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Enumeration of exoelectrogens in microbial fuel cell effluents fed acetate or wastewater substrates

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ABSTRACT

Effluents from well-acclimated microbial fuel cells (MFCs) have been widely used as inocula to start up new MFC reactors. However, the actual cell concentrations and cell viability of exoelectrogens in these MFC effluents have not been well examined. In this study, concentrations of exoelectrogens in the effluent from acetate- or wastewater-fed MFCs were examined using a quantitative polymerase chain reaction (qPCR) method specific for *Geobacter* spp. that are usually the dominant genus in MFCs, and a non-specific WO_3 nanocluster/most probable number (WO_3/MPN) method for enumeration of viable exoelectrogens. *Geobacter* spp. concentrations in acetate-fed MFC effluents based on qPCR were $1.3 \pm 0.2 \times 10^8$ cells/mL, slightly higher than those in the wastewater-fed MFC effluents ($9.3 \pm 3.5 \times 10^7$ cells/mL). However, exoelectrogen cell counts using the WO_3/MPN method were several orders of magnitude lower for both MFC effluents ($1.1 \pm 0.3 \times 10^4$ cells/mL for acetate-fed; $1.4 \pm 0.3 \times 10^5$ cells/mL for wastewater-fed). Live/dead cell staining suggested that most cells (85 %) in the effluents were inactive or dead, which could partly explain the lower numbers using the WO_3/MPN method. These results suggest that both acetate- and wastewater-fed MFC effluents contain high numbers of *Geobacter* spp. although a high percentage of cells are not viable.

1. Introduction

Exoelectrogenic microorganisms, such as *Geobacter* spp. and *Shewanella* spp., are the key microbes to use organic matter as a power source to run bioelectrochemical systems [1–3]. Extracellular electron transfer has enabled these microorganisms to be used in a variety of technologies for resource recovery from waste streams, such as electricity generation in microbial fuel cells (MFCs) or hydrogen and methane production in microbial electrolysis cells (MECs) [4,5]. Exoelectrogens can be found in many different environments, ranging from wastewater treatment plants to water and sediments in lakes and rivers [6]. While *Geobacter* spp. tend to be predominant in the biofilm of MFCs, exoelectrogenic microorganisms span the three domains of life, and thus there are many other microorganisms capable of exoelectrogenic activity. There have been a few studies of the abundance of exoelectrogenic microorganisms in different environmental samples or biological reactors [7–11].

Effluents from well-acclimated MFCs have been widely used by many research groups as inocula to start new MFCs or MECs [12–15]. It is expected that MFC effluents would contain a high proportion of exoelectrogens like *Geobacter* spp., based on previous studies that reported the dominance of exoelectrogens in the biofilm of well-acclimated MFCs [16–19]. However, there have been few studies that specifically address the concentrations of exoelectrogens in MFC effluents or use more than one method to assess exoelectrogen cell concentrations. The concentrations and diversity of exoelectrogens in MFC effluents are likely a function of the feed substrate, since the microbial diversity of biofilms in MFCs have been shown to vary considerably depending on the type of substrates used. For example, a relatively higher proportion of *Geobacter* spp. (31.7 %) was obtained in the biofilm of acetate-fed MFCs than other MFCs fed with glucose (18.0 %), propionate (1.9 %), and butyrate (13.6 %) [20]. For potato wastewater-fed MFCs, more than 60 % of microorganisms in anode biofilm were *Geobacter* spp., while only 7 % of *Geobacter* spp. were found in biofilms of MFCs fed with dairy wastewater [21]. Thus, it would be useful to examine cell concentrations of exoelectrogens in MFC effluents fed with different substrates as these effluents are commonly used as inocula for new MFC reactors. Also, few

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studies have examined the viability of cells in effluents from MFCs.

In this study, effluent samples were collected from MFCs acclimated to either acetate in a phosphate buffer or wastewater for a period of 6 months. Exoelectrogens in these MFC effluents were enumerated by two different methods and compared to total and viable direct cell counts. A quantitative polymerase chain reaction (qPCR) method was used to enumerate *Geobacter* spp. with a primer set specifically targeting the family *Geobacteraceae* [8,17], since there is no universal primer for exoelectrogens and *Geobacter* spp. are usually the predominant exoelectrogen in acetate-fed MFC biofilms [16,19]. A culture-based most probable number (MPN) enumeration method based on color changes in the presence of tungsten oxide (WO_3) nanoclusters [11] was used to estimate the total numbers of exoelectrogens in MFC effluents. Direct cell counts were performed using SYTO 9 and propidium iodide (PI) fluorescent dyes for total cell numbers and live/dead cell fractions to examine the possible impact of cell viability on the enumeration methods.

2. Materials and methods

2.1. MFC reactors and sampling

MFC effluent samples were collected from single-chamber MFCs (working volume, 28 mL) after multiple fed-batch cycles (~24 h for each cycle). The MFCs had a graphite brush anode and an activated carbon cathode with a polytetrafluoroethylene (PTFE) diffusion layer as previously described [22]. Four MFCs were used in this study and all MFCs were initially inoculated using MFC effluent (50 % v/v) from other well-acclimated MFC reactors. Two MFCs were fed with acetate as the sole electron-donor substrate in 50 mM phosphate buffer solution with nutrients as previously described [23]. Two other MFCs were fed with only primary effluent obtained from the Pennsylvania State University Wastewater Treatment Plant. All MFCs were acclimated to these two different feed solutions (i.e. acetate solution or primary effluent) for more than 6 months before collecting effluents to quantify exoelectrogens. Effluents were collected from the MFCs over multiple cycles to have sufficient volume for analysis methods. Collected effluents were analyzed as soon the sufficient volume was collected using the WO_3/MPN (in triplicate) and direct cell count (ten replicates) methods or stored at −20 °C for subsequent qPCR analysis (in triplicate).

2.2. Enumeration procedures

Geobacter sulfurreducens PCA (ATCC 51573) was purchased from ATCC and cultured in the ATCC Medium 1957 to create standard curves with plasmid insert DNA templates. Briefly, genomic DNA was isolated from the culture using the PowerSoil DNA isolation kit (MO Bio Laboratories, Inc.), and the 16S rRNA gene was amplified using a PCR Master Mix (Thermo Scientific) with the primer set *Geobacteraceae-494f* and *Geo825r* [10]. The PCR products were ligated into the pCR 2.1 vector and transformed into *E. coli* DH5α cells following the instructions of the kit (TA Cloning® Kit, with pCR™2.1 Vector and One Shot® TOP10 Chemically Competent *E. coli*, Invitrogen). Transformants were isolated using blue-white screening (Teknova LB Agar Plates with 150 μg/ml Ampicillin, 60 μg/ml X-Gal, and 0.1 mM IPTG), and plasmids were extracted and purified using a plasmid extraction kit (QIAprep Spin Miniprep Kit, Qiagen). The concentration of the prepared plasmids was determined using a Nanodrop 2000 (Thermo Scientific), and a dilution series was prepared from 10^{-2} to 10^{-12} (plasmid concentrations from 3.9 ng/μl to 3.9×10^{-10} ng/μl). qPCR was conducted in triplicate with the plasmid dilution series and extracted DNA (same DNA isolation kit described above was used) from the MFC effluent samples using the *Geobacteraceae*-targeted primer set at 10 nM concentration and the Power SYBR® Green PCR Master Mix (Applied Biosystems) following the instructions provided by the supplier. qPCR was conducted using an Applied Biosystems StepOne Plus (Grand Island, NY, USA), and cell numbers were estimated from gene copy numbers using an rRNA operon copy number of 2 [24].

WO_3 nanoclusters were prepared using a hydrothermal process with $Na_2WO_4 \cdot 2H_2O$ as previously described [25,26]. The WO_3 suspension (50 μL) and sterilized Luria-Bertani (LB) broth (100 μL) were loaded in each well of a 96-well plate. The MFC effluent samples were serially diluted (from 10^{-1} to 10^{-10}) and added into the wells (100 μL) with five replicates per dilution, and the wells immediately coated with a layer of oil (80 μL; Resolve microscope immersion oil high viscosity) to produce anaerobic conditions as previously described [11]. The prepared well plates were then placed in a constant temperature room (30 °C) for 48 h. The wells with blue color formation were considered positive and the MPN table (Table 9221.IV in Standard Methods) [27] was used to estimate the cell numbers based on the series of dilutions used for the sample.

Bacterial viability tests were conducted with a dual staining kit (BacLight™ Live/Dead Bacterial Viability Kit, L-7007, Molecular Probes) composed of two distinct fluorophores, SYTO 9 that penetrates intact cytoplasmic membranes and PI for membrane-compromised cells [28]. MFC effluent samples were collected and diluted 100×, followed by addition of the dyes and incubation as suggested by the bacterial viability kit supplier. After filtering through black Nucleopore Track-Etch Membranes (Whatman plc, UK), cell imaging was performed with an Olympus BX61 fluorescence microscope and a DP72 digital camera. Cell counts of diluted MFC effluent were performed by manual counting of 25 fields per sample with 15–30 cells per field.

3. Results and discussion

3.1. Enumeration of exoelectrogens in MFC effluents fed with different substrates

The number of *Geobacter* spp. in the acetate-fed MFC effluents based on qPCR was $1.3 \pm 0.2 \times 10^8$ cells/mL. This concentration was slightly higher but not significantly different ($p > 0.05$, student *t*-test) than the cell counts in the wastewater-fed MFC effluents ($9.3 \pm 3.5 \times 10^7$ cells/mL) using the qPCR method (Fig. 1). The total number of cells in the acetate-fed MFC effluents of $9.4 \pm 2.2 \times 10^7$ cells/mL (including both live and dead cells) based on SYTO 9 and PI staining was significantly higher ($p < 0.05$) than the cell counts in the wastewater-fed MFC effluents ($7.1 \pm 2.7 \times 10^7$ cells/mL) using the same method. In contrast, cell numbers based on WO_3/MPN were slightly higher in the wastewater-fed MFC effluents ($1.4 \pm 0.3 \times 10^5$ cells/mL) than acetate-fed MFCs ($1.1 \pm 0.3 \times 10^4$ cells/mL), though those concentrations were three or four orders of magnitude lower than the qPCR and direct count methods.

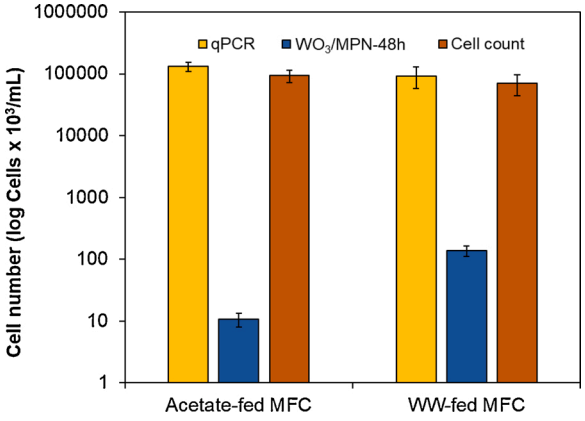

Fig. 1. Enumeration results by qPCR, WO_3/MPN (48 h), and direct cell counting methods from acetate- and wastewater-fed MFCs. Error bars indicate ± SD (n>3).

Similar cell concentration in both MFC effluents using qPCR and direct count methods indicate that *Geobacter* spp. were dominant in those effluents. Prior reports have shown that *Geobacter* spp. were dominant in the biofilm of well-acclimated MFCs, ranging from 72 to 90 % of cells in acetate-fed MFCs or MECs [16,19]. Also, MFCs inoculated with either wastewater or environmental samples (such as those in bogs) often become highly enriched by *Geobacter* spp. [17,18], and more than 60 % of *Geobacter* spp. were found in biofilms of MFCs fed potato wastewater [21]. A high proportion of *Geobacter* spp. in the biofilm of the well-acclimated MFCs most likely resulted in the largest cell counts in the effluents using qPCR, with the type of substrate (acetate or wastewater) not as important. However, the number of *Geobacter* spp. calculated using qPCR here was slightly larger (130–140 % of the number) than total cell counts. The use of qPCR to identify the number of cells based on specific sequences unique to *Geobacter* spp. has several limitations compared to total cells. Cell counts based on qPCR can include extracellular DNA and nonviable cells, overestimate cell numbers due to multiple copies of the targeted gene (which was accounted for in this study, but might be unknown for certain microbes of interest), and exclude genes or taxa not targeted by the primer sequences. The higher cell numbers using qPCR relative to direct counts obtained here was therefore most likely due to free DNA fragments in the effluent released from the anode biofilm during the cycle, as was observed in a previous study [29]. It is also possible that the total numbers of cells based on direct cell counts were underestimated due to the presence of small clumps or larger aggregates that made microscopic enumeration difficult.

The cell concentrations obtained using the WO_3/MPN method were much less than these other two methods. One problem was that aggregated particles were observed in the wells for the effluent from the wastewater-fed MFCs (as shown in Supplementary material). The presence of aggregated particles in the wells suggests that cell numbers were underestimated using this method, as the MPN approach assumes dilution-to-extinction of single cells. We further tested the WO_3/MPN method using pure cultures of *Geobacter sulfurreducens* PCA to examine the accuracy of both methods for an idealized test case. For pure culture samples, a cell concentration of 2.3×10^2 cells/mL was obtained for the original pure culture suspension, and a 10 times higher cell count (2.4×10^3 cells/mL) was obtained for the $\times 10$ concentrated pure culture suspension (as shown in Supplementary material). The cell counts by WO_3/MPN for pure culture samples therefore corresponded well with cell concentrations tested for pure cultures where no aggregates were observed in the wells.

3.2. Cell viability in acetate- and wastewater-fed MFC effluents

Live/dead cell staining was also conducted to examine the proportion of cells that could be considered viable, based on membrane integrity. In the effluent from the acetate-fed MFCs, 85 % of the total cells ($7.9 \pm 1.8 \times 10^7$ cells/mL) were classified as dead based on penetration of PI into the cells. A larger portion of microbes in the effluent of the wastewater-fed MFCs were also classified as dead (93 %, $6.6 \pm 2.6 \times 10^7$ cells/mL) using this analysis (Fig. 2). Exoelectrogens in MFCs are mostly active or live in the outer layer of the anode biofilm [30]. Once exoelectrogenic microorganisms become detached from the biofilm they may not remain viable due to the lack of a suitable electron acceptor. The larger portion of dead or inactive cells in the wastewater-fed MFC effluents might be due to the less favorable substrate (complex organic matter) and nutrients than the acetate feed solution with nutrients.

The large proportion of dead cells in both effluents partly explains the differences between the cell numbers of exoelectrogens measured using the WO_3/MPN method, which requires growth of cells, compared to that obtained with qPCR. However, assuming that only 15 % of cells were viable in the acetate-fed MFC effluent, the qPCR results would suggest 2.0×10^7 cells/mL, which is still three orders of magnitude

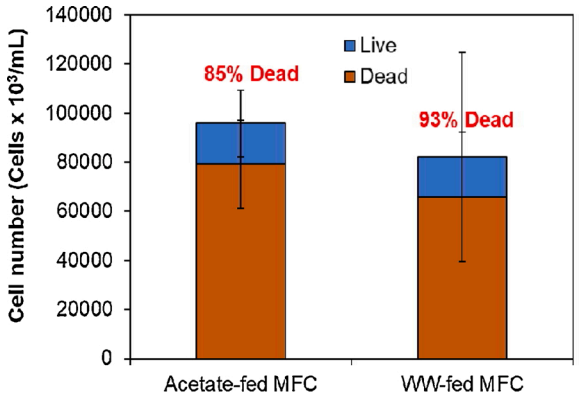

Fig. 2. Live and dead analysis for the effluents from acetate- and wastewater-fed MFCs. Error bars indicate ± SD (n>10).

larger than the WO_3/MPN method ($1.1 \pm 0.3 \times 10^4$ cells/mL) for the same effluent. The finding here that the number of exoelectrogenic microorganisms in the effluent were several orders of magnitude lower than total cell numbers is consistent with a previous WO_3/MPN study, where a three-order magnitude lower cell number was detected in the wastewater treatment plant effluent by the WO_3/MPN compared to the cell number by DAPI staining [11]. The lower cell number in that study might be due to the small fraction of active or live exoelectrogenic microorganisms in the wastewater treatment plant effluent. The method is based on sufficient growth of active exoelectrogens on the WO_3 nanoclusters to produce a change in color from white to blue as the nanoclusters are reduced by exoelectrogens. Thus, the short reaction time of 48 h may not be sufficient to enumerate low concentrations of cells or slow growing exoelectrogenic microorganisms. We therefore conducted additional tests using the WO_3/MPN method with a longer reaction time (96 h), and more wells were reduced and turned blue (as shown in Supplementary material). Also, in some wells instead of the development of a uniform blue color the color was a non-uniform blue suggesting that cells and particles aggregated during the test (Supplementary material). The dominance of active or live cells and impurity of samples will be crucial for the WO_3/MPN method since those cells would increase the chances of the reduction of the WO_3 nanoclusters in a set analysis time.

4. Conclusions

For the effluents from well-acclimated MFCs fed with synthetic (acetate in PBS solution with nutrients) and actual (primary effluent from wastewater treatment plant) substrates, qPCR that targeted *Geobacteraceae* and direct cell counting methods showed similar cell numbers, indicating the dominance of *Geobacter* spp. in those effluents. Cell viability testing suggested more than 85 % of cells in the effluent were dead, and a slightly higher portion (93 %) of cells was dead or inactive in the effluent from wastewater-fed MFCs likely due to the less preferable substrate and relatively insufficient nutrients. The results here show that the use of MFC effluents as inocula will be useful to start new MFC reactors due to the dominance of exoelectrogens like *Geobacter* spp.

CRediT authorship contribution statement

Kyoung-Yeol Kim: Conceptualization, Investigation, Formal analysis, Methodology, Data curation, Writing - original draft. **Ruggero Rossi:** Conceptualization, Investigation, Formal analysis, Methodology, Data curation, Writing - review & editing. **John M. Regan:** Methodology, Data curation, Writing - review & editing. **Bruce E. Logan:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2020.107816.

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