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# Immobilized multi-species based biosensor for rapid biochemical oxygen demand measurement

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ABSTRACT

To improve the practicability of rapid biochemical oxygen demand (BOD) method, we proposed a stable BOD sensor based on immobilizing multi-species BODseed for wastewater monitoring in the flow system. The activation time of the biofilm was greatly shortened for the biofilm prepared by BODseed in the organic–inorganic hybrid material. Some influence factors such as temperature, pH, and concentration of phosphate buffer solution (PBS) were investigated in detail in which high tolerance to environment was validated for the BOD sensor permitted a wide pH and PBS concentration ranges. The minimum detectable BOD was around 0.5 mg/l BOD under the optimized 1.0 mg/ml BODseed immobilized concentration. The as-prepared BOD sensor exhibited excellent stability and reproducibility for different samples. Furthermore, the as-prepared BOD biosensor displayed a notable advantage in indiscriminate biodegradation to different organic compounds and their mixture, similar to the character of conventional $BOD_5$ results. The results of the BOD sensor method are well agreed with those obtained from conventional $BOD_5$ method for wastewater samples. The proposed rapid BOD sensor method should be promising in practical application of wastewater monitoring.



## 1. Introduction

Biochemical oxygen demand (BOD) is one of the most important and widely used parameters for characterizing the organic pollution of water and wastewater, which is estimated by determining the amount of oxygen required by aerobic microorganisms for degrading organic matters in wastewater. Conventional BOD method is the well-known $BOD_5$ (APHA, 1997) which needs 5 days incubation at 20 °C in the dark. Apart from time consuming, it also involves tedious procedures, dedicated laboratory facilities and a high level of skill and experience to obtain accurate and reproducible results. Therefore, the rapid sensor method using a sensor is a very attractive alternative because it can conduct a measurement within several minutes.

Different kinds of BOD sensors for rapid measurement of BOD have been developed in last three decades since the biochemical oxygen demand (BOD) sensor firstly reported by Karube in 1977 (Karube et al., 1977; Lehmann et al., 1999; Chee et al., 2000, 2005; Catterall et al., 2003; Mason et al., 2006; Sakaguchi et al., 2007; Wang et al., 2010). Some commercial BOD instruments also have been developed for rapid BOD analysis (Nomura et al., 1998; D'Souza, 2001; Liu and Mattiasson, 2002; Jia and Dong, 2003). However, BOD sensor method is limited by its activation time, lifetime, stability, accuracy, etc. As the key roles played in BOD sensor, biofilm constructed with different kinds of microorganisms and various immobilization methods has been widely studied to circumvent the limitations of BOD sensors (Riedel et al., 1988, 1998; Tan et al., 1992, 1993; Jung et al., 1995; Sakai et al., 1995; Tan and Qian, 1997; Kim and Kwon, 1999; Tag et al., 2000; Yoshida et al., 2001; Chen et al., 2002; Rastogi et al., 2003; Kim and Park, 2004).

BOD sensors based on single strain microbe have the advantages of relatively good stability and long lifetime, but a low accuracy compared to $BOD_5$ which is restricted by its limited detection capacity for a wide spectrum of substrates. At the same time, the BOD sensors based on multi-species microorganisms have a wide spectrum of substrates, however, their applicability with respect to long-term stability relatively limited to the undefined interferences among immobilized microorganisms. On the other hand, the microbial cells are usually immobilized by physical adsorption on the polymer or hydrogel entrapment in aqueous solution. Physical adsorption method provides a loose circumstance for the microorganisms and the biofilm is facile to be activated, whereas the reproducibility and stability are usually poor due to leaking of microorganisms. Proper hydrogel entrapment method can provide a crosslinked matrix to protect the microorganisms from leaking for a long time, but the immobilization matrix might act as a barrier for the transfer of the substrate and oxygen to microorganisms, which leads to a long activation process of the sensor. In general,

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biofilms constructed with the two methods must be activated or preconditioned before use (Yang et al., 1996; Chan et al., 2000; Tan and Lim, 2005), and this process takes from 24 h to several days, which is a significant limitation for a rapid BOD method.

For the respiration rate type sensor, the detection principle is established in accordance with the rate of respiration that depends on the type of organic compounds (feed source) present in the sample. The analytical performance tends to be strong sample dependent. Typically, the methods perform very well with laboratory synthetic samples but they behave poorly when real samples are used. This is because some real wastewater samples contain very complex organic compounds that the immobilized microorganisms cannot uptake them within the short reaction time. This is a fatal drawback of respiration rate type BOD sensor. Consequently, the analytical results of real samples become unauthentic, because it is impossible to calibrate the sensor by a standard solution with the same degree of biodegradable components as the samples themselves (Liu et al., 2000).

Here we have developed an advanced organic–inorganic hybrid material based on silica sol and grafting copolymer of poly(vinyl alcohol) and 4-vinylpyridine (PVA-g-P(4-VP)) as a matrix (Dong et al., 1997) for the immobilization of microorganisms, which can prevent the silica glass from cracking during the sol–gel transition and eliminate the swelling of the hydrogel. The multi-species BODseed is a commercial microbial community, which is used as an alternative to conventional sewage, to get reproducible and comparable data in BOD determination. It contains 7 kinds of robust microorganisms isolated from activated sludge, and it was reported to be good consistency and reproducibility (Tan and Lim, 2005). Thus, the organic–inorganic hybrid material is used as a matrix to immobilize the BODseed to prepare a biofilm in this study.

The main purpose is to investigate the response characteristics of the proposed BOD sensor, including the effect parameters, the lifetime of the biofilm, and the repeatability and the reproducibility of the sensor BOD measurements. More importantly, this method was experimentally validated to process indiscriminate biodegradation ability to different kinds of organic compounds as $BOD_5$.

## 2. Experimental

### 2.1. Materials

Silica sol and PVA-g-P (4-VP) were prepared according to Dong et al. (1996, 1997). BODseed (Cole-Parmer E-05466-00), a commercial microbial consortium isolated from activated sludge, was selected for this study because of its wide spectrum and good reproducibility (Tan and Lim, 2005). The inclusion of a BODseed capsule was re-hydrated in 1500 ml of deionized water under bubbling with filtrated air at 20 °C for 1 h. Twenty-microlitre of the supernatant liquid were inoculated to the sterilized culture medium, containing 13 g/l of Oxoid CM1 nutrient broth. The mixture was left in a shaker bath to incubate at 37 °C for 7 h (Tan and Wu, 1999). Unless otherwise stated, all chemicals used in this study were of analytical-reagent grade and all solutions were prepared with deionized water.

### 2.2. Immobilization

The microbial consortium was harvested and washed twice with 5 mM phosphate buffer solution (PBS) by centrifugation (8000 rpm for 10 min) at room temperature. The supernatant liquid was then discarded and the remaining sediment was quantified by weight for construction of biofilms. One hundred microlitre of selected concentration of microorganisms was mixed adequately with 200 μl grafting copolymer PVA-g-P (4-VP) and 100 μl silica sol. Then 2 μl of the mixture was dropped on a piece of dialysis membrane (pore size 10 μm, Oxyphen, GmbH, Dresden, Germany), which was used as a carrier and also as a barrier to prevent the suspension species in samples. The prepared biofilms were dried at 4 °C for 6 h, and stored at 4 °C before use.

### 2.3. Buffer and standard

PBS (5 mM, pH 7.0) was used for dilution of the samples and rinsing of tubings and the biofilm unless otherwise stated. A glucose and glutamic acid (GGA) mixture with both of 150 mg/l is regarded as the calibration standard. The calibrated BOD value corresponds to 198 mg $O_2$/l. Another standard solution was prepared according to the Organization for Economic Cooperation and Development (OECD) standard (OECD, 1981). The OECD synthetic wastewater ($BOD_5$ = 1401 mg/l) contained peptone (1.6%, w/w), meat extract (1.1%, w/w), urea (0.3%, w/w), sodium chloride (0.07%, w/w), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$, 0.04%, w/w), magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$, 0.02%, w/w) and potassium monohydrogen phosphate ($K_2HPO_4$, 0.28%, w/w) in 1 l of water. The $BOD_5$ value of the sample was determined according to the standard dilution method (APHA, 1997).

### 2.4. Sensor design

A three-electrode system was used to detect dissolved oxygen, which consists of a gold disk (0.8 mm in diameter) as the working electrode, a Ag/AgCl (0.1 M KCl electrolyte) as the reference electrode and a platinum wire as the auxiliary electrode, respectively. The Teflon membrane (Orbisphere) was attached to the surface of the oxygen probe. The electrolyte was filled in the space between the Teflon membrane and the three-electrode system. The dialysis membrane with the biofilm side was then fixed on the surface of the working electrode by means of a rubber O-ring (Fig. 1a).

### 2.5. Sample solutions

Wastewater samples were taken from a municipal wastewater treatment plant with a small proportion of industrial wastewater from a car factory, which adopted a stepwise treatment method including primary effluent, biochemical tank and second effluent. Before analysis, all samples were adjusted to pH with concentrated PBS (1 M, pH 7.0) and the aliquot parts of the samples were used for both the sensor BOD method and the conventional $BOD_5$ test.

### 2.6. Instrumentation and procedures

Experiments were carried out with an XSF-1 online BOD analyzer manufactured by Jiangsu Jiangfen Electroanalytical Instrument Co. Ltd., China, which has a flow injection system with an automatic washing and measuring function by a precision pump and airtight tubes, a thermostatic bath for keeping the BOD sensor, the sample and the PBS at the same temperature, another precision pump specially for the supply of the PBS in the thermostatic bath as the same rate to its consumption, and some other units for online monitoring. The output current was set up at −0.7 V against Ag/AgCl.

The output steady-state voltage from BOD-free buffer was taken as the baseline voltage ($E_0$). When the wastewater sample or BOD check solution was pumped to the sensor, the system was equilibrated to a new steady-state voltage ($E_s$). The sensor response ($\Delta E$) corresponding to the $BOD_5$ value of the solution was defined as the difference between the two steady-state voltages ($E_0 - E_s$). The time taken for the sensor from $E_0$ to $E_s$ was regarded as the response time and the time from $E_s$ to the new baseline voltage $E_0$, in the flow system, was the recovery time (Fig. 1b).

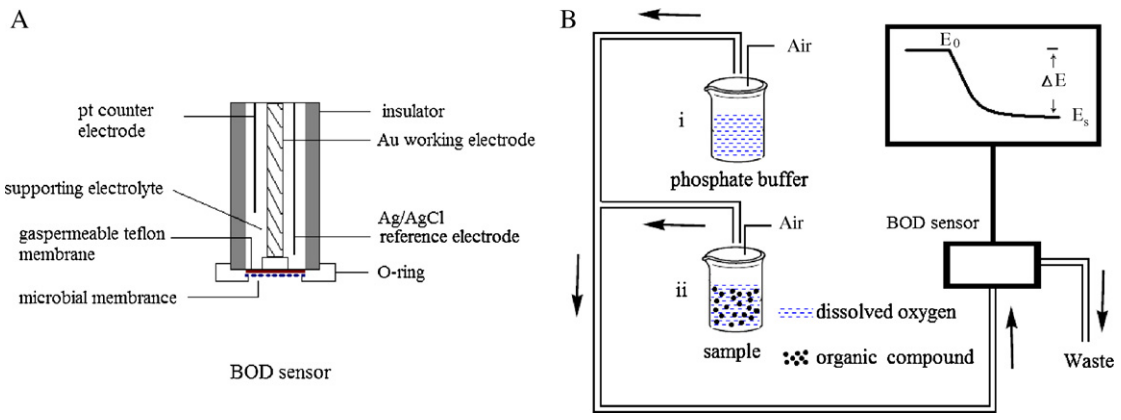


**Fig. 1.** Schematic diagram of the BOD sensor (A) and the flow injection system (B).

Generally, the response time is less than 10 min, and the recover time needs 30–60 min according to the concentration of samples. A fixed flow rate of 4 ml/min was set and the thermostatic bath was kept at 30 °C for the flow system unless otherwise stated. The PBS and sample solution were both placed in the thermostatic bath to keep consistent temperature unless otherwise stated. The PBS was aerated by air continuously, however, the sample solution needed to be aerated only 10 min before measurement with filtrated air at a flow rate of 3 l/min. GGA solution was used to check or calibrate before and after wastewater measurements. The BOD sensor was kept with circulating PBS when not in use.

## 3. Results and discussion

### 3.1. Activation time of the biofilm

The activation time was an important index for the rapid BOD method, which was affected greatly by the immobilization matrix and method. Generally, a shorter activation time by the physical adsorption method was needed comparing to the hydrogel entrapment method due to the lack of barrier of the matrix. However, by using the organic–inorganic hybrid material we had a notable result compared to the physical adsorption method (Tan and Lim, 2005). The prepared BODseed biofilms were pretreated by immerging in 16.0 mg/l GGA for 2, 8 and 12 h at 30 °C before use, respectively. The processes of activation were tracked. As shown in Fig. 2, sensor responses to the standard GGA solution rose up gradually and got to stable state from the third hour for all the three sensors. The sudden increase at the time of 2 h for the biofilm preconditioned 8 h could be deemed to an occasional deviation. The subsequent municipal wastewater measurements after 6.5 h activation processes further proved the advantages of the immobilized material. The relative standard deviations were 4.0%, 4.1% and 4.3% ($n = 5$) for the biofilm preconditioned for 2, 8 and 12 h, respectively. In addition, the maximal and minimal errors of a single measurement were observed at 14.5% and 1.7% compared to $BOD_5$. The use of this organic–inorganic hybrid material accelerated the activation process, which could be attributed to the porous structure of the biofilm which was not densely constructed, and also the large quantities of hydroxyl groups in the PVA-g-P(4-VP), which were favorable for maintaining the bioactivity of the immobilized microorganisms. Compared with the thermally killed BODseed sensor reported, short activation time was needed by the present BOD method, resulting in more convenient and feasible in portable and online application (Tan and Lim, 2005).

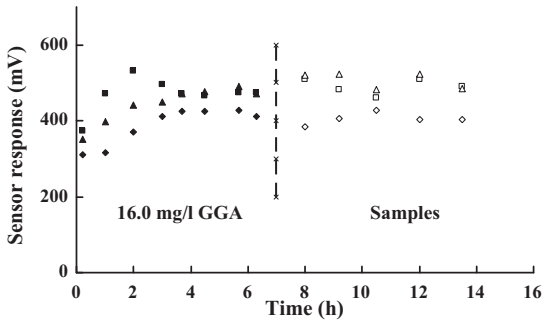


**Fig. 2.** Sensor response with time during activation process. Three biofilms were preconditioned in 16.0 mg/l GGA for 2 h (♦ and ◊), 8 h (■ and □), 12 h (▲ and △), respectively. Time 0–6.5 h: sensor responses to standard 16.0 mg/l GGA (solid); time 8–13 h: sensor responses to real samples (1:3 dilution) (hollow).

### 3.2. Optimization of cell loading

In order to have a sensitive signal, an optimal concentration of BODseed was investigated (Fig. S1). The more cells were immobilized, the more oxygen was uptake and the higher the signal would be shown. In addition, higher cell concentration increases the stability of the sensors. However, the diffusion rates of oxygen and substrates were proportional to the thickness of the layer. The thick layer with higher cell concentration hinders their diffusion. The decrease of the oxygen diffusion rate can be indicated by the decrease in the background current. Different concentrations of BODseed were used for immobilization in our tests. As illustrated in Fig. S1, the maximum response was observed with BODseed concentration at 1.0 mg/ml. Under this condition, the background current was almost the same as that of lower BODseed concentration biofilm. In contrast, the background current decreased a lot for the biofilm with higher BODseed concentration. The minimum detectable BOD was around 0.5 mg/l BOD at a signal-to-noise of 3, the sensitivity obtained was comparable to reported results (Rastogi et al., 2003).

### 3.3. Influence of measurement parameter

The effect of temperature on the catabolic activity of BODseed was studied by monitoring sensor response to various GGA concentrations. As shown in Fig. 3a, catabolic activity of microorganisms, indicated by the sensor response to 16 mg/l, increased gradually with increased temperature from 20 to 45 °C. The similar results to the activated sludge obtained by Kwok et al. (2005) were probably induced by the variety of the microorganisms, some of which were tolerant towards the high temperature.

The effect of pH on the catabolic activity of the microorganisms was examined by similar strategies as above except that the exper-

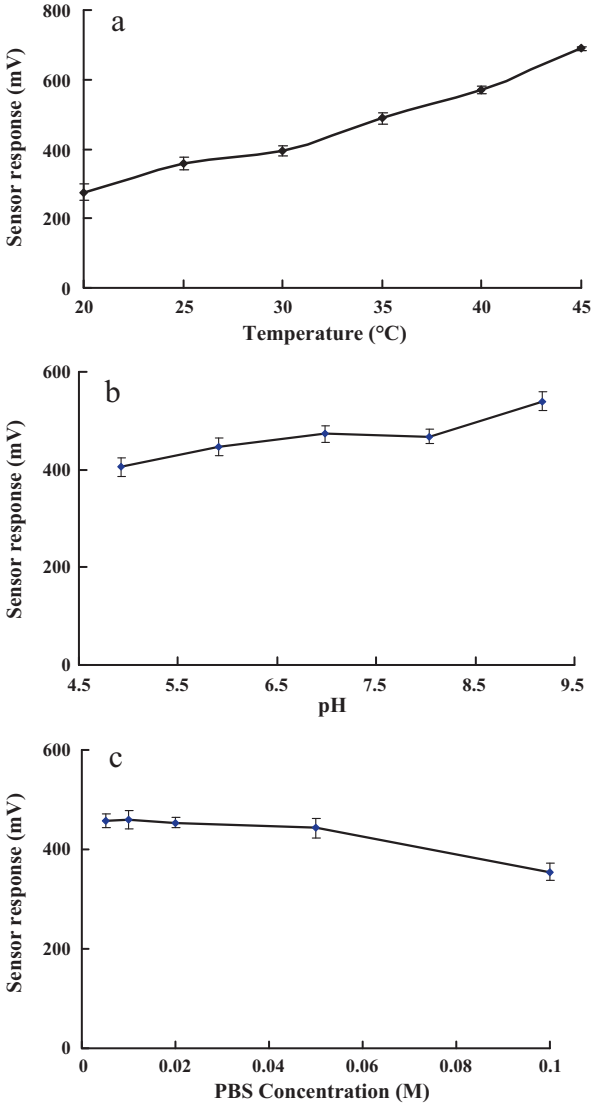


**Fig. 3.** BOD sensor responses to different parameters. (a) temperature between 20 and 45 °C; (b) pH between 4.9 and 9.2; and (c) PBS concentration between 5 and 100 mM, respectively. The error bars represent the result of three separate measurements.

iments were performed at 30 °C with the pH varied from 4.9 to 9.2 (Fig. 3b). Obviously, almost identical responses in the selected pH range were obtained. This phenomenon was remarkably different from previous reports in which the sensor response was strongly pH-dependent (Jia et al., 2003; Pang et al., 2007). It implied that the BODseed had a high tolerance towards the pH change of the environment.

The effect of PBS concentrations on the sensor responses was studied at pH 7.0 as shown in Fig. 3c. It was worth noting that the sensitivity was decreased if PBS concentration dropped below 10 mM in the previous work by using microorganisms cultivated in lab (Hikuma et al., 1979). But here, almost the same sensor responses were obtained for PBS concentration from 5 to 50 mM, and decreased when it was higher than 50 mM. This may be caused by the reason that the permeability of the immobilized microbial cells was affected by higher concentration of inorganic salt.

### 3.4. Repeatability and stability of one sensor

The repeatability of the analytical signal was investigated with different GGA concentrations. The relative standard deviations of

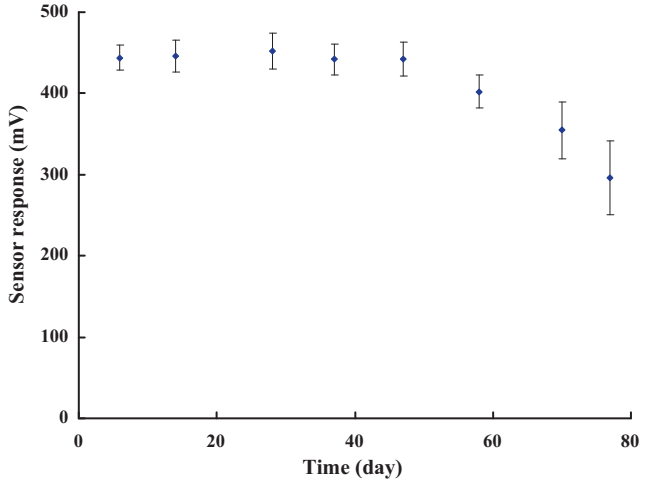


**Fig. 4.** Long-term stability of the BOD sensor. The error bars represent the result of six separate measurements.

seven successive tests were 3.9%, 3.2%, 3.0% and 2.8% for 4.0, 12.0, 25.0 and 40.0 mg/l GGA, respectively (Table S1). This sensor showed the same repeatability as previously reported BOD sensor based BODseed (Tan and Lim, 2005). Further more, long-term stability of the sensor for standard 16.0 mg/l GGA solutions was shown in Fig. 4. No detected decrease in the sensor response could be seen when the sensor was used to measure GGA standard until the 50 day, which showed an obvious difference to the activated sludge based BOD sensor (Liu et al., 2000; Kwok et al., 2005). It should be attributed to consistency and good reproducibility of BODseed and the advanced immobilization material. The bottleneck effect of the silica sol–gel prevented the microorganisms from leaking, and also the large quantities of hydroxyl groups in the PVA-g-P (4-VP) were favorable to maintain the bioactivity of the immobilized microorganisms (Jia et al., 2003; Liu et al., 2009). Gradual decrease was observed after the biosensor has been used over 50 days. During the investigation period, nearly 500 times to laboratory synthetic samples and real samples were analyzed. Our study showed that frequent measurements did not reduce the sensor response but the irregular and seldom measurements did. Deficient nutrition supply was regarded as an important reason for the loose of the bioactivity. This was the explanation why the stability decreased in the later period in Fig. 4.

### 3.5. Response to different kinds of organic compounds

The measurements for GGA and OECD with different concentrations by both BODseed sensor and $BOD_5$ methods were conducted. It was well-known that GGA was the most commonly selected standard solution in rapid BOD sensor studies, and OECD was usually used for calibration the real sample measurements because the organic compounds in it were more complicated (Jia et al., 2003; Pang et al., 2007). Of particular interests, the analytical results of the two synthetic wastewaters with different concentrations constructed a linear relationship with the fluctuant coefficient ($R^2$) at 0.9927. It revealed that the BODseed biofilm possessed similar biodegrade ability to the two samples though they were of much different components. Based on the principle of indiscriminate biodegradation, either GGA or OECD was alternative as the calibration solution in our method, and the calculated rapid results by standard curve method would not depend on the standard solution as reported (Liu et al., 2000). Further studies based on different kinds of artificial compounds validated this notable contribution of the proposed BODseed sensor. Five kinds of arbitrary organic compounds and their mixture with different concentrations were

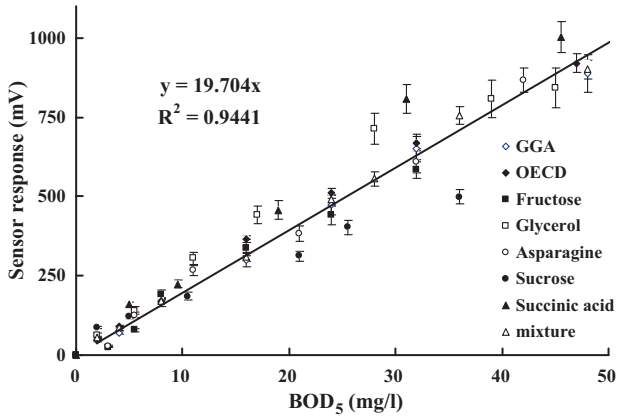


**Fig. 5.** BOD sensor responses to different kinds of organic compounds. The error bars represent the result of three separate measurements.

measured by both BODseed sensor and the $BOD_5$ method. The sensor responses against $BOD_5$ for these organic compounds along with GGA and OECD were described in Fig. 5. Regardless of the easily biodegradable compounds or the hardly ones, all the analytical signals were presented uniformly around the correlation equation, which showed that the sensor results were consistent with the $BOD_5$ value of different kinds of organic compounds. It could be inferred from the results that the BODseed have wide and indiscriminate biodegradation ability to different kinds of organic compounds. Preference to organic compounds for the individuals was indifferent compared with the cooperation in biodegradation each other in the multi-species BODseed. Benefit from these advantages, the BOD sensor constructed by BODseed could obtain reliable analytical results to single and multiple organic compounds as well as real samples though they were of different biodegradable characteristics. The relationship between the rapid BOD method and $BOD_5$ results sufficiently validated the indiscriminate biodegradation of the proposed rapid BOD method.

### 3.6. Relationship between the sensor BOD and $BOD_5$

Wastewaters from the primary effluent, biochemical tank, the second effluent and the different proportion of the three samples were adopted in this investigation. The calibration curve ($y = 19.0x + 19.7$, $R^2 = 0.9927$) was constructed by both GGA and OECD solution for BODseed sensor method. Five successive measurements for the BOD sensor method and two parallel experiments for the conventional $BOD_5$ method were carried out. No data were elided for the BOD sensor in calculating the standard deviations. As shown in Fig. 6, maximal and minimal standard deviations for the BOD sensor method were observed at 8.4% and 0.4% with the average values 75.2 and 14.0 mg/l ($n = 5$), respectively. The value of the correlation coefficient ($R^2$) was 0.9705 and the slopes of the fitted equations were 1.06. The result indicated that the BOD sensor based on BODseed biofilm was well practicable for the municipal wastewater determination. The consistency should attribute to the wide spectrum of substrates of BODseed, which was a commercial microbial consortium from activated sludge.

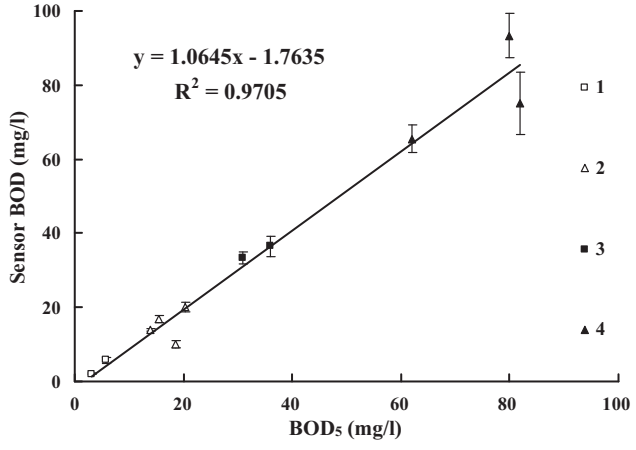


**Fig. 6.** Comparison of measurements by BOD sensor and $BOD_5$ for the samples from a municipal wastewater treatment plant. (1) wastewater from secondary tank; (2) wastewater from biochemical tank; (3) wastewater from both secondary tank and primary tank; (4) wastewater from primary tank. The error bars represent the result of five separate measurements.

## 4. Conclusions

Multi-species microorganisms BODseed were immobilized to fabricate a BOD sensor based on sol–gel derived composite materials. The BOD sensor was successfully used for nearly 50 days without obvious decrease in sensor responses. The biofilm proposed needs only a short activation time (about 2 h) before detection. Some influencing parameters were investigated and the immobilized BODseed was exhibited advantages such as tolerance of high temperature, insensitivity to pH and ionic concentrations. The as-prepared BOD sensor provided comparable results with those of conventional $BOD_5$. More importantly, the complex microbial culture BODseed was firstly validated to possess indiscriminate biodegradation ability among the respiration type BOD sensor. These results can certainly throw light on improvement of the analytical performance of rapid BOD in practical application.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.09.004.

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