

# Electroactive biofilms: how microbial electron transfer enables bioelectrochemical applications

Eric M. Connors , Karthikeyan Rengasamy, Arpita Bose

Department of Biology, One Brookings Drive, Washington University in St. Louis, MO, 63105, USA  
Correspondence should be addressed to: Arpita Bose. E-mail: [abose@wustl.edu](mailto:abose@wustl.edu)

**Abstract:** Microbial biofilms are ubiquitous. In marine and freshwater ecosystems, microbe–mineral interactions sustain biogeochemical cycles, while biofilms found on plants and animals can range from pathogens to commensals. Moreover, biofouling and biocorrosion represent significant challenges to industry. Bioprocessing is an opportunity to take advantage of biofilms and harness their utility as a chassis for biocommodity production. Electrochemical bioreactors have numerous potential applications, including wastewater treatment and commodity production. The literature examining these applications has demonstrated that the cell–surface interface is vital to facilitating these processes. Therefore, it is necessary to understand the state of knowledge regarding biofilms' role in bioprocessing. This mini-review discusses bacterial biofilm formation, cell–surface redox interactions, and the role of microbial electron transfer in bioprocesses. It also highlights some current goals and challenges with respect to microbe-mediated bioprocessing and future perspectives.

**Keywords:** Biofilms, Microbial electrosynthesis, Extracellular electron uptake, Bioelectrochemical systems, Extracellular electron transfer

## Biofilm Formation: Benefits of Living Together

The success of microbes on Earth in terms of biomass and habitat distribution can be attributed in large part to their phenotypic plasticity, which makes them resilient to environmental stimuli (Costerton et al., 1995). Part of this plasticity is the tendency of many bacteria to form biofilms. Biofilms can be found on organs and tissues [e.g. the human oral microbiome (Krzysciak et al., 2016; Macfarlane & Macfarlane, 2006)], in industrial settings [e.g. biocorrosion (Giorgi-Pérez et al., 2021) and biofouling (Banerjee et al., 2011)], or deep subsurface mineralogical deposits (Casar et al., 2021). For a review of bacteria–surface interactions, see Tuson and Weibel (2013).

Biofilms form via a two-step process initiated by reversible attachment via hydrodynamic and electrostatic interactions, followed by irreversible attachment via van der Waals interactions between the cells and surface (Costerton et al., 1995). In addition to cells, biofilms are comprised of extracellular polymeric substance (EPS) and extracellular organelles (flagella, pili, adhesins), which facilitate biofilm formation and adhesion (Costerton et al., 1995; Lewandowski, 2017). In contrast to free-floating planktonic cells, biofilms demonstrate resistance to externalities, including antibiotics (Mah, 2012; Mah & O'Toole, 2001), environmental stressors, and shear forces (Costerton et al., 1995; Donlan, 2002). Additionally, biofilms allow for dynamic nutrient exchange and cellular communication (Costerton et al., 1995; Donlan, 2002; Santos et al., 2018). Biofilm formation is influenced by numerous factors. For example, hydrophobic surfaces with a high surface area are most amenable to cellular attachment (Donlan, 2002; Simões et al., 2010). Also, the presence of EPS and external structures that support adhesion play important roles in attachment to surfaces (Donlan, 2002; Vu et al., 2009).

The relationship between electroactivity and biofilm characteristics is especially relevant for bioelectrochemical systems, as increased electroactivity can result in efficient product formation. Therefore, efforts to enhance biofilm formation on electrodes should be focused on either altering the electrode surface to encourage cellular attachment (e.g. increased surface area), or by engineering strains to encourage more robust biofilm formation (e.g. EPS formation).

## Electron Transfer Mechanisms Between Microbes and Surfaces

Redox reactions are the heart of cell–surface interactions. Extracellular electron transfer (EET) allows for the exchange of electrons between cells and surfaces, which drives biochemical processes necessary to convert substrates to products. One of the functions of biofilms is to mediate direct EET between surface-attached cells and redox-active surfaces (e.g. cathodes or redox-active minerals). This contrasts with indirect electron transfer, wherein electrons are transferred via conductive mediators (e.g. soluble electron shuttles). Broadly, the movement of electrons can be classified as electrode-reducing extracellular electron transfer (rEET) and electrode-oxidizing extracellular electron transfer (oEET). The former describes the transfer of electrons from cell to surface and the latter describes the transfer of electrons from surface to cell, which cells achieve via extracellular electron uptake (EEU). Bacteria mediate electron transfer using outer membrane cytochromes (Gupta et al., 2019; Tanaka et al., 2018); redox-active electron shuttles such as anthraquinone-2,6-disulfonate, natural organic matter, and flavins (Bai et al., 2020; Lin et al., 2018); conductive nanowires (El-Naggar et al., 2010; Reguera et al., 2005); and other conductive matrices comprised of redox active substrates

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(e.g. metallic nanomaterials or EPS) (Cui et al., 2020; Jia et al., 2021; Xiao et al., 2017).

Mechanisms for rEET have been explored in a variety of metal-respiring microbes, such as *Geothrix fermentans* (Coates et al., 1999), *Geovibrio ferrireducens* (Caccavo Jr. et al., 1996), *Thiobacillus ferrooxidans* (Pronk et al., 1992), and a range of other microbes, including Archaea (Vargas et al., 1998). The first metal-respiring bacteria studied in-depth were *Shewanella oneidensis* and *Geobacter sulfurreducens*. *S. oneidensis* MR-1 (MR-1), which can use iron or manganese as a terminal electron acceptor, encodes a metal-reducing (Mtr) pathway comprised of multiheme cytochrome *c* proteins (including MtrA and MtrC) and an outer-membrane porin (MtrA) that is essential for reduction of electrodes (Coursolle et al., 2010).

Efforts to understand rEET mechanisms of the iron-reducing bacterium *G. sulfurreducens* centered primarily around the roles of electrically conductive protein nanowires, referred to as e-pili, and cytochrome *c* proteins in mediating long-range electron transfer (Lovley & Walker, 2019). A number of early studies demonstrated that deleting the *pilA* gene encoding the *G. sulfurreducens* pilin monomer ablated the ability of cells to reduce Fe(III) oxides, inhibited biofilm formation, and decreased current production during growth on electrodes (Reguera et al., 2005, 2006). Coupled with data that *G. sulfurreducens* pili are electrically conductive, biofilm thickness is correlated with current production, and cells remain viable within the biofilm (Reguera et al., 2005, 2006), these studies suggest that *G. sulfurreducens* forms conductive networks comprised of metabolically active cells and nanowires to transfer electrons throughout surface-attached biofilms. Complicating this hypothesis, deleting genes encoding the outer membrane *c*-type cytochromes OmcS or OmcE inhibited Fe(III) and Mn(IV) oxide reduction in *G. sulfurreducens* (Mehta et al., 2005). Later work demonstrated that OmcS is localized along *Geobacter* pili (Leang et al., 2010). While this implies a possible role for *c*-type cytochromes in mediating long-range rEET, multiple lines of evidence support the superseding role of e-pili (Lovley & Walker, 2019). For example, denaturing *c*-type cytochromes in biofilms does not impact conductivity, and biofilm conductivity is negatively correlated with *c*-type cytochrome abundance while being positively correlated with *pilA* abundance (Malvankar et al., 2011, 2012). Moreover, OmcS spacing along pili precludes their ability to transfer electrons (Malvankar et al., 2012). Other lines of evidence include the ability of conductive magnetite to restore Fe(III) reduction in an *omcS* deletion mutant, but not a *pilA* deletion mutant (Liu et al., 2015), as well as the observation that OmcS cannot compensate for poorly conductive pili vis-à-vis Fe(III) oxide reduction (Liu et al., 2014; Tan et al., 2016). Despite recent claims to the contrary (Wang et al., 2019), these data suggest that OmcS is not essential for long-range rEET in *Geobacter*. Instead, protein nanowires appear to be the primary driver of long-range rEET in this organism (Lovley, 2012). Further work is required to understand the mechanisms responsible for electron transport along e-pili.

Although conductive protein nanowires are essential for long-range electron transfer between cells and solid surfaces in *Geobacter*, studies to date suggest a role for other protein complexes in rEET. Although deleting *omcS* inhibits Fe(III) and Mn(IV) oxide reduction, it does not ablate reduction of soluble electron acceptors, suggesting a potential substrate specificity (Mehta et al., 2005). Besides OmcS, other cytochrome *c* proteins are postulated to play important roles in *Geobacter* electron transfer, biofilm formation, and current production. In many cases, this involves integrating within transmembrane porin-cytochrome protein complexes or acting as periplasmic electron shuttles (Leang et al., 2003; Levar et al., 2014; Liu et al., 2014, 2015; Lloyd et al., 2003; Morgado et al., 2010; Qian et al., 2007; Shi et al., 2016; Zacharoff et al., 2016).

Additional studies are required to understand the role of cytochrome *c* proteins in mediating rEET. Moreover, efforts to understand the regulatory mechanisms governing electron transfer in *Geobacter* are needed. A recent study found that the  $\Delta$ gsu1771 mutant strain overexpresses *pilA*, *c*-type cytochromes, and develops thicker biofilms (Hernández-Eligio et al., 2022). This led to more efficient Fe(III) oxide reduction and approximately 100-fold higher current production in electrochemical reactors with fluorine-doped tin oxide electrodes. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and electrophoretic mobility shift assays indicate that GSU1771 directly binds to and controls the expression of *pilA*, *omcS*, *omcZ*, *omcE*, *dcuB*, and *frdC*, supporting its role as a transcriptional regulator important for rEET.

EEU has been studied primarily in autotrophs [see Gupta et al. (2020) for a review]. This includes phototrophic iron-oxidizing bacteria (photoferrotrophs) like *Rhodospseudomonas palustris* TIE-1 (TIE-1). Genetic, biochemical, and electrochemical studies revealed that TIE-1's phototrophic iron oxidation (Pio) operon encodes three proteins that mediate EEU: a multiheme cytochrome *c* (PioA), an outer membrane porin (PioB), and a periplasmic high-potential iron-sulfur protein (PioC) (Gupta et al., 2019; Guzman et al., 2019; Jiao & Newman, 2007). Homologs of *pilA* have been identified in related organisms, including *Rhodovulum* sp. PH10, *Blastochloris viridis*, *Rhodomicrobium udaipurense*, and *Rhodomicrobium vannielii*, suggesting that Pio operon homologs may be widespread among phototrophic bacteria (Gupta et al., 2019). As with EET, regulatory mechanisms controlling EEU processes remain elusive. Previous work suggests that the *pilABC* operon in TIE-1 is induced by anoxia (Bose & Newman, 2011). In the same study, deleting the upstream *fixK* gene led to decreased *pilABC* expression during phototrophic iron oxidation, suggesting it acts as a transcriptional activator during photoferrotrophy. However, the *fixK* deletion mutant showed increased *pilABC* expression under other growth conditions, which suggests that FixK represses *pilABC* transcription under non-photoferrotrophic growth. Other putative FixK target genes important for anaerobic phototrophic growth were identified, including a potassium uptake protein (*kup1*), photosynthetic genes, and iron metabolism genes. Additional work is required to uncover direct and indirect activation roles of FixK, as well as additional levels of *pilABC* regulation in TIE-1.

EEU mechanisms in other organisms remain elusive; *Sideroxydans lithotrophicus* ES-1 is hypothesized to oxidize iron via its *mto* gene cluster, though additional genetic and biochemical studies are needed to confirm this (Beckwith et al., 2015; Hädrich et al., 2019). A recent study revealed that the marine anoxygenic phototroph *Rhodovulum sulfidophilum* performs photosynthetic EEU from an electrode and that this process is linked to photosynthetic electron transport (Gupta et al., 2019). Using a transcriptomics-guided analysis of the environmental isolate *R. sulfidophilum* AB26, the authors determined that AB26 uses a previously unknown di-heme cytochrome *c* protein, EeuP, for photosynthetic EEU, but not photoferrotrophy. Interestingly, AB26 does not encode homologs of previously characterized electron transfer proteins known to play a role in iron oxidation of EET. This study also noted that phototrophic EEU and photoferrotrophy are common in marine phototrophs based on an analysis of 15 *R. sulfidophilum* environmental isolates enriched from an estuarine microbial mat (Gupta et al., 2019). EEU has also recently been demonstrated in an aerobically grown, non-phototrophic consortium enriched from seawater called Biocathode MCL (*Marinobacter-Chromatiaceae-Labrenzia*) (Bird et al., 2021; Strycharz-Glaven et al., 2013). Meta-omics analyses of Biocathode MCL identified *Candidatus Tenderia* electrophaga as the likely sole electroactive member of the biofilm and the most

active constituent of MCL biofilms grown on graphite electrodes (Eddie et al., 2016, 2017; Wang et al., 2015). More recently, Biocathode MCL was shown to increase the open circuit potential (OCP) of graphite, carbon cloth, and indium tin oxide (ITO) electrodes (Bird et al., 2021). Antibiotic treatments show that metabolically active cells, rather than enzymatic activity, are responsible for the OCP shift. The increase in OCP was coupled with a 10-fold increase in biomass relative to biofilms on nonconductive surfaces; importantly, only *Ca. Tenderia* demonstrated a significant increase in abundance on ITO relative to glass, supporting its role as the sole electroactive member of MCL. Moreover, because the OCP remains shifted positive in the presence of metabolically active cells, the authors propose that redox-active molecules in the growth medium likely recharge the electrode to supply sustained energy. This suggests that electroactive biofilms in the environment can use charge-storing conductive surfaces (e.g. metal-containing minerals) to acquire otherwise inaccessible electrons. Given that *Ca. Tenderia* is a biomarker of stainless steel corrosion (Trigodet et al., 2019), further research into this process may inform corrosion mitigation strategies.

Given the lack of understanding of EEU pathways with respect to their phylogenetic and geographic distribution as well as the molecular underpinnings of this process, it is essential to continue identifying novel EEU mechanisms and/or novel EEU-capable microbes. Recent examples of electrotrophic microbes enriched from the environment include a microbial consortium enriched from the sludge in a textile wastewater treatment plant, which was used to develop a biocathode for microbial fuel cell (MFC) applications (Mani et al., 2020); electroactive bacteria that were isolated from the roots and stems of sweet potato and angelica plants (Ling et al., 2020); and the first thermoacidophilic electroautotrophic community enriched from geothermal hot springs (Reiner et al., 2020). Other potential sources for electroactive microbes include anaerobic soils and sediments, deep-ocean coldseeps, marine and freshwater habitats, acid mining runoff, and other terrestrial and aquatic environments (Chabert et al., 2015). Therefore, efforts to explore untapped environments may reveal new and perhaps more efficient EEU mechanisms.

## Biofilm-Mediated Electron Transfer in Bioprocessing

Biofilms are relevant in a variety of industrial processes, including wastewater cleanup (Ahmad et al., 2022; Qureshi et al., 2005), bioremediation (Mohapatra et al., 2020), and the production of value-added products such as bioplastics and biofuels (Scientia, 2021). This latter category takes advantage of bioelectrochemical systems (BESs) in the form of MFCs, microbial electrolysis cells (MECs), and microbial electrosynthesis (MES) systems. All BES technologies are limited by (1) low electron transfer rates from cathodes to microbes, and (2) gaps in knowledge regarding the biology underlying EET and EEU between electrodes and microbes [for a review of EEU in MES applications, see Karthikeyan et al. (2019)]. Addressing these bottlenecks requires further exploration of biofilms as biocatalysts.

### Indirect versus Direct EEU

EET includes both reductive EET and oxidative EEU. Whereas reductive EET has been extensively studied, EEU mechanisms are not well-understood. Therefore, this section will focus on advancements in our understanding of biofilm-mediated EEU. For reviews of EET, see Kumar et al. (2017), Logan et al. (2019), and

Shi et al. (2016). Direct EEU relies on direct contact between cells and redox-active surfaces, while indirect EEU relies on intermediate electron shuttles. The latter includes soluble abiotic  $H_2$  generation at the cathode, which then acts as a soluble electron shuttle to transport electrons to cells (Blanchet et al., 2015). Direct EEU via biofilms is understudied, particularly with respect to EEU mechanisms. Importantly, direct EEU promises to be a more energy- and cost-efficient bioprocessing technology (Karthikeyan et al., 2019). Ultimately, both indirect and direct EEU require further examination, as both processes are at play in biofilms and BESs. For example, in electromethanogenesis, methanogens use  $H_2$  produced from water electrolysis to mediate indirect electron transfer for methane formation (Blasco-Gómez et al., 2017). However, they can also conduct direct EEU from a cathode (Cheng et al., 2009). This was explored in a study that sought to tease out the contributions of both processes to electromethanogenesis (Villano et al., 2010). Here, a bioelectrochemical system inoculated with a mixed methanogenic culture demonstrated simultaneous abiotic  $H_2$  evolution and biological direct EEU. This led to methane production at potentials lower than  $-650$  mV versus standard hydrogen electrode (SHE). The authors demonstrated a maximum contribution of direct EEU toward product formation at a cathode potential of  $-750$  mV. The relative contribution of abiotic  $H_2$  toward product formation was largely dependent on the cathode potential. Importantly, the authors note that the rate of direct EEU and its contribution to the overall electron transfer rate could be improved by enhancing electroactive biofilm formation.

More recently, the relative contributions of direct EEU and  $H_2$ -mediated EEU toward methane production were examined using a “cage” type cathode colonized by the methanogen *Methanosarcina barkeri* (Bai et al., 2020). This novel cathode design implemented a porous mixed cellulose ester membrane with a base of conductive carbon nanotubes, which concentrated the cells on the cathode surface to encourage the more energetically favorable direct EEU process. Methane production was facilitated by direct and indirect electron transfer processes at a range of cathode potentials; both direct and  $H_2$ -mediated indirect electron uptake occurred at potentials from  $-0.6$  to  $-1.2$  V, with indirect EEU's contribution increasing as cathode potential decreased. Only direct EEU was detected at  $-0.4$  V. This supports the notion that lower cathode potentials are required for  $H_2$ -mediated EEU. It also demonstrates that direct and indirect electron transfer co-occur with varying relative contributions, depending upon parameters such as potential.

Efforts to understand the electron uptake mechanisms of the methanogens *Methanococcus maripaludis* and *Sporomusa sphaeroides*, which mediate microbiologically influenced corrosion (MIC) and electrosynthesis, determined that direct EEU occurs via free, surface-associated redox enzymes such as hydrogenases and formate dehydrogenases (Deutzmann et al., 2015). Because loss of cellular integrity is a prerequisite for enzyme release, the tendency for a biofilm to induce cell lysis among its inhabitants may lead to accumulation of sorbed redox-active enzymes and therefore higher electroactivity. This is in line with later work showing that the methanogen *Methanosarcina barkeri* uses extracellular hydrogenases to conduct EEU (Rowe et al., 2019) and that multi-heme cytochrome *c* proteins are not widely conserved among methanogens that engage in direct interspecies electron transfer (Yee & Rotaru, 2020). Therefore, this enzyme-mediated electron transfer process may play an important ecological role in supporting methanogenic biofilms, although cytochrome-containing



methanogens like *M. barkeri* may employ other EEU mechanisms independent of extracellular enzymes (Rowe et al., 2019).

## Microscale Gradients Create Biofilm Microenvironments

Biofilms likely develop specialized subpopulations in different contexts (Häußler et al., 2003; Mann & Wozniak, 2012; Moormeier et al., 2013). Differential diffusion of signaling molecules, external stressors, nutrients, oxygen, and waste can lead to the development of microenvironments, which can drive changes in phenotypic diversity within the biofilm. The role of these subpopulations in biofilm development has been studied extensively in the clinical realm [for a review, see Bisht & Wakeman (2019)]. These studies have shown that heterogeneity within biofilms can manifest as temperature-dependent EPS gene expression and production, as seen in *Clostridium perfringens* (Obana et al., 2020); result in distinct transcriptomic profiles and phenotypes dictated by location within the biofilm, including antibiotic-tolerance and slow growth deep in a *Pseudomonas aeruginosa* biofilm (Williamson et al., 2012); and lead to heterogeneous quorum-sensing activation patterns according to external physical properties like flow and topography in *Vibrio cholerae* and *Staphylococcus aureus* (Kim et al., 2016). This is by no means an exhaustive description of the kinds of genotypic and phenotypic heterogeneity seen in biofilms. Instead, it underscores that biofilms are dynamic environments that respond to their environment.

With this in mind, it is likely that biofilms attached to electrodes develop heterogeneous genotypic and phenotypic expression in response to the environment (e.g. electrochemical gradients), and that this influences electron transfer processes (Beyenal & Babauta, 2012). Careful consideration of the biofilm microenvironment will reveal these microscale gradients, and lead to improvements in biofilm engineering and electrochemical system design. High-resolution analyses such as single-cell RNA sequencing can uncover underlying gene-expression profiles (Imdahl et al., 2020; Singh et al., 2019).

Diffusion limitations in response to biofilm morphology are an important consideration, as thick biofilms can result in lower diffusivity (Renslow et al., 2010). For example, current generation by anode-respiring bacteria (ARB) is influenced by buffer strength (Torres et al., 2008). Here, current generation by an ARB biofilm was limited by proton transport into and out of the biofilm. While electrons diffuse toward the anode, protons generated at the anode interface diffuse outward. A thick biofilm can limit buffer access to the bottom of the biofilm, resulting in proton accumulation and a lower pH near the anode interface. While this effect was mitigated using a stronger buffer, it suggests that the influence of biofilm thickness on diffusion kinetics can have a deleterious effect on current generation.

Also challenging the assumption that thick and conductive biofilms encourage high electroactivity, it was demonstrated that *G. sulfurreducens* biofilms have the highest electrochemical activity with a biofilm thickness of  $\sim 20 \mu\text{m}$ , with electroactivity decreasing beyond that and eventually ceasing at  $\sim 45 \mu\text{m}$  (Sun et al., 2016). This was attributable to the accumulation of inactive cells accumulating over time within the inner layer, resulting in high diffusion resistance. A recent study sought to overcome low electron transfer rates induced by diffusion-limited thick biofilms via quorum quenching (QQ), which is the process of disrupting autoinductor-mediated quorum sensing (QS) in microbes (Taşkan & Taşkan, 2021). QQ allows for the control of QS-mediated biofilm formation and has been used in membrane bioreactors to control

biofouling and increase membrane flux (Fakhri et al., 2021; Huang et al., 2019; Nahm et al., 2017; Oh & Lee, 2018). Here, QQ bacteria (*Rhodococcus* sp. BH14) were immobilized onto alginate beads and added to a dual chamber MFC. Using this approach, QS inhibition reduced EPS production, resulting in decreased biofilm thickness and increased electroactivity, with a maximum of  $1924 \text{ mW m}^{-2}$  (2.8-fold higher compared to control) at a biofilm thickness of  $26 \mu\text{m}$ . Interestingly, performance decreased past a certain point, with power generation falling to  $410 \text{ mW m}^{-2}$  (1.6-fold less than control) at a biofilm thickness of  $11.2 \mu\text{m}$ , suggesting that too significant of a decrease in thickness may negatively impact power generation.

Biofilm density is also an important consideration. A study of the ARB *G. sulfurreducens* demonstrated that higher biofilm density led to increased current generation (Otero et al., 2021). The authors demonstrated that a mutant lacking four of the five outer membrane cytochrome complexes (*extABCD*<sup>+</sup>) formed denser biofilms on an electrode with higher per-cell anabolic activity, leading to faster growth and  $\sim 1.4$ -fold higher current compared to wild-type. The mutant biofilm concentrated cells with higher anabolic activity near the electrode interface, which was supported by significantly higher diffusion rates in the mutant ( $\sim 30$ -fold faster electron diffusion in mutant relative to wild-type). Importantly, the study demonstrated that conductive biofilms are not homogeneous, as isotopic label incorporation showed that both mutant and wild-type biofilms contain subpopulations with decreasing anabolic activity further away from the electrode, while electron microscopy showed decreasing cell density with distance. In the same study, overexpression of *extABCD* did not increase current generation, suggesting that overexpressing components of electron transfer pathways does not necessarily yield improvements in electroactivity.

Other biofilm components can play an important role in determining the biofilm microenvironment and electroactivity. A recent study showed that deleting a component of the polysaccharide biosynthesis and export pathway genes (*GSU1501*) in *G. sulfurreducens* strain PCA yielded an EPS-deficient mutant with decreased current generation in a bioelectrochemical system (Zhuang et al., 2022). The EPS mutant had lower c-type cytochrome content, lower cell viability, and a thinner biofilm ( $14$  vs.  $27 \mu\text{m}$ ) relative to wild-type. Moreover, it demonstrated up to 50-fold higher expression of the *pilA* gene relative to wild-type, resulting in higher pili formation in the mutant. This allowed for later-stage biofilm formation. The mutant strain eventually developed a thicker biofilm ( $33 \mu\text{m}$ ) than wild-type after four batch cycles, suggesting a compensatory relationship between EPS and pili in biofilm formation.

## Remaining Questions and Challenges

One of the key challenges facing BES applications is electron transfer efficiency. Electrode material can be a significant factor in determining electron transfer rate. Typically, carbon electrodes have been employed, though novel cathode materials—including cathodes decorated with conductive metal oxides—are being explored (Paul et al., 2018; Xu et al., 2018). In a novel cathode design, a nanocomposite comprised of magnetite nanoparticles and reduced graphene oxide was electrochemically deposited on carbon felt. This modified cathode enhanced electron uptake  $\sim 5$ -fold relative to unmodified cathodes, resulting in  $\sim 4.2$ -fold higher polyhydroxybutyrate (PHB) production in the photoautotroph *R. palustris* TIE1-1. Other recent efforts to enhance electron transfer via electrode modifications include a porous mixed cellulose ester

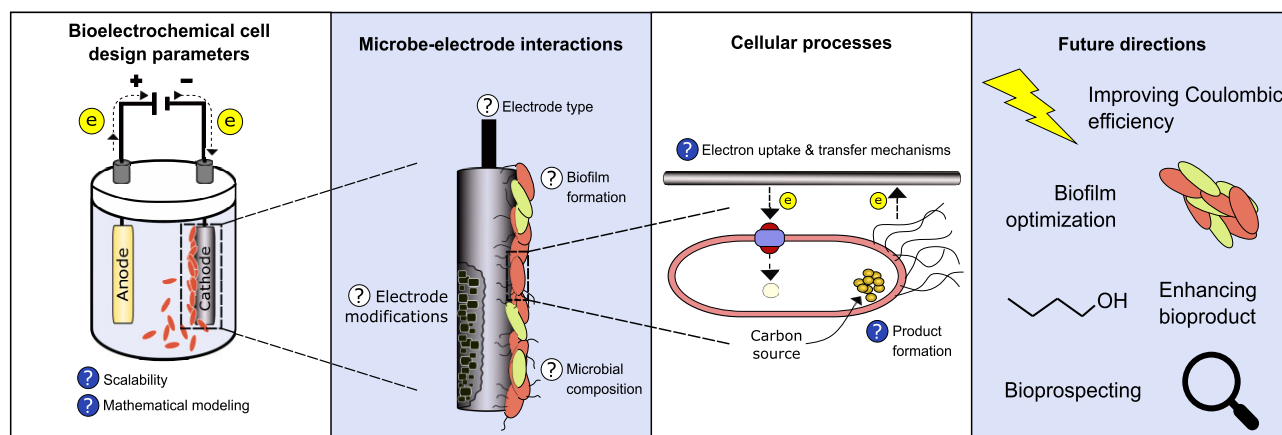
**Table 1.** Recent efforts at plastic (polyhydroxybutyrate) and fuel (hydrocarbon) production via microbial electrosynthesis

Organism	Genetic manipulations	Product	Cathode material + mediators	Substrate	Product yield (mg L <sup>-1</sup> )	Reference
<i>Ralstonia eutropha</i>	Heterologous Ribulose-1,5-bisphosphate carboxylase-oxygenase expression	PHB	Carbon cloth + Formate and neutral red redox mediators	CO <sub>2</sub>	485	Chen et al., 2018
<i>Rhodospseudomonas palustris</i> TIE-1 (TIE-1)		PHB	CF/rGO-MNPs	CO <sub>2</sub>	91.31	Rengasamy et al., 2021
<i>Cupriavidus necator</i> H16		PHB	Indium + indium nanoparticle	Formate	25.2	Al Rowaihi et al., 2018
TIE-1		PHB	Graphite	CO <sub>2</sub>	4.48	Ranaivoarisoa et al., 2017
TIE-1	Heterologous expression of <i>n</i> -butanol biosynthesis pathway; nitrogenase deletion	<i>n</i> -butanol	Graphite	CO <sub>2</sub>	0.91	Bai et al., 2021
Activated sludge from wastewater		Acetate/Ethanol	Toray Carbon Paper w/methyl viologen and neutral red redox mediators + polyaniline	CO <sub>2</sub>	4.42/0.48	Anwer et al., 2021
<i>Clostridium beijerinckii</i>		Butanol/H <sub>2</sub>	Graphite felt + neutral red redox mediator	Glucose	0.30 g g <sup>-1</sup> / 206.53 mL g <sup>-1</sup>	Zhang et al., 2021

membrane with a base of conductive carbon nanotubes, as described above (Bai et al., 2020); a cathode made of copper foam coated with reduced graphene oxide (Aryal et al., 2019); and a polyaniline/graphene-modified carbon cloth anode (Huang et al., 2016). For reviews discussing electrode modifications, see Bian et al. (2020), Hindatu et al. (2017), Kaur et al. (2020), and Yaqoob et al. (2020).

In another study, directed evolution of *Kyrpidia spormannii* EA-1 sought to enhance cathodic biofilm-forming capabilities (Jung et al., 2020). The adapted biofilm showed a 28% increase in thickness, 4-fold higher biofilm accumulation rate, more morphological homogeneity, and increased substratum coverage compared to the progenitor strain. Moreover, comparative genomics revealed adaptation to oxidative stress as a potential mechanism for enhanced biofilm development in the adapted strain. Variations were found in *mfd*, which encodes part of the DNA repair machinery, and CVV\_06825, which is a part of the Fur protein family consisting of metal ion homeostasis regulators and oxidative stress proteins. Further studies will be needed to clarify the potential role of oxidative stress in oxic electrosynthesis processes, as well as other genetic components of biofilm formation in electrosynthesis.

BES technologies are also limited by our understanding of biofilm dynamics. Mathematical models describing these may allow for more targeted applications. Recently, de Lichtervelde et al. (2019) developed an electrochemical transport model for conductive biofilms on electrodes. Their model considered substrate transport across the biofilm, oxidation of substrate in the bacteria, electron transfer (e.g. via pili), and charge transport to the anode of an MFC. Their model suggests that current production in an MFC is not limited by organic molecular transport or conductivity. Regarding biofilm conductivity, they demonstrate that electron transport efficiency decreases as conductivity is lowered, resulting in electron buildup via reduced cytochromes. These reduced cytochromes exist as a gradient across the biofilm, which is consistent with previous literature examining long-distance electron transfer by *G. sulfurreducens* (Liu & Bond, 2012). Other recent efforts to develop models of biofilm dynamics include mass transfer modeling in membrane biofilm reactors for wastewater treatment (Ghasemi et al., 2021), microalgal biofilm growth on photobioreactors (Huang et al., 2021), and multispecies oral biofilm development (Martin et al., 2017). In this vein, developing and validating models in other contexts will lead to improved reactor design.



**Fig. 1** Relevant areas of consideration for optimizing electroactive biofilms in bioelectrochemical applications. Efficient design and operation of bioelectrochemical systems rest on fine-tuning bioelectrochemical cell parameters using tools such as mathematical modeling to allow for scalability; investigation of microbe–electrode interactions and the effect of electrode type, electrode modifications, microbial composition, and biofilm formation on performance; and understanding the cellular processes underlying electron exchange and product formation. Future efforts to improve bioelectrochemical cell performance should focus on improving Coulombic efficiency, optimizing biofilm formation, enhancing bioproduct formation, and bioprospecting for novel electroactive strains and electron exchange mechanisms.

A further consideration is the implementation of multispecies biocatalysts. Microbial consortia are often implemented in BESs to enhance electrical efficiency, pollutant degradation, or product formation (Hassan et al., 2018). Consortia can be characterized by interspecies cooperation and competition that may impact bioprocessing outcomes, though this phenomenon is understudied. A recent study analyzed a microbial consortium composed of the *S. oneidensis* MR-1 and *Citrobacter freundii* An1 in a BES (Xiao et al., 2021). Using lactate as a substrate, the MR-1/An1 consortia generated a peak current of  $38.4 \mu\text{A cm}^{-2}$ , or 6-fold that produced of *S. oneidensis* MR-1 alone. The consortium's dynamic was characterized by competition at the electrode interface, which enhanced the metabolic activity of *S. oneidensis* MR-1 and facilitated biofilm formation, resulting in a competitive advantage for MR-1. The increased metabolic activity resulted in higher electron generation and more flavin production, the latter serving as a soluble electron shuttle that facilitated EET. Additionally, proteomics revealed that, relative to monoculture BESs, co-cultures had increased expression of proteins involved in biofilm formation, outer membrane cytochrome c, and lactate metabolism. Further studies of microbial consortia in the context of BESs may reveal additional dynamics at play and suggest strategies for designing microbial consortia or otherwise adjusting conditions to mirror the beneficial effects of a consortium.

## MES Applications: Bioplastics and Biofuels

Bioprocessing encompasses a variety of technologies with the shared goal of generating products using biological catalysts. Technologies falling under this umbrella include MECs, MFCs, and MES, among others [for reviews on these technologies, see Hasany et al. (2016), Obileke et al. (2021), Palanisamy et al. (2019), and Wang et al. (2015)]. Among other applications, these tools are being used for wastewater cleanup, bioremediation, hydrogen generation, plastic production, and fuel production (Ahmad et al., 2022; El-malek et al., 2020; Karthikeyan et al., 2019; Keasling et al., 2021; Mohapatra et al., 2020). The latter two products are especially important in the current environment. Global plastic waste may reach 155–265 Mt per year by 2060 if current trends continue (Lebreton & Andrady, 2019). The *World Economic Forum* predicts that

the plastics sector will account for 15% of the global annual carbon budget by 2050 (*The New Plastics Economy*, 2016). Similarly, fossil fuel consumption is a significant contributor to climate change (Hofmann et al., 2009). Therefore, developing sustainable alternatives to traditional fuels and plastics is of the utmost importance. This section will emphasize recent advancements in developing MES approaches to bioplastic and biofuel production (Table 1).

## Bioplastics

Polyhydroxyalkanoate (PHA) and its homopolymer PHB offer favorable alternatives to petroleum-based plastics. They are thermoresistant, moldable, biocompatible, and biodegradable polyesters produced intracellularly by some plants and bacteria, and have been used in agriculture, aerospace, medicine, infrastructure, and electrical engineering (El-malek et al., 2020). Additionally, biological PHA/PHB production uses renewable resources (e.g.  $\text{CO}_2$ , sunlight). However, biological production remains economically infeasible, and some PHA-producing organisms rely on organic feedstocks, which interferes with the global food supply and is more energy-intensive.

Challenges facing biological PHA production include organism choice and feedstocks (e.g. carbon sources, culture medium). Known PHA producers include bacteria (Ranaivoarisoa et al., 2017; Sindhu et al., 2011) and plants (Dobrogojski et al., 2018). Metabolically plastic bacteria hold promise as PHA producers, though output efficiency remains insufficient (Chen, 2010; El-malek et al., 2020). MES technologies utilizing  $\text{CO}_2$ -fixing bacteria as a chassis hold great promise, with the goal of establishing a continuous bioprocess using cathodic biofilms driven by renewable electrical energy and  $\text{CO}_2$  (Connors & Bose, 2021; Connors et al., 2022). Recent MES studies have demonstrated that PHB synthesis in the phototroph *R. palustris* TIE-1 can be improved via cathode modifications to enhance EEU. In one study, cathodes coated with an immobilized iron-based redox mediator—Prussian Blue—led to 3.8-fold higher cathodic current uptake and 1.4-fold greater PHB production in TIE-1 (Rengasamy et al., 2018). A second study found that magnetite nanoparticle-anchored graphene cathodes led to 5-fold higher electron uptake and 4.2-fold higher PHB production compared to unmodified carbon felt cathodes

(Rengasamy et al., 2021). Similar efforts to improve PHB production have been explored in other organisms. In a recent study, *Cupriavidus necator* grown on an indium-nanoparticle electrode produced PHB at a rate of 25.2 mgL<sup>-1</sup> (Al Rowaihi et al., 2018). Beyond this, more than 300 different bacterial strains produce PHBs, though a small fraction of those have been used at the industrial scale (McAdam et al., 2020). While this suggests an abundance of microbial chassis, the type of strain can have a significant impact on the mechanical quality of the PHB (McAdam et al., 2020; Peña et al., 2014). Moreover, the specific parameters guiding any given production platform may require uniquely tailored PHB-producing strains. With this in mind, it will be important to both develop existing PHB producers and identify novel ones. Considering the vital role of biofilms in mediating these processes, special care should be paid to the biofilm-forming abilities of these strains.

## Biofuels

Similar to bioplastics, biofuels represent an opportunity to take advantage of organisms that fix carbon dioxide, thus mitigating carbon emissions. Biofuel production methods include cellulosic and noncellulosic biomass-consuming organisms, photosynthetic microbes, and electron-consuming microbes (Keasling et al., 2021). Heterologous pathways in non-model organisms hold great promise, as these organisms offer several advantages to model microbes, including higher tolerance for end products (Smith et al., 2010; Yang et al., 2016) and metabolic versatility (Bai et al., 2021; Yang et al., 2016). A recent study achieved *n*-butanol production in a *R. palustris* TIE-1 strain encoding the *n*-butanol biosynthesis pathway and lacking its nitrogen fixation pathway (Bai et al., 2021). Using a bioelectrochemical cell platform with solar panel-generated electrons and halogen light (representing the solar spectrum), a maximum *n*-butanol production rate of 0.91 mg L<sup>-1</sup> was achieved. Importantly, this study demonstrated a proof-of-concept for sustainable, carbon-neutral biofuel production. Other recent studies to generate potential fuel precursors using electroactive biocatalysts include ethanol production via activated sludge enriched from a wastewater treatment plant (Anwer et al., 2021) and butanol and hydrogen production by *Clostridium beijerinckii* (Zhang et al., 2021). Future efforts geared toward isolating and engineering microbial chassis that are tolerant of end products and other toxins and have improved biofuel production relative to wild-type strains are paramount. The primary barrier to all bio-commodity production, including biofuels, is cost; strains that are more efficient fuel producers that can withstand variable conditions are best suited to tackle these challenges.

## Conclusion

Numerous efforts to use microbes as the centerpiece of bioprocessing technologies have revealed the importance of biofilms. Among other characteristics, biofilms facilitate redox reactions and are resilient against otherwise harmful products. Our understanding of the mechanisms underlying these traits in the context of bioprocessing is ongoing. As described in this review, there is a need for improvements with respect to the biology (e.g. bioprospecting and genetic engineering) and bioreactor design (e.g. electrode modification) (Fig. 1). As we turn toward transitioning these applications from the lab into industry, it is essential to address the barriers described here and elsewhere (Conners & Bose, 2021; Conners et al., 2022; Jourdin & Burdyny, 2021; Karthikeyan et al., 2019; Lee et al., 2021).

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## Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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