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Highlights

1 ZrCF is a suitable immobilization carrier for both *Saccharomyces cerevisiae* and *Escherichia coli*, which had the potential to immobilize other fungi and bacteria in the biosensor field.

2 The density of the biofilm strongly affects the performance of a BOD sensor including the sensitivity and linear response range.

3 The amount of immobilized microorganisms can be greatly improved when ZrCF were used as the immobilization carrier. The linear response range and the sensitivity of the sensor were improved as the increasing amount of immobilized microorganisms.

Microbial BOD sensors based on Zr (IV)-Loaded Collagen Fiber

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ABSTRACT

Biochemical oxygen demand (BOD) sensors based on Zr (IV)-loaded collagen fiber (ZrCF), a novel material with great porous structure, were developed. This novel material shows adsorbability by microorganisms. *Saccharomyces cerevisiae* and *Escherichia coli* were used for the construction of BOD sensors. Factors affecting BOD sensor performance were examined. The ZrCF-based BOD sensor showed different sensitivities and linear response ranges with different biofilm densities. The amount of microorganisms strongly affected the performance of the BOD sensor. Poor permeability of previously reported immobilization carriers were greatly circumvented by ZrCF. The service life of the ZrCF-based BOD sensor was more than 42 days. The immobilized microorganisms can be stored for more than 6 months under 4 °C in PB solution. There was good correlation between the results of the sensor method and the standard 5-day BOD method in the determination of pure organic substrates and real water samples.

Key words: ZrCF, Immobilization carrier, Microbial biosensor, BOD

Graphical abstract

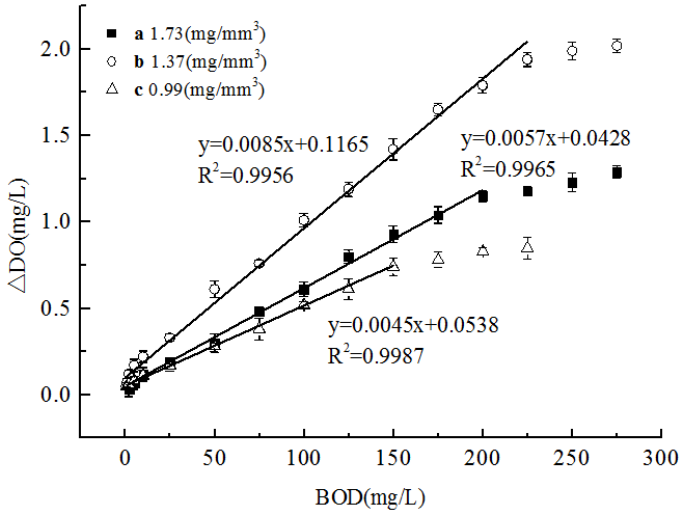

Figure 1. Influence of biofilm density on the sensor response

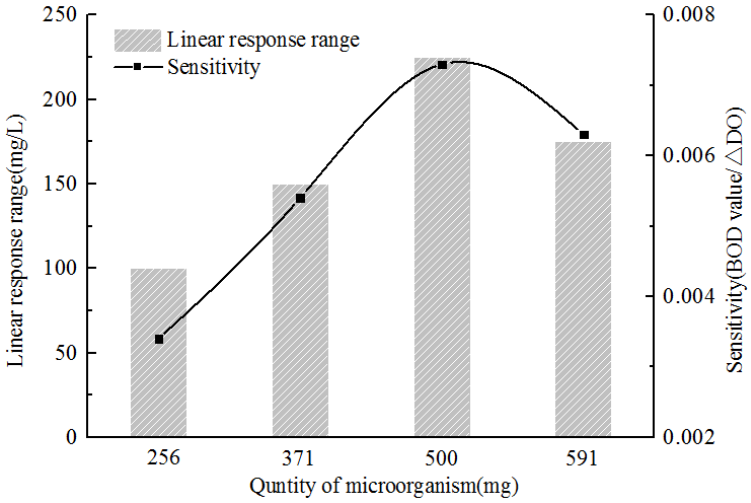

Figure 2. Influence of the amount of microorganisms on the sensor response

1. INTRODUCTION

Biochemical oxygen demand (BOD) is a widely used parameter for indicating the biodegradable organic pollutant content of wastewater. The conventional method for measuring BOD (5 days BOD, BOD_5) [1] is time-consuming, exhibits low reproducibility, involves tedious steps, and cannot provide an indication of pollution in a timely manner. Development of microbial BOD sensors has received considerable attention lately [2-4] because it can provide quick measurements, requires minimal labour, and can be controlled online.

The microbial film (immobilized microorganisms) is the key component of a microbial BOD sensor that greatly affects the sensor's performance, in terms of sensitivity, linear response range, response time, and service life. A range of methods (i.e., cross-linking, sandwich, absorbing, and embedding) have been applied for the immobilization of microorganisms [3, 4]. Different carriers, including polycarbonate [5], agarose [6], Ca-alginate [7], PCS [8], Al_2O_3 sol-gel [9], are involved in these processes. Almost all reported immobilization carriers possess an important feature (porous structure) that allows the substrates (e.g., dissolved oxygen, and organic compounds) to permeate the biofilm and react with the microorganisms, after which the signals can reach the electrode [3-4]. However, most of these materials are not sufficiently porous [10]; they show different degrees of compactness, which limits the spreading of the substrates in the biofilms, and restricts the thickness of the biofilms (i.e., the amount of immobilized microorganisms). Consequently, biosensor characteristics, such as sensitivity, response range, and response time are limited. To solve this problem, researchers have tried various approaches such as modification of the carriers [11, 12] and changing the structure of biosensors [10]. However, these methods have showed drawbacks of involving complicated procedure, high cost and low utilization of microorganisms.

Collagen fiber (CF) is a natural polymeric material extracted from animal skins (e.g., pig hide, and cattle hide). It has porous structure, excellent water binding capacity, and good bio-compatibility [13]. Zr (IV)-loaded collagen fiber (ZrCF) has many advantages, including excellent mechanical property, physical and chemical stability, anti-biodegradability, and adsorbability toward various biomaterials (proteins, enzymes, and microorganisms) [14, 15]. Therefore, ZrCF can be used as an immobilization carrier in biosensors.

Various microorganisms [3-4] have been used for the construction of BOD sensors. Fungi and bacteria are different in terms of their cytoderm structure. *Saccharomyces cerevisiae* (*S. cerevisiae*) and *Escherichia coli* (*E. coli*) are often used as the representatives of fungus and bacteria, respectively.

In this study , *S. cerevisiae* and *E. coli* were immobilized on ZrCF to prepare BOD sensors. The main purpose of this research was to investigate the effects of various factors, including temperature, pH, concentration of NaCl, density of the biofilm, and the amount of immobilized microorganisms on the ZrCF-based BOD sensor. Pure organic substrates and real water samples were determined by the sensor method and the 5-day BOD (BOD_5) method, respectively.

2. EXPERIMENTAL METHODS

2.1. Equipment and reagents

The equipments and reagents used in this study were as follows: dissolved oxygen meter (MP516, Shanghai Sanxin Instrument Company, China), thermostated water-jacket (self-made, temperature deviation was ±0.2 °C), nylon net (200 mesh, Sichuan GuangLong filter Industry Co.,

Ltd.), and metal rings (self-made).

All chemicals used in this study were of analytical reagent grade, and all solutions were prepared with deionized water.

2.2. Immobilization of microorganisms on ZICF

Both *S. cerevisae* and *E. coli* were provided by the Microbiology Lab of the College of Food Science in Sichuan Agricultural University (Ya'an China). CF was supplied by the National Engineering Laboratory for Clean Technology of Leather Manufacture (Chengdu, China).

ZrCF was prepared as follows: CF (15.0 g) was suspended in 300 mL of deionized water at 25 °C for 1.0 h. The pH of the mixture was adjusted to 2.0 by using H_2SO_4 solution (H_2SO_4:H_2O = 1:2, v/v). After the mixture was stirred at 25 °C for 2 h, 35.5 g of $Zr(SO_4)_2 \cdot 4H_2O$ was added to the above mixture and kept under constant stirring for another 4 h. Subsequently, a proper amount of $NaHCO_3$ solution (15%, w/w) was added dropwise into the reaction system within 4 h to increase its pH to 4.0 and reacted at 45 °C for another 4 h. When the reaction was completed, the product was collected by filtration, washed with deionized water, and dried at 45 °C for 4 h.

The *E. coli* was grown under aerobic conditions in a rotating shaker at 37 °C for 30 h in broth medium (Beijing Aoboxing Bio-tech Co., Ltd.). The culture broth was centrifuged at 8 000 r/min for 10 min at room temperature. *S. cerevisiae* was grown under aerobic conditions in a rotating shaker at 30 °C for 24 h in PD (potato dextrose) medium (2% glucose, 20% potato). The culture broth was centrifuged at 5000 r/min for 5 min at 20±5 °C. Both strains were washed twice with 0.07 mol/L phosphate buffer (Na_2HPO_4−KH_2PO_4, pH 6.2).

ZrCF (500 mg, dry weight) was soaked in sterilized water for 12 h. Then, the ZrCF was filtered and placed into 100 mL *E. coli* suspension (0.85% NaCl) with cell concentration in the range of 10^6–10^8 cfu/mL. The adsorption reaction was in a shaker at 37 °C for 5 h. Then, the immobilized *E. coli* was washed twice with normal saline (0.85% NaCl). The procedure for immobilization of *S. cerevisae* was the same as that for *E.coli* except for the adsorption temperature (30 °C). Then, the immobilized cells were stored in PB solution at 4 °C before use.

2.3. Preparation of solutions

Glucose and glutamic acid (GGA) solution is often used for calibration of BOD sensors. GGA only contains two organic substrates (i.e., glucose and glutamic acid), whereas the components of real water samples are often more complex. OECD synthetic wastewater has been used as the standard in other reports because this water is more similar to real water samples than GGA solution. GGA solution was prepared according to the standard procedure [1]. The BOD_5 value of this solution was 200 mg/L. OECD synthetic wastewater was prepared according to the OECD guideline [16], the BOD_5 value of this solution was 14000 mg/L. Higher strength standards were prepared by increasing substrate loading. Lower strength standards were prepared by appropriate dilution with distilled water.

PB solution (0.07 mol/L) was used as solvent to prepare various saline solutions. BOD_5 was determined by the standard method [1].

2.4. Instrumentation and measurement

The construction of the instrument is shown in Figure 1-a. The immobilized microorganisms were placed into a metal ring (with fixed height and the same area as the Teflon membrane on the

electrode). Then, they were sandwiched in two nylon meshes to create a biofilm (Figure 1-b). The biofilm was attached to the Teflon membrane side of the oxygen electrode and fixed with O-ring. The density of the membrane was controlled by changing the rings (different heights) with the same quantity of immobilized microorganisms.

The prepared electrode was inserted into the detection chamber containing 100 mL 0.07 mol/L PB solution saturated with dissolved oxygen and stirred with a magnetic bar. The temperature of the detection chamber was maintained using a thermostated water-jacket. The prepared electrode was left immersed in the PB solution at room temperature when not in use.

When the background current of the oxygen electrode reached a steady state, 10 mL sample solution was added to the PB solution. Then, the organic solutes were assimilated by the microorganisms in the biofilm, which consumed the dissolved oxygen and caused a decreasing current. After 2-10 min (depending on the organic concentration in the sample), the current reached another steady state. The decreased value was proportional to the biodegradable organic concentration in the samples. Then, the solution was replaced with fresh buffer for another estimation. All experiments were conducted thrice (n=3).

Figure 1-a here

Figure 1-b here

3. RESULTS AND DISSCUSSION

3.1. Effects of temperature, pH, and salt

The response of a microbial BOD sensor strongly depends on the quantity and activity of the microorganisms in the biofilm. The physiological state of microorganisms is affected by various factors. Therefore, the effects of temperature, pH, and salt on the response of the ZrCF-based BOD sensors were examined. The standard GGA solution of 100 mg/L BOD was used for these experiments.

3.1.1. Effects of temperature

The activity of the microorganisms increased with increasing temperature. Then, a drop in the current was observed at a certain temperature (Figure 2). The optimum temperatures in the *E. Coli*-based sensor and the *S. Cerevisiae*-based sensor were found to be about 36 °C and 30 °C, respectively. The *S. Cerevisiae*-based sensor was more sensitive to temperature changes than the *E. coli*-based sensor. These differences may be due to the biological properties of the respective strains. Therefore, these two optimum temperatures were selected for subsequent studies.

Figure 2 here

3.1.2. Effects of pH

The influence of pH value was investigated from pH 3.0 to pH 9.0. As shown in Figure 3, both currents peaked at pH 6.0, after which decreasing trends were observed with further increase in pH. In subsequent experiments, the samples were adjusted to pH 6.0 for both sensors.

Figure 3 here

3.1.3. Effects of salt

The effects of inorganic salts on sensor response were tested. As shown in Figure 4, the response of the sensors was maintained at a certain level until a certain concentration of salts in solutions was achieved. The output current of the *E. coli*-based BOD sensor decreased when the concentration of NaCl was higher than 0.3% (w/w) in the PB solution. Interestingly, the *S. Cerevisiae*-based BOD sensor showed a decreasing trend until the NaCl concentration reached 3.5% (w/w), which approximately equals the salt concentration of seawater [8]. The difference between the sensors may result from the different structures of the cell walls of each strain. Yeasts often show better tolerance of salts than bacteria. For this reason, yeasts (*A. adeninivorans* [8], *B. licheniformis* [9], and *S.cerevisiae* [17], etc.) are frequently used as recognition elements in BOD sensors. These sensors are applied in the processing of seawater or other high-salt concentration wastes. The *S. cerevisiae* used in this study is a promising strain that can be used in BOD sensors for seawater detection.

Figure 4 here

3.2. Selection of biofilm density

The density of the biofilm strongly affects the performance of a BOD sensor. Unlike other conventional carriers, the density of the biofilm made up of ZICF was adjustable because of the very porous structure of the collagen fibers, which can potentially overcome the poor permeability of the conventional materials.

The influence of biofilm density on sensor response was investigated. Immobilized *E. coli* (500 mg) was placed in several rings with different heights to form biofilms with various densities (**a** 1.73; **b** 1.37; **c** 0.99 mg/mm^3). As shown in Figure 5, the sensor showed the widest response range and the best sensitivity at density **b**. Higher or lower biofilm density caused poor performance of the BOD sensor, which is probably due to the altered permeability of the biofilm. Higher density of the biofilm hindered the diffusion of substrate and oxygen, i.e., lower amount of substrate reached the cells and lower signals were obtained. Low density led to excessive free movement of substrates in and out of the biofilm, thereby partly counteracting the biological reaction in the biofilm and causing poorer response of the sensor. Density **b** was then selected for subsequent experiments.

Figure 5 here

3.3. Select the amount of immobilized microorganisms

Five different volumes (251, 356, 500, 591, and 800 mg) of immobilized *E. coli* were tested as recognition elements in the BOD sensor. As shown in Figure 6, the linear response range and the sensitivity of the sensor improved with increasing amount of immobilized microorganisms. This result is differed from that obtained previously [18]. In the previous study, no increased sensitivity was observed with increasing amount of immobilized cells. This was likely due to increased resistance of the biofilm with increasing thickness. In this study, the resistance of the

biofilm showed no remarkable difference with various amounts of immobilized microorganisms. Higher amount of cells led to better biosensor sensitivity. This is due to the really porous structure of the ZrCF-based biofilm. The linear response range showed a drop at 591 mg immobilized *E. coli*, and the background current was suddenly too low to permit detection at 800 mg immobilized cells (not shown in Figure 6). These findings may due to the wall of rings that hinders the flow of substrates, thereby leading to very low background current (with 0.6-0.8 mg/L DO value) at 800 mg immobilized microorganisms. Porous rings should be developed for further research to achieve better outcomes.

Figure 6 here

3.4. Service life of the sensor

Service life is a very important property of a BOD sensor. The service life of the *E. Coli*-based BOD sensor was examined. As shown in Figure 7, the sensor exhibited good stability and long service life (42 days) mainly because of the good stability of ZrCF.

Regular use of the recognition element leads to better sensor stability, likely due to stabilization of the microorganisms in the biofilm. Regular use is conductive to stable quantity and activity of the microorganisms. If the sensor is not used for a long period of time (more than 10 days), the biofilm should be stored at <4 °C in PB solution. The immobilized microorganisms can be used after 6 months of storage.

Fig 7 here

3.5. Application of the ZrCF-based BOD sensors

3.5.1. Determination of organic substances

Various pure organic substances were investigated to determine the characteristics of the ZrCF-based sensors. As shown in Table 1, the sensors showed different responses to different organics, which is probably due to the special biological properties of each strain. Our findings indicate that ZrCF is a suitable carrier for *S. cerevisae* and *E. coli*, and ZrCF-based BOD sensors can be applied for BOD detection along with other fungi and bacteria. However, bubbles appeared in the chamber when sodium dodecyl sulfate (SDS) was tested. This might have resulted from chemical reaction between ZrCF and SDS. ZrCF-based biosensor may not be suitable for application in samples containing high SDS concentration.

Table 1 here

3.5.2. Application in real samples

The BOD values of different real samples were determined. Here, OECD synthetic wastewater and GGA solution were utilized as standards. Thus, the samples were determined by the sensor and BOD_5 methods. As shown in Table 2, most of the sensor BOD values of the examined organic substrates were comparable with the BOD_5 values. Thus, these sensors can be used for BOD determination of the samples. The sensor BOD values were more comparable with BOD_5 values when OECD synthetic wastewater was used as the standard. This may have been caused by the ease by which the the organic substrates in GGA solution were decomposed by microorganisms, which led to low normalized response of the sensors. Overall, these sensors have the potential to

be applied for BOD determination of river water and seawater.

Table 2 here

4. CONCLUSION

Here we report that the poor permeability of previously reported immobilization carriers could be circumvented by using ZrCF. The density of the biofilm and the amount of immobilized microorganisms could be controlled easily when ZrCF was used as the immobilization material in microbial BOD sensors. As a result, the sensitivity and linear response range of the BOD sensor were greatly enhanced. In addition, ZrCF offers several advantages such as low cost, ease of use, and long storage life, making it a promising carrier in the biosensor field.

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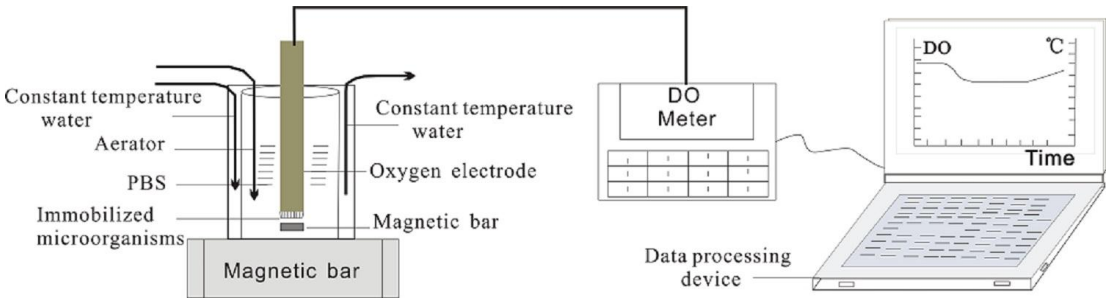

Figure 1-a Schematic diagram of the microbial BOD sensor

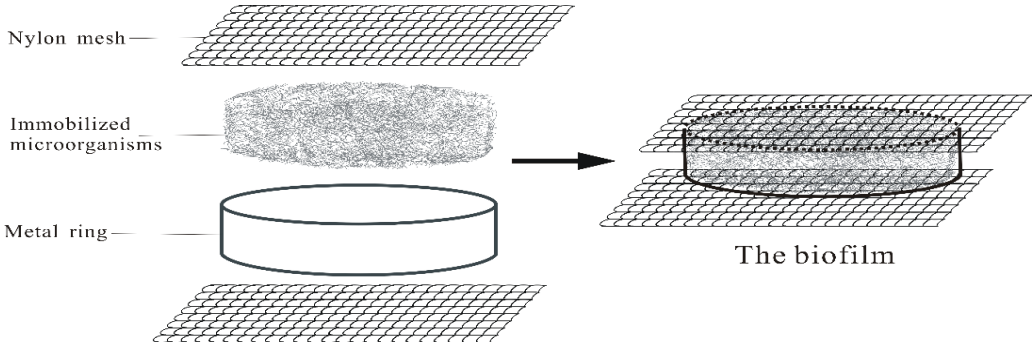

Figure 1-b Schematic diagram of the biofilm

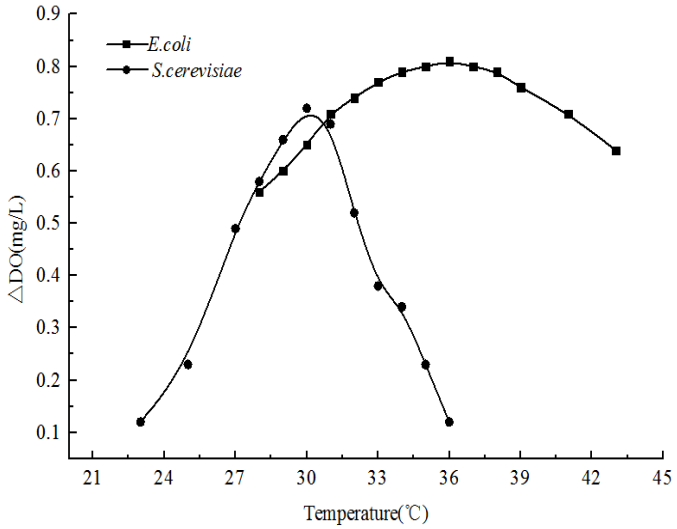

Figure 2 Influence of temperature on the sensor response

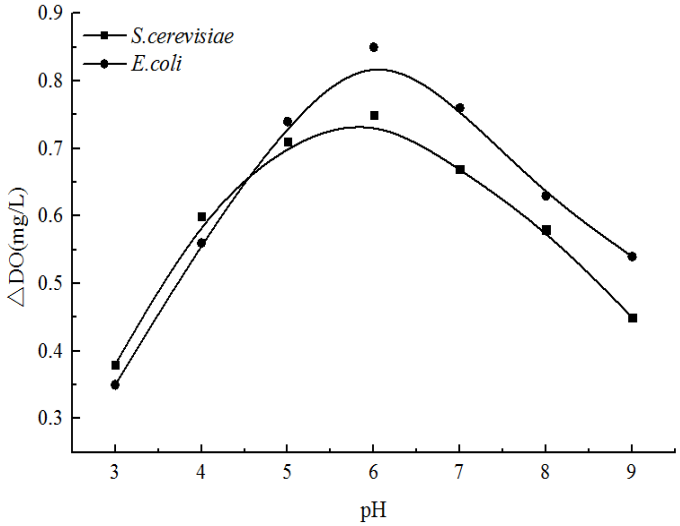

Figure 3 Influence of pH on the sensor response

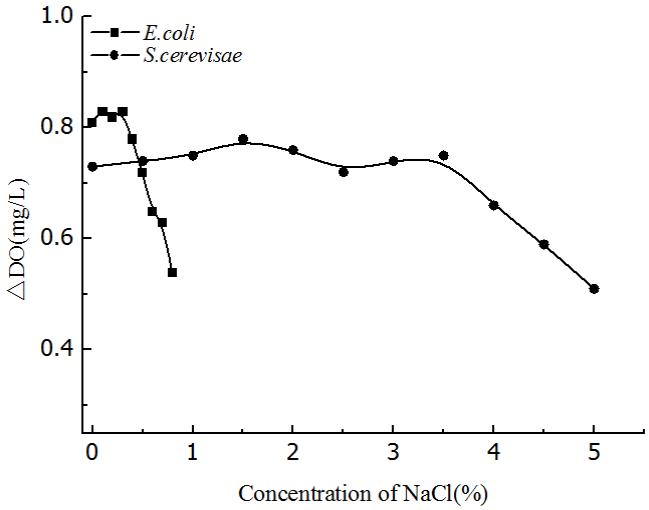

Figure 4 Influence of inorganic salts on the sensor response

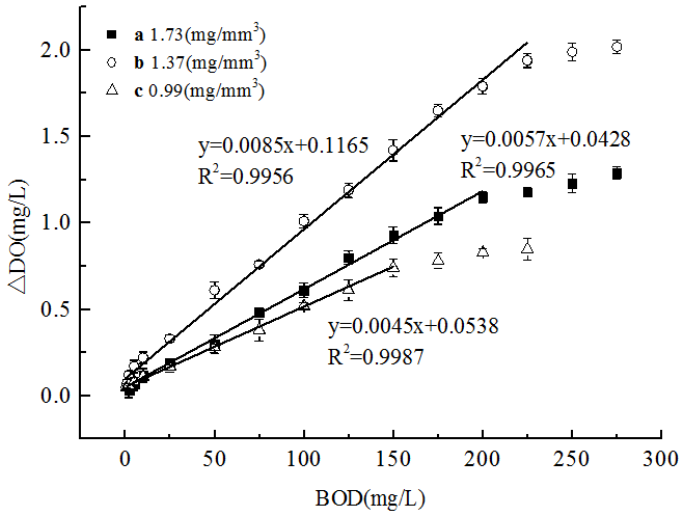

Figure 5 Influence of biofilm density on the sensor response

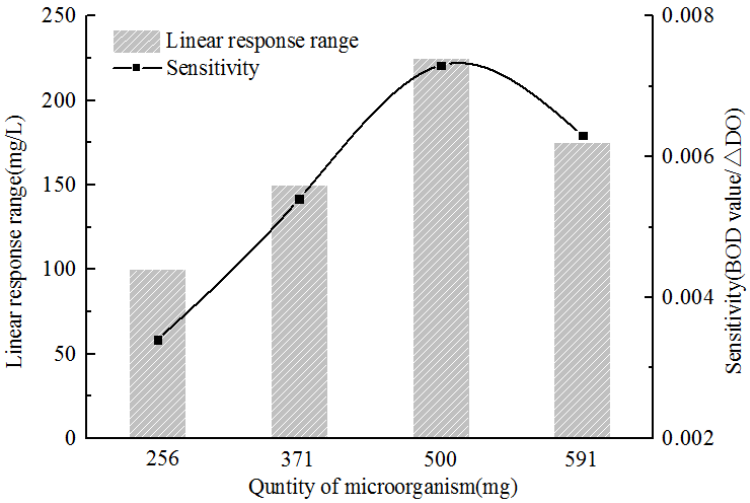

Figure 6 Influence of the amount of microorganisms on the sensor response

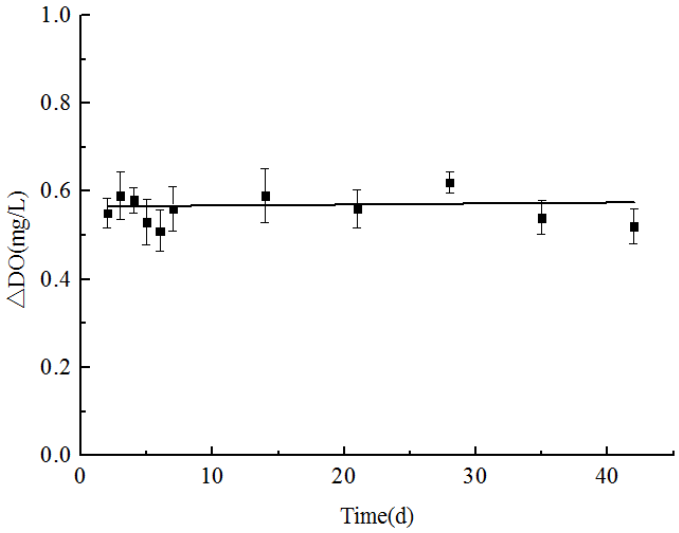

Fig 7 Service life of the ZICF based BOD sensor

Table 1 Comparison of different methods for the detection of pure organics

Substrate	BOD_{S1}(g/g)**a** (*Saccharomyces cerevisiae*)	BOD_{S2}(g/g)**a** (*Escherichia coli)*	BOD_5(g/g)**b**	BOD_S(g/g)**c** (*Pseudomonas fluorescens*) [19]	BOD_S(g/g)**c** (*Pseudomonas* sp. A_4) [20]
Glucose	0.80	0.72	0.3～0.6	0.72	0.30
Sucrose	0.21	0.55	0.28～0.44	0.36	0.11
Maltose	0.32	0.34	0.2～0.62	--	0.45
Lactose	0.15	0.11	0.4～0.63	0.04	0.00
Fructose	0.77	0.74	0.71	0.54	--
Glycine	0.35	0.49	0.55～1.30	--	0.23
Lactamic acid	0.17	0.27	0.55	--	--
Lactic acid	0.09	0.02	0.63～0.88	0.17	--
Glutamic acid	0.58	0.65	0.52～0.64	0.70	1.06
Citric acid	0.38	0.18	0.23～0.61	--	--
Mannitol	0.12	0.69	0.68	--	0.62
Glycerol	0.18	0.97	0.62～0.83	0.51	0.79

a Results of the ZrCF-based BOD sensors; **b** Results of BOD_5 method; **c** Results in reported papers

Table 2 Comparison between the sensor method and the BOD_5 method

Samples	BOD_5 (mg/L)	BOD_{s1}^{OECD} (mg/L)	BOD_{s1}^{GGA} (mg/L)	BOD_{s2}^{OECD} (mg/L)	BOD_{s2}^{GGA} (mg/L)
River water	7.1	7.7	8.5	9.2	11.7
Synthetic sea water	48.4	54	67.1	65	77.3
GGA solution	192	138	200	154	200
OECD synthetic wastewater	194	200	234	200	256

Note: $_{S1}$ rpresents the BOD sensor based on *S.cerevisiae*; $_{S2}$ represents the BOD sensor based on *E.coli*