 doi.org/10.52568/001285/JCSP/45.04.2023

Native Biofilm Used in Mediated Method for Toxicity Measurement

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(Received on 15th December 2021, accepted in revised form 25th May 2022)

Abstract: A native biofilm (NBF) bioreactor for monitoring total toxicity in water was developed in this article. Specific objectives include the investigation of (i) the NBF was introduced in toxicity measurement, and (ii) the immobilized microorganisms on carbon fiber felts are adopted for mediator toxicity assay. Culture conditions of NBF with 35 °C and 24 h were adopted, and a measuring condition with 45 mM ferricyanide (at pH 7) was optimized. Under the above conditions, NBF bioreactor was successfully employed to assess toxicities of four toxicants. By adopting the NBF, the IC50 values obtained were much lower than that of the single bacterium as the test microorganism. Furthermore, the long-term storage stability was examined. The result showed that the activity of the microorganisms of NBF was found to be roughly the same within 42 days. We confirmed that this NBF combined with mediator toxicity assay may be served as an early warning for affecting public health and avoiding environmental pollution.

Keywords*:* Native biofilm; Mediator method; Total toxicity; Carbon fiber felt.

Introduction

In recent years, environmental pollution has become a global problem, and the development of environmental monitoring and detection technology has become the focus of many scientists [1]. Existing standard bacterial toxicity tests based on the bioluminescence fading, such as MICROTOX, is the most widespread used toxicity detection and evaluation method. However, luminescence tests would cause the fluorescence scattering because of depending on optical detection, it is strictly limited by the number of cells and sample turbidity. Furthermore, Luminescent bacteria must add 3% NaCl in solution in order to maintain osmotic pressure. In the presence of NaCl, the solubility of some organic chemicals will be reduced.

In order to make up for the shortcomings of luminescence detection methods, electrochemical microbial sensors have been developed [2-5]. The method is considered to be a rapid and superior toxic screening tool because high concentrations of microorganisms and mediator are employed. Compared to healthy cells, the ability of the damaged cells for the electrons produced from cellular metabolism cells will be affected. These toxic effects can be easily converted into electrochemical signals and can be quantified as inhibition index.

However, the mediator-based toxicity assay mentioned above have some rooted disadvantages, such as complicated operation and tedious preparation [6]. Microbial cultivation is an necessary procedure which costs much time for most established methods [7–13]. Microorganism culture must be carried out before each measurement. As we all known, Microorganism culture process was time consuming which would spend more than 10 hours on every culturing. Recently, immobilized microbial cells are widely used in many fields including environmental pollution control. Cell immobilization has the advantages of strong resistance to toxic substances compared with suspended microorganism [14-17]. In addition, immobilized microorganisms can be used for multiple times without loss of activity [18, 19]. There are many works about immobilized microorganism technology for wastewater treatment in recent decades, but no report on immobilized microorganism technology for mediator toxicity assay.

On the other hand, the previous studies all adopted single bacterium as test microorganism which may generate a problem with low sensitivities because a certain bacterium is not sensitive to all toxicants [7-13, 20], such as the E. *coli*, is sensitive to As^{3+}, but is not sensitive to Hg^{2+} [9]. So using single bacterium as test microorganism is defined to detect the total toxicity. Fortunately, the compound strains microorganism can solve this problem. However, there is no work about using compound strains microorganism in mediator toxicity assay.

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Here, we proposed to use a native biofilm (NBF) for toxicity measurement. A simple way to immobilize the microorganisms on carbon fiber felt has been proposed. This immobilization method is maneuverable, and can provide a biocompatible condition in maintaining the bioactivities. The toxicity assessment of immobilized microorganisms based on mediator sensor will greatly simplify the detection process, shorten the operation time and reduce the cost. Furthermore, NBF contains a variety of microorganisms embedded by a polymeric matrix (EPS) and connected to the wet interfaces. NBF bioreactor has a high and sustainable native microbial population. Based on this, wide applicability and high sensitivity can be obtained. The approach has great potential in developing fast and cheap online environmental analysis instruments.

Experimental

Chemicals and solution

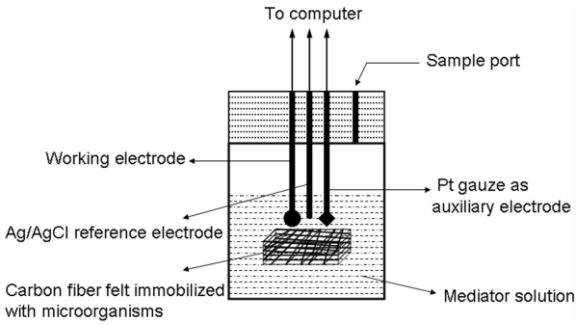
CASO broth medium was obtained from Fluka. Mercury chloride($HgCl_2$) and Arsenic trioxide(As_2O_3) were purchased from National Center of Analysis and Testing for Nonferrous Metals and Electronic Materials (NCATN), and Potassium dichromate($K_2Cr_2O_7$) was purchased from Sigma, 3,5-Dichlorophenol(3,5-DCP)(97%) was obtained from Sigma, Potassium hexacyanoferrate (HCF) (III) was purchased from Sinopharm Chemicals (China) and was freshly prepared before use. Unless otherwise state, ultrapure water was used in all experiments.

NBF culture conditions

The native biofilm (NBF) was cultured aerobically by using real water sample from South Lake in Changchun China. CASO (3 g) was put into the culture flask with 100 mL real water. Carbon fiber felts were put into the culture flask and grown aerobically in a shaker (100 rpm). To optimize the culture conditions, different factors, such as culture time and temperature, were adopted. Finally, time 24 h and temperature 35 ºC were adopted as the optimal conditions. After culturing, the NBF which formed on carbon fiber felt surface was moved into a 0.5 mL glass vial for fabricating a simple bioreactor and stored in the phosphate buffer solution (PBS, 0.12 M Na_2HPO_4/0.08M K_2HPO_4/0.1M KCl, pH 7).

Electrochemical detection

The final concentration of HCF(III) was 45 mM, the ultramicroelectrode array (UMEA) was used as working electrode，which consists of 20 Pt ultramicroelectrodes (25 μm), Ag/AgCl (saturated KCl) as reference electrode and a Pt gauze as auxiliary electrode. During the test, different concentrations of toxicants were injected into the bioreactor, and the oxidation of HCF(II) currents were monitored. Amperometric measurement was performed by applying a constant potential (+450 mV versus the reference electrode) on working electrode.



Scheme-1: Scheme of the biosensor system.

Toxicity calculation

Toxicity was monitored by measuring the current changes before and after contacted with toxicant by using CHI 832B electrochemical workstation (Chen Hua, Shanghai). The inhibition (*I*) of toxicant on *NBF* was calculated using the following equation:

$$I\,(\%) = \frac{i_{\lim control} - i_{\lim sample}}{i_{\lim control}} \times 100\% \qquad (1)$$

$i_{\lim\ control}$ *is* the limiting current of control and $i_{\lim\ sample}$ *is* the limiting current of sample, *I* is the inhibition percentage. IC50 represents 50% growth inhibition，which was calculated using OriginPro 8.

Results and Discussion

Characterization of the NBF bioreactor

The formation of NBF was studied by optical microscope. Fig 1A showed the optical microscopy image of the carbon fiber felt before microbial deposition, and Fig 1B showed the optical microscopy image after microbial deposition. Methylene blue staining was used for observation. The result showed that NBF has been successfully

deposited. The average diameter of carbon fiber obtained from twenty measurements was 14 µm. The 50 mg carbon fiber felt was about 70 cm^2 surface area. Obviously, high specific surface area was conducive to microbial deposition.

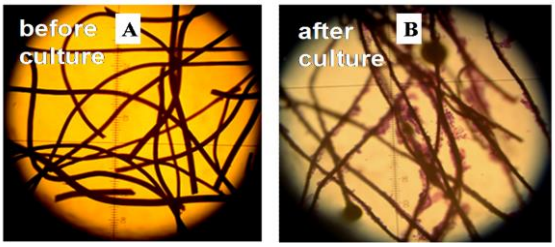


Fig. 1: Optical microscopy images of the carbon fiber felt before (A) and after (B) depositing of microorganisms.

Optimizing culture condition of the NBF bioreactor

Fig 2 shows the influence of different culture conditions for NBF growth. The limiting current was used here to quantify the reduced mediator (HCF(II)). Fig 2A showed that the analytical signal reached to the maximum at 24 h obtained by NBF. The result indicated that 24 h was a suitable culture time for NBF formation. Furthermore, the culture temperature has been selected from 20 ºC to 50 ºC, and the analytical signal reached to the maximum at 35 ºC for 24 h (Fig 2B). So the 35 ºC was selected for NBF growth on the carbon fiber felt.

Optimizing measurement condition of the NBF bioreactor

The pH and ferricyanide concentration may seriously affect the measurement results. Fig 3A showed that with the pH varied from 4 to 7, the HCF(II) produced by NBF increased gradually, and at pH 8 dropped sharply. It is possible that microorganisms in South Lake do not reproduce vigorously in alkaline solution. This result showed that pH 7 was the suitable measurement condition for NBF bioreactor. Fig 3B shows that when the ferricyanide solution was in the range of 45-65 mM, the responses reached a high response, and decreased above 75 mM. This result was consistent with the previous report that high concentrations of ferricyanide have toxic effect on cells [21].

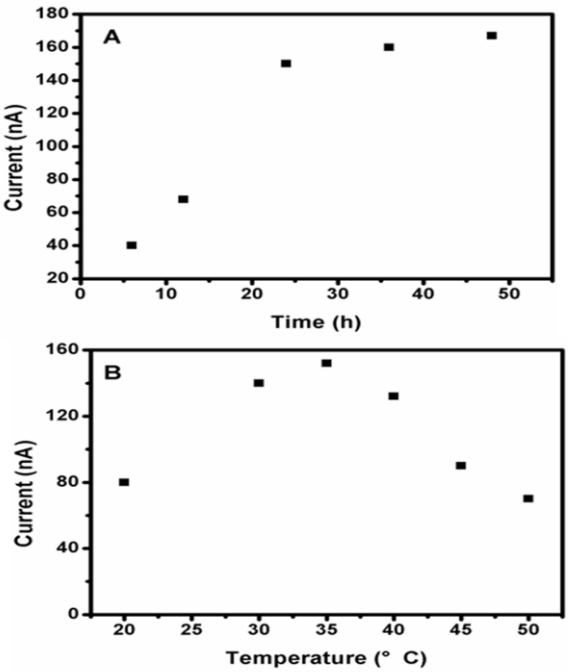


Fig. 2: Effect of culture condition of NBF formation. (A) Time (B) Temperature.

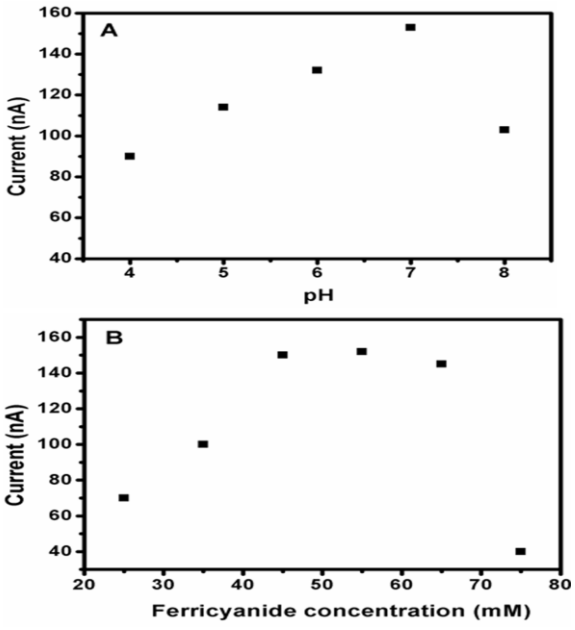


Fig. 3: Effect of (A) pH, and (B) ferricyanide concentration on the response of NBF bioreactor.

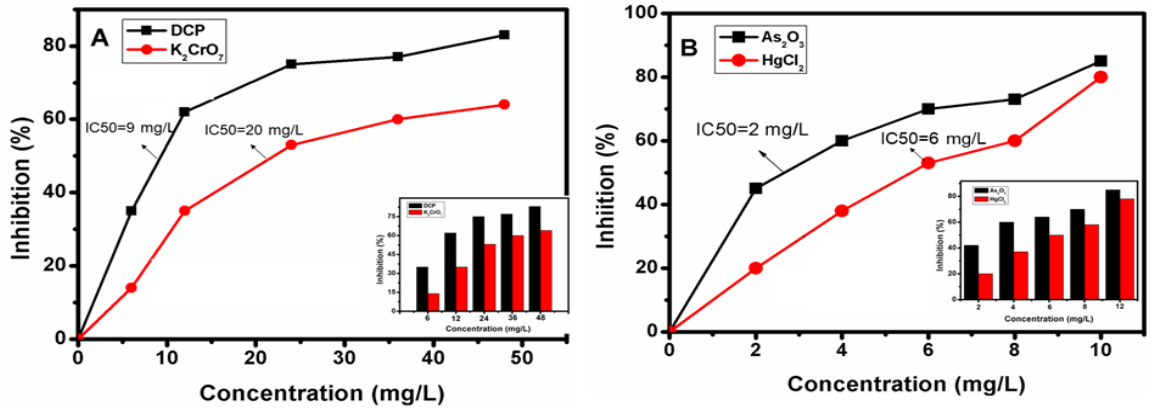


Fig. 4: The relationship between the inhibition and concentration of (A) DCP and $K_2Cr_2O_7$, (B) As_2O_3 and $HgCl_2$.

Application of the NBF bioreactor to detect the toxicity of four model toxicants

Toxicity assays of four toxicants were carried out using NBF bioreactor, and the results obtained were compared with that of using *E.coli* as test microorgnism. The different concentrations of toxicants were injected into the bioreactor and then a decrease in currents observed after one hour incubation. Fig 4 exhibited the relationship between the inhibition and concentration of these four toxicants. According to Fig 4A, the IC50 values of DCP and $K_2Cr_2O_7$ are 9 mg/L and 20 mg/L, respectively. Fig 4B showed that the IC50 values of As_2O_3 and $HgCl_2$ are 2 mg/L and 6 mg/L, respectively. We also used the single bacterium (*E. coli*) as the test microorganism to test these four toxicants. The IC50 values to DCP, $K_2Cr_2O_7$, As_2O_3 and $HgCl_2$ are obtained by *E. coli* as 10 mg/L, 18 mg/L, 17 mg/L and 36 mg/L, respectively. Obviously, the IC50 values of DCP and $K_2Cr_2O_7$ obtained by the two methods are similar, but the IC50 values of As_2O_3 and $HgCl_2$ obtained by NBF are much lower than that of *E. coli* as test microorganism. The reason is that NBF includes some bacteria which are more sensitive to As_2O_3 and $HgCl_2$ than that of *E. coli*. According to pervious reports, the *E. coli* as test microorganism has a good correlation with MICROTOX test [9, 13, 20, 22]. These results demonstrated that the NBF method can be used as an initial toxicity-screening tool and could be better than that of single bacterium as test microorganism.

Long-term stability of the NBF bioreactor

The storage stability is an important index of bioreactor for practical application. The NBF was stored in PBS (pH 7) at room temperature. For the determination of the storage stability of the NBF, the experiments were carried out periodically by detecting the signal decrease in the biosensor response. Approximately 10 measurements have been done during 60 days, making more measurements in a longer time period is possible. As evident from the Fig 5, the activity of the microorganisms was found to be fairly constant within 42 days. After 63 days storage, the biosensor lost only 30 % of its initial activity.

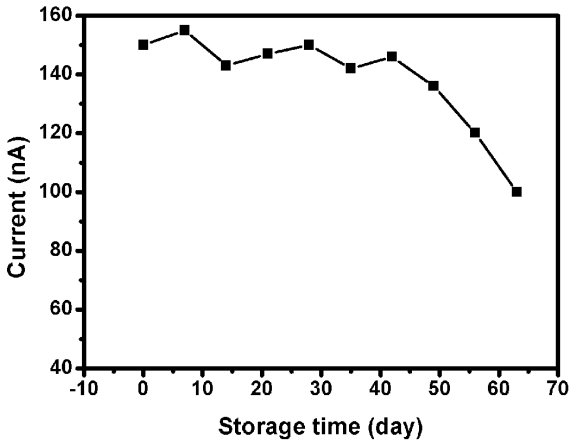


Fig.5: Long-term stability of the NBF biosensor.

Conclusion

NBF bioreactor used in toxicity mediator method have been successfully developed. There are two innovations. On the one hand, the immobilized microorganisms was used. Based on the immobilized approach, it did not need to culture the

microorganism for each measurement. The method has the long-term stability of bioreactor for up to 60 days. On the other hand, the native biofilm was adopted in toxicity measurement. The toxicity of four topical toxicants was detected via this method. It was demonstrated the result obtained by NBF method correlates well with that of classic method. More importantly, NBF method has a higher sensitivity than the single bacterium used as the test microorganism. This method provides a simple way to solve the problem of time consuming for microorganism cultivating tin toxicity mediator method, and will become a very practical way for the application of environmental analysis in the future.

Acknowledgement

This work was supported by the Excellent scientific and technological innovation team of Jiangsu Province (2021), Natural Science Foundation of Jiangsu Province (Grant No. BK20191220，BK20181219), Science and Technology Planning Project of Yangzhou (YZ2021171), the Program for the Young and middle-aged outstanding academic leaders of blue project in Jiangsu Province (2019，2021), and the Natural Science Foundation of Yangzhou Polytechnic Institute (2019xjzk001).

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