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AN IN-DEPTH STUDY OF SUBSTRATE EFFECT ON THE PERFORMANCE OF BOD SENSOR TYPE MICROBIAL FUEL CELL

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SUMMARY

Nowadays, instant assessment of the organic content in wastewater is an urgent requirement to reduce water pollution. Microbial fuel cells (MFCs) can be used as effective biosensors for rapidly measuring BOD concentration of wastewater. However, wastewaters from different sources may consist of diverse chemical components, which may affect the BOD-measuring performance of MFC-type biosensors. Therefore, in this research, we tested different input substrates for the BOD sensor type MFC (MFC_BOD) to investigate their effects on the performance of the MFC. The substrates belonging to diverse groups such as carbohydrates, organic acids, amino acids and some chlorinated compounds (xenobiotics) were tested at different concentrations equivalent to BOD levels from 10 to 200 mg L^{-1}. Concurrently, we also analyzed the alteration of the bacterial community in the anode of the MFC when tested with those different substrates by using PCR-DGGE. Our results showed that the MFC_BOD could have linear current-to-BOD responses (with the respective R^2 values >0.9) to more metabolizable substrates such as carbohydrates, organic acid and glycerol; while it responded less sensitively at different degrees to some amino acids (serine, threonine and methionine) and did not respond to chloroform and chlorobenzene (chlorinated compounds). PCA and bacterial community analysis results surprisingly imply that such different responses may be solely due to different bio(electro)chemical processes associated with the substrates but not due to changes in the composition of the bacterial community. The results suggest that, to enable the MFC_BOD to accurately sense the BODs of the wastewaters containing recalcitrant or toxic substrates, special procedures are required to enrich in the anode the bacterial communities acclimated to the substrates right from the beginning.

Keywords: BOD sensor, microbial fuel cell, operating parameters, substrate, wastewater

INTRODUCTION

One of the most common indicators of the pollution level of wastewater is Biological Oxygen Demand (BOD). BOD is the amount of oxygen required for aerobic heterotrophic microorganisms to metabolize organic compounds in water. Currently, the common method to measure BOD is BOD_5 measurement (Van Haandel, Van Der Lubbe, 2007). However, there are several drawbacks related to this method, including long measuring time, high cost and a complex procedure. Therefore, it is a requirement to find out simple and convenient methods for measuring BOD values of wastewater.

Recently, a novel BOD monitoring technology based on the use of microbial fuel cells has gained a lot of research attention due to

advantages that can enable it to meet the mentioned requirements (Do *et al.*, 2020). A microbial fuel cell (MFC) is a bioelectrochemical device operated by microbial electrochemical activity (Allen, Bennetto, 1993). Generally, an MFC includes one anode and one cathode, which is separated by a proton-exchange membrane. Microorganisms are enriched in the anode chamber, usually with organic compounds as fuel. They oxidize organic chemicals in the anode solution, producing electrons that go through electron transport chain (resulting in the production of ATP for cell survival) and are subsequently transferred to the anode functioning as the terminal electron acceptor (Logan, 2008). The two electrodes of an MFC are connected by an electric-consuming device, which in laboratory is replaced by a resistor. A membrane separating anode and cathode allows protons produced in the anode to go through to cathode. In the cathode, protons, together with electrons flowing from the anode to the cathode through the external circuit, interact with electron acceptor, usually being oxygen (Logan, 2008). The generated electrical current is thus correlating with the concentration of organic compounds in the anode influent, which is a unique characteristic making an MFC suitable for use as a BOD biosensor (Kim *et al.*, 2003).

In 1977, Karube *et al.* first reported a BOD sensor by using MFC operated by *Clostridium butyricum* fixed on the electrode. Subsequently, MFC-based BOD sensors have been well studied and developed. In 1999, Kim *et al.* reported that the electron mediators were not required for MFC operation, which rendered the study on MFC-based BOD sensors to become easier. Since then, significant progresses have been made with MFC-based BOD sensor research. For instance, miniaturized single-chambered MFC-based BOD sensors have been developed (Di Lorenzo *et al.*, 2009). Moreover, MFC-bassed BOD sensors capable of distinctively measuring BOD values of different types of wastewater have been also reported recently (Hsieh *et al.*, 2016).

BOD measurement of wastewater by MFC-typed biosensors is advantageous, compared with the conventional BOD_5 test. It takes a significantly shorter time for MFC-based BOD measurement compared with BOD5 test (minutes or hours compared with several days) and the MFC system can be maintained for long (up to 5 years) (Kim *et al.*, 2003). Therefore, the cost of MFC-based BOD sensor is highly competitive with other BOD measuring methods. Moreover, the MFC-based BOD measurement procedure can be simpler than the conventional BOD test. In addition, the detection range that MFC-based BOD sensor can offer is around 3–200 mg BOD L^{-1}, which makes this new technology quite competitive to the conventional.

In a MFC, the substrate is one of the most important factors affecting the electricity-generating capability of the MFC because it determines the nutrient source for anode bacteria to metabolize (Do *et al.*, 2020). Electrochemical bacteria are able to use different carbon sources to produce energy (Ghoreyshi *et al.*, 2011). Many studies chose pure compounds as carbon sources, such as glucose, ethanol or cysteine, for anode bacteria (Logan *et al.*, 2005; Kim *et al.*, 2007; Tuan *et al.*, 2014). Moreover, MFCs could be operated with mixed nutrient sources from wastewaters: e.g. those from beer factory (Feng *et al.*, 2008), paper factory (Lu *et al.*, 2009) or mixtures of them (Liu *et al.*, 2004). Those various substrates may predictively affect the BOD sensing ability of an MFC-based BOD sensor. Thus far there has been only one study about this matter but with an artificially constituted mixed culture in the MFC anode (Hsieh *et al.*, 2016). In that study, the responses of the MFC-based sensor to changes of the anode BOD concentration were relatively stable when the MFC was operated with different substrates. However, most MFC-based BOD sensors should be operated with a naturally enriched microbial consortium so that the device can practically work in open environments, avoiding complex handling (Chang *et al.*, 2004; Di Lorenzo *et al.*, 2009). Therefore, in this study, we investigated the effect of different inputs substrates on the

performance of a BOD sensor type MFC operated with a naturally enriched microbial consortium. Our interesting results suggested different responses of the device to different substrates. Simultaneously, we also studied the anode bacteria community shifts in response to different substrates. Based on that, more insights into the responses of the device to the substrates were provided.

MATERIALS AND METHODS

Fabrication and operation of BOD sensor type MFCs

Each BOD sensor type MFC (abbreviated MFC_BOD) used in this study was fabricated according to Tuan *et al.* (2014). Its design was actually modified from Chang *et al.* (2004). In brief, an MFC_BOD was constructed with two polyacrylic plastic inner frames (50 x 90 x 20 mm^3), covered and hold by two polyacrylic plastic outer frames of the same size. The two inner frames were carved to form the anode and cathode chambers each with the size of 10 x 50 x 15 mm^3. The two chambers were separated by a Nafion 117 membrane (DuPont Corp., USA). Inside each chamber, a graphite felt electrode (9 x 45 x 5 mm^3) was installed. A rubber sheet (50 x 90 x 2 mm^3) was placed between any two adjacent frames. The whole structure was assembled tightly with nuts and bolts until it was leakage-free. Each MFC_BOD was operated with a 10-Ω external resistor connecting to the electrodes through copper wire.

The anode bacteria in the MFC-BODs were enriched from multiples source, including: (i) soil samples from Fansipan (Lao Cai, Vietnam), Cuc Phuong National park (Ninh Binh, Vietnam); (ii) mud samples from Van Long (Ninh Binh, Vietnam), Xuan Thuy National park (Nam Dinh, Vietnam); (iii) sludge from Hanoi Beer company (Hung Yen, Vietnam); (iv) wastewater and sewage sludge mixture from multiple industrial villages, including Phong Khe paper industrial village, Yen Hoi metal recycling village and textile staining village (Bac Ninh, Vietnam), plastic can recycling village (Hung Yen, Vietnam). The enrichment of the anode bacteria was carried out with artificial wastewater containing glucose and glutamate as the substrates (and the BOD contents with the concentration of 10 mg L^{-1}), as stated in previous studies (Tuan *et al.*, 2014). Accordingly, the enrichment was considered complete when the currents generated by the MFC_BODs were stable. By default, the MFC_BODs were operated in continuous mode with the anode feeding rate of 0.3 mL min^{-1} and the cathode fed continuously with air-saturated tap water. Before the MFC_BODs were subjected to any test, they were operated with artificial wastewater containing glucose and glutamate (at 10 mg BOD L^{-1}). For the experiments testing the MFC_BODs with different substrates (see below), glucose and glutamate in the artificial wastewater were replaced with the respective substrates at the tested BOD-equivalent concentrations.

Experiments testing the BOD sensing responses of the BOD sensor type MFC with different substrates

By default, the MFC_BODs were maintained by 10 mg L^{-1} of each tested substrate for 3 days before testing so that the anode microbial population could adapt to the alteration. The test with each substrate was conducted by changing the substrate concentration in the anolyte from 10 to 200 mg L^{-1}. These substrate concentration changes were done by dilution using a stock solution containing 500 ± 10 mg BOD L^{-1} for each substrate, which was always checked by the BOD_5 method before the experiment. With each substrate, 2 MFC_BODs were tested as such while 1 control MFC_BOD was only operated with 10 mg L^{-1} of the substrate.

The tested substrates include some representatives of different organic contaminant groups. These are acetic acid and lactic acid (the organic acids that can be used as essential carbon sources by bacteria); threonine, methionine, and serine (amino acids); sucrose and starch (carbohydrates); and some other types of chemicals such as glycerol (a common substrate

used by many bacteria), benzoate (an aromatic compound), chloroform (a halogenated compound) and chlorobenzene (a halogenated aromatic compound).

PCR-DGGE analysis of the bacterial communities of the MFC_BODs tested with different substrates

After the tests (with different substrates), the anodic electrode of each MFC_BOD was sampled and genomic DNA of bacteria on the electrode was extracted, as previously described (Nguyen *et al.*, 2015). Briefly, a 10 x 10 mm^2 piece of the electrode was crushed and ground in about 1 mL of 0.9% NaCl solution before the resulted suspension was centrifuged at 5000 rpm for about 15 min. The pellet was subsequently resuspended in 500 µL phosphate buffer saline (pH 7, 0.9% NaCl) before mixed with 500 µL of a 25 phenol: 24 chloroform: 1 isoamyl alcohol solution (v/v/v). The genomic DNA was collected by ethanol precipitation after 15 min centrifugation at 14000 rpm at room temperature. The product was checked by electrophoresis in 1% agarose gel. The respective 16S rRNA gene fragments in the sample were amplified by PCR from the extracted DNA, using two primers: P63F (5'CAGGCCTAACACA TGCAAGTC3') and P1378R (5'CGGTGTGTAC AAGGCCCGGGAACG3'). The highly variable V3 fragments were amplified from the 16S rRNA gene fragments (the PCR products above) obtained, using the primers P338fGC and P518R (Muyzer *et al.*, 1993). The PCR products were also checked by electrophoresis.

For denaturing gradient gel electrophoresis (DGGE), the PCR products of interest were loaded into a 6% polyacrylamide gel, with a urea/formamide denaturing gradient from 45% to 60%. Electrophoresis was conducted in 1x TAE buffer with a DGGEK-2401 system (C.B.S Scientific, USA), at 38V, at 60°C for 16 hours. After electrophoresis, the gel was stained in HydraGreen Safe DNA Stain for 30 minutes and then observed by a LMW-20 UVP (UK).

Data acquisition and analysis

The cell voltage (U, in mV) was recorded automatically by a Keithley 2700 multimeter (Tektronix, OH, USA) every minute. The electrical current (I, in mA) was calculated according to the Ohm's law. The data were analyzed and plotted in graphs by using Microsoft Excel.

Input data for principal component analysis (PCA) include electrical current data and DGGE data. Electrical current data include the normalized current values, each of which is the factor by which the current of a MFC_BOD in the respective experiment (with a substrate at a certain BOD concentration) differed from that of the respective control (the MFC_BOD operated with 10 mg L^{-1} BOD of the respective substrate). DGGE data include the digitalized values of the bands in the DGGE patterns (where the bands at the same position were assigned the same number). PCA was done by R using DESeq2 package.

RESULTS

Sensing performances of MFC_BODs with some organic acids

We used acetic acid and lactic acid to investigate the sensing performances of the MFC_BODs to the organic acids that can be used as essential carbon sources by bacteria. The electrical currents of the tested MFCs increased in a concentration-dependent manner until the substrate concentration reached 200 mg L^{-1} (R^2 = 0.94 and 0.93 for the two MFC_BODs operated with acetate, and 0.96 and 0.92 for those with lactate), while that of the control MFC, for which the substrate concentration was constantly kept at 10 mg L^{-1}, did not (Fig. 1). Therefore, the alteration of the current was actually due to the change in substrate concentration. However, the response trend and range varied significantly between different MFCs tested with different organic acids. The optimal range of organic acid BOD that the MFCs could respond most sensitively was 10–150 mg L^{-1} for acetate (Fig. 1A) and 50–150 mg L^{-1} for lactate (Fig. 1B), respectively. Thus, it seems that the type of organic acid BOD can affect the BOD-sensing performance of the MFC_BODs.

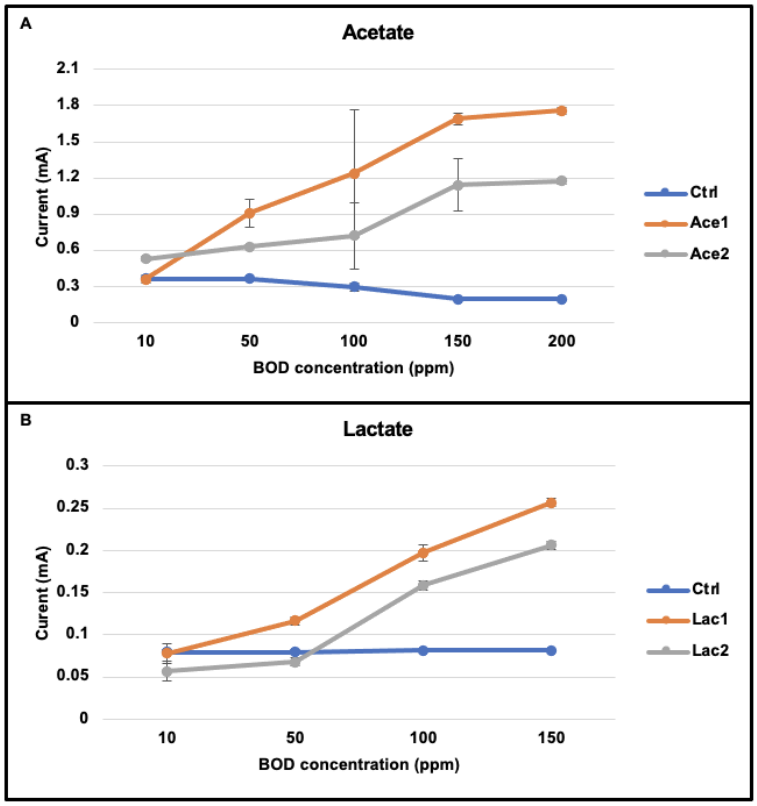

Figure 1. Electrical currents of the MFC_BODs in the test with some organic acids. (A) The test with different BOD-equivalent concentrations of acetic acid; (B) The test with different BOD-equivalent concentrations of lactic acid. Control (Ctrl) was maintained with 10 mg BOD L^{-1} throughout the experiment.

Sensing performances of MFC_BODs with amino acids

We tested the MFC_BODs with several amino acids, including threonine, methionine, and serine. The currents generated by the MFC_BODs increased when we increased the input BOD, while the current of the control MFC_BOD, which was maintained with 10 mg L^{-1} amino acid BOD, did not significantly alter (Fig. 2). By testing with threonine, we found that the current only reached approximately 0.02 mA when the input BOD was changed from 10 to 200 mg L^{-1} (Fig. 2A). Nevertheless, there was a linear correlation between input threonine concentration and the current (R^2 = 0.95 and 0.98, for the two MFC_BODs operated with threonine). Similar with the MFC_BODs operated with threonine, the MFC_BODs operated with methionine also generated increasing currents when the substrate concentration increased. However, the relationship between the methionine concentration and the current was not perfectly linear (R^2 = 0.83 and 0.98 for the two respective MFCs) (Fig. 2B). Interestingly, MFC_BODs seemed to be "numb" with serine when its concentration was lower than 100 mg BOD L^{-1}. The respective MFC_BODs started to respond when the input serine concentration reached 150 mg BOD L^{-1} and higher (Fig. 2C). Therefore, 100 mg BOD L^{-1} was the lower threshold for the MFC_BODs to detect serine. In conclusion, we can see that the sensing ability of the MFC_BODs varied with different amino acids as substrates. The optimal BOD range that the MFC_BODs could sensitively detect was from 10 mg L^{-1} for either threonine or methionine, and from 100 mg L^{-1} for serine.

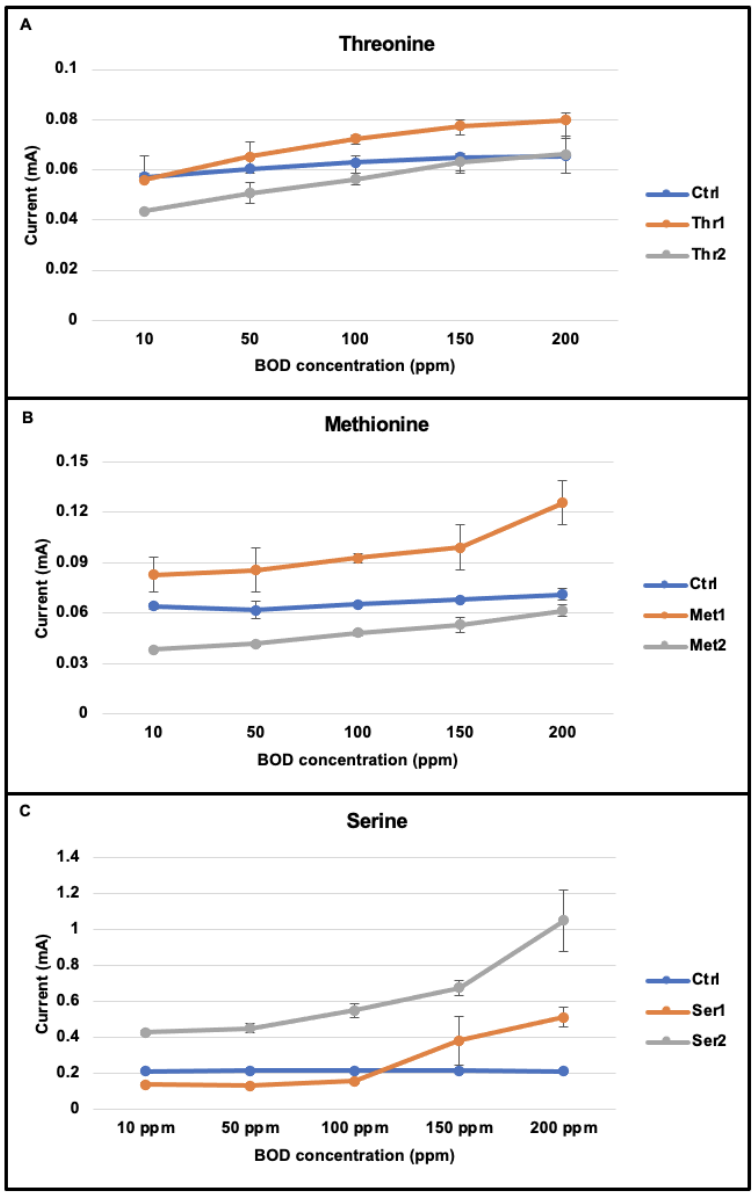

Figure 2. Electrical currents of the MFC_BODs in the test with some amino acids. (A) The test with different BOD-equivalent concentrations of threonine; (B) The test with different BOD-equivalent concentrations of methionine; (C) The test with different BOD-equivalent concentrations of serine. Control (Ctrl) was maintained with 10 mg BOD L^{-1} throughout the experiment.

Sensing performances of MFC_BODs with some carbohydrates

The performance of MFC_BODs in sensing carbohydrates was evaluated through testing with sucrose and starch. For both of those carbohydrate substrates, there was an obvious correlation between the generated current and the BOD concentration when the BOD concentration was increased, while no such correlation was observed with the control MFC_BOD (Fig. 3). Regarding the sensing range of the MFC_BODs with starch, the maximum BOD concentration that they could

detect was 120 mg L^{-1} (Fig. 3A). Within that range, the current and the BOD concentration had a nice linear correlation (R^2 = 0.96 and 0.93 for the respectively tested MFCs). When the input BOD was higher than 120 mg L^{-1}, the current slightly decreased and later seemed to stay steady (Fig. 3A). The responses of the MFC_BODs operated with sucrose were also similar: the currents increased with increasing input BOD concentrations to less than 120 mg L^{-1} (R^2 = 0.91 and 0.92) but did not increase further and even slightly decreased when the BOD reached 120 mg L^{-1} and higher (Fig. 3B). Therefore, we hypothesize that the maximum detection limit of MFC_BODs with sucrose and starch is approximately 100–120 mg BOD L^{-1}.

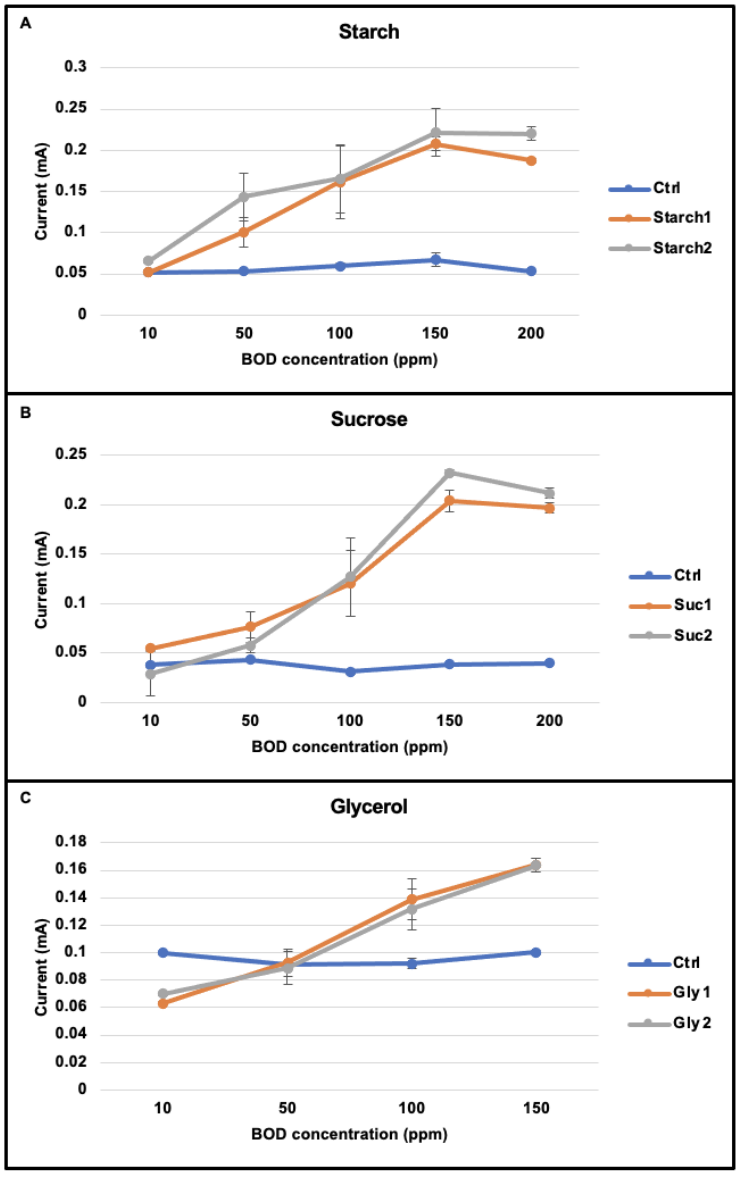

Figure 3. Electrical currents of the MFC_BODs in the test with some carbohydrates and glycerol. (A) The test with different BOD-equivalent concentrations of sucrose; (B) The test with different BOD-equivalent concentrations of starch; (C) The test with different BOD-equivalent concentrations of glycerol. Control (Ctrl) was maintained with 10 mg BOD L^{-1} throughout the experiment.

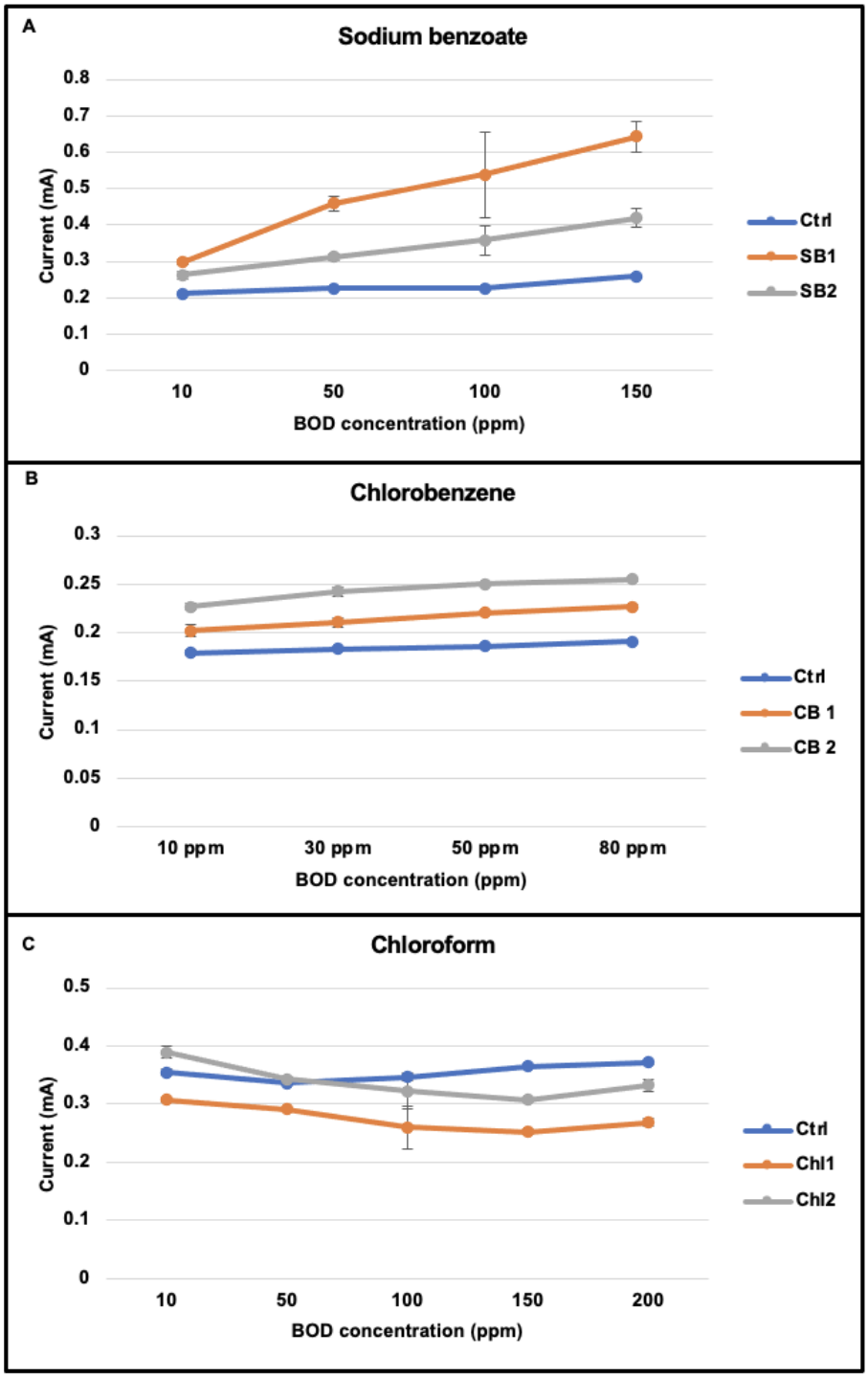

Figure 4. Electrical currents of the MFC_BODs in the test with some other chemicals. (A) The test with different BOD-equivalent concentrations of benzoate; (B) The test with different BOD-equivalent concentrations of chlorobenzene; (C) The test with different BOD-equivalent concentrations of chloroform. Control (Ctrl) was maintained with 10 mg BOD L^{-1} throughout the experiment.

Sensing performances of MFC_BODs operated with glycerol and other substrates

In terms of sensing glycerol, the MFC_BODs was more sensitive than those to the above-tested amino acids: the currents of the two tested MFC_BODs both increased 2 folds when the BOD concentration increased from 10 to 150 mg L^{-1}, in a nicely linear relationship (R^2 = 0.98 and 0.99 for the two MFCs) (Fig. 3C). Concurrently, the current of the control MFC_BOD, which was continuously maintained with 10 mg BOD (glycerol) L^{-1}, had no significant alteration (Fig. 3C).

When tested with sodium benzoate, the currents of the MFC_BODs also increased upon the increase of the input BOD concentration. The tested MFC_BODs responded well to the BOD-equivalent concentration of sodium benzoate in the range of 10–200 mg L^{-1}, in a good linear relationship (R^2 = 0.96 and 0.97) (Fig. 4A).

Contrary to the tests with other substrates, in the tests with chloroform and chlorobenzene, the MFC_BODs had no response to changes in BOD concentration (Fig. 4B and C). There was no significant difference between the responses of the currents of the tested MFC_BODs and those of the control (Fig. 4B and C). The average currents of the tested MFC_BODs when operated with chloroform or chlorobenzene could not exceed 0.1 mA, which is significantly lower compared with those obtained with other substrates. Therefore, it seemed that our MFC_BODs were less sensitive to and even inhibited by chloroform and chlorobenzene.

Principal component analysis (PCA) of the relationships among the MFC_BODs tested with different substrates by using the current level data

PCA was conducted to compare the BOD-sensing responses of the MFC_BODs operated with different substrates, based on their generated currents. From the result (Fig. 5), we can see that the data points corresponding to the MFC_BODs were relatively well-grouped by the substrates fed to them. Apparently, the data points of the MFC-BODs operated with the chlorinated aromatic compounds, chloroform and chlorobenzene, were close to each other and separate from the other data points (Fig. 5). This clearly indicates the different responses of the MFC-BODs operated with those chlorinated compounds, in comparison with those of the others MFC_BODs operated with the other substrates. This is also consistent with the observations of the current-to-BOD response patterns (Fig. 1–4). Furthermore, the PCA result (Fig. 5) indicates that the responses of the MFC_BODs operated with the amino acids were more similar to each other than to those of the other MFC_BODs. The same observation is for the responses of the MFC_BODs operated with essential carbon sources such as carbohydrates, acetate or lactate. Therefore, we can conclude that MFC_BODs display relatively different BOD-sensing performances when operated with substrates of different chemical natures.

Analysis of the bacterial communities in the anodes of the MFC_BODs tested with different substrates

Previous studies have proven that microorganisms at anodes of MFCs are solely bacteria (Logan, 2008). Therefore, in this study we only investigated how the bacterial communities at the anodes of the MFC_BODs changed after tested with different BOD substrates. Such investigation was done by DGGE analyses, which showed some changes in the bacterial communities after the MFC_BODs were tested with different BOD substrates (Fig. 6). However, a PCA of the DGGE results shows that most of the data points of the MFCs were close together (Fig. 7), suggesting that the respective bacterial communities were actually not so much different from each other. Only a community of one MFC_BOD tested with sucrose appeared to be dissimilar with the other (of the two such MFC_BODs) and with the rest of the communities (Fig. 6, Fig. 7). The same observation was also for a community of one MFC_BOD tested with methionine (Fig. 6, Fig. 7). The latter results are difficult to explain but

in general the PCA of the DGGE results suggests that the bacterial communities of the MFC_BODs did not significantly change after they were tested with different substrates.

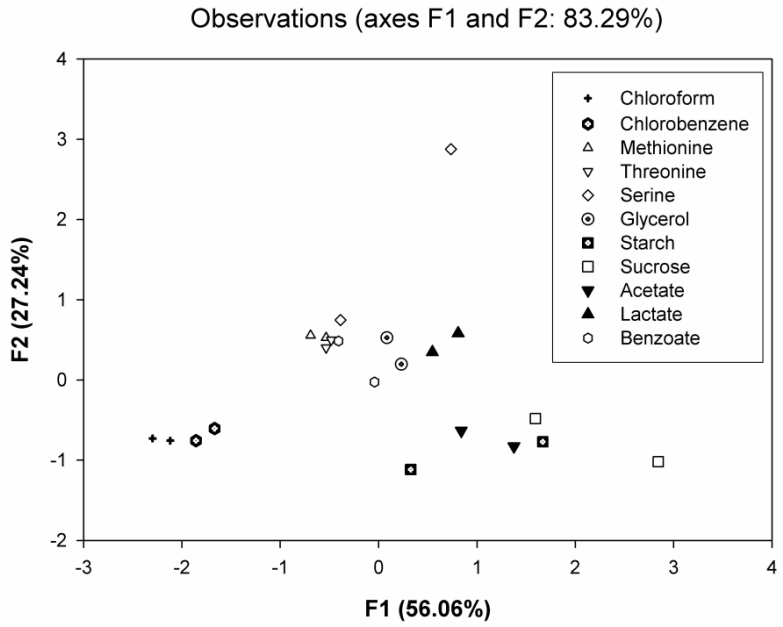

Figure 5. PCA of the current-to-BOD responses of different MFC_BODs tested with different substrates.

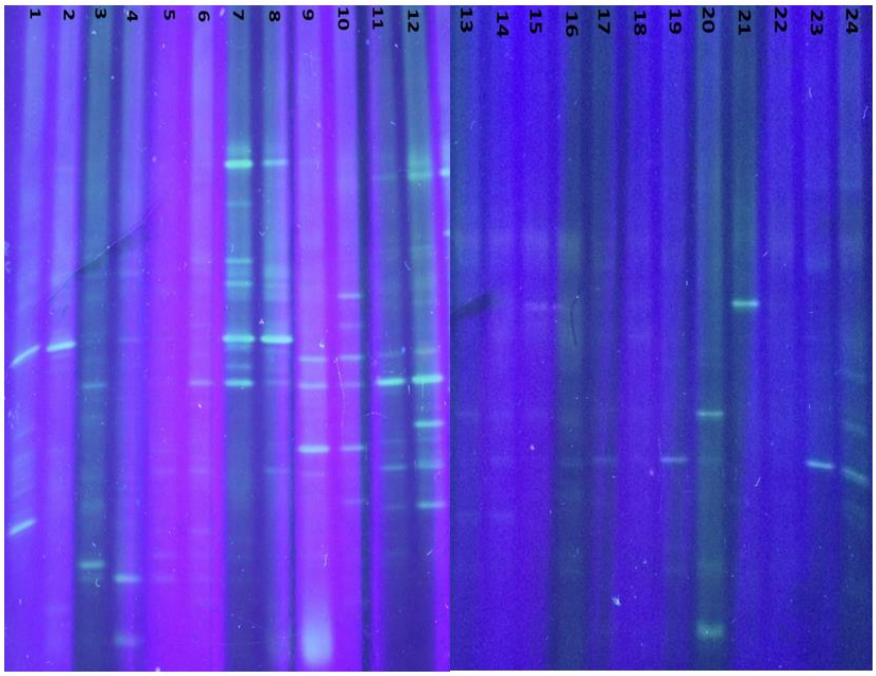

Figure 6. Denaturant gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments of the bacterial communities in the anodes of different MFC_BODs tested with different substrates. Lanes 1 and 2 indicate the originally enriched communities (enriched with glucose and glutamate) while other lanes indicate those of the MFC_BODs after tested with: acetate (lanes 3 and 4), lactate (lanes 5 and 6), sucrose (lanes 7 and 8), starch (lanes 9 and 10), methionine (lanes 11 and 12), glycerol (lanes 13 and 14), benzoate (lanes 15 and 16), threonine (lanes 17 and 18), serine (19 and 20), chloroform (lanes 21 and 22), chlorobenzene (lanes 23 and 24).

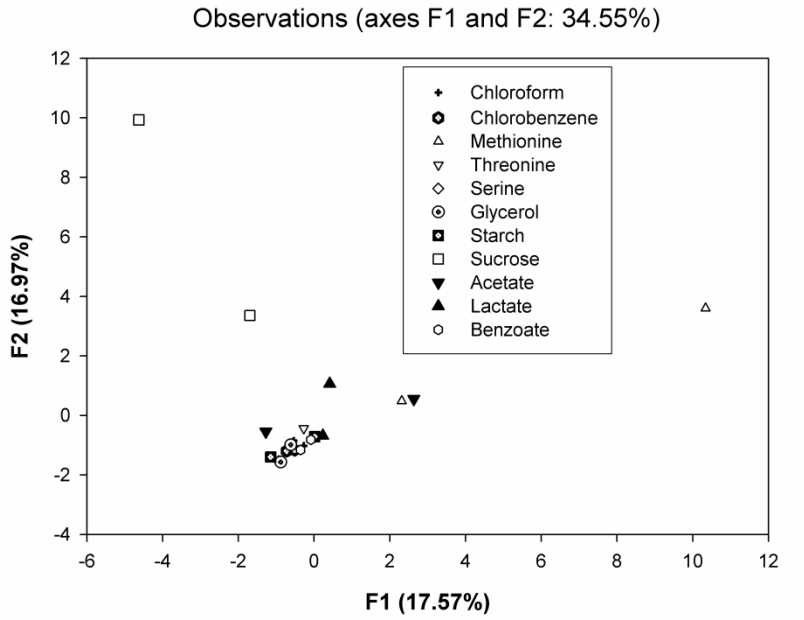

Figure 7. PCA of the compositions of the bacterial communities in the anodes of different MFC_BODs tested with different substrates based on their DGGE patterns.

DISCUSSION

Our results clearly demonstrated different BOD-sensing performances of MFC_BODs to different organic substrates. The results suggested that MFC_BODs can have pronounced sensing responses to organic acids, glycerol, benzoate and carbohydrates, in the range of BOD concentrations below 200 mg L^{-1} under laboratory conditions. However, the responses of MFC_BODs were less sensitive at different degrees to different amino acids or even "numb" to chlorinated compounds such as chloroform and chlorobenzene. Hsieh *et al.* (2016) also reported that carbohydrates were good fuel while amino acids such as methionine and phenylalanine were poor fuels for electricity generation by MFCs.

The above-mentioned observations are understandable because organic acids, glycerol, benzoate and carbohydrates seem to be more familiar and metabolizable substrates to bacteria, compared to amino acids and especially chlorinated compounds. Indeed, acetate is a favourable substrate of bacteria and can be metabolized by several metabolism pathways, while lactate can also be utilized by many bacteria (Madigan *et al.*, 2004). Similarly, carbohydrates and glycerol are easy-to-use carbon sources to various bacteria (Madigan *et al.*, 2004). The only surprising result is the responses of our MFC_BODs to benzoate, as benzoate is known as a preservative that inhibits bacteria (Madigan *et al.*, 2004). Actually, metabolism of benzoate has been reported feasible with complex microbial communities (Yadav *et al.*, 2021). Considering that the enriched bacterial consortia in our MFC_BODs are from multiple and diverse sources, it is quite possible that they can metabolize benzoate and thus the MFC_BODs can respond to it. In contrast, they cannot metabolize chlorinated compounds, probably due to the toxicity of these compounds (Tuan *et al.*, 2014). Regarding amino acids, as they are not major carbon sources for bacteria (Madigan *et al.*, 2004), the metabolism of these substrates by bacteria may not be stable to provide stable BOD-sensing responses for the MFC_BODs. Furthermore, different degrees of the BOD-sensing responses by the MFC-BODs to the different tested amino acids may be partly explained by the differences in their molecular

structures. Serine and threonine are both polar and simple molecules among those tested and can be therefore more metabolizable for bacteria. However, the response to serine by the MFC_BODs was not very sensitive, probably because this amino acid, with its very short carbon chain, provides little energy to be harvested. The response of the MFC_BODs to methionine was also poor, probably because the latter is not only non-polar but also large in molecular size. Moreover, it contains sulfur, which makes it even less favorable for bacteria to metabolize. It was already reported that methionine is not a good fuel for MFCs (Hsieh *et al.*, 2016). Altogether, the results suggest that the metabolizability of a substrate, which greatly depends on structural features of its molecule (Hsieh *et al.*, 2016), can significantly affect the BOD-sensing response of MFC_BODs.

The different BOD-sensing responses of MFC_BODs to different types of substrates in our study were not similar to the consistent current-to-BOD linear response to different substrates of the system previously reported by Hsieh *et al*. (2016), although these authors also mentioned the variation in the level of electricity generation with various substrates. Probably, in their study, Hsieh *et al*. (2016) used an artificially constituted mixed culture in their MFC anode, which might respond more stably with the more metabolizable substrates that they tested. In our system, a naturally enriched bacterial community was used, in order to enable the operation in open environments for practical use, and thus our system may respond more variously to various substrates.

Even when fed with metabolizable substrates, MFC_BODs seem to respond in a linear manner to BOD concentration changes only in a certain range, i.e. less than 200 mg L^{-1} in most of the cases. In fact, this "saturation" effect with similar BOD ranges was also reported with other BOD sensor-typed MFC systems by a number of studies (Chang *et al.*, 2004; Hsieh *et al.*, 2016). The reason for such an effect can be the BOD overload of the bioelectrochemical machinery in bacterial cells. It is also plausible that high BOD concentrations may by some means inhibit the bioelectrochemical activity of the anode bacteria, resulting in slight decreases of the current.

The general bioelectrochemical activity of the anode bacteria, rather than the composition of the bacterial community, might be the deciding factor to the BOD-sensing response of MFC_BODs, as demonstrated by PCA and community analysis results. Apparently, the result of the PCA of the electrical current responses versus the substrates pointed out that those responses were in association with the substrates (Fig. 5); while DGGE and DGGE-based PCA results showed that the compositions of the anode bacterial communities almost had no links with the substrates (Fig. 6 and Fig. 7). The differences in the responses of our MFC_BODs to the different substrates might be therefore: (i) only relating to the different metabolic processes that bacterial cells generally employed to deal with the substrates and (ii) not due to changes in the bacterial community. This finding, which has not been reported before, is actually interesting and provides an insight into the mechanism behind the BOD-sensing response of MFCs. Such an understanding will be helpful for both theory and application studies on MFC-based BOD sensors in the future.

Our study suggests that in terms of practical application, MFC_BODs can be used to monitor BOD more effectively with wastewaters containing more metabolizable substrates, such as food-processing wastewater or domestic wastewater. They will be less effective in monitoring BOD values of wastewaters containing recalcitrant or toxic chemicals (e.g. chlorinated compounds), such as industrial wastewaters. For more effective performances (i.e. with high correctness and broad detection range) of MFC_BODs in sensing BOD of those wastewaters, probably special enrichment procedures are required to obtain bacterial communities in the anode that can metabolize and hence sense recalcitrant or toxic compounds.

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