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

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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

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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.

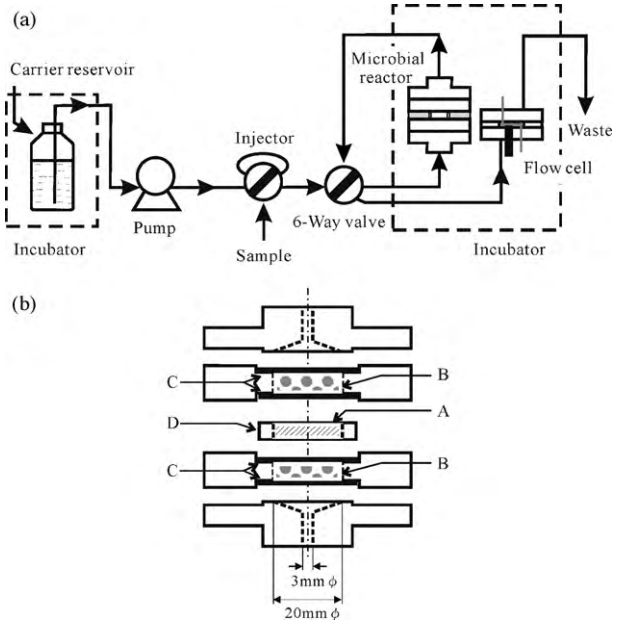

Fig. 1. Scheme of the BOD sensor system (a) and enlarged diagram of the microbial reactor (b). A: immobilized microbe, B: glass beads, C: stainless steel mesh screen, D: PTFE immobilized cell holder.

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 ϕ) 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