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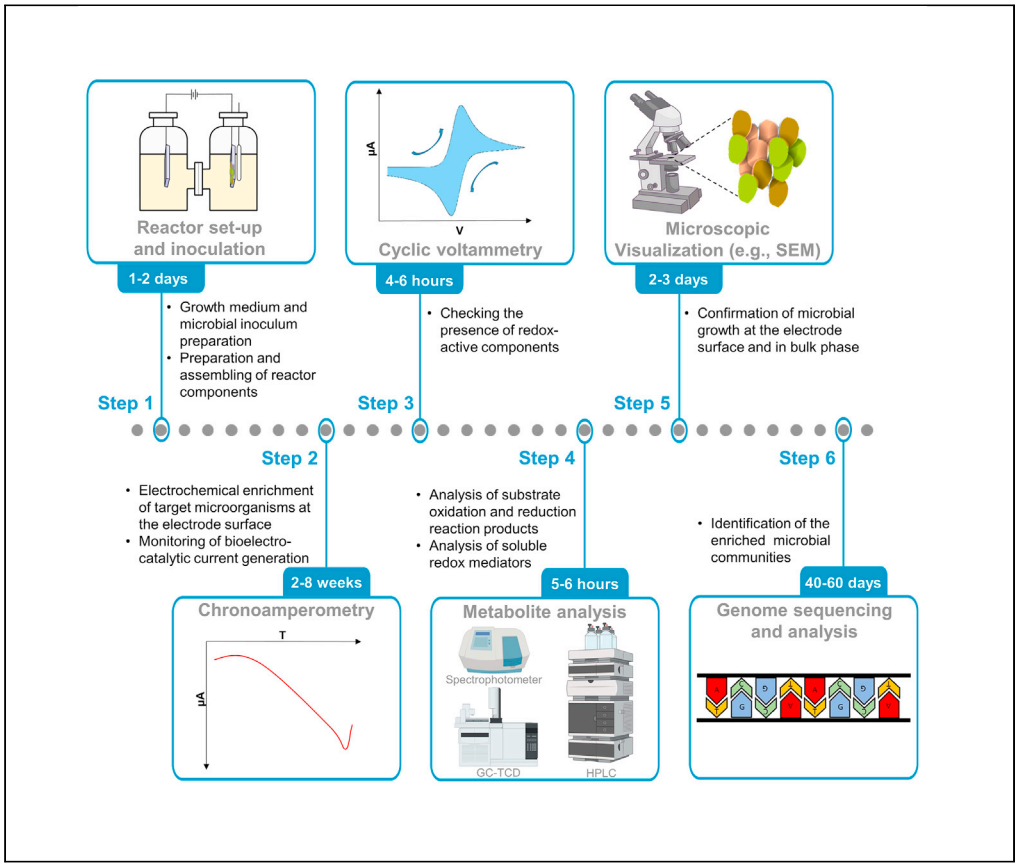
# Protocol for bioelectrochemical enrichment, cultivation, and characterization of extreme electroactive microorganisms




Electroactive microorganisms (EAMs) are a group of microbes that can access solid extracellular electron donors or acceptors via extracellular electron transfer processes. EAMs are useful in developing various microbial electrochemical technologies. This protocol describes the use of bioelectrochemical systems (BESs) to enrich EAMs at the cathode from an extreme haloalkaline habitat. It also provides information for a detailed characterization of enriched cathodic biofilms via various cross-disciplinary techniques, including electrochemical, analytical, microscopic, and gene sequencing techniques.

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## Highlights

Detailed protocol for the electrochemical enrichment of extreme microorganisms

Useful for cultivating different microbes at cathode of bioelectrochemical systems

Protocols for characterizing electrotrophic biofilm and metabolic products provided

These include electrochemical, analytical, microscopic, and gene sequencing techniques

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# Protocol for bioelectrochemical enrichment, cultivation, and characterization of extreme electroactive microorganisms

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## SUMMARY

**Electroactive microorganisms (EAMs) are a group of microbes that can access solid extracellular electron donors or acceptors via extracellular electron transfer processes. EAMs are useful in developing various microbial electrochemical technologies. This protocol describes the use of bioelectrochemical systems (BESs) to enrich EAMs at the cathode from an extreme haloalkaline habitat. It also provides information for a detailed characterization of enriched cathodic biofilms via various cross-disciplinary techniques, including electrochemical, analytical, microscopic, and gene sequencing techniques.**

**For complete details on the use and execution of this protocol, please refer to Chaudhary et al. (2021).**

## BEFORE YOU BEGIN

Electromicrobiology is an emerging sub-discipline under the domain of microbiology. It pertains to studying microorganisms capable of extracellular electron transfer (EET) processes to or from a solid-state electron acceptor or donor and their implications in different environments (Lovley, 2012; Obi and Asogwa, 2016; Nealson and Rowe, 2016). Depending upon the mode of EET, EAMs are categorized as exoelectrogens and electrotrophs. Exoelectrogens can transfer electrons to a solid-state electron acceptor, and electrotrophs can oxidize a solid-state electron donor (Logan et al., 2019; Yadav and Patil, 2020). Bioelectrochemical systems (BESs) are routinely used in laboratories to study the electroactive nature of microorganisms and explore them for various applications (Yee et al., 2020). While exoelectrogens have been studied well, electrotrophs remain poorly investigated mainly due to a lack of well-designed protocols or methodology (Kiran and Patil, 2019; Logan et al., 2019; Yee et al., 2020). Moreover, most work in electromicrobiology has been conducted with EAMs from non-extreme environmental conditions (Dopson et al., 2016; Shrestha et al., 2018; Yee et al., 2020). Studying EAMs from extreme habitats is essential to advance the electromicrobiology discipline and find novel strains possessing electroactivity. For this purpose, unique modifications need to be applied to the existing experimental protocols according to the extreme condition and target group of electrotrophic microorganisms.

This protocol describes the electrochemical enrichment and characterization of nitrate-reducing electrotrophs from the highly saline and alkaline environment of Lonar Lake. The protocol has been adapted from various other papers on the enrichment of different types of EAMs (e.g., Karbelkar et al., 2019; Gregory et al., 2004; Yadav and Patil, 2020). It mainly targets the growth of cathodic

microbial biofilm, provided with a suitable terminal electron acceptor (TEA), i.e., nitrate. It can be adapted to target other electrotrophs by using different TEAs such as sulfate ($SO_4^{2-}$), carbon dioxide ($CO_2$), oxygen ($O_2$) or oxidized heavy metals. It can also be modified to enrich exoelectrogens at the anodic surface.

Refer to "key resources table" for a list of resources needed for this protocol.

### Preparation of calibration curves for the detection of different ionic components

Timing: 1 week

1. 5-point calibration curves, obtained by following different UV-Vis spectrophotometric protocols, are used to detect different ionic components, *namely*, nitrate, ammonium, phosphate, sulfate, and chemical oxygen demand (COD) (Rice et al., 2012).

2. Gas analysis: Gases such as $H_2$, $CH_4$, and $N_2$ are detected using a Gas Chromatograph.
   a. Resources required:
      i. A gas chromatograph system equipped with a thermal conductivity detector (GC-TCD, Agilent 490 Micro GC) is used to perform the gas analysis of the samples at a temperature of 80°C. The mentioned GC-TCD consists of three different channels for detecting different gases. Channel 1 contains a Column-Molecular sieve for $H_2$ detection (retention time (RT) of 0.4 min) with Ar as the carrier gas. Channel 2 contains a Column-Molecular sieve for the detection of $O_2$ (RT of 0.4 min), $N_2$ (RT of 0.6 min), CO (RT of 0.8 min), and $CH_4$ (RT of 1.1 min) with He as the carrier gas. Channel 3 contains Column-Pora plot U for $CO_2$ (RT of 0.5 min) and $H_2S$ (RT of 0.9 min) detection, and the carrier gas in this channel is He. A sample volume of 1–2 mL is injected for analysis.
      ii. 1 mL or 2.5 mL capacity sterile syringes (depending upon the required volume of samples for injection) can be used to collect the gas samples from the reactor headspace.

   *Note:* We recommend using syringes equipped with a gas-tight lock for this purpose. Alternatively, syringes with needles should be placed/inserted in the butyl rubber stoppers to avoid any gas exchange until the sample is injected for analysis, or the samples should be analyzed immediately.

      iii. Standard gas cylinders (Chemtron Science Laboratories Pvt. Ltd.) are used to calibrate the GC-TCD for different gases. Alternatively, the gas cylinders of high purity can be obtained and diluted using the dynamic gas dilution method using zero air or an inert gas like nitrogen. It can be done using a standard gas calibration unit (GCU) and a mass flow meter or controller to maintain stable flow rates (Kumar et al., 2020).
   b. Procedure:
      i. Perform 3-point calibration using the standard gas cylinders.
      ii. Pump the gas samples through the sample port and analyze them using the accompanying instrument software.

3. Organic compound analysis: C1-C4 organic compounds (i.e., acids and alcohols) are detected using the High-Performance Liquid Chromatograph (HPLC).
   a. Resources required:
      i. A High-Performance Liquid Chromatograph equipped with a refractive index detector (HPLC-RID) and Hiplex H column-C18 (Agilent Technologies) - The mobile phase is 5 μM sulfuric acid ($H_2SO_4$) (HPLC-grade, Sigma-Aldrich) at a flow rate of 0.5 mL/min at a temperature of 50°C.
      ii. HPLC vials are used to collect the samples.

iii. Standard solutions: Standard solution of each organic compound is made using HPLC-grade chemicals (Sigma-Aldrich).

b. Procedure:

i. Prepare 1 mL calibration standards in the range of 1–50 mg/L and 50–1000 mg/L for each organic compound. Different calibrations with low as well as high concentration ranges can be decided to efficiently detect and quantify the concentration of various organic compounds, including acetic acid, formic acid, propionic acid, butyric acid, and citric acid. One can also use a wider calibration range to quantify highly concentrated organic acids.

ii. Filter 1 mL samples using 0.2 μM syringe filters in 2 mL HPLC vials.

iii. Analyze the samples via HPLC-RID.

4. Bradford Assay: Protein content-based biomass estimation is based on the Bradford assay (Redmile-Gordon et al., 2013).

a. Resources required:

i. Bovine serum albumin (BSA) is required to make a calibration standard, effective between 0.050 mg/L to 250 mg/L.

ii. Bradford reagent-mix* from Sigma-Aldrich is used for the analysis.

iii. A microplate spectrophotometer (e.g., Multiskan SkyHigh Microplate Spectrophotometer, Thermo Fisher) is required to record the absorbance.

b. Procedure:

i. Sonicate the sample at 30 KHz amplitude using an Ultra-point-sonicator (500 Watt ultrasonic processor VC 505). Set the instrument at 5 s pulse-on and 10 s pulse-off intervals for a total period of 10 min.

*Note:* These settings have been optimized for this specific study and may vary accordingly.

ii. Add 20 μL of the Bradford reagent mix to 200 μL of the sample** in the 96-well Bradford assay plate and incubate it in dark for 10 min.

iii. Record $OD_{595}$ using the spectrophotometer and measure the concentration of the protein content in the sample using the calibration curve.

**Note:* The reagent is light sensitive, so it must be preserved under dark conditions or in an amber-colored bottle.

***Note:* The accuracy of the Bradford assay decreases with an increasing concentration of the protein content in the sample.

### Sediment collection and characterization

Timing: 1–2 weeks

5. Sediment collection:

a. Resources required:

i. Amber-colored 1 L bottles

ii. Nitrogen gas

b. Procedure: Collect the subsurface sediment samples (e.g., from a depth of 20–25 cm) in 1 L amber-colored bottles/containers (previously flushed with nitrogen) from different locations and store them at 4°C. To maintain the anoxic conditions, flush the headspace with $N_2$. Alternatively, a gravity corer can also be used to avoid sediment mixing.

6. The physicochemical characterization of the sediment samples is done to analyze different parameters present in them. These may include pH, salinity, conductivity, ammonia, chemical oxygen demand (COD), phosphate, sulfate, and nitrate. These parameters may vary depending on the research interest. These parameters are quantified by following the standard analytical

**Table 1. Composition of minimal growth medium**

| Reagent | Final concentration (mM) | Amount (g/L of distilled water) |
|---|---|---|
| Disodium hydrogen phosphate ($Na_2HPO_4$) | 30.5 | 4.33 |
| Sodium dihydrogen phosphate ($NaH_2PO_4$) | 22.4 | 2.69 |
| Sodium chloride (NaCl) | 342.2 | 20 |
| Sodium carbonate ($Na_2CO_3$) | 40.6 | 4.3 |
| Potassium chloride (KCl) | 1.75 | 0.13 |
| Ammonium chloride ($NH_4Cl$) | 5.8 | 0.31 |
| Vitamins[a] (Table 3) | N/A | 12.5 mL |
| Trace elements[a] (Table 4) | N/A | 12.5 mL |
| Adjust pH to 9.5. | | |

[a]**Note:** The pH and ammonium concentration in the medium should be checked before and after autoclaving. Autoclaving vitamins and trace elements can affect the heat-sensitive components; hence they must be added to the growth medium using sterile syringe filters with a pore size of 0.2 μM. The composition of vitamins and trace elements solutions is given in Tables 3 and 4, respectively.

***Note:*** The complete medium should be used immediately. If unavoidable, it can be stored at 4°C for a couple of days. However, precautions must be taken to avoid microbial contamination in the medium. Only buffer medium without vitamins and trace elements can be stored for extended periods.

protocols mentioned in "Standard Methods for the Examination of Water and Wastewater" (Rice et al., 2012). This preliminary characterization helps to decide the microbial growth conditions and operating parameters for the bioelectrochemical enrichment experiments.

a. The procedure for the detection of pH, salinity, and conductivity is as follows:
   i. pH: Record the pH using the pH meter (Jenway 3510, Jenway®).
   ii. Conductivity (C): Record the conductivity of the sediment solution using a conductivity meter (Oakton 2700, Oakton®).
   iii. Salinity (S): Calculate this by using the conductance value in the given formula:

$$S = kC,$$

where k = 0.4665 (Rusydi, 2018).

## Preparation of the basal growth medium for microbes

**Timing: 2 h**

7. A minimal growth medium (Table 1; based on a modified M9 medium) can be used to support the growth of the target microbial group.
   a. Resources required:
      i. 99.999% inert $N_2$ or He gas cylinders are required for flushing the media. In this specific case, the evolution of nitrogen via complete reduction of nitrate (i.e., denitrification) needs to be detected, so an inert gas, namely helium, is used. Apart from helium, argon can also be used.
      ii. Chemicals mentioned in Tables 1, 3, and 4 are required to make different media. In addition to these, potassium nitrate ($KNO_3$—as nitrate source)* and sodium bicarbonate ($NaHCO_3$—as carbon source)** are also required.
      iii. Instruments including autoclave (CL-32L, ALP Co. Ltd.), weighing balance (AS 220.R2, Radwag®), stirrer (EcoStir, DLab Scientific Inc.), and pH meter (Jenway 3510, Jenway®) are required. ***

*_Note:_ For the enrichment of different microorganisms, both exoelectrogens and electrotrophs, the experiments can be modified by varying the possible electron donors (like organics such as acetate and lactate, inorganics such as hydrogen sulfide, hydrogen, etc.)

**Table 2. Composition of anolyte**

| Reagent | Final concentration (mM) | Amount (g/L of distilled water) |
|---|---|---|
| Disodium hydrogen phosphate ($Na_2HPO_4$) | 30.5 | 4.33 |
| Sodium dihydrogen phosphate ($NaH_2PO_4$) | 22.4 | 2.69 |
| Sodium chloride (NaCl) | 342.2 | 20 |
| Potassium chloride (KCl) | 1.75 | 0.13 |
| Ammonium chloride ($NH_4Cl$) | 5.8 | 0.31 |
| Adjust pH to 7. | | |

**Note:** The anolyte can be stored at 4°C for 2–3 months. However, precautions must be taken to avoid any contamination in the anolyte.

and electron acceptors (like sulfate, oxygen, carbon dioxide) sources. For instance, exoelectrogens capable of oxidizing various organics such as acetate and lactate, inorganics such as hydrogen sulfide, hydrogen, etc. can be enriched over the anode by polarizing it at the appropriate potential.

****Note:*** $NaHCO_3$ can be replaced by other C-sources according to the desired mode of growth of the microorganisms (i.e., autotrophic or heterotrophic).

*****Note:*** All the instruments mentioned above can be of any reliable make.

b. Procedure:
   i. The medium composition is presented in Table 1 (Yadav and Patil, 2020; Chaudhary et al., 2021). Dissolve the components in 500 mL distilled water, and then make the final volume to 1000 mL. In bioelectrochemical experiments, this is used as the catholyte.
   ii. Autoclave all the solutions and media and make them anaerobic by flushing with $N_2$ or He gas for at least 20 min simultaneously with agitation to remove the dissolved oxygen. Subject each sealed serum bottle containinng specific medium components to at least 20 gassing-degassing cycles before autoclaving.
   iii. Prepare and autoclave 1M anaerobic stock solutions of sodium nitrate and sodium bicarbonate separately and add them to the growth medium before starting the experiments to make a final concentration of 10 mM each.

**Table 3. Composition of vitamin solution (Greening and Leedle, 1989)**

| Reagent | Final concentration (mM) | Amount (mg/L of distilled water) |
|---|---|---|
| Sodium ascorbate | 0.05 | 10 |
| Biotin | 0.016 | 4 |
| Folic acid | 0.09 | 4 |
| Pyridoxine hydrochloride | 0.097 | 20 |
| Thiamine hydrochloride | 0.03 | 10 |
| Riboflavin | 0.026 | 10 |
| Nicotinic acid | 0.08 | 10 |
| DL-calcium Pantothenate | 0.02 | 10 |
| Vitamin B12 | 0.0002 | 0.2 |
| p-Aminobenzoic acid | 0.072 | 10 |
| Lipoic (thioctic) acid | 0.048 | 10 |
| Myo-inositol | 0.055 | 10 |
| Choline chloride | 0.071 | 10 |
| Niacinamide | 0.08 | 10 |
| Pyridoxal hydrochloride | 0.049 | 10 |

**Note:** Store the solution at 4°C when not in use. It can be used for 2–3 months by taking necessary precautions to avoid any microbial contamination in the medium.

**Table 4. Composition of trace elements solution (Balch et al., 1979)**

| Reagent | Final concentration (mM) | Amount (g/L of distilled water) |
|---|---|---|
| Nitrilotriacetic acid (dissolve with KOH; pH 6.5) | 7.85 | 1.5 |
| Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) | 14.7 | 3.0 |
| Manganese chloride dihydrate ($MnCl_2.2H_2O$) | 3.1 | 0.5 |
| Sodium chloride (NaCl) | 17.25 | 1.0 |
| Ferrous chloride ($FeCl_2$) | 0.79 | 0.1 |
| Cobalt chloride ($CoCl_2$) | 0.77 | 0.1 |
| Calcium chloride dihydrate ($CaCl_2.2H_2O$) | 0.68 | 0.1 |
| Zinc chloride ($ZnCl_2$) | 0.73 | 0.1 |
| Copper chloride ($CuCl_2$) | 0.07 | 0.01 |
| Aluminum chloride hexahydrate ($AlCl_3.6H_2O$) | 0.041 | 0.01 |
| Boric acid ($H_3BO_3$) | 0.163 | 0.01 |
| Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) | 0.04 | 0.01 |

**Note:** Store the solution at 4°C when not in use. It can be used for 2–3 months by taking necessary precautions to avoid any microbial contamination in the medium.

iv. The composition of anolyte (pH 7) (Table 2) is the same as catholyte except for sodium carbonate ($Na_2CO_3$), vitamins, and trace elements, which are not added to the anolyte.

### Preparation of electrodes

**Timing: 1–2 days**

8. In this experiment, graphite* electrodes are used as the working electrodes.
   a. Resources required:
      i. Graphite sheet/plate/rod for making the electrodes.
      ii. Garnet sandpaper with grit designation no. P220 can be used to polish the electrodes.
      iii. 1M solutions of hydrochloric acid (HCl) and sodium hydroxide (NaOH) for the pretreatment of electrodes.
      iv. Titanium wire (1 mm thickness and 99.7% purity; Alfa Aesar) and carbon conductive cement (Alfa Aesar) are used to establish connections with the electrodes.
   b. Procedure:
      i. Cut the graphite rod or plate as per the desired dimensions using an electric cutter or a hack saw.
      ii. Use sandpaper (220 grit or other specifications) to polish the electrodes and make the surface rough.
      iii. Pre-treat the electrodes by the acid-alkali method before use. Briefly, dip the electrodes sequentially in a 1M solution of hydrochloric acid (HCl) and sodium hydroxide (NaOH) for 2–3 h each (Yee et al., 2020).
      iv. Wash the electrodes by soaking in distilled water for ~30 min.
      v. Attach the titanium wires as current collectors to the electrodes using carbon conductive cement (Alfa Aesar) and allow them to dry overnight (12–14 h) in an oven at ~60°C. On drying, measure the resistances of the only electrode, only titanium wire, and whole electrode using a multimeter, and only use the electrodes with resistance less than 1 ohm for experimentation. The connection between the current collector and electrode is not assayed in the experiments by keeping it out of the electrolyte. Alternatively, the connection can also be insulated by coating with non-conductive resins/glues to ensure that the electrode only contributes to the reaction.

*_Note:_ As cathode, different types of materials can be used. The dimension or projected surface area of the electrodes is selected according to the reactor type and experimental goal. Here the protocol is provided considering graphite, which is the most commonly used electrode material in bioelectrochemical systems.

### Proton Exchange Membrane treatment

Timing: 5–6 h

9. The Proton Exchange Membrane (PEM) is used to connect the two reactor chambers (anodic and cathodic) to enable the transfer of $H^+$ ions from the anodic to the cathodic chamber.

*Note:* PEM is required when working with a double-chambered reactor and not needed for a membraneless single chamber reactor.

a. Resources/Reagents required:
   i. Proton Exchange Membrane (PEM) (Nafion 117, Sigma-Aldrich)* is used.
   ii. 2% hydrogen peroxide ($H_2O_2$) solution is made by diluting a 30% hydrogen peroxide ($H_2O_2$) reagent.
   iii. 1N sulfuric acid ($H_2SO_4$) is prepared by diluting 99.8% pure sulfuric acid ($H_2SO_4$) reagent.
b. Procedure:
   i. Cut the PEM according to the connecting junction dimensions of anode and cathode chambers.
   ii. Pre-treat it by sequentially heating at 70°C–80°C for 1 h each in distilled water, 2% hydrogen peroxide ($H_2O_2$), distilled water, 1N sulfuric acid ($H_2SO_4$), and distilled water (Fan and Zhang, 2018).

**Note:* Other types of ion exchange membranes can also be used.

⚠ CRITICAL: The treated PEM has to be stored in a closed container in distilled water at 4°C until further use.

### Preparation of microbial inoculum

Timing: 1.5–2 h

10. Prepare the microbial inoculum by suspending sediment or soil samples in the minimal growth medium (Table 1). Sparge the suspension with $N_2$ for at least 10 min to make it anaerobic, followed by sedimentation for around an hour. Then use the intermediate layer between the settling sediment particles and the supernatant top layer as an inoculum.

## KEY RESOURCES TABLE

| REAGENTS/RESOURCES | SOURCE | IDENTIFIER |
|---|---|---|
| Chemicals, peptides, and recombinant proteins | | |
| Acetic Acid (HPLC-Grade) | Sigma-Aldrich | Cat#45754-500ML-F |
| Aluminium chloride hexahydrate | Sigma-Aldrich | Cat#237078-100G |
| Ammonium chloride | Sigma-Aldrich | Cat#213330-1KG |
| Biotin | Sigma-Aldrich | Cat#B4639-5G |
| Boric acid | Sigma-Aldrich | Cat# 1001641000 |
| Butyric acid (HPLC-Grade) | Sigma-Aldrich | Cat#19215-5ML |
| Calcium chloride | Sigma-Aldrich | Cat#499609-10G |
| Choline chloride | Sigma-Aldrich | Cat#C7527-100G |
| Cobalt chloride | Sigma-Aldrich | Cat#44976-5G |
| Copper chloride | Sigma-Aldrich | Cat#451665-25G |
| Disodium hydrogen phosphate | Sigma-Aldrich | Cat#1065851000 |
| DL-calcium Pantothenate | Sigma-Aldrich | Cat#P5155-100G |

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| REAGENTS/RESOURCES | SOURCE | IDENTIFIER |
|---|---|---|
| Ferrous chloride | Sigma-Aldrich | Cat#450936-25G |
| Folic acid | Sigma-Aldrich | Cat#F8758-5G |
| Formic acid (HPLC-Grade) | Sigma-Aldrich | Cat#5438040100 |
| Glutaraldehyde | Sigma-Aldrich | Cat#G5882 |
| Helium gas (99.9999%) | Sigma gases, India | N/A |
| Hydrochloric acid (HPLC-Grade) | Sigma-Aldrich | Cat#320331-500ML |
| Hydrogen gas (99.9999%) | Sigma gases, India | N/A |
| Hydrogen peroxide | Sigma-Aldrich | Cat#349887-4L |
| Lipoic(thioctic) acid | Sigma-Aldrich | Cat#T1395-5G |
| Magnesium chloride hexahydrate | Sigma-Aldrich | Cat#M2393-100 |
| Manganese chloride | Sigma-Aldrich | Cat#244589-10G |
| Myo-inositol | Sigma-Aldrich | Cat#I7508-50G |
| Niacinamide | Sigma-Aldrich | Cat#N0636-100G |
| Nicotinic acid | Sigma-Aldrich | Cat#N0761-100G |
| Nitrilotriacetic acid | Sigma-Aldrich | Cat#N9877-100G |
| Nitrogen gas (99.9999%) | Sigma gases, India | N/A |
| p-Aminobenzoic acid | Sigma-Aldrich | Cat#A9878-25G |
| Paraformaldehyde | Sigma-Aldrich | Cat#158127 |
| Potassium chloride | Sigma-Aldrich | Cat#1049361000 |
| Potassium nitrate | Sigma-Aldrich | Cat#221295-500G |
| Propanoic acid (HPLC-Grade) | Sigma-Aldrich | Cat#94425-5ML-F |
| Pyridoxal hydrochloride | Sigma-Aldrich | Cat#P6155-5G |
| Pyridoxine hydrochloride | Sigma-Aldrich | Cat#47862 |
| Riboflavin | Sigma-Aldrich | Cat#1076090010 |
| Sodium acetate | Sigma-Aldrich | Cat#S2889-250G |
| Sodium ascorbate | Sigma-Aldrich | Cat#A4034-100G |
| Sodium carbonate | Sigma-Aldrich | Cat#223484-500G |
| Sodium chloride | Sigma-Aldrich | Cat# S9888-1KG |
| Sodium dihydrogen phosphate | Sigma-Aldrich | Cat#1063461000 |
| Sodium hydroxide | Sigma-Aldrich | Cat#06203-500G |
| Sodium molybdate dihydrate | Sigma-Aldrich | Cat# 480967-25G |
| Standard Gases for Gas Chromatograph (99.9999%) | Chemtron Science Laboratories Pvt. Ltd. | N/A |
| Sulfuric acid (HPLC-Grade) | Sigma-Aldrich | Cat#5438270250 |
| Thiamine hydrochloride | Sigma-Aldrich | Cat#T1270-25G |
| Tri-sodium citrate | Sigma-Aldrich | Cat#1110371000 |
| Vitamin B12 | Sigma-Aldrich | Cat#V6629-5G |
| Zinc chloride | Sigma-Aldrich | Cat#429430-25G |
| **Critical commercial assays** | | |
| Bradford Assay | Sigma-Aldrich | Cat#2740-1KIT |
| FastDNA 2 mL SPIN Kit for Soil | MP biomedicals | Cat#116560200 |
| DNeasy PowerSoil® Pro kit | QIAGEN | Cat# 47016 |
| **Biological samples** | | |
| To enrich electroactive microbial biofilms at the electrode surfaces, the inoculum source can vary according to the target group of microorganisms. In general, anoxic soil/sediment/water samples from specific environments can be used as the inoculum source. | Specific/selected environment site (e.g., subsurface sediments from aquatic environments, anaerobic sludge, etc.) | N/A |
| **Deposited data** | | |
| Raw data | Chaudhary et al. (2021) | NCBI-SRA: SRR12506991 |
| **Software and algorithms** | | |
| EC Lab | BioLogic Science Instruments, France | N/A |

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*Continued*

| REAGENTS/RESOURCES | SOURCE | IDENTIFIER |
|---|---|---|
| Other | | |
| 500 Watt ultrasonic processor VC 505 | Sonics | https://www.sonics.com/liquid-processing/products/vibra-cell-processors/vc-505-vc-750/ |
| Ag/AgCl (3.5 M KCl) electrode | BioLogic Science Instruments, France | Cat#RE-1B |
| Amber-colored bottles (Wide mouth HDPE) | Tarsons | Cat#581350 |
| Autoclave, CL-32L | ALP Co. Ltd. | http://alpco.co.jp/en/auto |
| Biosafety cabinet, Logic+ A2 | Labconco Corporation | https://www.labconco.com/category/biological-safety-cabinets-enclosures |
| Conductive Cement | Alfa Aesar | Cat#AA41212-15 |
| Conductivity meter, Oakton 2700 | Oakton | http://www.4oakton.com/proddetail.asp?parent=53&prod=371&TotRec=5 |
| Customized Fuel Cell Glass Reactors (each chamber 250 mL volume) | Jain Scientific Glass Works | N/A |
| Electrospray Ionization High-Resolution Mass Spectrometry (HR-MS-ESI): Waters Synapt G2-Si Q ToF Mass Spectrometer | Waters | https://www.waters.com/waters/en_IN/Mass-Spectrometry/nav.htm?cid=514257&locale=en_IN |
| FEG-SEM-EDS: Hitachi SU8010 series | Hitachi | https://www.hitachi-hightech.com/global/about/news/2011/nr20110214.html |
| Gas Calibration Unit | Ionicon Analytik GmbH | Cat#GCU-s/GCU-a |
| Gas Chromatograph: GC-TCD, Micro GC, Agilent 490 | Agilent Technologies | https://www.agilent.com/en/product/gas-chromatography |
| Graphite Plate (0.5 cm thickness) for working electrode | Ipgi Instruments | N/A |
| HPLC: Agilent 1260 Infinity II, Hiplex H column | Agilent Technologies | https://www.agilent.com/en/product/liquid-chromatography |
| MiSeq/HiSeq/NovoSeq/NExtSeq sequencing platforms | Illumina | https://www.illumina.com/systems/sequencing-platforms.html |
| Isopore Membrane Filter (pore size 0.2 µm, hydrophilic polycarbonate membrane, 25 mm diameter) | Sigma-Aldrich | Cat#GTTP02500 |
| JEOL JEC-1600 Auto-Fine Coater | JEOL Ltd., Japan | https://www.jeol.co.jp/en/ |
| Mass Flow Controller (MC-100SCCM-D-DB9/5M) | Alicat Scientific | N/A |
| Multiskan SkyHigh Microplate Spectrophotometer | Thermo Fisher Scientific | https://www.thermofisher.com/in/en/home/life-science/lab-equipment/microplate-instruments/microplate-readers/multiskan-sky-microplate-spectrophotometer |
| Nafion 117 Proton Exchange Membrane (PEM) | Sigma-Aldrich | Cat#274674-1EA |
| Nanodrop: Genova Nano – 4359 | Jenway | http://www.jenway.com/product.asp?dsl=885 |
| Nanopore | Oxford Nanopore Technologies | https://nanoporetech.com/products |
| Nylon 66 Filter Membranes (pore size 0.45 µm, diam. 47 mm, pkg of 50 ea) | Sigma-Aldrich | Cat#58067 |
| pH meter: Jenway 3510 pH meter | Jenway | http://www.jenway.com/product.asp?dsl=285 |
| Potentiostat (VMP3) | BioLogic Science Instruments, France | N/A |
| Pyrosequencing | QIAGEN | https://www.qiagen.com/us/product-categories/instruments-and-automation/pyrosequencing/instruments/ |
| Qubit fluorometer: V.3.0 | Thermo Fisher Scientific | Cat#Q33238 |
| Sandpaper (220 grit) | Norton LION | https://www.indiamart.com/proddetail/polish-paper-16279696148.html |

(*Continued on next page*)

*Continued*

| REAGENTS/RESOURCES | SOURCE | IDENTIFIER |
|---|---|---|
| Scanning Electron Microscope: JEOL JSM-6010 PLUS-LS | JEOL Ltd. Japan | https://www.jeol.co.jp/en/ |
| Serum Bottle: volume 100 mL, O.D. 51.7 mm × H (94.5 mm) | Sigma-Aldrich | Cat#33110-U |
| Stirrer, EcoStir | DLab Scientific Inc. | Cat#8010194000 |
| Titanium electrode coated with mixed metal oxides (MMO-coated Ti) for water oxidation reaction (5 cm X 2.5 cm) with titanium wire current collector ( 1 mm thickness) | IPGI Instruments | N/A |
| Titanium wire (1 mm thickness and 99.7% purity) | Alfa Aesar | Cat#AA45485 |
| UV-Vis spectrophotometer: PhotoLab® 7600 | Xylem | https://www.xylemanalytics.com/en/general-product/id-364/spectrophotometer-photolab%C2%AE-7600-uv-vis—wtw |
| Weighing Balance, AS 220.R2 | Radwag | Cat# WL-104-0177 |

N/A– Not Applicable.

## STEP-BY-STEP METHOD DETAILS

### Preparation of growth media

Timing: 1 day

1. As explained earlier, a suitable terminal electron acceptor (TEA) should be supplemented to the growth media based on the targeted microbial group.
   a. Procedure:
      i. Prepare the growth medium as described in the earlier sections.
      ii. Select the TEA after analyzing the sediment samples as well as according to the desired reaction, as explained above. Analysis of the Lonar lake sediment samples revealed high concentrations of nitrate ions, which indicated the presence of nitrate-reducing bacteria in these samples (Chaudhary et al., 2021). Thus, nitrate is supplemented as the sole TEA in the growth medium in this study.

### Setting up of double-chambered electrochemical reactors

This section describes steps to set up the H-shaped double-chambered reactor with a three-electrode configuration.

Timing: 6–7 h

2. The steps are as follows.
   a. Resources required:
      i. An autoclave instrument (CL-32L, ALP Co. Ltd.) is required to sterilize different components.
      ii. A two-chambered reactor consisting of glass chambers and all accessories such as stoppers and clamps.
      iii. A pre-treated PEM.
      iv. Pre-treated working electrodes (i.e., cathode).
      v. Reference electrodes such as Ag/AgCl and standard calomel electrode.
      vi. A graphite-based electrode can be used as the anode. However, we recommend using MMO-coated Ti anode since it is a high-performing material for water splitting reaction compared to the graphite electrode.
   b. Procedure:

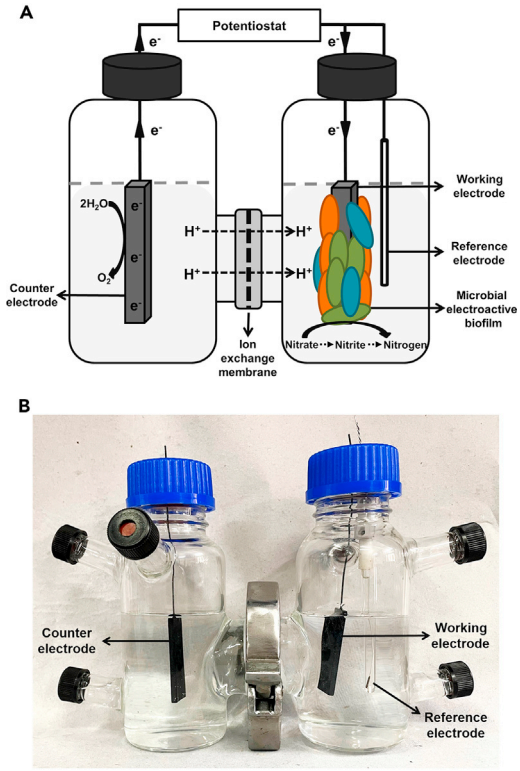


**Figure 1. Two-chambered bioelectrochemical reactor**

(A) Schematic representation

(B) Digital image

i. Join the autoclaved chambers by placing the pre-treated PEM between the junction. The representative schematic (Figure 1A) and an image (Figure 1B) of the reactor are presented in Figure 1. Maintain the aseptic condition by autoclaving everything before use (except the reference electrode, which is sterilized by 70% ethanol) and setting up everything under sterile conditions in the laminar hood (Logic+ A2, Labconco Corporation). It is necessary to avoid any contamination in the electrochemical setup. Alternatively, in the initial phase of the enrichment experiments, a bench flame/bunsen burner can be used to achieve aseptic conditions to some extent.

*Note:* Autoclaved distilled water can be used in both chambers to check for any leakage.

ii. Place all three electrodes, viz. cathode (working electrode), anode (counter electrode), and reference (Ag/AgCl), by using butyl rubbers stoppers along with the chamber ports/openings/head.
iii. Place both the cathode and anode to face the PEM with the largest exposed surface area.
iv. Add both catholyte (same as the minimal growth medium; Table 1) and anolyte (Table 2) in the respective chambers. Flush the cathodic chamber with 99.999% inert $N_2$/ He at a flow rate of 0.5 L/min (He gas is used in case $N_2$ production is checked in the headspace) for about 15–20 min. Then seal off all reactor openings or sampling ports using butyl rubbers or other stoppers to make the chamber air-tight.

## Monitoring of bioelectrocatalytic current generation

**Timing:** Varies according to the target group and growth cycle of microorganisms. For example, for exoelectrogenic microbial biofilms, the initial enrichment duration may be

shorter for a few days to weeks. However, for electrotrophic microbial biofilms, mostly longer duration of several days to weeks may be needed.

Based on the target reaction and literature survey, a suitable electric potential is applied at the working electrode using the chronoamperometry (CA) technique, and the current generation is recorded at regular time intervals. This section describes the steps to conduct these experiments.

3. The procedure to conduct bioelectrochemical experiments is as follows:
   a. Procedure:
      i. Connect the electrodes of the BES to the potentiostat, and apply a fixed potential of −0.3 V* (*vs.* Ag/AgCl sat. KCl) at the working electrode, i.e., cathode in this case.
      ii. Record the Cyclic Voltammetric (CV) profiles before and after inoculating the reactors in a potential window of −1.0 V–0.0 V** at a scan rate of 1 mV/s (Labelle and Bond, 2005; Harnisch and Freguia, 2012). Alternatively, different scan rates (higher or lower) can also be used. However, higher scan rates will mask the presence of redox peaks, while the lower scan rates may disrupt the biofilm leading to decreased performance (Ruiz et al., 2020). Low scan rates can also affect the repeatability of the bioelectrochemical experiments. These control CVs are conducted to check the presence of any redox-active components in the growth medium and at the electrode surface.
      iii. Analyze all the CVs using the EC-Lab software accompanied with the potentiostat (Biologic - VMP3 Multichannel Potentiostat). Plot and calculate the first derivative for the turnover CV by the same software to look for the presence of any redox peak.

   ***Note:*** Other electrochemical workstations and accompanying analysis software such as that from Gamry Instruments, Metrohm, CH Instruments, etc. can also be used for bioelectrochemical experiments .

      iv. Then perform CA at the fixed applied potential of −0.3 V *vs.* Ag/AgCl at pH 9.5. After completing each batch cycle***, replenish the reactor with a fresh medium by maintaining anaerobic conditions.
      v. Along with this, set two control experiments, namely, abiotic and biotic. In abiotic control, to monitor the (electro)chemically-induced nitrate reduction in the medium, keep all the conditions identical to the main experimental setup except inoculating the reactor with microbes. In biotic control, inoculate the reactor but do not connect it to the potentiostat to observe the microbial growth in the absence of an electron donor.

   **Note:* The −0.3 V potential is selected to target the nitrate reduction reaction at the cathode. It can be altered according to the desired reaction. For example, Pozo et al. applied a fixed potential of −1.105 V (*vs.* Ag/AgCl at pH 7) to grow the sulfate-reducing mixed microbial community in the cathodic chamber (Pozo et al., 2015). Similarly, Yadav and Patil (2020) have used a potential of 0.2 V (*vs.* Ag/AgCl at pH 9.5) to enrich acetate oxidizing haloalkaliphilic exoelectrogens at the anode surface.

   ***Note:* The potential window for recording cyclic voltammograms is selected in a manner to avoid any undesired redox reactions at the electrodes and according to the target reaction.

   ****Note:* The completion of a batch cycle is considered when the bioelectrocatalytic current approaches zero and almost all substrate is cosumed. The time taken by each cycle of the CA profile can vary depending upon the life cycle of the enriched microorganisms and the concentration of the nutrients.

4. Along with the CA profiles, regular sample analysis is conducted.
   a. Procedure:

i. Monitor different parameters, including $OD_{600}$, pH, nitrate and nitrite concentrations in the medium, and $N_2$ gas in the headspace, at a regular time interval.
ii. Quantify the microbial growth in the bulk phase of the BES via protein estimation using Bradford assay, as described earlier.

*Note:* Initial polarization at a fixed potential allows the electrode to acclimatize it as an analog to a solid-state electron donor and eliminate any redox chemicals present on its surface (Gregory et al., 2004; Karbelkar et al., 2019).

### Electrochemical characterization of the cathodic biofilm

Timing: 4–6 h

5. To check the presence of any redox-active moieties or components involved in the electron transfer mechanisms, for indirect (medium) and direct (electrode-biofilm interface) after the completion of the experiment, cyclic voltammetry (CV) is recorded at different conditions. These include bare electrode (i.e., abiotic control), substrate turnover and non-turnover conditions, and filtered spent medium in the new electrochemical set-up (Harnisch and Freguia, 2012; Labelle and Bond, 2005). Different conditions at which CVs may be recorded are described below.

   a. *Substrate turnover:* The growth condition when the enriched EAMs utilize the substrate at their maximum capabilities results in the highest current production values. So, upon completing at least three* batch cycles, perform a CV at this condition. The resulting CV will most likely depict the presence of redox peaks associated with the components involved in the electron transfer process.
   b. *Substrate non-turnover:* The growth condition when all the substrate is completely consumed results in very low or close to zero current response. This CV is performed to confirm the presence of redox moieties actively involved in electron transfer, even in the absence of substrate.
   c. *CV with filtered spent media:* After completing a batch cycle and replacing the spent medium with a fresh medium, record a CV in the filtered spent medium in a new electrochemical set-up to check the presence of any soluble mediators involved in the electron transfer processes**.

   **Note:* This is to ensure sufficient growth of microbial biofilm at the electrode surface.

   ***Note:* For detecting the presence of any soluble mediators, mass spectrophotometry may be conducted for samples (biofilm and reactor suspension) and blank medium (catholyte) using HR-MS (Waters Corporation, Agilent Instruments, Thermo Scientific, and Zevo). In this study, HR-MS (Waters Corporation) recorded mass spectrometric profiles for each sample are compared against the blank and are also referred to the masslynx software. For the same purpose, other mass spectrometric-based techniques such as GC-MS, LC-MS, and HPLC-MS can also be used.

### Testing of the use of hydrogen as the electron source

Timing: 6–7 days

A trace amount of $H_2$ gas produced (note: depends on the set cathode potential and medium conditions) in the headspace could serve as an electron source for the microorganisms. A methodology to check if the target group of microbes (here, nitrate-reducing microorganisms grown at the cathode and in suspension) uses hydrogen as an alternate electron donor is summarized in this section.

6. Two different experiments which can be performed to confirm this are as follows.
   a. Procedure for experiment 1:

i. Set a BES by following the same procedure as explained earlier.
ii. After biofilm development at the cathode surface, electrically disconnect the bio-electrochemical set-up from the potentiostat.
iii. Then add pure hydrogen gas (e.g., 20% [v/v] of total headspace) in the cathode chamber of the unconnected BES.
iv. Monitor all the parameters, including $OD_{600}$, pH, nitrate/nitrite concentrations in the bulk phases, and $H_2$ and $N_2$ gases in the headspace.

b. Procedure for experiment 2:
i. For the anaerobic cultivation of the enriched biofilm, set serum flasks with $H_2$ as the sole electron donor and nitrate as the TEA under autotrophic conditions.
ii. To monitor the growth of the enriched culture, analyze parameters including $OD_{600}$, pH, nitrate/nitrite concentrations, and $H_2$ and $N_2$ gases in the headspace.

### Anaerobic cultivation of enriched microbes with different electron donors in serum flask

Timing: 15–20 days

The growth of enriched electrotrophic microbial culture is tested with soluble electron donors, selected based on their presence in sediment samples. It can reveal the capabilities of electroactive microorganisms to grow using soluble electron donors and suggest the role of enriched microorganisms in the cycling of chosen elements in the natural habitat and their respiratory capabilities.

7. Triplicate experimental set-ups in serum flasks are started. At least three growth batch cycles are conducted to validate the microbial growth profiles of the inoculated electrotrophic culture (Yee et al., 2020; Stieglmeier et al., 2009).
   a. Resources required:
      i. Five 100 mL serum flasks are required for each experimental setup.
      ii. 1M autoclaved anaerobic solutions, each of nitrate, citrate, and acetate.
      iii. 99.999% He gas is required for flushing.
      iv. UV-Vis spectrophotometer and pH meter are required to analyze different parameters.
   b. Procedure:
      i. Flush each serum flask containing 40 mL growth medium with 99.999% He gas for 10–15 min. In this study, two soluble electron donors, namely citrate and acetate, are used.
      ii. Set two controls, namely, biotic and abiotic, with each main experiment to observe the microbial growth in the absence of electron acceptor (i.e., nitrate) and monitor the chemically-induced nitrate reduction in medium, respectively.
      iii. After flushing, seal off the serum flasks using butyl rubbers and crimp seals to maintain anaerobic conditions.
      iv. Autoclave all the serum flasks before inoculating them with the enriched microbial culture.
      v. Monitor $OD_{600}$, pH, and nitrate/nitrite concentrations every 24 h, and analyze $N_2$ gas in the headspace and citrate/acetate concentrations on the $0^{th}$ and last day of each growth cycle.

### Microscopic observations for cathodic biofilm confirmation and visualization

Timing: 2–3 days

The microbial growth at the cathode surface and in the bulk phase of BESs and serum flasks can be confirmed by observing the samples by microscopic techniques such as fluorescence microscopy, confocal microscopy, and electron microscopy (scanning electron and transmission electron microscopy). Here, a scanning electron microscope (SEM) protocol is described.

8. The samples are prepared following a series of steps described below (Khan et al., 2014).
   a. Reagents required:

i. Make 2% glutaraldehyde and 2.5% paraformaldehyde* solutions beforehand by dissolving the chemicals in distilled water.
ii. Prepare different solutions (30%, 50%, 70%, 80%, 90%) of ethanol by diluting 100% ethanol.
iii. Use 0.2 μM isopore membrane filters (Sigma-Aldrich) for filtering the cell suspension.

b. Procedure:
i. Take the biocathode or suspension samples** and incubate overnight (12–14 h) in a fixative solution of 2% glutaraldehyde and 2.5% paraformaldehyde at 4°C.
ii. Then sequentially dehydrate the fixed samples using 30%, 50%, 70%, 80%, 90%, and 100% ethanol solutions for 20 min each.
iii. After dehydrating the samples with alcohol, keep them overnight (12–14 h) in a silica desiccator for further dehydration. A critical point drying method and equipment can also be used for this purpose.
iv. After dehydration, coat the samples with gold nanoparticles using JEOL JEC-1600 Auto-Fine Coater (JEOL Ltd., Japan) at 20 mA for 45 s. Then, observe the coated samples under a scanning electron microscope (JEOL JSM-6010 PLUS-LS, JEOL Ltd. Japan).
v. Subject the biocathode samples to an element analysis via FEG-SEM-EDS (Hitachi SU8010 series) to check the presence of any chemical compound deposition on its surface which might be toxic or involved in any chemical reaction.

**Note:* Paraformaldehyde takes ~2 days to completely dissolve in the distilled water. Thus, it should be prepared beforehand.

***Note:* Electrode samples are fixed, and the suspension samples are collected on 0.22 μM polycarbonate membrane filters.

### Microbial community analysis in the enriched biofilm

Timing: 40–60 days

To know and understand the microbial communities enriched in any specific experimental condition, various amplicon and metagenomic sequencing approaches including V3-V4, V2-V4, complete 16S and 18S rRNA, and whole-genome metagenomics can be used.

9. The steps to achieve this are as follows (Yadav and Patil, 2020; Chaudhary et al., 2021).
a. Resources required:
i. A genomic DNA extraction kit is required (e.g., Fast DNA SPIN kit (MP biomedicals, France); DNeasy® PowerSoil® Pro kit (Qiagen, Germany))
ii. A nanodrop and Qubit fluorometer are required for DNA quantification and gel electrophoresis for DNA quality check.
iii. A next-generation sequencing platform, such as Illumina (MiSeq/HiSeq/NovoSeq/NextSeq), Pyrosequencing, and Nanopore is required.
iv. Bioinformatic analysis is performed via different computer programs and tools such as Linux, Anaconda, Miniconda, and R-packages.
b. Procedure:
i. For a detailed procedure, refer to Marizzoni et al., (2020) and Jo et al. (2020).

## EXPECTED OUTCOMES

The given protocol is helpful for successfully enriching electroactive microorganisms, particularly nitrate-reducing electrotrophs from a highly saline and alkaline habitat. The electrochemical characterization of the enriched electrotrophic microbial biofilm reveals the formal potentials of redox-active moieties associated with the electron transfer, along with the nature of electron transfer between the microorganisms and the electrode. Moreover, the mass spectrometry-based analysis

helps to detect any soluble mediators, if present, in the medium. Different experiments conducted with hydrogen and well-developed biofilm in BES confirm the role of hydrogen as the electron donor. The microbial growth with different soluble electron donors reveals their potential role in element cycling in the chosen extreme environments. Scanning electron microscopy reveals the microbial morphology and growth as a biofilm at the electrode surface. Notably, the electrochemical enrichment protocol can be adapted to enrich different electroactive microorganisms according to target reactions and microbial groups.

## LIMITATIONS

The time required to complete the given protocol will vary according to target microorganisms and the concentration of used electron acceptors. Microorganisms grown under electroautotrophic conditions are generally slow-growing, and hence the enrichment experiments may take longer than the mentioned duration in this protocol. The particular microbial group involved in the direct electron transfer process cannot be identified in the mixed community unless isolated in pure cultures. Similarly, the redox-active peaks observed in the cyclic voltammograms of bio-electrodes cannot be attributed to a particular microbial group in the mixed community.

## TROUBLESHOOTING

### Problem 1

Standardization of calibration curves for different analytical methods and tools (before you begin, step 1-4).

### Potential solution

The detection limits of different analytical methods and tools should be known before conducting any analysis. The calibration curves can then be prepared considering the detection limits and the used range of concentrations in the experiments.

### Problem 2

Maintenance of anaerobic conditions in the system (before you begin, step 7 and step-by-step method details, step 3, 6, 7).

### Potential solution

Different electrochemical reactions may vary according to the conditions in which the experiments are run. It is necessary to maintain the desired conditions such as anaerobic or aerobic in the reactor chambers according to the target reaction and microorganisms. For instance, the reactor headspace gas composition can be regularly analyzed via gas chromatography (GC-TCD). Reducing agents such as cysteine or redox indicators such as resazurin can be added to the medium.

### Problem 3

Selection of potential window for recording cyclic voltammograms (step-by-step method details, step 3, 5).

### Potential solution

The safe working potential window for recording cyclic voltammograms needs to be selected beforehand to avoid undesired reactions at different potentials at the electrodes. The target reaction or process at the electrodes will determine the safe working potential window.

### Problem 4

Selection of the potential to be applied on the working electrode (step-by-step method details, step 3).

### Potential solution

The applied working potential for obtaining chronoamperometric profiles can be selected depending on the target substrate reaction and considering different experimental conditions such as pH and used reference electrode.

### Problem 5

Variation in the applied potential at the working electrode (step-by-step method details, step 3).

### Potential solution

The set potential at the electrode via potentiostat should be cross-checked with a multimeter to ensure stable applied potential at the working electrode.

### Problem 6

Precipitation of salts during visualization of microorganisms by microscopy (step-by-step method details, step 8).

### Potential solution

The growth media used in this protocol is rich in salts which can precipitate and thus can interfere in the visualization of microorganisms by microscopy techniques. The sample can be washed thoroughly by phosphate buffer saline to overcome this issue.

### Problem 7

Working with faulty reference electrodes (step-by-step method details, step 2).

### Potential solution

Some common issues related to the reference electrode are mentioned below, and these should be addressed before starting experiments and must also be checked regularly during the experiment.

Potential drift

Potential fluctuation

Reference electrode fouling

Bubbles in the frit

Loose connections with the reference electrode

Electrolyte bleeding

> *Note:* The potentials of the reference electrode should be cross-checked with a new or standard reference electrode before starting, during, and after completion of the (bio)electrochemical experiments using a multimeter. It will help monitor the potential drift and fluctuation of the reference electrode. Acute visualization is recommended to check the health (fouling, bubbles in the frit, and electrolyte bleeding) and loose connection of the reference electrode at regular intervals throughout the experiment.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Sunil A. Patil (sunil@iisermohali.ac.in; sunilmicro12@gmail.com).

### Materials availability

This study did not generate unique reagents. All reagents are available as specified in the key resources table.

### Data and code availability

The raw sequencing data files obtained in Chaudhary et al. are available on the NCBI short-read archive under the SRR12506991 accession number (Chaudhary et al., 2021). The same article includes all data generated or analyzed during this study.

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## AUTHOR CONTRIBUTIONS

S.A.P. conceived and designed the study. R.S., S.C., and S.Y. conducted the experiments and acquired and analyzed the data. All the authors wrote and approved the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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