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Native biofilm cultured under controllable condition and used in mediated method for BOD measurement

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ABSTRACT

In this article, we developed a native biofilm (NBF) bioreactor used for biochemical oxygen demand mediated method (BOD_{Med}). There were two innovations differed from previous BOD_{Med} assay. Firstly, the immobilization of microorganisms was adopted in BOD_{Med}. Secondly, the NBF was introduced for BOD measurement. The NBF bioreactor has been characterized by optical microscopy. A culture condition of NBF with 24 h, 35 °C and pH 7 was optimized. Furthermore, a measuring condition with 35 °C, pH 7 and 55 mM ferricyanide in 1 h incubation were optimized. Based on the optimized condition, the real wastewater samples from local sewage treatment plant had been measured. Performances of the NBFs proposed at different culture conditions were recorded for 110 d, and the results indicated that long-term storage stability was obtained. With the proposed method, an uncontaminated native microbial source solution can be obtained from a wastewater treatment plant. In this way, we can ensure that the microbial species of all in the NBF are same as that in the target to be measured.

1. Introduction

The biochemical oxygen demand (BOD) is a significant environmental parameter, because it is a biofeedback of environmental infection. The international legislated standard test 5-d biochemical oxygen demand assay (BOD_5) is not suitable for rapid feedback information in practice. So the rapid BOD biosensors were developed. In those BOD biosensors, oxygen was used as the terminal electronic acceptor and which concentration in water (8.7 mg/L at 25 °C) was a limited factor for biosensors [1]. Based on the high solubility, synthetic electron acceptor (called mediator) has been explored for BOD measurement in the last decade. In 2000, the ferricyanide-mediated microbial reaction has been used for BOD measurement [2]. Subsequently, the mediated method for BOD measurement (BOD_{Med}) gained great attention, because the high solubility of synthetic electron acceptor overcame the limitation from lower oxygen solubility [3–8]. So far, numerous works of BOD_{Med} method have been reported and successfully established [9–15].

However, in recent years, the BOD_{Med} method developed slowly. In fact, BOD_{Med} method is labour-intensive and making rapid analysis of the bioreaction very difficult [6]. One important barrier to the use of BOD_{Med} method is that the microorganisms suspended in solution is generally adopted [16–19]. Microorganism culture was required before BOD measuring. As is known, microorganism's culture process was tedious and time consuming, so the immobilization of microorganisms to form a biofilm on substrate is the unique way to solve the problem [20]. It is known that an insufficient broad spectrum of organic substrates could be biodegraded by the single species. Obviously, the ideal microbial consortium employed for the BOD biosensor fabrication is the same as that truly reflecting the biodegradation on the real wastewater sample [21–23].

Here, we proposed to fabricate the bioreactor using a native biofilm (NBF) cultured from native resource wastewater for BOD measurement. Such a NBF bioreactor possesses an extremely high and sustainable native microorganisms' population hence ensuring a satisfying biodegradation of native microbial source wastewater. Based on this, the high biodegradation ability, sensitivity and accuracy, superior reliability and wide applicability can be expected. The present approach has significant potential for developing of rapid and inexpensive instruments for on-line environmental measurement.

2. Experimental

2.1. NBF culture conditions and bioreactor

The native biofilm (NBF) was cultured aerobically by using real wastewater from a local sewage treatment plant. CASO broth

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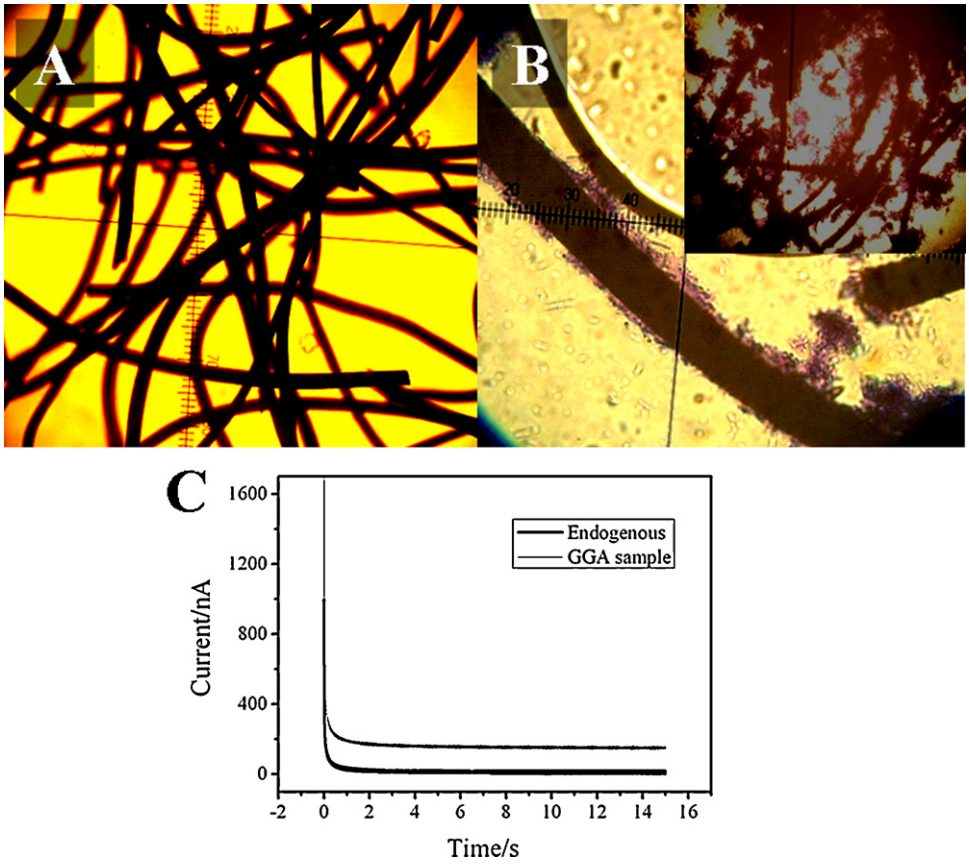

Fig. 1. Optical microscopy images of carbon fiber felt before (A) and after (B) depositing microorganisms with 50× objective. Inset: 10× objective. (C) Amperograms recorded at a Pt microarray electrode (25 μm × 4) for BOD^{200} GGA solution and an endogenous control after 1 h incubation with NBF bioreactor.

medium was bought from Fluka (Fluka Chemie GmbH CH-9471 Buchs). The 3 g of CASO substrate was pulled into the culture flask with 100 mL real wastewater. Carbon fiber felt was immersed in the flask and shacked at 100 rpm. To optimizing the culture conditions, the different culture time (under 35 °C and pH 7), temperature (under 24 h and pH 7) and solution pH (under 24 h and 35 °C) were adopted. After culture, the NBF was filled into a 0.5 mL eppendorf tube for fabricating a simple bioreactor and stored in the phosphate buffer solution (PBS, 0.12 M Na_2HPO_4/0.08 M K_2HPO_4/0.1 M KCl, pH 7).

2.2. BOD_{Med} measurement and electrochemical response

Test solution with different concentrations was prepared by appropriate dilution of a BOD^{1980} (1500 mg/L glucose and 1500 mg/L glutamic acid) standard solution with PBS [5,6]. The symbol that BOD with a superscript means a solution was assigned with a corresponding BOD value. For example, the abbreviation BOD^{1980} means a solution with the BOD value 1980 mg O/L. Endogenous control solutions were prepared by adding PBS in place of the test organic substrate. Potassium ferricyanide solutions were prepared by 0.33 M PBS. All chemicals used in this study were of analytical reagent grade and all solutions were prepared with deionized water being sterilized. The temperature was controlled by water bath. To terminate the reaction, the samples were pumped out by syringe and centrifuged with 10,000 rpm for 3 min. The supernatant solution was then used for analysis of microbially produced ferrocyanide. Electrochemical responses were measured with a CHI 832 electrochemical analyzer (CHI Co., Shanghai, China). The setup was conducted in amperometric mode. The detailed operation was introduced in our previous report [24]. The platinum array microelectrode (2 × 2 microdiscs of 25 μm diameter each) was used as working electrode, and piece of Pt was used as the counter electrode, and Ag/AgCl reference electrode was applied to the Pt-working electrode throughout all measurements. The results calculated referred previously report [25].

2.3. Measurement of real polluted wastewater

Mixture of 55 mM ferricyanide (final concentration) and real wastewater were added into the NBF bioreactor. The sample solutions were incubated at 35 °C for an optimized time (1 h for the present work). The operation for terminating the reaction and obtaining the analytical signal were referred in Section 2.2.

3. Results and discussion

3.1. Characterization of the NBF bioreactor

In the present work, NBF was cultured from a sewage treatment plant. Optical microscopy was used for investigating the NBF formation. Fig. 1A and B presets optical microscopy images of the carbon fiber felt before and after deposition of microorganisms, respectively. The microorganisms were stained by methylene blue for observation. The result indicated that NBF has been deposited on the carbon fiber felt. Which carbon fiber felt with NBF could be used for bioreactor fabrication. The diameter of carbon fiber was 14 μm in average obtained from twenty measurements. The bioreactor volume was 0.5 mL, and the surface area of 70 mg carbon fiber felt was estimated ~90 cm^2. Obviously, the high specific surface area is benefit for depositing microorganisms.

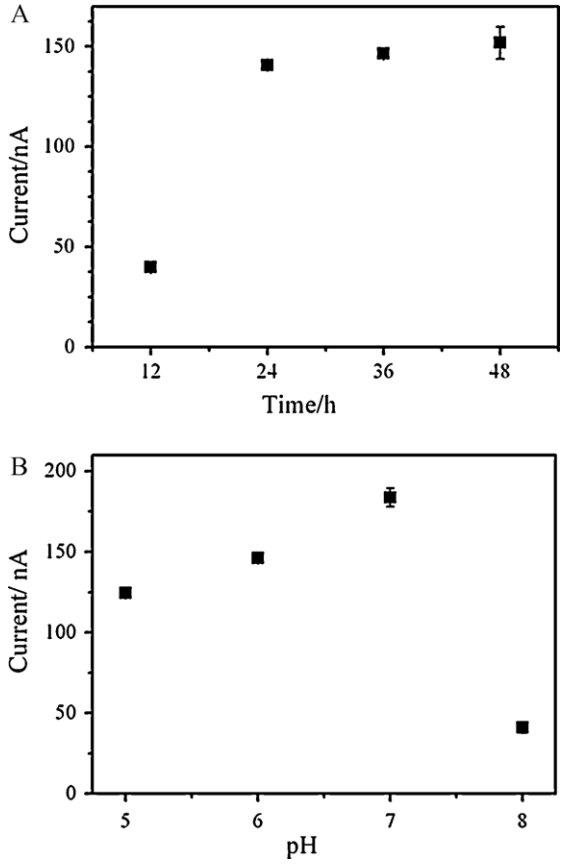

Fig. 2. Effect of culture condition of NBF formation. (A) Time, and (B) pH.

Fig. 1C showed the amperometric responses for the BOD^{200} GGA and an endogenous control after 1 h incubation with NBF bioreactor. The final concentration of ferricyanide was 55 mM. This result indicated two points. On the one hand, the NBF was able to utilize endogenous substrate for respiration. On the other hand, there was a distinguished analytical signal between sample and endogenous substrate. This result indicated a satisfactory performance of the present NBF.

3.2. Optimizing culture condition of the NBF bioreactor

The culture time of NBF was varied from 12 h to 48 h at 35 °C in pH 7 solution. The performance of NBF was evaluated by biodegrading a BOD^{200} GGA solution. Fig. 2A showed that the signal of mediator reduced by NBF have reached to the maximum at 24 h and got to a stable state. As we know, a higher temperature of culture can accelerate the growth of microorganisms and promote the cell aging finally. The results indicated that 24 h was a suitable culture time for NBF formation.

pH of nutritional substrates for culturing NBF was varied from 5 to 8 at 35 °C for 24 h. The catalytic ability evaluation was accorded to culture time-dependent experiments. Fig. 2B showed that the reduced mediator generated by NBF increased gradually with the pH varied from 5 to 7, and sharply dropped at pH 8. It is possible that the reproduction of microorganisms from the real wastewater was not vigorous in alkaline solution. This result indicated that pH 7 was a suitable condition for NBF formation. Furthermore, the culture temperature has been selected at 28 °C and 35 °C in pH 7 solution for 24 h, and the analytical signal obtained at 35 °C was six-fold of that at 28 °C (data were not shown). So the 35 °C was selected for culturing NBF on the carbon fiber felt.

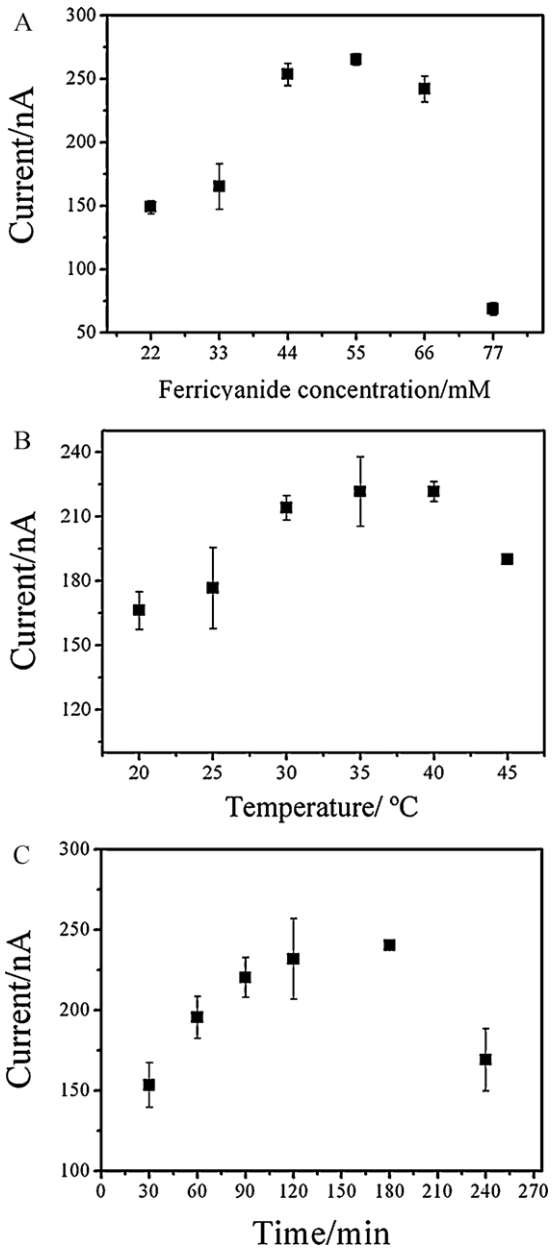

Fig. 3. Effect of (A) ferricyanide concentration (endogenous control values have not been reduced), (B) incubation temperature and (C) time on the response of NBF bioreactor.

3.3. Optimizing measurement condition of the NBF bioreactor

The effect of ferricyanide concentration on the response of NBF bioreactor has been valuated by using BOD^{200} GGA solution. Fig. 3A shows that the analytical signal reached a high response when the ferricyanide concentration was in the range of 44–66 mM. The responses decreased when the ferricyanide concentrations were below 33 mM or above 77 mM. We presumed the reason of the present result was that the microorganism viability was decreased by the toxicity of artificial electron acceptor, when the concentration of ferricyanide was above 66 mM. When the ferricyanide concentration was below 33 mM, the reaction could be limited by the availability of the artificial electron acceptor. This result was also in good agreement with the previous report [7].

The effect of incubation temperature on the response of NBF bioreactor was then evaluated by using BOD^{200} GGA solution. Fig. 3B shows that the analytical signal reached a high response

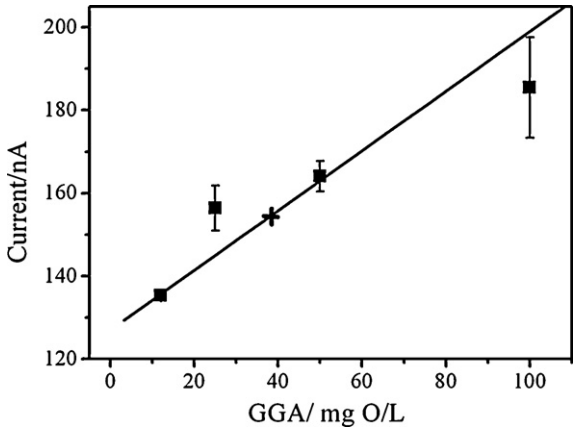

Fig. 4. Calibration curve of GGA standard solution. The point of intersection was BOD_{Med} value of real polluted wastewater.

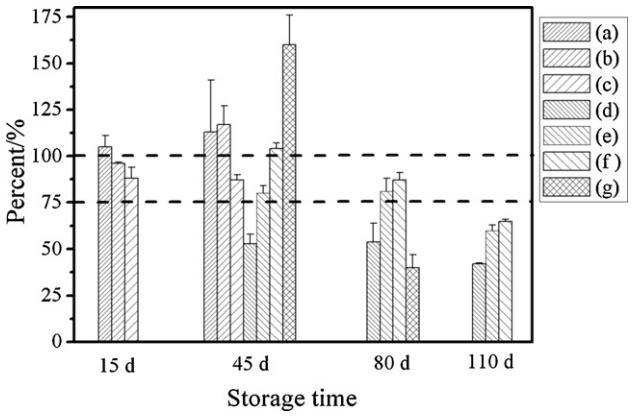

Fig. 5. Long-term stability of NBFs formed in different culture conditions. (a) 36 h, pH 5, 35 °C; (b) 36 h, pH 6, 35 °C; (c) 36 h, pH 7, 35 °C; (d) 48 h, pH 7, 35 °C; (e) 24 h, pH 7, 35 °C; (f) 12 h, pH 7, 35 °C; (g) ~60 h, pH 7, 28 °C.

when the incubation temperature was in the range of 35–40 °C. The responses decreased when the incubation temperature was below 25 °C or above 45 °C. The results suggested that the microorganism viability was temperature-dependent.

The incubation time has been valuated by using BOD^{200} GGA solution. Fig. 3C shows that the analytical signal was reached a high response when the incubation time was in the range of 60–180 min. The responses were decreased at the incubation time below 30 min and above 180 min. It is known that the damage of bacteria was related with the incubation time. The morphology variation could be referred our early work [26]. So we selected 1 h for all measurements in subsequent experiments.

3.4. Calibration and measurement of real polluted wastewater

The linear range of GGA solution with the NBF bioreactor was further studied. A response range was obtained from BOD^{12} to BOD^{400} GGA organic substrate (see supporting information, Fig. S1), but the linear correlation was obtained only between BOD^{12} and BOD^{100}. All measured signals were obtained by subtracting the background current. A linear regression equation of $y = 125 + 0.79x$ ($R = 0.9710$) was calculated, where y is the difference of current response (ΔI) and x is the concentration of the standard solution. The trendline of GGA curve from BOD^{12} to BOD^{400} would be predicted as a logarithm (Fig. S1). The result revealed that the bacterial degradation efficiency gradually decreased with extending the concentration of organic substrate. All the characters revealed by the present results were the same as that with mediator in solution reported by our group previously [24]. The measurements of real wastewater were carried out by the NBF bioreactor. As shown in Fig. 4, the BOD_{Med} value 37 mg/L was recorded as the point of intersection of calibration curve. Compared with BOD_5 value 42 mg/L, the RSD of ±15% for BOD measurement was acceptable. So the present result indicated that the present NBF bioreactor could be used for measuring real wastewater.

3.5. Long-term stability of the NBF bioreactor

An important index of bioreactor is the long-term stability. The species and amount of microbial consortium of NBF could be affected by their growth conditions, and the biodegradation activity of NBF could be affected. So the three batches of NBF formed under different conditions were recorded by Fig. 5. The NBF bioreactors were stored in pH 7 PBS with 20% BOD^{1980} GGA solution (v/v). The storage stabilities of NBF formed in different pH were recorded by (a)–(c). The signals of pH 5 and pH 6 NBFs were increased over 100% of initial activity in the first 45 d storage. We assumed the reason was that some species of microorganism were limited at the culture condition low pH (5 and 6) and re-grown at storage condition pH 7. The result indicated that a more appropriate pH for NBFs were formed in low pH and stored in pH 7 condition. The storage stabilities of NBF formed in different culture times were recorded by (d)–(f). The 12 h and 24 h NBFs exhibited relatively good performance, and the signals remained about 80% for 80 d and 60% for 110 d. The performances of 48 h NBF was non-ideal after 45 d storage. We assumed the reason of the sharply reduction of analytical signals was the growing of NBFs so rapidly and the biofilm not well-knitted. This result indicated that the excessively high culture temperature was not beneficial for NBF storage. The signals of 28 °C NBF were enhanced strongly in the first 45 d for storing, which possibly because the microorganisms of NBF (based mainly on yeast) divided continually and some new bacteria reproduced. This result indicated that the lower temperature NBF was beneficial for storage. To use NBF effectively, the suitable culture conditions would be selected based on different aims.

4. Conclusion remarks

We developed a NBF bioreactor used in BOD_{Med} method. There are two innovations. On one hand, we used the immobilized microorganisms in mediated method. Based on the immobilized approach, the microorganism culture was not needed for measurement each time. The present method possessed a long-term stability of bioreactor as long as 110 d. On the other hand, we introduced the native biofilm in BOD measurement to fabricate a bioreactor. Design, fabrication and optimization of the cultivated NBF bioreactor were achieved by creating a highly biocompatible reactor surface via using the carbon fiber felt to facilitate the biofilm formation.

The biofilm can be readily formed by cultivating the native microbial source solution and the resultant biofilm should possess the same microbial composition as that of sample solution. Consequently, the measured BOD from this type of natively cultivated NBF should reflect the actual impact on the sample. In other word, the test microbial consortium (the NBF bioreactor) is specifically designed for the test sample. The present strategy offers a simple way for resolving the problem of microorganism cultivating multi-times in BOD_{Med} method, and it will become a much practical approach in future application for environmental analysis.

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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.talanta.2011.02.025.

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