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A Simple and Low-Cost Procedure for Growing *Geobacter sulfurreducens* Cell Cultures and Biofilms in Bioelectrochemical Systems

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Abstract

Anaerobic microorganisms play a central role in several environmental processes and regulate global biogeochemical cycling of nutrients and minerals. Many anaerobic microorganisms are important for the production of bioenergy and biofuels. However, the major hurdle in studying anaerobic microorganisms in the laboratory is the requirement for sophisticated and expensive gassing stations and glove boxes to create and maintain the anaerobic environment. This appendix presents a simple design for a gassing station that can be used readily by an inexperienced investigator for cultivation of anaerobic microorganisms. In addition, this appendix also details the low-cost assembly of bioelectrochemical systems and outlines a simplified procedure for cultivating and analyzing bacterial cell cultures and biofilms that produce electric current, using *Geobacter sulfurreducens* as a model organism.

Keywords

biofilm; anaerobes; gassing station; bioelectrochemical systems; microbial fuel cell; *Geobacter sulfurreducens*

INTRODUCTION

There are a variety of methods and systems available to grow anaerobic microorganisms at the bench as well as in bioelectrochemical systems (BES; Rabaey et al., 2009). Many such methods have been previously reviewed in detail. For example, Reguera and colleagues (see *APPENDIX 4F*; Speers et al., 2009) reviewed the principles and techniques used in anaerobic gassing stations. This equipment is an integral part of anaerobic research in a laboratory because it facilitates the maintenance of an anaerobic environment for the growth and transfer of cultures routinely on a bench without any need for cumbersome anaerobic glove chambers (glove boxes). In addition, gassing stations allow rapid preparation of anaerobic medium. However, many of these methods are cumbersome and expensive. Sophisticated anaerobic glove chambers and manually built gassing stations are necessary to create and maintain an anoxic environment. In this appendix, a simple design for constructing a gassing station that can be followed by an inexperienced investigator using items that can be purchased off the shelf is presented for cultivation of anaerobic

microorganisms. This appendix describes a simplified protocol to grow anaerobic microorganisms using this gassing station.

BES have gained considerable interest in the past few years due to their ability to harness the catalytic activity of anaerobic microorganisms for the production of commodity chemicals, bioenergy, and biofuels (Logan and Rabaey, 2012; Lovley and Nevin, 2013). This appendix presents the low-cost assembly of bioelectrochemical systems and outlines a simplified procedure for cultivating and analyzing bacterial cell cultures and biofilms that produce electric current. The methods provided here can be readily expanded to other anaerobic microorganisms and to BES with different configurations.

Geobacter sulfurreducens is chosen as a model microorganism because of its importance in several biogeochemical processes (Lovely et al., 2011). *Geobacteraceae* are among the few pure-culture microorganisms that have been studied extensively in BES for the last decade to identify the mechanism of electron transfer through biofilms grown on the anodes of the apparatus. This appendix also describes the basic techniques to maintain and grow *G. sulfurreducens* in the BES.

Basic Protocol 1 introduces and describes how to assemble a functional gassing station. In Basic Protocols 2 and 3, this gassing station is used to prepare anaerobic medium and inoculate a culture of *Geobacter sulfurreducens*, respectively. Basic Protocol 4 outlines how to assemble a bioelectrochemical system (BES); its operation as a microbial electrolysis cell (MEC) is described in Basic Protocol 5.

CAUTION: *G. sulfurreducens* is a common soil microorganism with Biosafety Level 1 (BSL-1). Follow sterile practices to avoid contamination, and follow your institutional guidelines for handling and safety while working with BSL-1 microorganisms. Refer to *UNIT 1A.1* (Burnett et al., 2009) and other pertinent resources (*APPENDIX 1B*) for instructions on safe handling of microorganisms.

BASIC PROTOCOL 1. PREPARATION OF THE SIMPLIFIED GASSING STATION

A gassing station is the most common method for producing anoxic medium. Many modern gassing stations are derivatives of the Hungate technique that was originally developed in 1969 by Hungate and Nottingham. Gassing stations remove O₂ by sparging (“bubbling”) inert gases (traditionally N₂ and CO₂) into medium with a sealed cap (described in detail below). The inert gases remove dissolved O₂ from the medium and the headspace of the vessel. While many investigators use variations of this technique, gassing stations have traditionally needed to be built manually. The need for manual construction is a barrier for inquiring scientists looking to incorporate anaerobic components into their investigations. In this protocol, we describe the assembly of a gassing station with a simplified gassing manifold that we have designed and made commercially available. This manifold significantly reduces assembly complexity and resolves complications of the Hungate technique (discussed below in the Commentary).

The pre-assembled gassing manifold (Fig. A.4K.1, Table A.4K.1) leaves a new investigator only with the task of connecting the manifold to a gas cylinder, mounting the manifold in the lab, and connecting the tubing for dispensing gas. The most cumbersome component of the gassing station is the gas cylinder, which should be strapped to the lab bench so it cannot move or fall over. Alternatively, multiple gas cylinders can be stored in a gas cabinet and copper tubing can be used to supply gases to individual gassing stations on every lab bench. The copper tubing connects the gas cylinders (80:20 N₂:CO₂ for this work) to the mounted manifold gas inlet.

Materials

Inert gas cylinder (80:20 N₂:CO₂ for this work)
Pressure regulator
Gassing station manifold (Swagelok)
Copper tubing, 1/8-in. O.D. (Swagelok, cat. no. CU-T2-S-030-50)
Rubber tubing (Cole Parmer, cat. no. 06508-14)
Male luer-lock fittings 1/16-in. (Cole Parmer, cat. no. 41507-26)
Sterile 1-ml plastic syringe

1. Connect the pressure regulator to a full gas cylinder.

In this system, the regulator on the gas cylinder determines the pressure of the system, and the gas flow rate can be adjusted using the flow meter on the front of the manifold. The front of the manifold control panel contains a switch with four positions (Fig. A.4K.1E):

1. The “toggle valve” setting passes flow control to the toggles above each port. This valve is typically used in the preparation of anaerobic medium.
2. The “needle valve” setting is used for finer control of the gas flow. This is typically used for a syringe port to inoculate and transfer cell cultures.
3. The “vent” setting is used to remove any residual gas from the apparatus once experiments are finished.
4. The “closed/off” setting is used when the gassing station is not in use.

For the gassing stations that are only connected to BES to maintain anaerobic environments in the anode and cathode chamber, a simpler version with only the needle valve setting can be used.

2. Connect the regulator to the inlet tube of the gassing station manifold using 1/8-in. copper tubing.

Ensure that the copper tubing will be long enough to connect the gas cylinder and manifold in their final positions before cutting the tubing.

3. Mount the gassing station on a lab bench at an appropriate height such that there is room below the station for medium bottles.

We recommend mounting one gassing station on the lab bench and another in a walk-in incubation room so that anaerobic medium can be made in the lab and microbial electrolysis cells (BES) can be kept anaerobic during biofilm growth. Alternatively, the gassing station can be relocated back and forth for the two processes.

4. Connect rubber tubing to the hose connectors at the bottom of the gassing station and fit them with either 1/16-in. male luer-lock fittings or inexpensive 1-ml plastic syringes so that each hose can dispense gas to appropriate connectors.

BASIC PROTOCOL 2. PREPARATION OF ANAEROBIC MEDIUM USING THE GASSING STATION

This protocol presents the steps for preparing anoxic medium, a critical component for successful growth of anaerobic bacteria. In brief, medium prepared following the instructions in the Reagents and Solutions section of this appendix are sparged (“bubbled”) with inert oxygen-free gas to remove dissolved oxygen from the solution and headspace of the vessel. Vessels are then autoclaved to further sterilize them before use. The vessels are sealed with butyl rubber stoppers and aluminum crimps that keep them anaerobic for several months, allowing anaerobic medium to be prepared in bulk. While the protocol uses NBAF medium as an example, this protocol should be followed for all media and solutions that need to be made anaerobic.

Materials

NBAF medium (see recipe)

10-ml pressure tubes (Chemglass, cat. no. CLS-4209-10) *or* 125-ml serum bottles (Fisher Scientific, cat. no. 06-406K)

Assembled gassing station (Basic Protocol 1)

Cannulas (reusable stainless steel needles; Cadence Science, cat. no. 7937)

Inert gas cylinder (80:20 N₂:CO₂ for this work)

Blue butyl rubber stoppers (Chemglass, cat. no. CLS-4209-14)

Aluminum crimps (Fisher, cat. no. 06-406-15)

Crimper (Chemglass, cat. no. CG-4930-20 or equivalent)

1. Dispense 10 ml NBAF medium into 10-ml pressure tubes or 100 ml into 125-ml serum bottles.

Always use glass vessels specifically designed for anaerobic culturing.

2. Connect the gassing station hosing to cannulas. An example of the connection of tubing to the plastic syringe and then of the syringe to the cannula is presented in Figure A.4K.2A-B. Insert the cannulas into the glass vessels so that they touch

the bottom. Switch the gassing station control valve to the toggle valve position and sparge with 80:20 N₂:CO₂ for 6 min for pressure tubes and 30 min for serum bottles (Fig. A.4K.2A).

3. Close the vessels with butyl rubber stoppers while keeping the cannulas at the bottom of the vessels, and sparge pressure tubes with 80:20 N₂:CO₂ for 6 min, or serum bottles for 15 min (Fig. A.4K.2B).

We recommend periodically checking the vessel during the sparging process to make sure the butyl stopper stays in place.

4. While holding the butyl stopper in place, lift the cannula tip into the headspace (air just above the level of the medium) and sparge pressure tubes for 3 additional min and serum bottles for 15 additional min.

For vessels of 1 liter and more, use a sparger (Ace Glass, cat. no. 6453-109) instead of cannulas for more effective bubbling. Connect the sparger to 1/16-in. rubber tubing with a female luer-lock (Cole Parmer; see Fig. A.4K.2C) so that it can be connected to the gassing station hose. Bubble the 1-liter medium bottle with the sparger for at least 1 hr with the cap open (Fig. A.4K.2A). After the 1 hr of bubbling, replace the sparger with a cannula, close the vessel with a butyl stopper, and sparge in the headspace for at least 30 min before use (Fig. A.4K.2B).

5. Seal the vessels with aluminum crimps using a crimper. This provides a tight seal for long-term storage of the anaerobic medium.
6. Autoclave using a fast/dry cycle for 30 min. Do not tighten the bottle caps of the 1-liter bottles completely, to avoid build-up of pressure inside bottles (the aluminum crimps are left on the 10-ml pressure tubes and 125-ml serum bottles). Always add water to the trays used for autoclaving. Remove medium the 1-liter medium vessels immediately after the autoclave cycle is complete to avoid loss of medium due to prolonged evaporation.

BASIC PROTOCOL 3. INOCULATION AND GROWTH OF *GEOBACTER* CULTURE

The *Geobacter* culture is initially grown in anaerobic vessels (pressure tubes and serum bottles) and then transferred into the BES. Initially growing the bacteria in anaerobic vessels gets the cells to mid-exponential growth phase, which increases probability of survival during the transfer into the BES. Furthermore, several transfers are completed between anaerobic vessels to dilute the 100 µl dimethyl sulfoxide (DMSO) present in 1 ml freezer stocks. This protocol describes how to grow *Geobacter* to an acceptable optical density from a freezer stock.

Materials

70% and 100% ethanol

100 mM cysteine (see recipe)

NBAF medium (see recipe) in 10-ml pressure tube (see Basic Protocol 2)

G. sulfurreducens strain PCA (ATCC #515733), frozen stock

Glass syringe filter (See Support Protocol 2), autoclaved

Tripod stand

Sterile 1-ml syringe and 25-G needle

Sterile 3-ml syringe and 25-G needle

Spectrophotometer for determining OD₆₀₀

125-ml serum bottles (Fisher Scientific, cat. no. 06-406K)

Additional reagents and equipment for preparing glass syringe filter (Support Protocol 2)

Prepare an anoxic syringe

- 1 Wearing gloves that have been sterilized with 70% ethanol, mount the glass syringe filter on a tripod stand for ease of access (Fig. A.4K.3B).
- 2 Sterilize the front of the glass syringe filter by placing a drop of 100% ethanol on it and burning it with a Bunsen burner.
- 3 Fit a sterile 1-ml syringe with a sterile 25-G needle.
- 4 Insert the needle into the tip of the glass syringe, ensuring a good seal so that the gas pumps directly into the plastic syringe and slowly pushes the plunger back.
- 5 Once the plunger is close to the end, remove the needle from the glass syringe and depress the plunger to expel the gas.

Pay attention to not allow the plunger to be fully expelled from the syringe.
- 6 Repeat ~10 times.

Prepare the medium with cysteine

- 7 Use the syringe to take up 0.1 ml of 100 mM cysteine stock.
- 8 Sterilize the top of a 10-ml NBAF medium pressure tube by placing a drop of 100% ethanol on the butyl stopper and flaming it.
- 9 Transfer the cysteine solution into the 10-ml of NBAF medium.

Cysteine acts as a reducing agent for medium prepared with resazurin and should change the color of the medium from pink to colorless within 1 hr.

Inoculate and grow culture

- 10 Thaw a freezer stock of *G. sulfurreducens* culture on ice. Once thawed, transfer the culture into a cysteine-reduced, "colorless" NBAF pressure tube using an

anoxic, sterile 3-ml (see steps 1 to 6 above, but use a 3-ml syringe instead of a 1-ml syringe) syringe fit with a 25-G needle.

- 11 Keep the culture at 30°C for ~2 days. Once growth is observed (the medium will become opaque) and the optical density (OD₆₀₀ in a disposable plastic cuvette) is 0.2 to 0.3, transfer 3% of the culture into another 10-ml reduced NBAF pressure tube and let it grow at 30°C until the OD₆₀₀ again reaches 0.2 to 0.3 indicating the mid-exponential phase.
- 12 For BES cell inoculum, transfer 10 ml of cell culture in the mid-exponential phase into 100 ml NBAF (in a 125-ml serum bottle) and incubate at 30°C.

Once the culture grows to OD₆₀₀ ~0.2, the culture is ready for injection into the BES (described in detail below). A total of 25 to 50 ml culture can be injected into the anode chamber of BES for rapid growth of bacterial cells.

BASIC PROTOCOL 4. CONSTRUCTION OF THE BIOELECTROCHEMICAL SYSTEM (BES)

Bioelectrochemical systems (BES) use microorganisms to catalyze an oxidation and/or reduction reaction at an anodic and/or cathodic electrode, respectively (Fig. A.4K.4; Rabaey et al., 2009; Babauta et al., 2015; Speers and Reguera, 2015). BES have gained considerable interest in the past few years due to their ability to harness the catalytic activity of anaerobic microorganisms for the production of commodity chemicals, bioenergy, and biofuels (Rabaey et al., 2009; Babauta et al., 2015; Speers and Reguera, 2015). The principles and operations of BES have been reviewed in detail in multiple previous publications (Rabaey et al., 2009; Babauta et al., 2015; Speers and Reguera, 2015), and the Commentary section of this appendix also provides an overview of the essential concepts and terminology in BES. This protocol provides instructions for assembling a reusable, low-cost BES for growing current-producing *Geobacter* biofilms. Additionally, we aim to reduce the inherent confusion that accompanies assembling a new apparatus. While the sheer number of parts appears daunting, we have constructed several resources to make things clear: Figure A.4K.5 presents the parts of a BES prior to assembly, Figure A.4K.6 presents an assembled BES, and Figure A.4K.7 provides a tabulation of equipment with a picture adjacent to each item for reference during construction. Furthermore, the materials section below includes the solvents, reagents, and special equipment that are used to operate a BES.

Materials

0.5 M HCl

0.5 N NaOH

Double-distilled H₂O

100% and 70% ethanol

Inert gas cylinder (80:20 N₂:CO₂ for this work)

FWAF medium, anaerobic (see recipe)
FWNN medium, sterile, aerobic (see recipe)
10% bleach
Items in Figure A.4K.7
Drill
25-G needle
Assembled gassing station (Basic Protocol 1)
Autoclaved 0.2- μ m filter (Pall, cat. no. 4423)
Rubber tubing (Cole Parmer, item no. EW-06508-14)
25-G needle
21-G BD Vacutainer Eclipse Blood Collection Needle (BD, cat. no. 368607)
Known standard electrode
Vinyl tape (of type used in anaerobic glove boxes, e.g., 3 M, cat. no. 471)

Prepare the electrodes

- 1 Drill a hole (2 mm diameter, 2 cm depth) into the top of each graphite electrode (Fig. A.4K.7, item no. 7).
- 2 Insert the watertight XSA-BC–connector (Fig. A.4K.7, item No. 8) into the hole.

Before every use, clean the electrodes by placing them overnight in 0.5 M HCl and then 0.5 N NaOH. Rinse thoroughly with double-deionized water by running the water over them overnight. Take care to minimize water contact with the metal pins of the XSA-BC connector.
- 3 Connect the electrodes to the wires.
- 4 Insert each glass chimney (Figure A.4K.7, item no. 3) into an orange rubber stopper (Fig. A.4K.7, item no. 4).
- 5 Drill a hole into a blue butyl stopper (Fig. A.4K.7, item no.13) that is just big enough to let the watertight electrode connector wire through, while also small enough to seal it very tightly against oxygen diffusion.
- 6 Thread the wire through the chimney and the blue butyl stoppers. Then, position the graphite electrode in the chamber at the level of the membrane, but high enough to allow the stir bar to rotate (Figs. A.4K.5K and A.4K.6).

Prepare the membrane

- 7 Cut a 5 $\times$ 5–cm piece of the Nafion 117-membrane (Fig. A.4K.7, item no. 10).
- 8 Put the cut membrane into a glass beaker filled with double-distilled water. Take care to completely cover the membrane with water.

Once the membrane makes contact with water, it should always stay solvated (at least on one side); otherwise it will dry and be destroyed.

- 9 Heat the water with a hot plate until it starts to boil, then remove the heat source and allow it to cool to room temperature.

Always use a new Nafion membrane for assembly. If the BES needs re-autoclaving, replace the membrane with a fresh one.

Assemble the cell

- 10 Seal the three sideways apertures of the glass chambers with the blue butyl stoppers (Fig. A.4K.7, item no. 5) and crimp them closed with aluminum seals (Fig. A.4K.7, Item No. 6).
- 11 Fill one chamber with double-distilled water. Put the O-ring (Fig. A.4K.7, item no. 9) on the joint of this chamber.
- 12 Take the membrane out of the water-filled beaker and lay it onto the O-ring. Take care to minimize loss of contact with water.
- 13 Take the other chamber and hold its junction against the membrane, and clamp the chambers together with the pinch clamp (Fig. A.4K.7, item no. 2).
- 14 Fill each chamber with approximately 150 ml double-distilled water until the membrane is totally covered.
- 15 Place a stir bar (Fig. A.4K.7, item no. 11) into each chamber.
- 16 Insert both electrode/wire/rubber stopper/chimney constructions made in the electrode preparation steps above (see Fig. A.4K.5K) into the glass chambers and fix them into place with the red screw caps.
- 17 Peel back the aluminum cap of the top sideways aperture of each chamber halfway, insert a 25-G needle, and cover it with aluminum foil.

This allows pressure equilibration during the autoclave process.
- 18 Check for water leaks by placing the microbial electrolysis cell (BES) on a piece of dry paper.
- 19 Autoclave the fuel cell for 20 min on a fast/dry cycle. Remove the fuel cell immediately after the autoclave cycle is complete to avoid excessive water evaporation, which can expose the membrane to air and dry it out.

The fuel cell is ready for use after cooling. Alternatively, the fuel cell can be stored for several days for future use.

Fill the cell

- 20 Select one chamber as the anode chamber and the other as the cathode chamber. Label the chambers accordingly with marking tape.
- 21 Fit the hose from the gassing station with an autoclaved 0.2- μ m filter and a 25-G needle.

- 22** Sterilize the bottom sideways rubber stopper of the MEC by placing a drop of 100% ethanol on it and flaming the end. Insert the needle in the stopper and flush out the oxygen by bubbling both chambers with 80:20 N₂:CO₂ gas for ~30 min.
- 23** Connect the vacuum pump (Fig. A.4K.7, item no. 15) to the anode chamber with rubber tubing (Cole Parmer, item no. EW-06508-14) and a sterile 25-G needle, which is typically injected into the bottom sideways aperture (Fig. A.4K.8). Turn on the pump to pressurize the cell and pump the water out of the anode chamber. During the vacuum pumping, simultaneously flush the chamber with filter-sterilized anaerobic gas.
- The needle in the top sideways aperture allows pressure equilibration and prevents bursting of the membrane.
- 24** After the emptying process is complete, fill the anode chamber with anaerobic FWAF medium by following these steps:
- a.** Pressurize the FWAF media bottle with sterile anaerobic gas and connect it to the top sideways aperture of the MEC with a 21-G BD Vacutainer Eclipse Blood Collection Needle.
 - b.** Allow the bottle to fill the anode chamber until the medium completely covers the graphite electrode (total volume of 200 to 250 ml).
- 25** While continuing to bubble the anode chamber with sterile, anaerobic gas, empty the cathode chamber using the vacuum pump.
- 26** Fill the cathode chamber with sterile, aerobic FWNN medium using the same method as for the anode chamber.

Insert the reference electrode

Always store reference electrodes in an electrolyte solution or deionized water.

- 27** Test the stability of reference electrode (Fig. A.4K.7, Item No. 12) by measuring the potential against a known standard electrode.
- Sterilize gloves with 70% ethanol while preparing the reference electrode.
- 28** Sterilize the reference electrode by placing it in freshly prepared 10% bleach for 5 min. Wash thoroughly with autoclaved double-deionized water.
- 29** Insert the sterilized reference electrode into a previously-autoclaved blue butyl rubber stopper with a hole (Fig. A.4K.7, item no. 13; also see Fig. A.4K.6).
- 30** By slightly tilting the BES towards the cathode chamber, replace the middle blue stopper with the above reference electrode–stopper assembly. Seal the stopper with vinyl tape.

BASIC PROTOCOL 5. MICROBIAL ELECTROLYSIS CELL (MEC) OPERATION

When a BES is run by maintaining a constant potential between the electrodes, it is termed a microbial electrolysis cell (MEC). This work uses a potentiostat to maintain a constant potential at the anode (working electrode) so that the bacteria can use the electrode as a terminal electron acceptor. Operate the MEC with the following three considerations:

1. Use FWAF mode for the initial growth of the bacterial cells in the MEC. In this mode, bacteria use fumarate in the medium as a terminal electron acceptor in addition to the anode.
2. Use FWA batch mode to promote biofilm growth on the electrodes by removing fumarate from the medium, so that the only remaining terminal electron acceptor is the anode (which is kept at a constant potential with the potentiostat).
3. Use flowthrough mode with a peristaltic pump to replenish the medium with an uninterrupted supply of medium with electron donor (acetate) and to remove planktonic cells. Flowthrough mode is critical for the growth of multilayer biofilms that can be hundreds of micrometers thick and produce high electric current. The maximum current observed with *G. sulfurreducens* strain PCA is ~12 mA with the MEC setup described in this appendix.

Materials

Serum bottle containing at least 60 ml of cells in mid-exponential phase ($OD_{600} \sim 0.3$; see Basic Protocol 3)

100% ethanol

FWAF medium, anaerobic (see recipe)

FWA medium, anaerobic (see recipe)

DL vitamins (see recipe)

DL minerals (see recipe)

10% bleach

Assembled gassing station (Basic Protocol 1)

25-G needles

21-G BD Vacutainer Eclipse Blood Collection Needle (BD, cat. no. 368607)

Thermo Cimatec Poly 15 stir plate (Thermo Scientific)

Glass syringe filter prepares as in Support Protocol 2

Ag/AgCl reference electrode (Electrolytica, cat. no. C-925)

Potentiostat (Gamry Instruments, cat. no. 992-00088, Interface 1000E Potentiostat or equivalent)

Spectrophotometer for determining OD_{600}

Masterflex PharMed BPT tubing 3/16-in. (Cole Parmer, cat. no. EW-06508-15)

Autoclaved rubber stopper (size 6.5) with two holes

Sparger (Ace Glass, cat. no. 6453-109)

Female luer-lock hose connector (Cole Parmer, cat. no. EW-41507-30)

Fisherbrand Instant Sealing Sterilization Pouch (Fisher, cat. no. 01-812-57, or equivalent)

10-liter jug (Corning, cat. no. 1395-10L)

Male Luer-lock connectors (Cole Parmer, cat. no. EW-41507-26)

Pump tubing (2-STOP PHARMED, 1.30 mm I.D., Cole Parmer, cat. no. EW-95713-32)

Peristaltic pump (Figure 7C.1.7, item 14; Cole Parmer, cat. no. EW-78001-42)

Walk-in culture room set to 25°C and 30% humidity for optimal growth

FWAF mode

This mode enables the planktonic growth of bacterial cells inside the MEC chamber that can be later used for the biofilm grown using the electrodes as terminal electron acceptor. For the FWAF mode:

- 1 Fit the end of one of the gassing station hoses with a sterile 25-G needle, and turn on the gas flow.
- 2 Sterilize the cap of a serum bottle that has at least 60 ml of cells in mid-exponential phase ($OD_{600} \sim 0.3$) by placing a drop of 100% ethanol on the butyl stopper and flaming it.
- 3 Insert the needle attached to the gassing station hose (step 1) into the side of the butyl stopper of the serum bottle to pressurize the vessel.

Stick the needle through the side of the butyl stopper to allow more room for the double-sided needle that will be used for transferring the culture into the MEC in a later step.
- 4 Stick one end of a double-sided needle (21-G BD Vacutainer Eclipse Blood Collection Needle) through the center of the pressurized serum bottle.
- 5 Insert the other end of the double-sided needle into the top sideways aperture of the anode chamber (filled with FWAF medium) and dispense roughly 50 ml of the cells.

If the medium level is now higher than the highest sideways aperture and the pressure of the cell is high, some of the medium will flow out of the cell upon removal of the double-sided needle. Remedy this by temporarily inserting a sterile 25-G needle into the top butyl stopper to release the pressure.

- 6 Move the cell onto the stir plate and turn the stirrer on so that there is gentle flow in each chamber (speed of 150 for Thermo Cimarac Poly 15).
- 7 Attach a glass syringe filter (prepared as in Support Protocol 2) to the gassing station hose line.
- 8 Attach a sterile 25-G needle to the end of the glass syringe filter and insert it into the bottom sideways aperture of the cathode chamber.

Sparging the chamber at ~1 bubble per second is enough. Sparge only the cathode chamber during the FWAf fumarate mode and FWA batch mode. Sparging the anode chamber during these modes can disrupt the initial biofilm growth.
- 9 Stabilize the rubber stopper, then insert a sterile 25-G needle into the top sideways aperture of the cathode chamber to allow the pressure to equilibrate.
- 10 Connect the working electrode cable of the potentiostat to the MEC anode, the counter electrode cable of the potentiostat to the MEC cathode, and the reference electrode cable of the potentiostat to the MEC reference electrode.
- 11 Apply 0.3 V versus Ag/AgCl (typical value for *G. sulfurreducens*) and set the potentiostat to continuously record the current using the potentiostat's data-acquisition program.
- 12 Check the OD₆₀₀ regularly (1 to 2 times a day) and once it reaches ~0.2, move onto FWA-batch mode.

FWA batch mode (no fumarate)

This mode eliminates the soluble electron acceptor fumarate and forces the bacteria to grow using the anode as the terminal electron acceptor.

- 13 Replace the medium in the anode chamber with FWA medium following the emptying/filling steps outlined above.
- 14 Continue to monitor the current until it reaches ~0.2 mA or reaches a maximum and starts to drop, then move onto flowthrough mode.

Flowthrough mode

This mode provides a continuous supply of fresh medium to remove planktonic cells and enables formation of thick biofilms that produce high current density in MEC.

- 15 Pass two sets of 3/16-in. Masterflex PharMed BPT tubing through a rubber stopper (size 6.5) that has two 3/16-in. holes in the top.

The tubing should be long enough that when spargers are connected to the ends they will be at the bottom of the 10-liter jugs (~3 feet is adequate).
- 16 For both of the tubes, connect the bottom end (that will eventually go into the medium) with a sparger and the top end with a female luer-lock hose connector.

- 17 Autoclave this assembly on a fast/dry cycle for 30 min using an instant sealing sterilization pouch.
- After autoclaving, the assembly can be stored for several days.
- 18 Add 100 ml each of filter-sterilized DL vitamins and DL minerals to a 10-liter jug filled with 9800 ml FWA medium, next to a lit Bunsen burner, which reduces the possibility of contamination from airborne contaminants.
- Make sure the jug has been autoclaved and cooled down before adding in the DL vitamins and DL minerals.
- 19 Seal the jug with the rubber stopper–tubing assembly prepared in steps 15 to 17, being careful to not touch the bottom half of the tubing that will be immersed in the medium.
- 20 Connect the gassing station to a glass syringe filter prepared as in Support Protocol 2, and then connect it to one of the female luer-locks so that the medium jug can be sparged. Sparge the jug for 45 to 60 min.
- 21 Connect the anode chamber of the MEC to the 10-liter FWA medium jug using pump tubing (2-STOP PHARMED, 1.30 mm I.D.) that has male Luer-lock connectors at both the ends.
- One end connects to the female luer-lock of the medium jug and the other is fit with a sterile 25-G needle and is inserted into the top sideways aperture of the anode chamber (after flame sterilizing the butyl rubber stopper).
- 22 Lay the pump tubing down onto the peristaltic pump and use the stopper to lock the tubing down to ensure a proper flow rate. Set the pump to flow at 0.5 ml/min.
- 23 To collect used medium as the MEC fills up, fit autoclaved rubber tubing with a sterile 25-G needle and insert it into the top sideways aperture, and put the other end of the tubing in a waste jug (See Fig. A.4K.9). Add 10% bleach to the jug to reduce contamination and empty the waste jug daily. Use bleach to clean the jug.

SUPPORT PROTOCOL 1. TESTING OF MEDIA

A batch of medium should always be tested before use. Below are the testing protocols for NBAF, FWAF, and the 100 mM cysteine media used in the protocols above.

Materials

Pressure tubes and serum bottle with NBAF or FWAF medium to be tested (see recipes and Basic Protocol 2)

100 mM cysteine (“good batch”; already tested according to the steps below)

“Happy” wild-type NBAF culture of *Geobacter* (Basic Protocol 3; do not allow to stand in stationary phase > 2 days)

Serum bottle with 100 mM cysteine to be tested (see recipe and Basic Protocol 2)

Litmus paper

For NBAF and FWAF

If the medium was made on the same day, testing two pressure tubes will suffice. If the medium was made on separate days, test both two pressure tubes and a 125-ml serum bottle.

- 1a** Reduce the two 10-ml pressure tubes and a 125-ml serum bottle of NBAF or FWAF by adding 0.1 ml or 1.0 ml of 100 mM cysteine (from a good batch), respectively.

The medium should change from purple/pink to colorless within 1.5 hr.

- 2a** Inoculate one pressure tube with 0.1 ml and the other with 0.5 ml of a “happy” wild-type NBAF culture of *Geobacter* (a culture that has not sat in stationary phase for more than 2 days). Inoculate the 125-ml serum bottle with 5 ml of the happy culture.
- 3a** To pass inspection, the pressure tube inoculated with 0.5 ml culture should reach an $OD_{600} > 0.6$ within 24 hr. The pressure tube inoculated with 0.1 ml should reach an $OD_{600} > 0.6$ within 2.5 days. For the serum bottle, the OD_{600} should surpass 0.4 within 24 hr.
- 4a** If the NBAF passes inspection, record its success on a medium batch chart and transfer the medium from untested to tested medium drawers.
- 5a** If the NBAF fails inspection, record its failure on the medium batch chart and throw out the entire batch.

For 100 mM cysteine

- 1b** Take one serum bottle of 100 mM cysteine and reduce two pressure tubes of NBAF (from a good/inspected batch) by adding 0.1 ml of 100 mM cysteine per each 10-ml pressure tube.

The medium should go from purple to colorless within 1.5 hr.

- 2b** Inoculate one pressure tube with 0.1 ml and the other with 0.5 ml of a “happy” wild-type NBAF culture of *Geobacter* (a culture that has not sat at their stationary phase for more than two 2 days).
- 3b** The tube inoculated with 0.5 ml cell culture should reach and $OD_{600} > 0.6$ within ~ 24 hr. The tube inoculated with 0.1 ml should reach $OD_{600} > 0.6$ within ~ 2.5 days.
- 4b** Withdraw 1 ml and check that the pH is ~ 7 with litmus paper.
- 5b** If the cysteine passes inspection, record its success on a medium batch chart and transfer the medium from untested to tested medium drawers.
- 6b** If the cysteine fails inspection, record its failure on a medium batch chart. Throw out the entire batch.

SUPPORT PROTOCOL 2. PREPARE GLASS SYRINGE FILTER ASSEMBLY

Plastic syringes can be used to transfer anaerobic medium and the bacterial cultures, but special care must be taken to ensure that an anaerobic and sterile environment is maintained. A filtering system attached to the end of the gas tubing is a common safeguard against possible contaminants. While filters are commercially available (Pall, cat. no. 4423), and are occasionally used in our laboratory, in the following protocol we describe the assembly of an inexpensive glass-syringe filter system that can be built in-house and that has worked effectively.

Materials

Glass syringe (Cadence Science, cat. no. 5024, or Fisher, cat. no. 14-825-3B)

Glass wool (Fisher, cat. no. AC38606-0500)

Rubber stopper (size #1)

18-G needle

1. Fill 75% to 90% of a glass syringe with glass wool.
2. Cap the syringe with a rubber stopper (size #1; Fig. A.4K.3A).
3. Penetrate the stopper with an 18-G needle.
4. Autoclave the assembly for 30 min using fast/dry cycle.

The syringe filter can be used immediately or stored in a covered container.

The syringe is now ready to either flush out oxygen from syringes for the inoculation and transfer of cell cultures (described in Basic Protocol 3) or be fitted with a needle to provide anoxic, sterile gas for sparging the BES (described in Basic Protocol 4).

SUPPORT PROTOCOL 3. MEC MAINTENANCE

If the peristaltic pump is set to 0.5 ml/min, an 8-liter medium jug can be used for approximately 11 days for one MEC. A lower pumping speed can be used for longer-term operations. Multiple MECs can be connected to a common jug using a splitter (Fig. A.4K.9) where the glass joint is prepared in a local glass shop and connected with BPT tubing and female luer-lock connectors as described earlier for the medium jug. While connecting the jug, make sure that there are no air bubbles and the medium is flowing smoothly from the jug through the MEC to the waste jug. The glass syringe filter assembly with the glass wool needs to be replaced periodically if it is blocking the gas supply. Also, monitor for possible water leaks regularly.

REAGENTS AND SOLUTIONS

Use deionized (DI) water for all solutions.

Cysteine, 100 mM—Table A.4K.2 presents the ingredients needed to make the 100 mM cysteine stock. Make the 100 mM cysteine stock solution by adding 17.56 g of cysteine HCl to 800 ml deionized water. If the solution will be made anaerobic (see Basic Protocol 2) with pure N₂ gas, adjust the pH to 7.0 with NaOH; if the solution will be made anaerobic with 80:20 N₂:CO₂ gas, adjust the pH to 7.5 before bubbling to take into account the drop in pH from the CO₂ introduced during the sparging process. After the pH adjustment, bring the volume to 1000 ml with deionized water and make the solution anaerobic following the procedure discussed in the manuscript. Aliquot the stock solution into 10-ml pressure tubes and 100-ml serum bottles, and make them anaerobic using the steps presented in Basic Protocol 2. Store the batch in drawers marked “untested” until the medium testing protocol (See Support Protocol 1) is followed. Store up to 1 month at room temperature.

DL mineral mix—Table A.4K.3 presents the components of the DL mineral mix. To make the DL mineral mix stock solution, start by adding 1.5 g nitrilotriacetic acid trisodium salt (NTA) to 800 ml deionized water. Then, add the other components of Table A.4K.3 to the solution, mix, and complete the volume to 1000 ml with deionized water. Store the solution up to 1 month at 4°C.

DL vitamin mix—Table A.4K.4 presents the components of the DL vitamin mix. To make the DL vitamin mix stock solution, add the components of Table A.4K.4 to 800 ml deionized water, mix, then complete to 1000 ml with deionized water. Final pH should be around 7. Store the bottle out of direct sunlight in an opaque bottle up to 1 month at 4°C.

FWA medium—Table A.4K.5, part A lists the components for 1 liter Fresh Water Acetate (FWA) medium. FWA includes the components of Table A.4K.5 and electron donor acetate, but does not contain an electron acceptor. To make 1 liter FWA, add the components of Table A.4K.5A to 800 ml deionized water. The 1.38 g sodium acetate trihydrate corresponds to a final concentration of 10 mM acetate. Mix the components, then bring the volume to 1000 ml. Final pH should be around 7. The medium can be stored in Pyrex bottles, which are sealed by a chimney/rubber stopper/screw-cap construction identical to that for the electrode preparation step (Basic Protocol 4), just without the electrode and the hole in the butyl stopper. Before using the medium, autoclave the containers on 30 min fast or dry cycle. Store up to 1 month at room temperature.

FWA medium (10 liter) for medium jugs—Table A.4K.5, part B lists the components for 10 liters FWA medium. To make 10 liters FWA medium for jugs used in the flowthrough MEC mode (see Basic Protocol 5), add the components of Table A.4K.5B to 8000 ml deionized water. The 13.8 g sodium acetate trihydrate corresponds to a final concentration of 10 mM acetate. Mix the components, then bring the volume to 9800 ml. Final pH should be around 7. Do not add vitamins and minerals yet. Autoclave for 30 min in the liquid cycle with the cap loosely closed. Just before using the medium in flowthrough mode, add 100 ml each of filter-sterilized DL vitamins (see recipe) and DL minerals (see recipe) to the autoclaved medium near a lit Bunsen burner to reduce contamination. Add the bottle-top shown in Fig. A.4K.8D and sparge for at least 30 min to make it anaerobic. Remove air from

the tubes using a 30-ml syringe before connecting to the MECs. Refer to Basic Protocol 5, "flowthrough mode" for details. Store up to 1 month at room temperature.

FWAF medium—Table A.4K.6 lists the components of Fresh Water Acetate Fumarate (FWAF) medium. FWAF medium contains the components of Table A.4K.6 in addition to electron donor acetate (sodium acetate trihydrate, 10 mM final concentration) and electron acceptor fumarate (fumaric acid, 40 mM final concentration). To make FWAF, add the solid components of Table A.4K.6 to 800 ml deionized water, mix the components, and then bring the volume to 1000 ml. Check that the final pH is 7.0. The medium can be stored in Pyrex bottles, which are sealed by a chimney/rubber stop/screw cap construction identical to construction in the electrode preparation step (see Basic Protocol 5), just without the electrode and the hole in the butyl stopper. Before using the medium, autoclave the containers on 30 min fast or dry cycle. Store up to 1 month at room temperature.

FWNN medium—Table A.4K.7 lists the components of FWNN medium. "FW" stands for "Fresh Water," the first "N" stands for no electron donor, and the second "N" stands for no electron acceptor. To make the medium, add the components of Table A.4K.7 to 800 ml deionized water, mix the solution well, then bring the total volume to 1000 ml. Final pH should be around 7. The medium can be stored in Pyrex bottles, which are sealed by a chimney/rubber stop/screw cap construction identical to construction of the electrode preparation step (see Basic Protocol 5), just without the electrode and the hole in the butyl stopper. Before using the medium, autoclave the containers on 30 min fast or dry cycle. Store up to 1 month at room temperature.

Na₂SeO₄, 1 mM—Dissolve 18.9 mg Na₂SeO₄ in 90 ml water, mix well, then bring to the solution to 100 ml with water. Store the solution up to 3 months at 4°C.

NB mineral elixir—Table A.4K.8 lists the components of the NB mineral elixir. Start by adding 2.14 g nitrilotriacetic acid trisodium salt (NTA) to 800 ml deionized water. Add the other components of Table A.4K.8 to the solution, mix, then complete the volume with deionized water to 1000 ml. Store up to 1 month at 4°C.

NBAF medium with resazurin—Table A.4K.9 lists the components of NBAF medium. Add 4.64 g fumaric acid to 800 ml deionized water in a beaker with a stir bar. Adjust the pH of the solution to ~5.0 with 10 N NaOH. As the pH approaches 5, switch to a lower-normality base such as 5 N or 2 N NaOH and bring the pH to 7.0. Then, add the rest of the components of Table A.4K.9 and bring the solution to 1000 ml with deionized water. Aliquot 10 ml of the stock solution into 10-ml pressure tubes (Chemglass, cat. no. CLS-4209-10) and 100 ml into the 125-ml serum bottles (Fisher Scientific, cat. no. 06-406K) and make them anaerobic using the steps presented in Basic Protocol 2. Store the batch in drawers marked "untested" until the medium testing protocol (See Support Protocol 1) is performed.

NB salts, 100×—Table A.4K.10 presents the components of the 100× NB salts stock. To make the 100× NB Salts stock solution, add the solid components of Table A.4K.10 to 700

ml deionized water, mix the components until they dissolve, then complete the volume with deionized water to 1000 ml. Store the solution up to 1 month at 4°C.

COMMENTARY

Background Information

Rationale for using a gassing station—There are a variety of methods for growing anaerobic microorganisms. Many such methods have been reviewed in detail previously. For example, Reguera and colleagues have previously reviewed the principles and techniques used in anaerobic gassing stations (*APPENDIX 4F*; Speers et al., 2009). This equipment is an integral part of anaerobic research in a laboratory because it facilitates the maintenance of anaerobic environment for the growth and transfer of cultures routinely on a bench without any need of cumbersome anaerobic glove chambers (glove box). In addition, gassing stations allow rapid preparation of anaerobic media. The Hungate technique has remained a common tool for most laboratories studying anaerobic microbiology (*APPENDIX 4F*; Speers et al., 2009). To purify the inert gases, the traditional Hungate technique requires pretreatment of the copper column with H₂ and heating the column to 350°C before the sparging process can be carried out. The gassing manifold presented in this appendix replaces the need for H₂ pretreatment and heating of a copper column by including an oxygen trap (Supelco supelpure-O, SKU 22449; 1/8-in. connector) in the gas line (Fig. A.4K.1A-B). However, a gassing station typically requires a complex assembly of multiple parts, which represents a major hurdle for an inexperienced researcher interested in studying anaerobic microbiology. For example, in order to create oxygen-free environment, copper-filled glass columns wrapped in a heating tape are commonly used, which need to be pre-reduced with hydrogen (*APPENDIX 4F*; Speers et al., 2009). A typical gassing station also requires separate gas cylinders for nitrogen, hydrogen, and carbon dioxide, making the whole assembly cumbersome. This appendix presents a simple design developed by the authors that eliminates the use of flammable gases such as hydrogen as well as glass columns filled with copper filings that are typically powered by bulky rheostats. The gassing station with this new design can be purchased off the shelf and readily mounted on a lab bench. A simple procedure to grow anaerobic microorganisms using this gassing station is also presented in this appendix.

Bioelectrochemical systems (BES) and microbial electrolysis cell (MEC)—

Bioelectrochemical systems (Rabaey et al., 2009) (BES) have gained considerable interest in the past few years due to their ability to harness the catalytic activity of anaerobic microorganisms for the production of commodity chemicals, bioenergy, and biofuels (Logan and Rabaey, 2012; Lovley and Nevin, 2013). Electrochemically active biofilms grown on electrodes play a central role in the operational performance of BES (Franks et al., 2010; Babauta et al., 2015). Therefore, this appendix presents low-cost assembly and operation of a reusable BES. A typical two-chambered BES uses microorganisms to catalyze an oxidation and reduction reaction at an anodic and cathodic electrode, respectively. The two electrodes are separated by a proton-exchange membrane (Nafion 117, Electrolytica Inc.) for the selective passage of protons generated at the anode chamber. Current generation at the anode relies upon oxidation of organic and/or inorganic electron donors. Common electron

donors are sodium acetate, waste organics, and sulfides. At the cathode, an electron acceptor, such as oxygen, nitrate, or potassium ferricyanide is reduced. In a bioelectrochemical system, the anode and the cathode are connected through an electrical circuit. If electrical energy is generated from this circuit, the system is called a Microbial Fuel Cell (MFC). On the other hand, if electrical energy is supplied to the electrodes to change the potentials of the anode or the cathode, the system is called a Microbial Electrolysis Cell (MEC). Alternatively, both electrodes can be placed in a single chamber. However, the protons accumulated in the chamber as well as other reactive species present in the medium can undermine BES performance by affecting microbial growth or introducing other limitations. Therefore, this appendix will focus on the two-chambered MECs.

Microbial fuel cell (MFC) versus microbial electrolysis cell (MEC)—In order to generate electricity in an MFC, the anode and cathode need to maintain appropriate electrical potentials that are thermodynamically favorable for electron transfer from anode to cathode, without use of any external power supply. The reduction potential of the cathode is maintained by selecting an appropriate catholyte. Oxygen is commonly used because it is inexpensive and has high theoretical reduction potential ($E^\circ = 0.805$ V versus SHE), but it has low solubility in water and requires a continuous supply of air through the liquid in the cathode chamber. Potassium ferricyanide ($E^\circ = 0.361$ V versus SHE) is an alternate electron acceptor and shows improved BES performance due to the availability of a good electron acceptor at high concentrations, but it needs to be replaced regularly and is not sustainable. In MFCs, the anode and the cathode are connected by an external resistor ($\sim 560 \Omega$) that serves as a load and also regulates the anode potential.

On the other hand, in an MEC, the potential of the anode and the cathode is maintained by applying voltage using an external power supply. For electricity generation, a 3-electrode setup is typically used for better potential control, employing a reference electrode where the anode serves as a working electrode and the cathode serves as counter electrode. A commonly used reference electrode is Ag/AgCl (3 M KCl; Electrolytica, cat. no. C-925) because of its relatively low cost, small size (1/8-in. I.D.) and durability. A potentiostat (e.g., Gamry Instruments, cat. no. 992-00088, Interface 1000E Potentiostat) is commonly used to control the potential between the working and reference electrodes and monitor the current between the working and counter electrodes. This potentiostat can be interfaced with a multiplexer (e.g., Gamry Instruments, cat. no. 992-00108 ECM8 Electrochemical Multiplexer) to apply potential to multiple MECs for long-term experiments. The Gamry ECM8 can apply potential to eight MECs simultaneously and measure current from each MEC in series. In addition to the measurements of electrical current in chronoamperometry mode, the potentiostat can be used to perform several electrochemical experiments such as cyclic, square wave, and differential pulse voltammetry, as well as AC impedance spectroscopy.

Specific microbe: *Geobacter sulfurreducens*—*Geobacter sulfurreducens* (Lovely et al., 2011) is chosen as a model organism for the demonstration of the BES (Speers and Reguera, 2015) for the following reasons. Microbial reduction of insoluble electron acceptors, such as Fe(III) or Mn(IV), has a significant impact on the geochemistry of

submerged soils and sediments and on water quality and the growth of submerged crops such as rice (Lovely et al., 2011). *Geobacter* species are the predominant metal-reducing microorganisms in many of these environments (Lovely et al., 2011) and have evolved strategies to export electrons outside their cell body to extracellular electron acceptors using metalloproteins (c -type cytochromes) and conductive protein appendages called pili displayed on their surface (Malvankar and Lovley, 2012, 2014). As the electrodes in BES are similar to insoluble metal oxides found in their natural environment, the mechanisms of electron transfer in BES share similarities with metal reduction in soils and sediments (Lovely et al., 2011). *Geobacter* species are most often the predominant microorganisms on electrodes harvesting electricity from wastewater (Lovely et al., 2011). Furthermore, *Geobacteraceae* are among the few pure culture microorganisms that have been studied extensively for the last decade to identify the mechanism of electron transfer through biofilms grown on the anodes of BES (Speers and Reguera, 2015). In addition, with the availability of genetic systems and genome sequences, *Geobacter* species are attractive candidates for molecular-level mechanistic studies. Furthermore, *Geobacter sulfurreducens* are capable of generating electricity in BES without any artificial mediators or soluble shuttles (Lovely et al., 2011). Therefore, this appendix presents a detailed protocol to cultivate and analyze *G. sulfurreducens* biofilms in BES. The methods provided here can be expanded to other anaerobic microorganisms and also to BES with different configurations.

Biofilm analysis—Once the biofilm is completely grown and producing stable current, the MEC can be disassembled by disconnecting the electrical connections and tubings. Remove the pinch clip and reference electrode. Always store the unused reference electrode in double-deionized water. Carefully detach the anode from the connectors and place in a petri dish for further analysis.

1. For analysis of an intact biofilm, biofilms grown on electrodes can be imaged either with Scanning Electron Microscopy (Malvankar et al., 2012a) (SEM) or Laser Confocal Scanning Microscopy (LCSM; Malvankar et al., 2011; Malvankar et al., 2012b). Both methods can provide information about parameters such as biofilm thickness, electrode coverage area, and inhomogeneity in the biofilm growth. LCSM requires staining the bacterial cells with fluorescent live-dead stains (e.g., BacLight viability kit by Invitrogen). For the measurement of intrinsic biofilm parameters such as electrical conductivity, a slightly modified design of BES is used and the protocol is described in detail elsewhere (Malvankar et al., 2015).
2. For analysis of total biomass (Malvankar et al., 2011) or extraction of pili (Tan et al., 2016) and cytochrome proteins (Malvankar et al., 2012c,d) from the electrode-grown biofilm, the biofilm is scraped off the electrode gently using a cell scraper and isotonic wash buffer. The extracted biomass is then used for further analysis such as pili purification (Yang et al., 2016) or the measurement of total protein or cytochrome content (Malvankar et al., 2012c,d).
3. For analysis of electrical properties of biofilms such as electrical conductivity, charge transfer resistance, capacitance, and electron transport rate, a detailed

protocol for the experimental design, instrumentation and data analysis is presented elsewhere (Malvankar et al., 2015).

Critical Parameters/Troubleshooting

Preparation of anaerobic media—Maintaining anaerobic conditions and using sterile equipment are crucial when preparing anaerobic media and transferring cultures. Therefore, limiting the time between flame cleaning a butyl stopper and extracting/inserting solutions is a good practice to help increase efficiency.

BES setup—When setting up the BES, take special care to not let the Nafion membrane dry out; otherwise it will not function well. If the BES starts to leak, check that the two chambers are aligned well and tighten the clamp.

MEC operation—During operation, constantly bubble the cathode chamber with sterile anaerobic gas. Do not bubble the anode chamber during the FWAf or FWA-batch modes, to minimize the turbulence to bacterial cells. Start bubbling the anode chamber once the MEC is switched to flowthrough mode, but keep the flow rate low. To avoid contamination, the gas outlets can be fit with sterile filters or glass-syringe filters (Support Protocol 2).

While the bacteria require relatively low maintenance during growth periods in the MEC, special care should be taken to regularly check (one to two times a day) that the sparging is still sufficient. There could be several causes of interrupted sparging, including an empty gas cylinder or a clogged needle. If the needles are suspect, sterilize the butyl rubber stopper and replace them with sterile needles. When in FWA mode, regularly check (about two to three times a day) the MEC current to catch the moment when the current starts to decrease, a sign that the acetate has run out and FWAf needs to be replaced with FWA medium.

Anticipated Results

This appendix details how to assemble the equipment necessary to grow electroactive biofilms in an anaerobic environment. For the *G. sulfurreducens* example used, the wild-type biofilms reach the maximum current of ~12 mA within a few days (Fig. A.4K.10).

Prolonged growth produces higher biomass but the current remains stable. Figure A.4K.10 compares a wild-type *G. sulfurreducens* PCA strain with a mutant strain (Aro5) where the five key aromatic amino acids in the *G. sulfurreducens* pilA monomer have been substituted with alanine (Vargas et al., 2013). This strain produces pili filaments that are morphologically similar to wild-type pili but with significantly lower conductivity (Vargas et al., 2013). This strain does not generate high current due to lack of conductive pili (Vargas et al., 2013).

Time Considerations

When growing *G. sulfurreducens*, an OD₆₀₀ is typically reached within 1 to 2 days in FWAf or NBAF medium. Upon switching to FWA mode in the MEC, the current starts to drop after 2 to 4 days. After switching to flowthrough mode, maximum current output is usually attained after 3 to 5 days.

The bulk of the time considerations revolve around planning the inoculation. While waiting for the initial bacterial growth, the BES can be assembled so it is ready when the culture has grown to the right OD₆₀₀. Furthermore, while waiting for the initial growth phases in the MEC, the 10 liters of FWA medium, DL vitamins, and DL minerals can be made, so that once the current output starts to dip, the MECs can be switched to flowthrough mode.

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Figure A.4K.1.

Gassing station manifold design. (A) Design and (B) picture of manifold back. (C) Design and (D) picture of manifold front. (E) Zoomed picture of control panel switch.

Figure A.4K.2.

Preparation of anaerobic medium in a pressure tube, serum bottle, and 1-liter bottle using the gassing station. Sparging of medium with (A) the vessel open and (B) the vessel sealed by a butyl stopper. (C) Sparger for bubbling large volumes of medium.

Figure A.4K.3.

(A) Close-up of glass syringe filter system. (B) Glass syringe assembly connected to the gassing station where the oxygen can be flushed out of a plastic syringe.

Figure A.4K.4.

BES diagram. In the cathode chamber, C_{ox} and C_{red} refer to the oxidized and reduced forms, respectively, of the particular catholyte being used in the experiment.

Figure A.4K.5.

All parts needed for building a BES. (A) Glass chamber for the anode and cathodes. (B) Pinch clamp. (C) Glass chimney. (D) Orange rubber stopper with center hole. (E) Blue butyl stopper. (F) Aluminum seal. (G) Graphite electrode. (H) Watertight electrode connector. (I) O-ring. (J) Pyrex cap with center hole. (K) electrode/wire/rubber stopper/chimney assembly. Not pictured in this photograph is the magnetic stir bars, the Nafion PEM, the reference electrode, and the blue butyl stopper with a center hole.

Figure A.4K.6.
Assembled BES.

Item No.	Item	Picture
1	Two glass vessels for the anode and the cathode chamber. These glass vessels can be manufactured by the local glass shop to reduce cost or can be purchased by commercial vendors. (see e.g., Adams & Chittenden Scientific Glass, Berkeley, CA, http://adamschittenden.com/microbial%20fuel%20cells). Each vessel has a screw thread aperture, OD (Outside Diameter) = 50 mm, made of a PYREX glass bottle (Fisher scientific), a glass joint (tubing OD = 28 mm, ID (Inside Diameter) = 25 mm, ACE Glass Incorporated, Order no. 7646-12, and 3 apertures located sideways, OD of tubing = 15 mm, to be sealed by blue butyl stoppers and Aluminum seal.
2	One pinch clamp size 35, with screw lock (ACE Glass Incorporated, cat. no. 7669-14).
3	Two glass chimneys, manufactured by the local glass shop from anaerobic culture tubes (18 mm x 150 mm, Chemglass # CLS-4209-10).
4	Two orange rubber stoppers, 45 mm diameter, (Wheaton # 1053995) with 18 mm hole for the glass chimneys.
5	Six blue butyl stoppers, 10 mm diameter (Chemglass # CLS-4209-14), for sealing the chambers sideways.
6	Six Aluminum seals (Chemglass # CG-4930-20 or equivalent).
7	Two Graphite electrodes: 1 3/8 sticks of sanded graphite grade g10 (Mercen # UOFMA-001).

8	Two threaded watertight connectors, Item # XSA-BC (Teledyne Impulse, San Diego, Calif.)
9	One O-ring, D = 35 mm, KALREZ, size -217, Ace Glass, cat. no. 7855-640 (provided together with glass joints).
10	One Proton exchange membrane (Nafion 117, Electrolytica Inc.) cut into 5 cm x 5 cm size.
11	Two small magnetic stir bars that fit into the chambers.
12	Reference electrode Ag/AgCl (3 M KCl) (Electrolytica # C-925).
13	Blue butyl stopper (Chemglass # CLS-4209-14) with a hole in the middle to insert reference electrode during assembly.
14	Peristaltic pump for the flow of new media. A multi-channel pump can be used (see e.g., 24-channel pump Cole Parmer # EW-78001-42) to reduce the cost and for simultaneous supply of media to multiple MECs. Typical flow rate of 0.5 ml/min. Make sure that the tubing diameter is calibrated with the pump for proper flow rate.
15	Oil-free vacuum pump for filling and emptying the BESs with media. The pump is connected to the waste jug (1000 ml filtration flask, Pyrex Part no. 5340-1 liter) fitted with a cork (stopper size # 8) and a 18 G needle via a rubber tubing 5/16 ID (Fisher Cat. No. 14175D).

Figure A.4K.7.
Components required for BES assembly

Figure A.4K.8.
(A) Emptying and (B) filling the fuel cell.

Figure A.4K.9.

Flowthrough mode setup. (A) MECs. (B) Peristaltic pump. (C) Glass splitter with tubing to supply medium to multiple MECs simultaneously. (D) Medium jug with FWA, DL vitamins, and DL minerals. (E) Waste jug.

Figure A.4K.10.

Current production by MEC inoculated with wild-type *G. sulfurreducens* cells (red) and Aro-5 mutant cells in which five key aromatic amino acids in PilA, structural pilin subunit, are substituted by alanine (green).

Table A.4K.1

Parts for a Gassing Station

Item no.	Part no.	Description	Quantity
1	Custom made	3/16-in. aluminum back plate—2 foot × 1 foot	1
2	Cole Parmer, E-03217-28	150-mm correlated flowmeter, with valve, aluminum, 8678 ml/min air	1
3	Supelco Supelpure-O, SKU 22449	Oxygen moisture trap, 1/8-in. size connector	1
4	McMaster-Carr, 8981T75	Vibration-damping clamp with SBR cushion, zinc-plated steel, 1-3/8-in. I.D.	2
5	Swagelok B-43ZF2	5-way switching valve with detents	1
6	Swagelok B-OGS2	OG series toggle valve	10
7	Swagelok B-OGS2	Needle valve	1
8	Swagelok CU-T2-S-030-50	Copper tube 1/8-in. O.D.	10 feet
9	Swagelok B-201-PC	Port connector	11
10	Swagelok B-200-3	Union tee	11
11	Swagelok B-2-HC-A-201	Tube adapters hose barb	11
12	Swagelok B-200-11-2	1/8-in. MNPT × –200 BULKHEAD	2
13	Swagelok B-200-1-2	Male connector (tapered thread)	3
14	Swagelok B-2-P	1/8-in. pipe plug	1

Table A.4K.2

100 mM Cysteine Recipe (1 Liter)

Component	Amount
Deionized H ₂ O	800 ml
Cysteine HCl	17.56 g
Adjust pH to	7.0
Bring volume to	1000 ml

Table A.4K.3**DL Mineral Mix Components**

Component	Amount
Deionized H ₂ O	800 ml
Nitrilotriacetic acid trisodium salt (NTA)	1.5 g
MgSO ₄ (anhydrous)	3.0 g
MnSO ₄ •H ₂ O	0.5 g
NaCl	1.0 g
FeSO ₄ •7H ₂ O	0.1 g
CaCl ₂ •2H ₂ O	0.1 g
CoCl ₂ •6H ₂ O	0.1 g
ZnCl ₂	0.13 g
CuSO ₄ •5H ₂ O	0.01 g
AlK(SO ₄)•12H ₂ O	0.01 g
H ₃ BO ₃	0.01 g
Na ₂ MoO ₄ •2H ₂ O	0.025 g
NiCl ₂ •6H ₂ O	0.024 g
Na ₂ WO ₄ •2 H ₂ O	0.025 g
Bring volume to	1000 ml

Table A.4K.4

DL Vitamin Mix Components

Component	Amount
Deionized H ₂ O	800 ml
Biotin	0.002 g
Pantothenic acid	0.005 g
Cyanocobalamin (B-12)	0.0001 g
<i>p</i> -Aminobenzoic acid	0.005 g
Thioctic acid	0.005 g
Nicotinic acid	0.005 g
Thiamine	0.005 g
Riboflavin	0.005 g
Pyridoxine HCl	0.01 g
Folic acid	0.002 g
Bring volume to	1000 ml

Table A.4K.5**FWA Medium Components**

Component	Amount
<i>A. For 1 liter</i>	
Deionized H ₂ O	800 ml
NH ₄ Cl	0.25 g
KCl	0.1 g
NaH ₂ PO ₄ •H ₂ O	0.06 g
NaHCO ₃	2.5 g
Sodium acetate trihydrate	1.38 g
DL minerals ^a	10 ml
DL vitamins ^a	10 ml
Bring volume to	1000 ml
<i>B. For 10 liters</i>	
Deionized H ₂ O	8000 ml
NH ₄ Cl	2.5 g
KCl	1 g
NaH ₂ PO ₄ • H ₂ O	0.6 g
NaHCO ₃	25 g
Sodium acetate trihydrate	13.8 g
Bring volume to	9800 ml

^aSee recipe in Reagents and Solutions.

Table A.4K.6**FWAF Medium Components**

Component	Amount
Deionized H ₂ O	800 ml
Fumaric acid ^a	4.64 g
Bring pH to ^a	7.0
NH ₄ Cl	0.25 g
KCl	0.1 g
NaH ₂ PO ₄ •H ₂ O	0.06 g
NaHCO ₃	2.5 g
Sodium acetate trihydrate	1.38 g
DL minerals ^b	10 ml
DL vitamins ^b	10 ml
Check final pH is	7.0
Bring volume to	1000 ml

^a As the pH reaches 7.00, more of the fumaric acid will dissolve into solution, changing the pH. If there are any particles still visible below pH 6, you may want to break them up with a spatula so that they dissolve faster.

^b See recipe in Reagents and Solutions.

Table A.4K.7**FWNN Medium Components**

Component	Amount
Deionized H ₂ O	800 ml
NH ₄ Cl	0.25 g
KCl	0.1 g
NaH ₂ PO ₄ •H ₂ O	0.06 g
NaHCO ₃	2.5 g
DL minerals ^a	10 ml
DL vitamins ^a	10 ml
Bring volume to	1000 ml

^aSee recipe in Reagents and Solutions.

Table A.4K.8**NB Mineral Elixir Components**

Component	Amount
Deionized H ₂ O	800 ml
Nitrilotriacetic acid trisodium salt (NTA)	2.14 g
MnCl ₂ •4H ₂ O	0.1 g
FeSO ₄ •7H ₂ O	0.3 g
CoCl ₂ •6H ₂ O	0.17 g
ZnSO ₄ •7H ₂ O	0.2 g
CuCl ₂ •2H ₂ O	0.03 g
AlK(SO ₄)•12H ₂ O	0.005 g
H ₃ BO ₃	0.005 g
Na ₂ MoO ₄ •2H ₂ O	0.09 g
NiSO ₄ •6H ₂ O	0.11 g
Na ₂ WO ₄ •2H ₂ O	0.02 g
Bring volume to	1000 ml

Table A.4K.9**NBAF Medium Components**

Component	Amount
Deionized H ₂ O	800 ml
Fumaric acid	4.64 g
Bring pH to ~5.0 with 10 N NaOH	
Bring pH to 7.0 with 2 N NaOH	
100× NB salts ^a	10 ml
100× NB mineral elixir ^a	10 ml
DL vitamins ^a	15 ml
CaCl ₂ •H ₂ O	0.04 g
MgSO ₄ •7H ₂ O	0.1 g
NaHCO ₃	1.8 g
Na ₂ CO ₃ •H ₂ O	0.5 g
1 mM Na ₂ SeO ₄ ^a	1.0 ml
Sodium acetate•3H ₂ O	2.04 g
0.1% Resazurin	0.5 ml
Bring volume to	1000 ml

^aSee recipe in Reagents and Solutions.

Table A.4K.10

100× NB Salts Components

Component	Amount
Deionized H ₂ O	700 ml
KH ₂ PO ₄	42.0 g
K ₂ HPO ₄	22.0 g
NH ₄ Cl	20.0 g
KCl	38.0 g
NaCl	36.0 g
Bring volume to	1000 ml