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Stable and high energy generation by a strain of *Bacillus subtilis* in a microbial fuel cell

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ABSTRACT

In this study, the Gram-positive aerobic bacterium *Bacillus subtilis* has for the first time been employed in a microbial fuel cell (MFC). A glucose-fed MFC with M9 minimal medium in the anode chamber was operated for 3 months, establishing a highly active MFC using filtered M9 medium as the catholyte, carbon cloth as the anode and a 20% platinum electrode as the cathode. The bioelectrical responses of the MFC were characterized by the circuit potential, measured at an average value of 370 mV. A potential of 115 mV appeared to characterize the maximum power produced from a polarization test was 1.05 mW cm^{-2} at a resistance of 0.56 kΩ. *In situ* cyclic voltammograms with and without biofilm anodes were performed in the growth phase and showed that redox metabolites were produced, which varied with physiological status. Voltammograms obtained from a comparative study of broth, supernatant and resuspended bacterial cells revealed that the electrochemical activity in the anode chamber arose from the redox compounds in the supernatant. The results show that the microorganism *B. subtilis* is electrochemically active and that the electron transfer mechanism is mainly due to the excreted redox compounds (mediator) in the broth solution and not to the membrane-bound proteins.

1. Introduction

Microbial fuel cells (MFCs) are bio-electrochemical systems that generate electricity by oxidation of organic or inorganic substrates catalyzed by microorganisms [1,2]. Microorganisms that can oxidize substrates such as glucose, acetate, butyrate or wastewater to produce electricity have been reported [3–5]. The electrons generated from the oxidation of organic substrates by microbes are generally transferred to a high potential electron acceptor such as dissolved oxygen in the medium. In MFCs, electrons are transported to an insoluble electrode (anode) through an electrical circuit to reach the cathode, where electron acceptors are reduced [5]. As the current then flows over a resistance, electrical energy is directly generated from the MFC [6].

Some researchers have reported the generation of electricity using sludge as the source of microorganisms [4]. However, the sewage sludge that has been used in MFCs is confined to a specific location, and electricity produced cannot be reproduced by other researchers without the same source. In addition, when using a mixed community, the electrochemical activity of a few bacterial species enhances the power output of the whole system [7]. Hence, it becomes difficult to ascertain the mechanisms and roles of the individual microorganisms contributing to power generation.

Several mechanisms of electron transfer from bacteria to the insoluble electrode have been proposed, which include: (i) use of external mediators such as thionine and neutral red, which are very expensive and toxic; (ii) direct electron transfer from the bacterial cell wall to the anode; (iii) use of mediators produced by the bacteria [5]; and (iv) electrically conductive bacterial nanowires produced by *Shewanella oneidensis* were also found to be in direct response to electron-acceptor limitation [8]. Exogenous mediators have to be used to facilitate the electron trasfer process in cases of electrochemically inactive bacteria [9]. It has been reported that *Desulfitobacterium hafniense* strain DCB2 generates electricity only after the addition of humic acids or humate, which is analogous to anthraquinone-2,6-disulfonate (AQDS) [10]. Direct electron transfer through outer membrane cytochromes is well known for *S. putrefaciens*, *Rhodoferax ferrireducens* and *Geobacter sulfurreducens* [3,4,11]. Pure strains of *Geobacteraceae* and *Escherichia coli* have been found as a noval mediatorless systems in MFCs [12,13]. Mediators produced by the bacteria have also reported as secondary

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metabolites: for instance, it has been demonstrated that pyocyanin, which functions as a mediator produced by *Pseudomonas aeruginosa*, has been excreted [6,14]. In MFCs, *in situ* production of mediators by bacteria is of great interest.

In this paper, a two-chambered microbial fuel cell that utilizes the soil bacterium *Bacillus subtilis*, to generate electricity using a glucose substrate has been developed. Recently, it has been shown that *B. subtilis* can grow anaerobically, either by using nitrate or nitrite as a terminal electron acceptor or by fermentation [15]. A comparative study of cyclic voltammograms of anodes with and without biofilm performed in the growth phase and in the broth, supernatant and resuspended bacterial cells was made in order to elucidate the mechanism of electricity generation.

2. Experimental details

2.1. Microorganism and culture conditions

B. subtilis (BBK006), a wild type and was used for electricity generation in this study. Prior to inoculation into MFC, the microorganisms were grown in 25 mL of M9 medium (with 0.2% glucose) in a 250-mL flask for 24 h on an orbital shaker (200 rpm) at 30 °C in order to produce a seed culture.

The composition of the M9 medium used as an electrolyte in the MFC was given by Miller [16] as 0.2% glucose in mineral salts (1 g L^{-1} NH_4Cl, 3 g L^{-1} KH_2PO_4, 6 g L^{-1} Na_2HPO_4, 5 g L^{-1} NaCl, 1 mmol L^{-1} $MgSO_4$, and 0.1 mmol L^{-1} $CaCl_2$). Before sterilization, the medium pH was adjusted to 7.0 with 0.5 mol L^{-1} NaOH. The medium was sterilized at 121 °C for 20 min without glucose, which was filter-sterilized (Millipore membrane PVDF, 0.22 μm filter unit; Millipore, Watford, UK) and added afterwards. The catholyte of the MFC was the sterilized M9 medium without glucose.

2.2. Microbial fuel cell setup

A double-chambered membrane MFC was constructed from two glass bottles (800 mL capacity) joined together with a glass bridge containing a proton exchange membrane (PEM; inner diameter = 3 cm), clamped between the flattened ends of the two glass bottles fitted with rubber gaskets. The materials used for the anode and cathode were carbon cloth and carbon paper containing platinum catalysts, respectively. Both electrodes were 2.5 cm × 4.5 cm, with a projected area of 22.5 cm^2, and were pierced with copper wire projecting outside to provide the connections to an external electrical circuit, through which the electrons were transported. Immediately after transferring the bacterial sample to the anodic compartment, air purging was permitted in the anode chamber in order for the bacteria to grow aerobically. On the second day, the anode compartment began showing turbidity, implying the growth of bacteria. The air supply in the anode was discontinued at this stage and the joined parts of anode chamber were sealed using silicone gel in order to provide an anaerobic environment. After 2 days, the formation of a layer of biofilm, air was continuously purged (flow rate: 0.6 mL min^{-1}) to avoid the insufficiency of oxygen reduction at cathode chamber. A constant temperature of 30 °C was maintained by circulating water through jacketed glass anode and cathode chambers. Each chamber contained three ports at the top to hold the electrode wires

2.3. Data acquisition and the electrochemical technique used for analysis

The electrode circuit potential and real time data were continuously monitored and recorded by a computer, the anode and cathode having been connected directly to a PicoLog recorder using PicoLog® v. 5.09.4 recorder software (Pico Technology) with an RS232 interface connected to an ADC 20-21, A–D converter (Pico Technology Ltd., Cambridgeshire, UK). A circuit was permanently connected, with an external load of 1 kΩ. To obtain the polarization curve, a different external load was used for a complete batch cycle, with a variable resistance of 10, 8.2, 6.8, 5.6, 4.7, 3.3, 2.2, 1, 0.56, and 0.22 kΩ. The microbial fuel cell was then operated for a long period of time (more than 6 days), under the application of a single constant resistance, and the current and anodic and cathodic potentials were measured with respect to time.

2.4. Cyclic voltammetry

Cyclic voltammetry was performed using a potentiostat (CHI 627C; CH Instrument, USA) connected to a personal computer (CHI627C, Electrochemical Analyzer) which was used at a scan rate of 25 mV s^{-1} ranging from −450 to 600 mV. Voltammograms were generated in duplicate for each sample with a 1.5% variability. The data were recorded using a conventional three-electrode arrangement consisting of a working electrode, reference electrode (an Ag/AgCl electrode) and counter electrode (a platinum wire). Two carbon cloths of the same size, 22.5 cm^2, were used as working electrodes: one formed the biofilm anode; the other was a new anode that was cleaned in ethanol and deionized water prior to use. All three electrodes were inserted into the MFC, avoiding any contact between the electrodes.

In situ cyclic voltammetry was performed for the growth phase as described by Rabaey et al. [6]. The active biofilm anode was grown in fresh nutrient broth in a glucose-fed fuel cell and tested electrochemically in the spent broth at different optical readings of the suspended bacteria (600 nm). To obtain a measurement with and without the components released into the solution at the end of the batch operation, the bacteria were centrifuged (10 min at 11,000 × *g*) and resuspended in an equal amount of fresh medium solution. The original broth, the supernatant and the resuspended bacteria were tested using cyclic voltammetry. For the analysis, another new anode was placed into the microbial fuel cell to complete the same cyclic voltammetry measurement.

2.5. Scanning electron microscopy (SEM)

Anodes with biofilm were gently placed on a silicon wafer. The samples were fixed with 2% glutaraldehyde and 1% osmium tetroxide, and then dehydrated in increasing concentrations of ethanol. Observations were performed with a Hitachi, S-4700, Type II scanning electron microscope

3. Results and discussion

3.1. Stable and long-term power generation

An example of the sequence of substantial voltage increments by the MFC for the initial glucose concentration is shown in Fig. 1. The variation of cell voltage with time in the MFC with this substrate was measured for 93 days. On day 1, the anode chamber was loaded with *B. subtilis* and the system was maintained aerobically for bacterial growth. The voltage by the sterile medium was less than 93 mV, which might be due to biological and chemical factors based on the difference in potential between the two chambers. Thereafter, the anode chamber was sustained anaerobically for electricity generation. After the second day, an initial circuit voltage in the range of 275 mV was immediately generated after the addition of glucose, following which there was a grad-

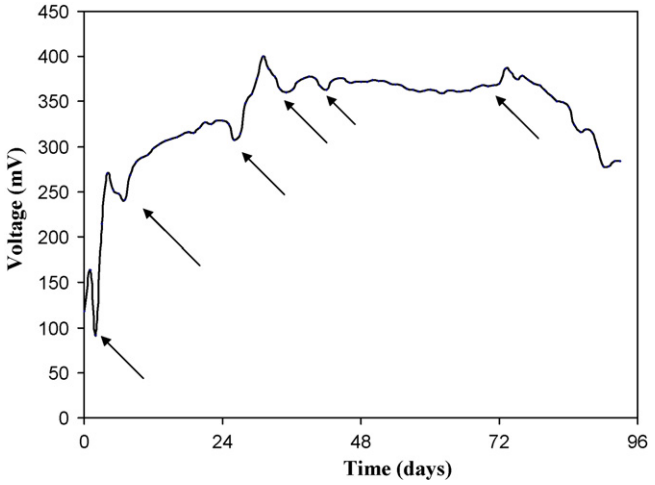

Fig. 1. Steady state circuit potential established in the MFC with *B. subtilis* followed by subsidiary addition of glucose, as shown by arrows.

ual decrease in the voltage as a function of time (after day 5); however, the addition of glucose on day 7 resulted in a substantial increase in potential up to 328 mV. The highest cell voltage recorded was approximately 400 mV, which occurred after the glucose boost on day 26. Analysis implied that the behavior of circuit voltage with time was due to the utilization of nutrients from the medium by microbes. The cell voltage remained almost constant (360 ± 20 mV) throughout the remainder of cultivation up to day 72, and subsequently a gradual decrease was observed, despite the addition of glucose on the same day. These results demonstrated that there was a substrate limitation after the long cultivation, which affected the microbial metabolism and therefore the voltage output. In addition, the final pH of 6.2 caused by volatile acids accumulation might be another factor for voltage decrease. In our study, the voltage obtained was consistent for more than 90 days without mediators. In some cases, electron transfer without the addition of mediators has been described as unfeasible [17]. With the incorporation of electron mediators, current production was found to be enhanced significantly [17]. In addition, the experiment using a well-studied strain (*B. subtilis* 168) was also carried our under the identical experimental conditions and the setup. The less voltage of 200 ± 20 mV generated was found.

Power, current and cell potential measurements were performed as a function of external electrical circuit resistance. Following system stabilization after the generation of constant potential, in order to demonstrate the cell potential, maximum power and current output of the system, the external electrical circuit was subjected to loads (resistance) of 10, 8.2, 6.8, 5.6, 4.7, 3.3, 2.2, 1, 0.56 and 0.22 kΩ, as shown in Fig. 2. The measurements were recorded for 30 min intervals. After applying resistance, a classical cell potential trend was observed, as shown in Fig. 2. The cell potential increased with external resistance, and then reached a value of 200 ± 25 mV (corresponding to 3.3 kΩ; Fig. 2). The power curve shows a maximum of 24 mW at a low resistance of 0.56 kΩ, then decreases with increasing external resistance (Fig. 2). At a higher resistance (10 kΩ), a relatively lower power (0.28 mW cm^{-2}) output was observed. While the maximum power 1.05 mW cm^{-2} was observed at a resistance of 0.56 kΩ. The rate of oxidation of substrates by microbes was observed to be higher at lower resistances as compared with that at higher resistances, where microbes donate electrons to the anode as the electrons are discharged in a closed circuit.

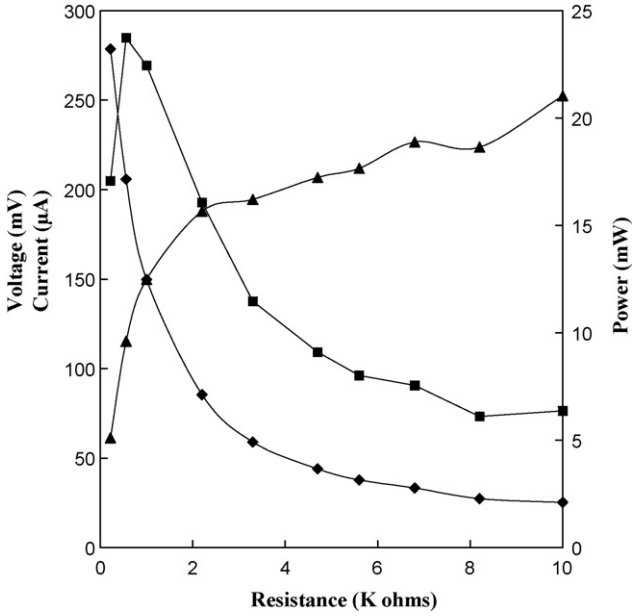

Fig. 2. Cell potential (▲), power (■) and current (♦) measurements in the microbial fuel cell with the application of various external resistances.

3.2. Polarization curve

A polarization curve describes voltage as a function of current and is a powerful tool for the analysis and characterization of MFC [1]. The polarization curve shows an OCV of 372 mV at zero current (Fig. 3). There was an initial steep decrease in the voltage between the OCV of 226 mV, which indicated activation losses. The subsequent slope of the polarization curve was almost linear over the range of 0.08–0.27 mA, which is due to ohmic losses. The internal resistance calculated from the polarization curve was found to be 29 Ω cm^{-2}. However, at higher current densities, no rapid fall in voltage was observed, indicating a lower mass transport limitation at the electrode. This observation implies that the bacterial biofilm is electrochemically competent and capable of solving diffusional and electrochemical limitations. Fig. 3 shows a typical power curve calculated from the polarization curve. There was no power produced when no current was flown under open circuit conditions,

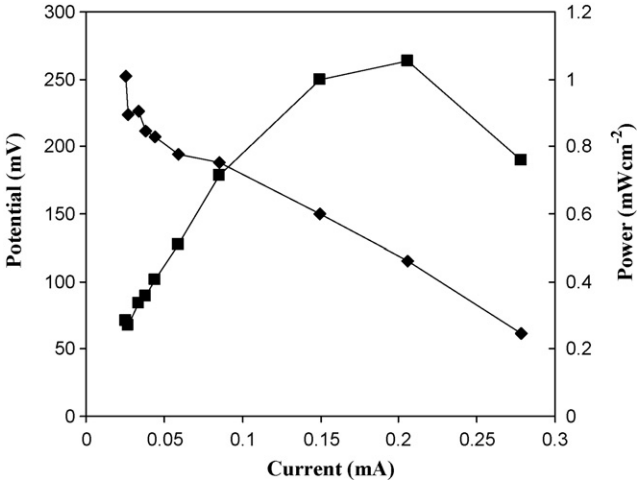

Fig. 3. Polarization of the microbial fuel cell system as a function of cell voltage (♦) and power output (■).

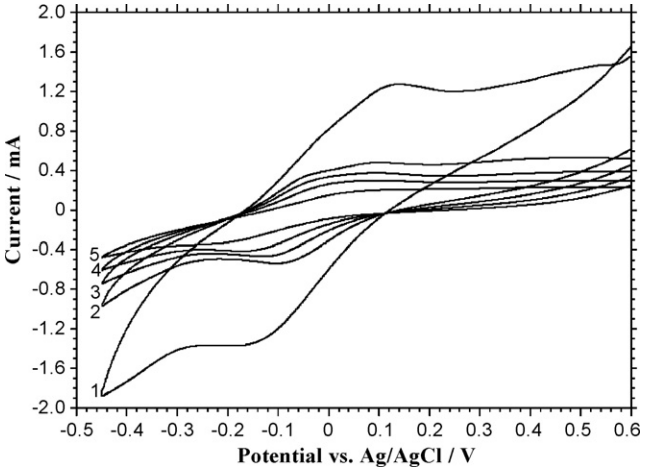

Fig. 4. Cyclic voltammograms of bacterial growth phases at OD readings of 0.0 (1), 0.31 (2), 0.60 (3), 0.71 (4) and 4.7 (5), using an anode with biofilm.

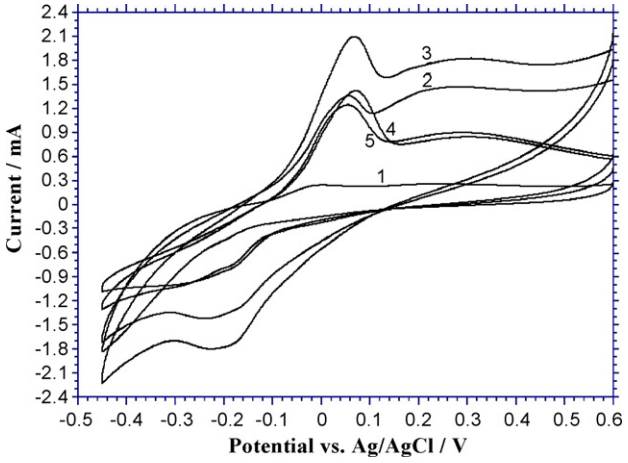

Fig. 6. Cyclic voltammograms of bacterial growth phases at OD readings of 0.0 (1), 0.31 (2), 0.60 (3), 0.71 (4) and 4.7 (5), using a new anode without biofilm.

followed by an increase in power output to a maximum of 24 mW, corresponding to a current of 0.206 mA, at a potential of 115 mV at low resistance (0.56 kΩ).

3.3. Cyclic voltammograms

3.3.1. Electrochemical activity of the bacterial growth phase

The influence of the bacterial growth phase for the biofilm anode at different OD (optical density) readings of 0.0, 0.31, 0.60, 0.73 and 4.7 is shown in Fig. 4. As can be seen from Fig. 4, when the OD is zero, an oxidation peak in the forward scan of the voltammogram was observed at 0.14 V (*vs.* Ag/AgCl, 1.3 mA). During the reverse scan, a reduction peak was found at −0.4 V (*vs.* Ag/AgCl, 1.25 mA). These observations indicate that redox components (i.e., mediators) might have been released in the anodic biofilm at the beginning of the batch test when there were no suspended bacteria. These mediators, if present, would be held in the biofilm. Surprisingly, the voltammogram decreased with increasing bacterial density of the broth, and no redox couple was found when the OD reached 0.71. The voltammogram remained inactive throughout the remainder of the culture time. SEM showed a thicker biofilm observed at an OD of 4.7, implying that there was spatial obstruction caused by the biofilm (Fig. 5), resulting in diffusion limitation between the solution and the anode electrode. Fig. 6 shows the results obtained after placing a new anode in the same medium. In contrast, no voltammograms of redox couples were obtained when a new anode was used in the clear medium (Fig. 6), which is obviously due to the absence of any microbial community and mediator in the medium. This is in good agreement with the performance of MFCs reported by Liu et al. [18], who demonstrated that no redox couple was found when the biofilm anode was replaced by a new anode [18]. However, a redox couple was observed in the voltammogram (−0.22 and 0.06 mV). The voltammogram increased with bacterial density of the broth, reaching a maximum intensity of 2.1 mA at an OD of 0.6, and decreased after an OD of 0.71. The explanation for this is that the amount of metabolites in solution varied with physiological status in the growth phases, which influences conductivity and capacity. This is not in accordance with the performance of voltammograms in glucose catabolite for MFCs as reported by Rabaey et al. [19]. They also reported the electrochemical activity of the bacterial growth phase (lag, log and stationary phases) using the bacteria consortium on day 155. Cyclic voltammograms were performed with the new anode (without biofilm) at time zero, after 30 min and after 2 h after the addition of 1 g of glucose per liter. At time zero, a redox couple was found at 170 ± 10 mV, which disappeared after 30 min and reappeared after 2 h. According to their findings, there was a temporal disappearance of a component out of reach of the electrode. Care was taken with the choice of the growth phase and anode, as we found that the electrochemical activity varied with growth phase, which represents different bacterial physiological status. Rabaey *et al.* (2004) found that the component active at −180 mV was apparently affected by the feeding regimen [6]. This can be explained by the electrochemical activity being due to the same production by bacteria at the same growth phases as those of day 155. In our study, M9 minimal medium supplemented with glucose was employed to culture bacteria in the anode with biofilm. Using this design, we are sure that the effects of electrochemical activity assays do indeed result from compounds produced by the bacteria, as we checked the electrochemical activity of M9 minimal medium and found it to be zero (Fig. 6, line 1).

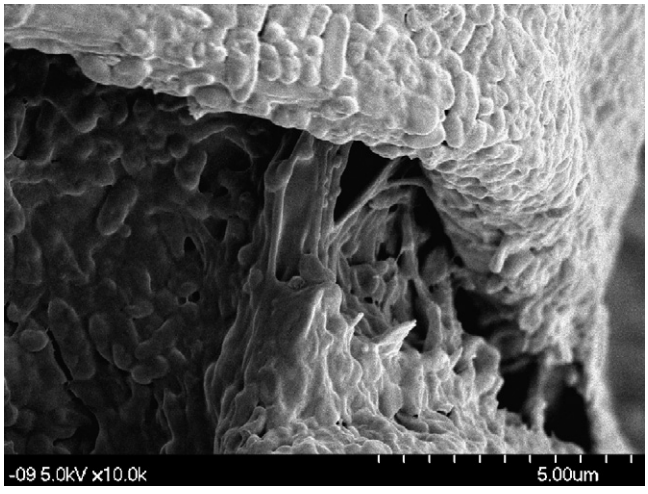

Fig. 5. SEM of biofilms formed by *B. subtilis* on day 93.

3.3.2. Verification of soluble redox compounds secreted by bacteria

In order to investigate the mechanism of extracellular electron transfer, which is either through membrane-bound proteins or via the secretion of redox components, further voltammetric study was conducted with a biofilm anode and a new anode (without biofilm) in spent broth, supernatant and bacterial cells

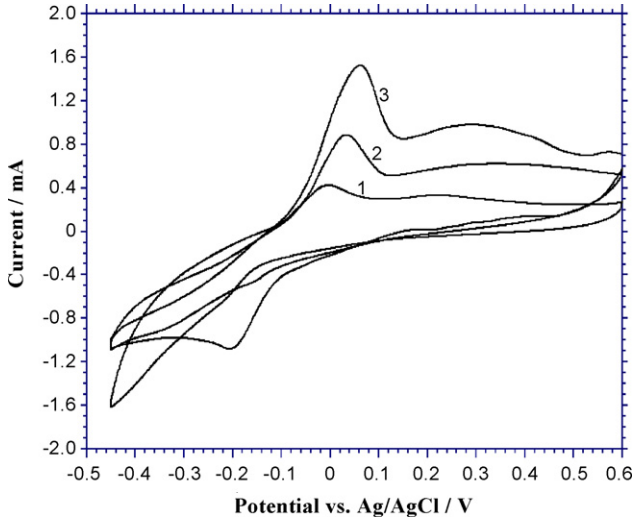

Fig. 7. Cyclic voltammograms of a new anode with resuspended bacteria (1), supernatant (2), and original broth (3).

resuspended in clear medium after centrifugation. All tests were carried with the anolyte at the end of the batch test. The characteristic electrochemical activity of the redox compound for spent broth, supernatant and resuspended bacterial cells (Fig. 7). An oxidation peak of approximately 0.06 V (1.5 mA) was observed during the forward scan for the spent broth with a new anode (Fig. 7) along with a reduction peak during the reverse scan at −0.2 V (−1.1 mA). Kim et al. [2] demonstrated the formation of redox couples, which revealed the presence of mediators that are reversibly oxidized and reduced during CV tests [1]. These results indicate the presence of active redox compounds responsible for the electrochemical activity in the broth solution. The voltammogram of the new anode with the supernatant also produced an oxidation peak during the forward scan at 0.04 V (0.9 mA), which shifted slightly to the left of the first peak. In addition, the intensity of the peak was lower than that obtained from the spent broth, which implies involvement of bacterial cells in the electrochemical activity. Therefore, the voltammogram of bacterial cells suspended in the new medium found at −0.01 V (0.45 mA) was confirmed. Rabaey et al. used a pure culture of *P. aeruginosa* and an isolated KRA3 to study electrochemical activity [19]. In that study, the CVs of *P. aeruginosa* and an isolated KRA3 exhibited peaks at −100 and 200 mV, respectively, which disappeared when the culture was centrifuged and resuspended in the physiological solution [6]. Our results, particularly the CV of the supernatant, verified the conclusion that the electrochemical activity of the bacteria is governed by the excreted metabolites rather than the bacterial cells themselves.

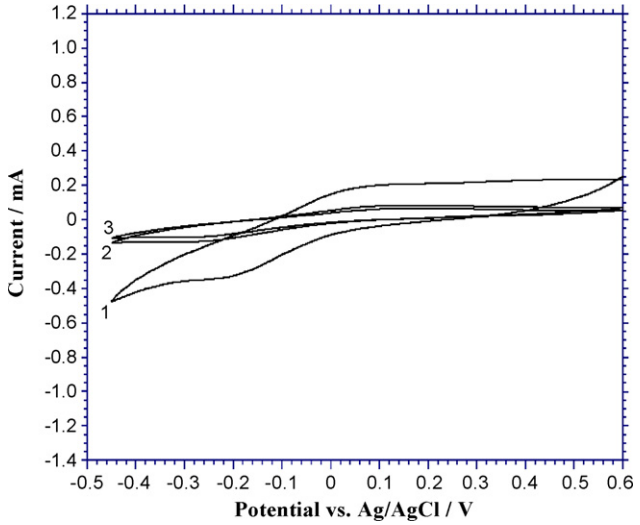

Fig. 8. Cyclic voltammograms of the biofilm anode with resuspended bacteria (1), supernatant (2), and original broth (3).

3.4. Electrochemical behavior of the biofilm anode

Subsequent cyclic voltammograms were conducted with the biofilm anode placed in a solution identical to that described in the above section. It can be seen from Fig. 8 that no active oxidation peak was observed during either the forward or reverse scan in the original broth, supernatant and bacterial cells in clear medium. This result is in accordance with the performance of MFCs at the end of electricity generation as reported by You et al. [20], who suggested that the electrons are carried away from a respiratory enzyme in the biofilm, which determines the potential of the anode.

4. Conclusion

We have demonstrated that the aerobic Gram-positive species *B. subtilis* was able to grow anaerobically and produce a biofilm in a microbial fuel cell, which generated a long-term power output. The *in situ* voltammogram of the bacterial growth phase showed that the electrochemical activity was mainly due to excreted redox mediators, which were affected by physiological status. The voltammograms of the broth, supernatant and resuspension with a new anode and with a biofilm anode showed the involvement of dissolved components but not of the biofilm. The *B. subtilis* strain used in this study is a member of an industrially important species. This species is one of the most commonly used hosts in fermentation production, because it is simple to cultivate and its products (protein and metabolites) are often secreted in the growth medium. Our results demonstrate significant application potential and provide insight into the mechanism of MFCs.

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