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Comparative analysis of microbial fuel cell based biosensors developed with a mixed culture and *Shewanella loihica* PV-4 and underlying biological mechanism

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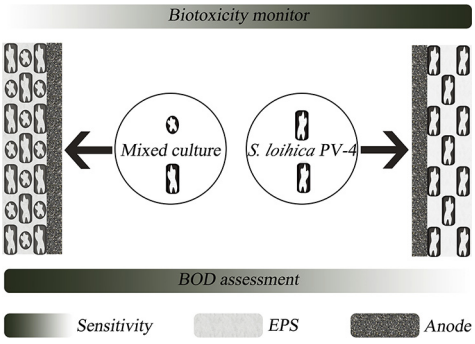
GRAPHICAL ABSTRACT



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ABSTRACT

Microbial fuel cell based biosensors (MFC-biosensors) utilize anode biofilms as biological recognition elements to monitor biochemical oxygen demand (BOD) and biotoxicity. However, the relatively poor sensitivity constrains the application of MFC-biosensors. To address this limitation, this study provided a systematic comparison of sensitivity between the MFC-biosensors constructed with two inocula. Higher biomass density and viability were both observed in the anode biofilm of the mixed culture MFC, which resulted in better sensitivity for BOD assessment. Compared with using mixed culture as inoculum, the anode biofilm developed with *Shewanella loihica* PV-4 presented lower content of extracellular polymeric substances and poorer ability to secrete protein under toxic shocks. Moreover, the looser structure in the *S. loihica* PV-4 biofilm further facilitated its susceptibilities to toxic agents. Therefore, the MFC-biosensor with a pure culture of *S. loihica* PV-4 delivered higher sensitivity for biotoxicity monitoring. This study proposed a new perspective to enhance sensor performance.

1. Introduction

Water quality monitoring is paramount for the guarantee of ecological safety and public health. Available monitoring items mostly focus on physical and chemical indicators such as pH and chemical oxygen demand. Only a few biochemical indexes represented by biological oxygen demand (BOD) are included (SEPA, 2002). Among these parameters, physical and chemical indicators enable the accurate

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determination of the concentration of specific substance in aquatic environment, but the detection process requires skilled and time-consuming operation. Moreover, these indicators fail to provide a comprehensive assessment of biotoxicity and cumulative toxicity, which makes these methods unsuitable for in situ and online monitoring. BOD index, which relates to the biodegradability of water, is evaluated by the consumption of dissolved oxygen after incubation for a fixed period (usually 5 d) (APHA, 1998). However, tediously long detection period of BOD measurement disagrees with the demand of prompt alert and leads to the deficiency of fast-response detection method. Therefore, research on novel technology and equipment for timely and efficient water quality monitoring is of crucial importance.

Microbial fuel cells (MFCs) are devices which convert chemical energy from organic matters into electricity with the catalysis of electroactive microorganisms (Logan, 2009). Previous studies have validated the feasibility to use MFC based biosensor (MFC-biosensor) to monitor BOD concentration and biotoxicity in water environment (Kim et al., 2003; Kim et al., 2007; Modin and Wilén, 2012). Kim et al. (2003) developed a dual-chamber MFC for BOD quantification and observed a good linear relationship between Coulombic yield and BOD concentration. Thereafter extensive efforts have been made to improve sensor performance from the aspects of configuration design and operation parameter. For instance, scaling down the anode volume from 25 mL to 5 mL and the optimization of fuel-feeding rate could reduce the response time of BOD test to 36 min (Moon et al., 2004), while another study managed to extend the linear range to 1280 mg/L by applying an external voltage to MFC-biosensor (Modin and Wilén, 2012). Research on the assessment of biotoxicity via MFC-biosensor started later than BOD sensor. The first MFC based toxicity sensor was reported by Kim et al. (2007). They installed it in a wastewater treatment plant and successfully alerted the shocks of several heavy metals and polychlorinated biphenyl. Since then, significant progress has been made to enhance the sensitivity and stability of detection process (Shen et al., 2013; Lorenzo et al., 2014; Jiang et al., 2015; Xu et al., 2015).

Anode biofilm is considered as the main sensing element for both BOD concentration and biotoxicity monitoring. Fluctuations in water quality would affect the metabolic activities and electron transfer processes of electroactive microorganisms and further lead to visible changes of output current or voltage (Jiang et al., 2018). Hence, electroactive biofilm colonized on the anode surface is a key factor to sensor performance. MFC-biosensors in most of researches were constructed with mixed cultures (Liu et al., 2014; Lorenzo et al., 2014; Wu et al., 2014; Xu et al., 2015; Yu et al., 2017). Only a few publications concerned about the application of pure strain as sensing element. In a recent study, Atci et al. (2016) developed a MFC-biosensor with a pure culture of *Geobacter sulfurreducens* and observed a good linear correlation between current signal and acetate concentration. As for biotoxicity sensor, a pure culture of *Shewanella oneidensis* MR-1 was employed for the first time to detect formaldehyde (Wang et al., 2013). However, the influence of the two types of inocula on the performance of MFC-biosensors has not been revealed yet.

When studying the temporal-spatial changes of biofilms in microbial electrolysis cells, Sun et al. (2015) reported that various inocula (mixed culture and *Geobacter anodireducens*) would result in discrepancies in biofilm structure, viability and biomass density and further affected electricity generation directly. Since electricity generation was an important factor for BOD quantification via MFC-biosensor (Modin and Wilén, 2012), different inocula might impact the sensor performance for BOD determination. On the other hand, the contents of extracellular polymeric substances (EPS) in biofilms might vary with different inocula as well. Due to the protective effect of EPS against toxic agent invasion (Miao et al., 2017), various inocula might also influence the sensitivity of MFC-biosensor for biotoxicity monitoring.

Therefore, this study aimed to investigate the effect of inoculum on the sensitivity of MFC-biosensor, which has not been revealed yet and might propose a new perspective to enhance the sensor performance. *Shewanella loihica* PV-4, which was a widely applicable strain of electroactive microorganisms with the superior capacity of extracellular electron transport (Kim et al., 1999; Newton et al., 2009), was selected to represent the pure culture inoculum and to develop pure culture MFC-biosensors. Meanwhile, the effluent taken from a stably operating MFC based on mixed culture was utilized to construct traditional mixed culture MFC-biosensors. Herein, a systematic analysis of the effect of inoculum on sensor sensitivity was provided by employing the two types of MFC-biosensors for both BOD and biotoxicity determination. Furthermore, anodic biofilm properties and the production of EPS were both assessed to illustrate the underlying biological mechanism.

2. Materials and methods

2.1. Chemicals and reagents

The inoculum of the mixed culture was collected from the effluent of an acetate-fed MFC which had been steadily operated for over one year. *S. loihica* PV-4 was purchased from American type culture collection and preserved in a −80 °C refrigerator. Before inoculation, *S. loihica* PV-4 was twice activated in a shaking flask (23.0 °C, 200 rpm) containing Luria-Bertani medium overnight. During all the experiments, the same anolyte and catholyte were used in these two types of MFCs. The anolyte used in this study consisted of 2.50 g $NaHCO_3$, 0.08 g $CaCl_2{\cdot}2H_2O$, 1.00 g NH_4Cl, 0.20 g $MgCl_2{\cdot}6H_2O$, 10.00 g NaCl and 7.20 g HEPES in 1.00 L deionized water. Specific volume of substrate stock solution, which contained 112.00 g/L sodium lactate and 50.00 g/L yeast extract, was added to fresh anolyte as carbon sources. Anolyte was sterilized prior to use and preserved in a clean bench (SW-CJ-1F, Airtech, China). The catholyte consisted of 10.00 g NaCl and 7.20 g HEPES in 1.00 L deionized water. For biotoxicity determination, $CdSO_4$ and chlortetracycline hydrochloride (CTC), which respectively represented the pollution of heavy metal and antibiotic, were individually dissolved in deionized water to prepare 1.0 g/L toxic stock solutions and kept at 4.0 °C after filtration. Toxic and non-toxic anolyte were then prepared by adding a specific volume of toxic stock solution and the same volume of solvent in fresh anolyte, respectively.

2.2. MFC construction and start up

Eight MFCs (MFC1-8) with dual-chamber configuration were constructed and divided into two groups. MFC1-4 were designed to investigate the effect of inoculum on sensor sensitivity for BOD and biotoxicity determination while the other group (MFC5-8) was set for the analysis of biofilm. The total volumes of anode and cathode chambers were 14 mL and 42 mL, respectively, and the two chambers were separated by a proton exchange membrane (Nafion 117, Hesen Electric Corporation, China). A 3.0 cm × 2.5 cm piece of carbon cloth (HCP330, Hesen Electric Corporation, China) with ammonia pretreatment was used as anode while cathode consisted of two pieces of 0.5 mg/cm^2 Pt loaded carbon paper with an area of 2.0 cm × 2.0 cm. Anode and cathode were connected with an external resistor of 330 Ω via titanium wire. After fabrication, all the MFCs were sterilized and inoculated in the clean bench. Then, both mixed microbial consortium and *S. loihica* PV-4 were diluted to the same viable cell concentration, and were immediately used to inoculate MFC1-8 with the same inoculum size of 30% (v/v). MFC1-2 and MFC5-6 were inoculated with the mixed consortium while the others with *S. loihica* PV-4. All the anode chambers were finally filled with sterile anolyte containing 1.12 g/L sodium lactate and 0.50 g/L yeast extract as fuel, and all the operation parameters were consistent in each MFC except for inoculum. Self-circulation at a flow rate of 2 mL/min was operated inside the anode chamber via a peristaltic pump (BT100-1L, LongerPump, China) to obtain a hydraulic retention time of 7 min. Anolyte was forced to work in the flow-by-anode mode to improve sensor performance (Jiang et al., 2015). In addition, all MFCs were refreshed simultaneously in

sterile environment during the startup process, and all the experiments were conducted at a temperature of 25.0 ± 0.5 °C.

2.3. Biosensor tests

After startup process and stable operation for 10 d, biosensor tests for the determination of BOD and biotoxicity were performed under the continuous flow mode. Before each test, the continuous flow pipelines were sterilized by 3% hydrogen peroxide. For BOD monitoring, different volumes (0.0 mL, 0.5 mL, 1.0 mL, 1.5 mL) of substrate stock solution were added to 1.0 L sterile anolyte respectively. Anolyte containing various concentrations of organic matters were all sampled for BOD quantification according to the standard method (MEPC, 2009) and then continuously fed into the anode chamber after nitrogen sparging in proper sequence until a stable voltage was observed. In toxic agent tests, the concentrations of Cd^{2+} and CTC were 1.5 mg/L and 1.0 mg/L with reference to a previous study (Kim et al., 2007), and the concentration of the substrate stock solution in non-toxic and toxic anolyte was both switched to 1.50 mL/L during the test period to enhance the sensor sensitivity (Jiang et al., 2016). Each biotoxicity test included the following procedures. Firstly, non-toxic anolyte was continuously pumped into the anode chamber to obtain a stable baseline of output voltage. Secondly, the influent was switched to the toxic anolyte and the toxic shock would last for 1 h. Finally, the toxic anolyte was evacuated and the non-toxic anolyte was continuously pumped into the anode chamber to avoid irreversible damages to the biofilms induced by residual toxic agents. Output voltage and internal resistance of each MFC were both measured after each test to ensure the complete recovery of the sensors. Each test was performed in triplicate for each MFC and output voltages during all the tests were recorded for subsequent analysis.

2.4. Analysis of biofilm properties

3.0 cm × 0.3 cm pieces of carbon cloth colonized with biofilms were taken from MFC5-8 after 10 d of stable operation for the assessment of the effect of inoculum on anode biomass, respectively. Subsequently, another 3.0 cm × 0.3 cm pieces of anodes were sampled from MFC5-8 to measure the biofilm thickness, cell viability and EPS content before and after the toxic shocks as described in Section 2.3. Sterilized scalpel was used to obtain the biofilm sample from the same position of the anode in each MFC.

For the quantification of biomass, the anode samples were washed with 100 mM phosphate buffer saline (PBS) and then electrode-attached microbes were collected using a vortex shaker (1000 rpm). Total bacterial biomass was determined based on adenosine triphosphate (ATP) by a bioluminometer (Model 3560, New Horizons Diagnostics, USA).

The latter pieces of anode samples were divided into 2 parts. One part (0.3 cm × 0.3 cm) was used for the measurement of the biofilm thickness and cell viability. Firstly, the biofilm samples were stained with fluorescein diacetate (FDA) and propidium iodide (PI) to display alive and dead microbes (Xiao et al., 2011). Then, images were acquired with a confocal laser scanning microscope (CLSM, TCS SP8, Leica, Germany) with the specific excitation/emission wavelengths (480/530 nm for FDA and 488/660 nm for PI). Microscope photos were all obtained under Z-stack mode with a step of 1 μm and the set of images was processed with Leica Application Suite X for 3D reconstructions. The thickness and integrated optical density of three-dimensional biofilm structure were analyzed using the software ImageJ. For each sample, photos were taken at least three different locations.

The other part (2.7 cm × 0.3 cm) of the anode samples was utilized to determine the EPS content in biofilms. The anode microorganisms were firstly removed from biofilm samples by vortex oscillation at 1000 rpm. Then EPS was extracted using a modified heat extraction method with a water bath of 80 °C for 30 min (Li and Yang, 2007). Prior to the quantification of polysaccharide (PS) and protein (PN), samples were concentrated via a vacuum centrifugal concentrator (RVC2-18, Christ, Germany). Subsequently, PS components were determined by anthrone-sulfate method (Roe, 1955) with glucose as the standard while PN components were measured based on Bradford method (Bradford, 1976) with bovine serum albumin as the standard.

2.5. Illumina sequence of the mixed culture biofilm

High-throughput sequencing technology was employed to analyze the microbial composition of the mixed culture biofilm. After startup process and stable operation for 10 d, two pieces of carbon cloth (3.0 cm × 0.3 cm) were collected from the anode of MFC5-6. Sampling technique was mentioned above and the sample was stored at −80 °C for further analysis. To avoid the interference on other subsequent experiments induced by the difference in anode area between the two inocula, the same size of carbon cloth was also cut down from MFC7 and MFC8, respectively. DNA extraction and the quality control of the DNA sample were performed according to a previous work (Xie et al., 2016). Then a pair of primers (338F, 806R) was used to amplify the hypervariable region V3-V4 of the 16S rRNA gene by polymerase chain reaction (PCR) and the PCR product was sequenced by Illumina Hiseq platform at Biomarker Technologies Co., Ltd., China.

2.6. Analysis

Output voltage of MFC was recorded by a data acquisition system (USB-1608FS, Measurement Computing Corporation, USA) with a sampling interval of 1s. Electrochemical impedance spectroscopy (EIS) was used to evaluate the internal resistance of MFC in an electrochemical workstation (Zennium E, Zahner, Germany). Impedance spectrum was obtained in the dual-electrode system with a frequency range of 100 mHz–10 KHz and a signal of 10 mV amplitude. EIS data was then fitted with an equivalent circuit model (CPE_1/R_a + R_s + CPE_2/R_c) to classify internal resistance. In this model, R_s represented ohmic resistance; R_a and R_c accounted for the polarization resistance of anode and cathode respectively; CPE_1 and CPE_2 are both constant phase elements simulating nonideal capacitors (Zhu et al., 2013). To eliminate the interference of baseline signal, the inhibition ratio (IR) of MFC voltage was employed to assess the sensor sensitivity and calculated according to Eq. (1). It was defined as the ratio of voltage decay to output voltage before toxic shock:

$$IR = (V_n - V_t)/V_n * 100\% \quad (1)$$

where V_n was the voltage before the toxic shock and V_t was the voltage after exposure to the toxic pollutant for 1 h. Viability of biofilm (VB) was characterized by the proportion of live cells based on Eq. (2):

$$VB = Int_L/(Int_L + Int_D) * 100\% \quad (2)$$

where Int_L and Int_D referred to the integrated optical densities of living cells and dead cells respectively.

3. Results and discussion

3.1. Effect of inoculum on startup process and internal resistance

A remarkable difference in the output voltages of the two types MFCs during startup process could be observed from Fig. 1a. An apparent voltage about 10.4 mV was produced immediately after the inoculation of *S. loihica* PV-4. This phenomenon suggested an ability to generate electricity by suspended bacteria of this strain (Newton et al., 2009). The output voltage of *S. loihica* PV-4 MFC increased rapidly in the first batch and achieved a maximum value of 83.3 mV, which might be due to that exoelectrogens could adhere to anode surface and developed redox components to accomplish extracellular electron transfer in a short time (Stöckl et al., 2016). In contrast, only a faint voltage (less

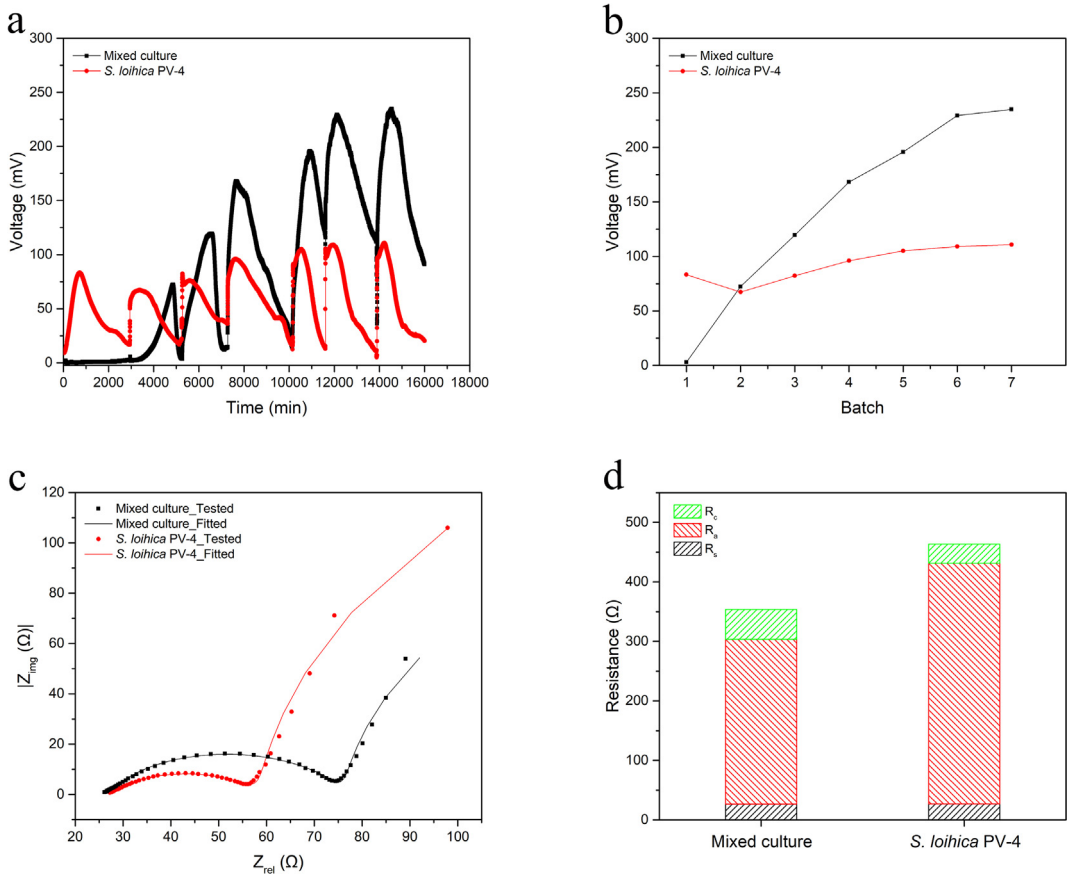

Fig. 1. Effect of inoculum on startup process and internal resistance: (a) evolution of voltage generation in the first seventh batch; (b) maximum output voltages in the first seventh batch; (c) Nyquist impedance spectra and fitting curves; (d) components of internal resistance obtained by model simulation.

than 1.0 mV) appeared in the MFC with the mixed culture after inoculation and it sustained for approximate 3000 min. This indicated that the planktonic cells of the mixed culture were barely able to generate electricity. The long lag period after the inoculation of mixed culture suggested that electroactive microorganisms needed some time for the stepwise adaptation to the anode environment and finally occupied a dominate proportion. This might be due to that the change of substrate (from acetate to sodium lactate and yeast extract) caused complex interactions inside the microbial community (Bakonyi et al., 2018).

Moreover, the startup time for MFCs to produce repeatable current was different between the two inocula. As shown in Fig. 1b, except a little decrease in the second batch, the maximum voltage of *S. loihica* PV-4 MFC slightly rose and remained stable after the fourth batch. However, as for the MFC with the mixed consortium, after the long lag period, sharp increase of the maximum voltage could be observed in each batch until it stabilized in the seventh batch.

After startup process, a higher voltage (234.9 mV) across the same external resistor (330.0 Ω) was observed in the MFC developed with the mixed culture. Since both MFC configuration and operation conditions were identical, this discrepancy might derive from various anode microbes, which could be also testified by the results of EIS as shown in Fig. 1c. It could be seen that the experimental data were well fitted using the circuit model mentioned above. The components of the internal resistance were present in Fig. 1d. The total internal resistance was 353.52 Ω for the mixed culture, compared with 463.44 Ω for *S. loihica* PV-4. Notably, the R_a of the mixed culture MFC was obviously lower than that of the *S. loihica* PV-4 MFC while both R_c and R_s remained similar. Given that higher R_a was generally considered to be indicative of poorer electrochemical activity (He et al., 2008), the difference in R_a was probably the reason why the MFC using the mixed culture produced a higher voltage. Previous studies also reported the superior current-generating capability in MFCs inoculated with complex microbes (Rabaey et al., 2004; Ishii et al., 2008; Hassan et al., 2012). The reason for this phenomenon might be ascribed to the more sophisticated metabolic pathway and correspondingly more diverse extracellular electron transfer process. However, some studies presented different results when comparing the bioelectricity production by mixed and pure cultures. This discrepancy could be due to the different inocula of mixed cultures which might lack electroactive microorganisms (Nimje et al., 2012; Varanasi et al., 2016). In this study, the effluent of a well-operated MFC, which was favorable for the enrichment of exoelectrogens (Kim et al., 2005), was used as inoculum for the mixed culture MFCs, and electroactive microorganisms such as *Geobacter* and *Shewanella* were found to play a dominant role in the anode biofilms (Supplementary Material). To sum up, different inocula could affect the start-up process and electricity performance (Kumar et al., 2017), which might further impact the sensor performance (Modin and Wilén, 2012).

3.2. Effect of inoculum on sensor sensitivity

When stable operation, differently inoculated MFCs were fed with anolyte at several BOD values (0.00–65.25 mg/L) successively. Fig. 2a displayed the voltage responses to various BOD concentrations. It was clear that output voltage could rapidly change to a novel steady state (defined as stable voltage, V_s) when a different concentration was applied. However, the relationship between V_s and BOD concentration followed different patterns between the two types of MFCs (Fig. 2b). For the mixed culture MFC, V_s continuously ascended along with the increase of BOD value in the range of 0.00–65.25 mg/L. Yet for the *S. loihica* PV-4 MFC, only a slight variation in V_s could be observed when

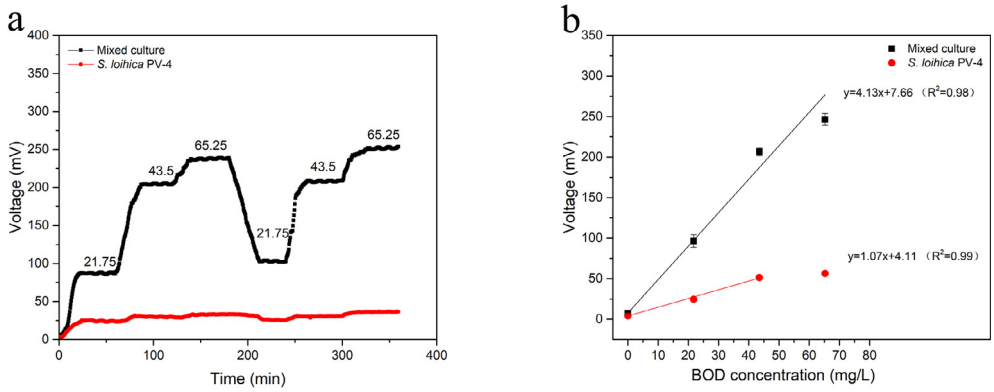

Fig. 2. Effect of inoculum on sensor sensitivity for BOD assessment: (a) voltage responses to various BOD concentrations; (b) stable voltage as a function of BOD concentration.

the BOD value increased from 43.50 mg/L to 65.25 mg/L, which suggested that exoelectrogens attained the maximum metabolic rate due to the substrate saturation. This result indicated that MFC-biosensor developed with the mixed culture achieved wider response range than that with the pure culture of *S. loihica* PV-4 when monitoring BOD. Two good linear relationships were observed between V_s and BOD concentrations from 0.00 mg/L to 65.25 mg/L for the mixed culture MFC and from 0.00 mg/L to 43.50 mg/L for the *S. loihica* PV-4 MFC. The sensitivity was further analyzed by comparing the slope of the linearly fitted line (Lorenzo et al., 2014). As shown in Fig. 2b, the sensitivity of the mixed culture MFC was 3.86 times that of the MFC developed with *S. loihica* PV-4, which implied a better performance in the MFC-biosensor constructed with the mixed culture. Similar phenomenon was also reported by Anam et al. (2017) when comparing complex microbes and artificially designed bacterial consortia as sensing elements. This result was probably due to the higher bioactivity and substrate consumption rate of the anode biofilm with a mixed culture. Overall, higher sensitivity and wider response range made the mixed culture MFC more favorable for the assessment of BOD concentration.

Cd^{2+} and CTC were employed as toxic agents to study the effect of inoculum on the sensor sensitivity for biotoxicity determination. Fig. 3a displayed the voltage responses to the shock of 1.5 mg/L Cd^{2+}. It could be seen that output voltages of both types of MFCs maintained steady states with variances less than 5% before the toxic event. Once exposed to 1.5 mg/L Cd^{2+}, MFCs presented varying degrees of decreases in output voltages. As shown in Fig. 3b, the IR of the mixed culture MFC was less than half of *S. loihica* PV-4 (9.83% vs. 22.77%). In the case of 1.0 mg/L CTC, higher sensitivity was also obtained in the MFC developed with *S. loihica* PV-4 with an IR of 25.12%, which was 1.78 times that of the mixed culture MFC. These results both indicated that inoculum could obviously affect sensor sensitivity for biotoxicity assessment. So far, the utilization of a pure culture in MFC as sensing element was seldom concerned and comparative analysis of sensor performance for toxicity monitoring with a mixed culture was still unexplored. This study demonstrated for the first time that MFC-biosensor developed with a pure culture might be more favorable for the assessment of biotoxicity.

However, microbiological contamination might be a threat when pure culture MFC-biosensor is applied in the practical application. Thus, the pretreatment of water sample is necessary. Strainer together with fine filtration through 0.22 μm pore size membrane might be applied to remove natural microflora in water samples (Reiche and Kirkwood, 2012; Bakonyi et al., 2018). Besides, whether the pure culture MFC with mature anodic biofilm can resist microbiological contamination should be considered. Thus, the predominance of *S. loihica* PV-4 in anode microbial composition after invasion of microbes would be assessed in the future studies.

On the other hand, the pure culture could also be considered as bioaugmentation for mixed culture MFC to enhance the sensitivity and to avoid microbiological contamination. Previous studies have proved the feasibility of employing *Shewanella* for bioaugmentation on MFC performance (Raghavulu et al., 2012; Kumar et al., 2016). In this study, *Shewanella* accounted for 5.65% of the anode microbial population in the mixed culture MFC (Supplementary Material). This might suggest that the bioaugmentation of *S. loihica* PV-4 could also enhance the sensitivity of mixed culture MFC-biosensor for biotoxicity monitoring. Future studies should focus on the optimization of inoculum size as well as modes of culture addition (i.e. batch vs. sequential).

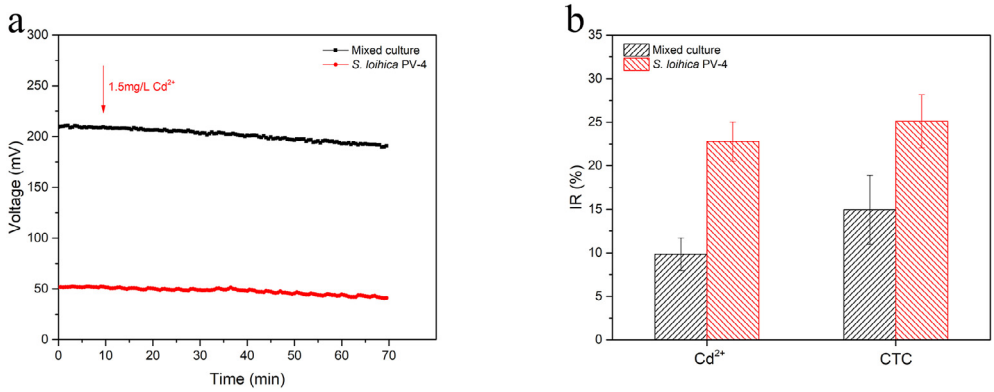

Fig. 3. Effect of inoculum on sensor sensitivity for biotoxicity assessment: (a) voltage responses to the shock of 1.5 mg/L Cd^{2+}; (b) inhibition ratios of output voltages induced by toxic shocks.

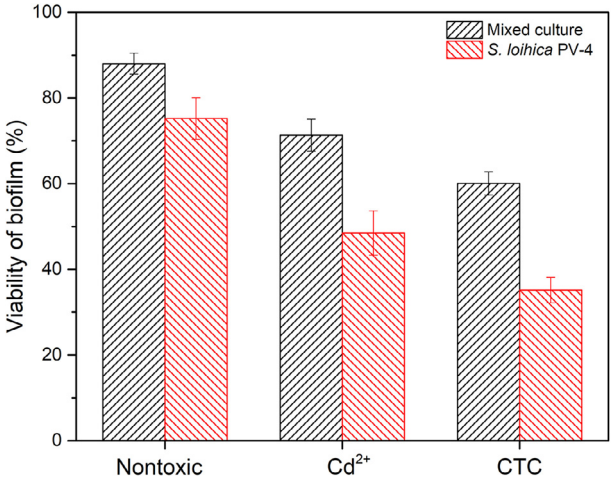

Fig. 4. Biofilm viabilities of MFCs developed with different inocula before and after toxic shocks.

Table 1
Biofilm properties of MFCs developed with different inocula.

Biocatalyst	Thickness (μm)	Integrated optical density	Biomass density (CFU/cm^2)
Mixed culture	29.62 ± 3.74	1.84E-03 ± 2.36E-04	4627 ± 515
S. loihica PV-4	34.00 ± 1.76	8.60E-04 ± 6.15E-05	2414 ± 496

3.3. Biological mechanism of discrepancies in sensor sensitivity between two types of inocula

To investigate the effect of inoculum on biofilm properties and their changes after toxic shocks, FDA/PI staining and CLSM imaging were employed to discriminate live/dead bacteria. Under stable operation, the anodes of MFCs with mixed and pure cultures were both completely colonized by biofilms (Supplementary Material). Additionally, a two-layer structure was clearly observed from the side view of CLSM images, which could be due to the relatively low anodic current density (Sun et al., 2015). This phenomenon suggested that not all of the anode biofilm was metabolically active. Moreover, the biofilm viability showed obvious difference in the two types of MFCs. The proportion of live cells in the mixed culture biofilm (88.0 ± 2.4%) was larger than that in the *S. loihica* PV-4 biofilm (75.2 ± 4.9%), indicating a higher viability in the MFC with the mixed culture (Fig. 4).

Biomass density was another important parameter of biofilm properties and it could be estimated by the integrated optical density of living cells in CLSM image (Liu et al., 2016). According to Table 1, the Int_L of the mixed culture biofilm was approximately twice as high as that of *S. loihica* PV-4. Moreover, the results of ATP bioluminescence assay presented that the biomass densities of mixed and pure culture biofilms were about 4627 ± 515 CFU/cm^2 and 2414 ± 496 CFU/cm^2, respectively, which accorded well with the data from the CLSM images. Since higher biomass density could promote the electrochemical activity of anode biofilm (Li et al., 2016a), higher biomass density and biofilm viability might lead to superior electrochemical activity and voltage-generating ability in the mixed culture MFC. This was probably the reason why the MFC-biosensor developed with the mixed culture was favorable for BOD assessment.

EPS was a vital component of microbial biofilm and served to prevent damages from toxic agents (Miao et al., 2017). As displayed in Fig. 5, the content of EPS in the MFC with *S. loihica* PV-4 was lower when compared with the mixed culture. The PS and PN contents in *S. loihica* PV-4 biofilm were only 42.19% and 67.41% of those in the mixed culture under nontoxic condition, respectively. This was probably one important reason why the MFC-biosensor developed with *S. loihica* PV-4 gave better responses to the shocks of Cd^{2+} and CTC (Fig. 3). Moreover, the two kinds of toxic shocks both promoted the secretion of PN and PS in the two types MFCs (Fig. 5), which was considered as a self-protective mechanism of bacteria to resist the invasion of toxic agents (Wang et al., 2014). Besides, the PN/PS ratios in the EPS of both biofilms increased after toxic shocks. This phenomenon was probably due to that PN components contained some functional groups which could bind toxic agents and decrease the toxicity (Wang et al., 2018). However, a poorer ability to secrete PN in *S. loihica* PV-4 was observed when compared with the mixed culture. After 1.0 mg/L CTC shock, PN content in the *S. loihica* PV-4 biofilm increased by 166.06% while that in the mixed culture elevated by 212.51% compared with respective initial content. Similar phenomenon was also observed after 1.5 mg/L Cd^{2+} shock. This result indicated an inferior self-protect capability in the *S. loihica* PV-4 biofilm, which might be another reason why the MFC-biosensor developed with *S. loihica* PV-4 gave better responses to toxic shocks.

Moreover, the biomass density of *S. loihica* PV-4 biofilm was only half that of mixed culture while the thickness of biofilms was almost equal (Table 1), which implied that the pure culture biofilm formed a looser structure. With looser structure and lower EPS content, it was expected to favor the permeation of toxic agents inside the pure culture biofilm (Shen et al., 2013). CLSM images could verify this conjecture. Meanwhile, the variances of biofilm viability showed obvious differences in these two types of MFCs after toxic shock. As presented in Fig. 4, the proportions of living cells decreased by 16.72% and 27.96% after shocks of Cd^{2+} and CTC in the mixed culture MFC while the ratios dropped by 26.73% and 40.01% with *S. loihica* PV-4, indicating a more serious damage in the pure culture biofilm. Hence, it could be conclude that lower EPS content, poorer ability to secrete PN and looser biofilm structure all accounted for the higher sensitivity in the MFC-biosensor with *S. loihica* PV-4 for biotoxicity monitoring.

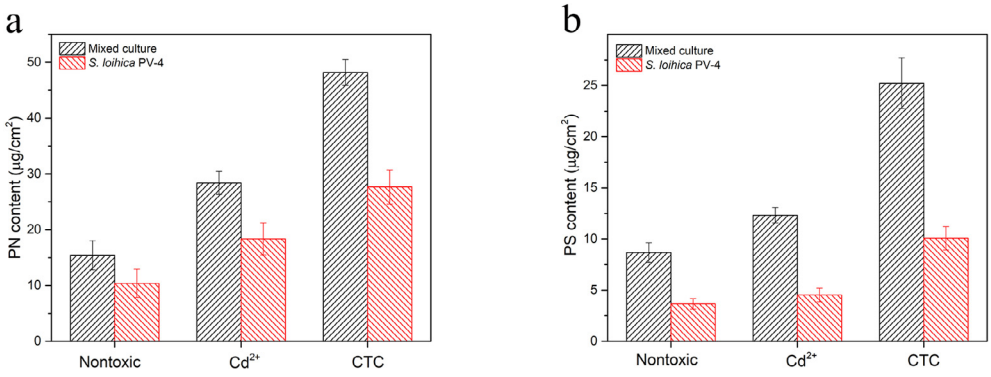

Fig. 5. EPS contents of MFCs developed with different inocula before and after toxic shocks: (a) protein component; (b) polysaccharide component.

4. Conclusions

This study demonstrated that different inocula could significantly affect the sensitivity of MFC-biosensor. Higher biomass density and cell viability of the mixed culture biofilm resulted in better performance for BOD assessment via the mixed culture MFC. Yet for biotoxicity monitoring, the *S. loihica* PV-4 MFC presented higher sensitivity due to the lower EPS content, poorer ability to secrete PN and looser biofilm structure of the pure-culture biofilm. Future studies would keep on investigating sensor performances in MFC-biosensors constructed with other exoelectrogens.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.06.037.

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