration curve was 6.6% (average of R.S.D.s). Under the other two conditions, the detection limit was compromised due to the high background responses caused by the endogenous activity of the yeast. In subsequent experiments, 0.2 mM menadione was adopted as the final concentration.

2.5. Sensor responses to organic substances

The BOD sensors should have the ability to detect a wide spectrum of organic substances. Therefore, the BOD values for various kinds of pure organic substances were measured using our BOD_{DM} sensor to confirm the characteristics of this method. The BOD values obtained by the sensor were determined using the procedure described above for the GGA measurement. Each pure organic substance was prepared at 300 mg L^{-1} so that it would be within the range of the calibration curve. Table 1 shows the substrate specificity of the BOD_{DM} sensor method relative to the reported data from BOD_{SPV} [15], BOD_{DO} [5], BOD_5 [21], BOD_{LUM} [14] and BOD_{SM} [16] for the compounds. Due to differences in the dissolved oxygen concentration, microorganism type, BOD standard solution, and incubation time for the BOD_5 method, it was not possible to obtain accurate comparisons of the six methods.

In Table 1, the values from our BOD_{DM} sensor method for the substances evaluated show that the glucose response was significantly higher than the other substances. A microbe metabolizes glucose through glycolysis. Glycolysis is generally faster than the TCA cycle; therefore, the major factor of the response might be glycolysis in a short response time. There is no consumption of oxygen in glycolysis, and the production of only two molecules of NADH requires a small amount of oxygen in the following reaction of ATP production. Compared to the TCA

Table 1
Comparison of BOD values of various organic samples^a

Substrate	BOD_{DM}^b Eukaryote (<i>S. cerevisiae</i>) ^c	BOD_{SPV} [15] Eukaryote (<i>T. cutaneum</i>) ^c	BOD_{DO} [5] Eukaryote (<i>T. cutaneum</i>) ^c	BOD_5 [21] Consortium ^c –	BOD_{LUM} [14] Prokaryote (<i>P. phosphoreum</i>) ^c	BOD_{SM} [16] Prokaryote (<i>P. fluorescens</i>) ^c
Glucose	1.13	0.66	0.72	0.50–0.78	0.62	1.54
Fructose	0.74	0.73	0.54	0.71	0.57	0.35
Sucrose	0.40	0.45	0.36	0.49–0.76	0.50	0.07
Lactose	0.07	0.04	0.06	0.45–0.72	0.31	0.02
Soluble starch	0.03	0.07	0.07	0.22–0.71	0.02	–
Asparagine	0.28	–	–	0.58	0.48	0.29
Alanine	0.25	–	–	0.55	–	0.73
Glycine	0.25	0.36	0.45	0.52–0.55	0.50	–
Glutamic acid	0.15	0.40	0.70	0.64	0.73	0.59
Histidine	0.63	0.34	0.35	0.55	–	0.27
Citric acid	0.18	0.18	0.72	0.63–0.88	–	–
Lactic acid	0.11	0.14	0.17	0.40	0.32	0.66
Propanol	0.31	–	0.28	0.47–1.50	0.28	0.29
Glycerol	0.23	0.44	0.51	0.62–0.83	0.53	0.05

^a Values are expressed in $\text{mg O}_2 \text{mg}^{-1}$ substrate.

^b Concentration of each pure organic substances were 300 mg L^{-1} for each substance.

^c Microbe.

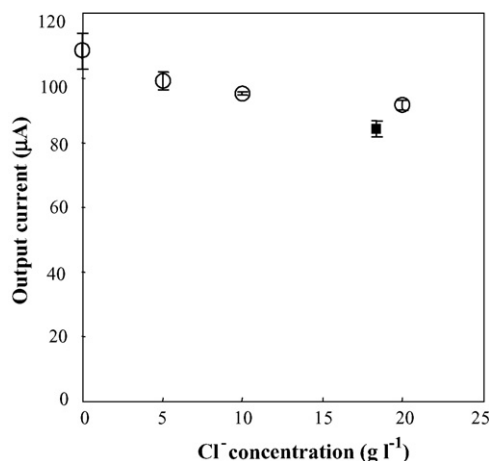


Fig. 6. Influence of the chloride ion. (○) Cl[−] ion in a NaCl solution, (■) artificial seawater.

cycle, glycolysis makes only a small contribution to oxygen consumption in the oxygen electrode method. Thus, a relatively small response would probably be obtained for compounds that are metabolized in the TCA cycle. In addition, the sensor methods using yeast, including our method, showed quite similar profiles to various kinds of pure organic substances. While the most important requisite for the microorganisms used with the BOD sensor is to have a wide substrate spectrum for degradation, it would be extremely unlikely to have the same spectra for different microbial species. Therefore, this is sufficiently adequate to demonstrate that the BOD_{DM} method is valid for the BOD determination.

2.6. Influences of chemicals

The influence of the chloride ion on the sensor responses of 220 mg O₂ L^{−1} GGA was investigated from 5.00 to 20.0 g L^{−1} Cl[−] ion. In Fig. 6, the responses were slightly influenced by the increasing concentration of the Cl[−] ion. In addition, the influence of artificial seawater (26.5 g NaCl, 3.26 g MgCl₂, 2.07 g MgSO₄, 1.36 g CaSO₄, and 0.714 g KCl in 1 L water) on the sensor responses of 220 mg O₂ L^{−1} GGA was also investigated, and the responses had a relative value of 77% of that of the control. However, *S. cerevisiae* is known to be a salt-tolerant microbe; therefore, these results suggest that the BOD_{DM} sensor has potential for the BOD evaluation of seawater when a standard solution prepared with artificial seawater is used.

Most real samples, such as river water, seawater, and effluent, contain various heavy metal ions, such as Fe³⁺, Cu²⁺, Mn²⁺, Cr³⁺, and Zn²⁺, and the presence of these heavy metal ions in the sample may interfere with the activity of microorganisms [22]. Thus, the influence of heavy metal ions on the response was investigated at 1 mg L^{−1} because the highest concentration of these ions in a polluted Japanese river should be below 1 mg L^{−1} [23]. To perform the examinations, chlorides were used as a counter ion of heavy metal ions. The BOD of 220 mg O₂ L^{−1} GGA was used as a control. The results shown in Table 2 reveal that Fe³⁺, Cu²⁺, Mn²⁺, Cr³⁺, and Zn²⁺ (1 mg L^{−1}, each) have no effect on the response of the sensor.

Table 2

Influences of heavy metal ions on the biosensor response to 220 mg O₂ L^{−1} BOD

Heavy metal ion (1 mg L ^{−1})	Relative value (%)
Control	100
Fe ³⁺	98.8
Cu ²⁺	102
Mn ²⁺	103
Cr ³⁺	99.1
Zn ²⁺	99.9

2.7. Investigation of the yeast storage condition

The storage conditions for living yeast cells were examined next. For this purpose, two storage conditions of yeast suspensions (OD₆₀₀ = 45) were examined, namely, at 28 °C with a rotating speed of 120 rpm and 4 °C, respectively. In the case of storage at 28 °C, the responses after 1 day were significantly reduced relative to those obtained on the first day, although responses to the GGA sample could be obtained by the residual activity of living yeast (data not shown). On the other hand, stable results as reproducible responses to 220 mg O₂ L^{−1} GGA were obtained under storage at 4 °C for 14 days. The responses after 14 days decreased to 93% from their original values, and the reproducibility of the responses obtained from 6 testing days was 9.1% (R.S.D.). In addition, the responses after 30 days decreased to 73% from their original values. These results demonstrate once again the high survival ability of the yeast.

2.8. Real sample application

Samples of both river water and seawater with BODs of 0, 75, and 150 mg O₂ L^{−1} as a result of the added GGA solution were measured using our BOD_{DM} sensor and the BOD₅ method (Fig. 7). Here, a GGA solution with a 10% volume was added to the sample solution. For the seawater sample, the GGA solution was prepared using artificial seawater containing 2.65% NaCl, 0.326% MgCl₂, 0.207% MgSO₄, 0.136% CaSO₄, and 0.0714% KCl. The river water was sampled from the Yudono River in Hachioji, Tokyo (24.8 °C, pH 7.2, DO

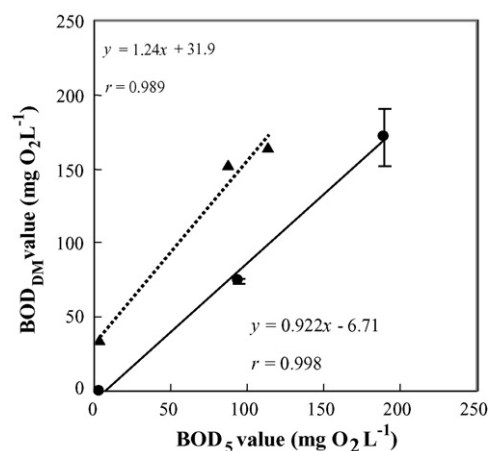


Fig. 7. Correlation of the BOD value calculated from the response of the BOD_{DM} method with the BOD₅ method for river (●) and sea (▲) samples.

8.8 mg L⁻¹, COD_{Mn} 6 mg L⁻¹, BOD₅ 4.22 mg L⁻¹; conductivity, 0.24 mS cm⁻¹; salinity, 0‰). An excellent correlation coefficient of 0.998 for these results using river water was obtained for the BOD_{DM} sensor with two sets of measurements and for the BOD₅ method with one set of measurements. The seawater was sampled from Tokyo Bay in Odaiba, Tokyo (25.4 °C, pH 8.0, DO 7.6 mg L⁻¹, COD_{Mn} 7.5 mg L⁻¹, BOD₅ 3.61 mg L⁻¹; conductivity, 29.6 mS cm⁻¹; salinity, 3.28‰). The correlation coefficient for these results using seawater was 0.989 for both the BOD_{DM} sensor and the BOD₅ method with one set of measurements. The BOD₅ values were lower than those obtained using the BOD_{DM} sensor, probably due to insufficient microbial activity in the BOD₅ samples. The seawater sample with a BOD of 150 mg O₂ L⁻¹ as a result of the added GGA solution showed relatively high values with the present sensor methods. These results might be expected as the averaged correlative variances for the BOD_{DM} sensor. These results demonstrate the potential of our BOD_{DM} sensor method as a salt-tolerant BOD determination method.

3. Conclusions

In this study, we demonstrated the use of a BOD sensor mediated by the eukaryote *S. cerevisiae* by employing the DM system. First, we designed a microbial sensor device equipped with a micro-stirrer system to determine the potassium hexacyanoferrate(II), which formed as a result of the assimilation of organic substances by *S. cerevisiae* in the presence of FC and menadione. The sensor device showed excellent response characteristics to hexacyanoferrate(II), and sensor responses to organic substances were obtained only when the reaction mixture contained *S. cerevisiae* in the presence of FC and menadione. By optimizing the BOD_{DM} sensor, a calibration curve with a wide range was obtained by the GGA solution. Next, we examined the sensor responses to pure organic substances and compared them with those obtained with the conventional 5-day method and other BOD sensor methods. In this investigation, we found that the BOD_{DM} method offers a wide range of assimilability to several categories of organic substances. To investigate the effects of chemicals on the sensor responses, chloride ion, seawater, and heavy metal ions were employed, and the responses were not significantly influenced by the chloride ion and seawater. Furthermore, they were not affected by the heavy metal ions. Thus, we found that our BOD_{DM} sensor system was tolerant to naturally existing chemicals. Finally, we demonstrated the possibility of measuring real samples by comparing them with the conventional BOD₅ method using both fresh water and seawater as real samples.

In this study, we proposed a new BOD estimation method using a eukaryote microbe. This BOD_{DM} sensor can be easily applied to practical mobile system employing a disposable DM sensor chip because it takes advantage of some of yeast's specific

properties: wide availability, ease of handling, omnivorousness to a wide range of organic substances, and high survival ability with high resistance to the influences of naturally existing chemicals. In addition, many possibilities for developing different kinds of DM sensors were shown. As an example, the development of a sensitive ethanol sensor employing this DM microbial sensor principle is in progress. Consequently, this study is important for the development of microbial sensors using a eukaryote microbe as a new generation.

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