

The Electron Transport Chain of *Shewanella oneidensis* MR-1 can Operate
Bidirectionally to Enable Microbial Electrosynthesis

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Running Head: Energetics of Electrosynthesis in *S. oneidensis*

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Abstract

Extracellular electron transfer (EET) is a process by which bacterial cells can exchange electrons with a redox active material located outside of the cell. In *Shewanella oneidensis*, this process is natively used to facilitate respiration using extracellular electron acceptors such as Fe(III) or an anode. Previously, it was demonstrated that this process can be used to drive microbial electrosynthesis of 2,3-butanediol (2,3-BDO) in *S. oneidensis* exogenously expressing butanediol dehydrogenase (Bdh). Electrons taken into the cell from a cathode are used to generate NADH, which in turn is used to reduce acetoin to 2,3-BDO via Bdh. However, generating NADH via electron uptake from a cathode is energetically unfavorable, so NADH dehydrogenases couple the reaction to proton motive force. We therefore need to maintain the proton gradient across the membrane to sustain NADH production. This work explores accomplishing this task by bidirectional electron transfer, where electrons provided by the cathode go to both NADH formation and O₂ reduction by oxidases. We show that oxidases use trace dissolved oxygen in a microaerobic bioelectrical chemical system (BES), and the translocation of protons across the membrane during O₂ reduction supports 2,3-BDO generation. Interestingly, this process is inhibited by high levels of dissolved oxygen in this system. In an aerated BES, O₂ molecules react with the strong reductant (cathode) to form reactive oxygen species, resulting in cell death.

Importance

Microbial electrosynthesis is increasingly employed for the generation of specialty chemicals such as biofuels, bioplastics, and cancer therapeutics. For these systems to be viable for industrial scale-up, it is important to understand the energetic

requirements of the bacteria to mitigate unnecessary costs. This work demonstrates sustained production of an industrially relevant chemical driven by a cathode. Additionally, it optimizes a previously published system by removing any requirement for phototrophic energy, thereby removing the additional cost of providing a light source. We also demonstrate the severe impact of oxygen intrusion into bioelectrochemical systems, offering insight to future researchers aiming to work in an anaerobic environment. These studies provide insight into both the thermodynamics of electrosynthesis and the importance of bioelectrochemical systems design.

Introduction

As reliance on fossil fuels becomes increasingly unsustainable from an economic and environmental perspective, researchers work toward alternative energy sources. One solution is microbial electrosynthesis, which is the microbially catalyzed transfer of electrons from an electrode to cells to drive a biochemical reduction reaction(1–3).

Microbial electrosynthesis can be catalyzed by electroactive bacteria capable of interfacing with an electrode surface in a BES or by using the electrode to generate electron carriers, such as H₂ or formate, that can be taken up by bacteria(4–6). When using electroactive bacteria, a potential is applied to the system to drive oxidation of an electron donor (typically H₂O) at the anode, and the electrons liberated are taken up by bacteria at the cathode surface. These electrons are used for the reduction of a feedstock, such as CO₂, to the desired product. When CO₂ is the reactant, microbial electrosynthesis becomes a carbon sink, acting as a carbon-neutral platform to produce biofuels, bioplastics, or specialty chemicals(7, 8). Because CO₂ is the ideal feedstock for microbial electrosynthesis, much of the existing research focused on bacterial strains or

communities with the capacity for autotrophic growth. However, these microbes can be inefficient due to slow growth rates and poor interaction with electrodes(9–11). We have chosen to focus on developing a microbial electrosynthesis platform using the well-understood electroactive bacterial chassis, *Shewanella oneidensis* MR-1. In this way, we can focus on understanding the energetics associated with inward electron transfer and optimizing product generation(12, 13). This will also contribute to understanding of respiration and basic physiology of this organism. Additionally, recent advances in engineering autotrophy raise the possibility of expanding the applications of microbial electrosynthesis beyond the need for native autotrophy or mixed microbial populations(14–17).

S. oneidensis MR-1 is a metal-reducing bacterium with a well-characterized extracellular electron transfer pathway using MtrCAB(12, 18–21). The Mtr pathway allows *S. oneidensis* to respire anaerobically using extracellular, insoluble electron acceptors such as Fe(III) oxides, Mn(IV) oxides, and electrodes(22). As with aerobic respiration, electrons are passed into the quinol pool (menaquinol and ubiquinol) by dehydrogenases in the electron transport chain that oxidize metabolites such as lactate, formate, and NADH. For extracellular electron transfer, the reduced quinones (quinols) are oxidized by the inner membrane bound cytochrome CymA(23–26). This protein acts as an electron hub, depositing electrons onto periplasmic electron carriers, such as fumarate reductase (FccA) and a small tetraheme cytochrome protein (CctA), to be shuttled onto terminal oxidoreductases. During respiration with an extracellular electron acceptor such as an anode, electrons are transferred to the Mtr pathway, a three-

protein complex that spans the outer membrane and extends into the extracellular space(27, 28).

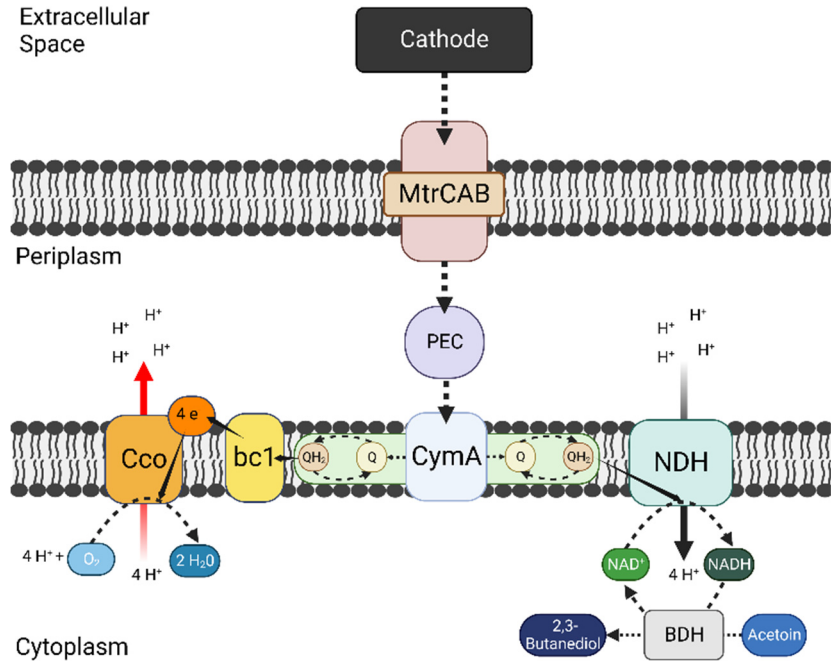


Figure 1 Overview of Bidirectional Electron Transfer to 2,3-BDO. *S. oneidensis* cells are incubated on a cathode poised at -500 mV_{Ag/AgCl}. Electrons are taken up by the cell via outer membrane MtrCAB pathway and passed to the inner membrane quinols via various periplasmic electron carriers (FccA, CctA) and CymA. Electrons from the quinol pool are used to reduce NAD⁺ to NADH, catalyzed by NADH dehydrogenases (NDHs) coupling the reaction to proton movement across the inner membrane. The electrode produced NADH is used to reduce exogenous acetoin to 2,3-BDO via butanediol dehydrogenase (BDH).

Importantly, the Mtr pathway is reversible, allowing inward electron transfer from a cathode into the cell(2, 27, 29–33). Electrons that are taken up via the Mtr pathway reduce respiratory quinones, and the cell can use the quinols as the electron donor for NAD⁺ reduction by reversing NADH dehydrogenases. NADH can be used to drive a wide variety of intracellular reduction reactions. As a proof-of-concept, we previously demonstrated that reducing power from the electrode can be used to drive the NADH-dependent reduction of acetoin to 2,3-BDO via the heterologous enzyme butanediol

dehydrogenase (Bdh) (**Figure 1**). By measuring the accumulation of 2,3-BDO in the system, we can assess the rate and efficiency of electron uptake(29, 34).

Inward electron transfer requires a continuous supply of electrons and an electron sink. In this system, acetoin is provided as the electron sink and electrons are supplied by a cathode poised at $-0.5 \text{ V}_{\text{Ag}/\text{AgCl}}$. At this electrode potential, electron transfer from the cathode to the MtrCAB complex is thermodynamically favorable. All subsequent reactions in the pathway from electrode to menaquinol are also freely reversible. However, there is a significant energy barrier for electron transfer from menaquinol ($-80 \text{ mV}_{\text{SHE}}$) to form NADH ($-320 \text{ mV}_{\text{SHE}}$) due to the large difference in reduction potential. To overcome the energy barrier, the reaction is catalyzed by ion-coupled NADH dehydrogenases working in reverse. *S. oneidensis* uses both H^+ -coupled and Na^+ -coupled NADH dehydrogenases. In the forward direction, these enzymes couple NAD^+ reduction to the movement of ions down the electrochemical ($\Delta\psi$) and proton or sodium gradient (ΔpH or $\Delta[\text{Na}^+]$) known as proton or sodium motive force (PMF or SMF) across the inner membrane(35). The movement of ions down these gradients into the cytoplasm provides the energy needed to power unfavorable chemical reactions; examples of this include formation of ATP via F_0F_1 -ATP synthase or, as in this case, NAD^+ reduction by NADH dehydrogenases. In nature, reverse NADH dehydrogenase activity is a means to prevent the potentially lethal overreduction of the quinol pool by generating NADH(26, 33, 35–37).

To enable electron uptake from the electrode, *S. oneidensis* cells are incubated in a BES in the absence of an organic substrate or native terminal electron acceptor. Under these conditions, the cells do not generate PMF via NADH dehydrogenases (Nuo

or Nqr) or succinate dehydrogenase (Sdh) because no substrate for these complexes is available(35, 36). Similarly, the absence of a terminal electron acceptor prevents forward electron transport chain flux. Because the reduction of NAD^+ to NADH requires the free energy provided by PMF, continuous PMF regeneration is necessary for the sustained production of 2,3-BDO. This concept is supported by the prior observation that addition of CCCP (carbonyl cyanide m-chlorophenyl hydrazone), an ionophore that dissipates PMF, results in the cessation of 2,3-BDO production(34). This result underscores an important consideration for microbial electrosynthesis design; how the cell will maintain PMF to continuously drive the reduction reaction forward. To maintain PMF in previous experiments, we introduced proteorhodopsin (PR). PR is a light-driven proton pump that moves protons against the proton gradient into the periplasm, sustaining PMF. Active PR and illumination resulted in an increase of both 2,3-BDO production and cathodic current(29). However, relying on PR as a source of PMF is not a viable solution for scale-up due to well-known issues with light penetration in industrial photobioreactors, and the additional energy cost associated with continuous illumination(29). Understanding this, we sought to utilize bidirectional electron transfer so electrons from the electrode are used for generating both NADH and PMF.

Oxygen typically is excluded from cathodic systems due to the risk of reactive oxygen species (ROS) forming. However, there is an increased interest in developing microbial electrosynthesis with oxygen, either to expand on potential products or new species as biocatalysts(38–40). Additionally, recent work suggests that oxic microbial electrosynthesis can be as efficient as gas fermentation or using photosynthetic organisms(41). For this work, oxygen in this system will instead be used to maintain

energy balance via bidirectional electron transfer. During this process, electrons taken up by the cell go towards both the generation of NADH and the reduction of a terminal electron acceptor, e.g., oxygen.

This coupling of electron uptake to oxygen reduction by terminal oxidases for PMF generation was first described in *S. oneidensis* by Rowe et al.(33). They found that under carbon-starvation and aerobic conditions, *S. oneidensis* on a cathode generated "non-growth-linked energy" in the form of PMF via terminal oxidase activity. Evidence indicated that PMF was used for production of ATP and reduced cytoplasmic electron carriers (FMNH₂, NAD(P)H). However, it is still unknown whether this process could continuously generate reducing power for use in MES as there was no sink for NADH. By implementing the Bdh-based system, we sought to determine if bidirectional electron transfer could sustain PMF generation to drive 2,3-BDO generation.

Results and Discussion

Eliminating electrode-independent 2,3-BDO Production

We previously demonstrated that the combination of an electrode and active PR led to higher levels of 2,3-BDO production than without either of these components. Before exploring the possibility of using bidirectional electron transfer instead of PR to drive PMF generation, we reexamined 2,3-BDO production in wild-type (WT) *S. oneidensis* MR-1 with and without active PR. Importantly, this experiment was done using an updated version of the previously described experiment protocol. In previous experiments, 35-50% of 2,3-BDO production was independent of the electrode, likely generated using NADH from organic carbon oxidation. To address the high background, we altered the protocol to promote residual organic carbon in the presence of an

electron acceptor (anode) to reduce the availability of alternative sources of NADH. Briefly, in the updated protocol *S. oneidensis* is pre-grown in minimal medium (M5) with 20 mM lactate aerobically (or anaerobically with 40 mM fumarate as described later) for 18 hours, followed by inoculation into the BES under aerobic and anodic conditions (+0.2 V_{Ag/AgCl}). After six hours, N₂ sparging is started to switch the cells from using oxygen to the anode as the electron acceptor. In the current study, this anaerobic, anodic phase continued for 40 hours (versus 18 hours in the previous protocol) before the potential is switched to cathodic (-0.5 V_{Ag/AgCl}). After this modification, we observed elimination of electrode-independent butanediol production (**Figure 2**, No Potential).

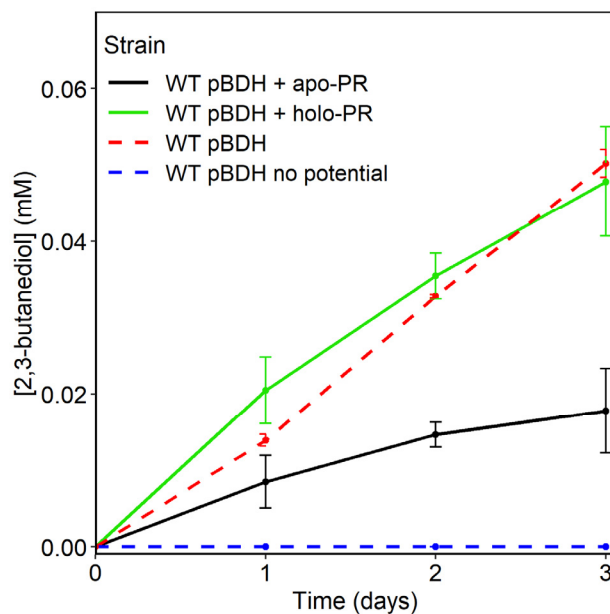


Figure 2 All 2,3-Butanediol Production is Electrode Dependent. Measurement of 2,3-butanediol in BES experiment with modified protocol. WT cells with pBDH or pBDH-PR with (holo-) or without (apo-) retinal as a cofactor, were pre-grown aerobically, washed, and inoculated into anodic BES (Table 1). After 40 hours potential was switched to cathodic, and acetoin was added to a final concentration of 1 mM (T=0). Samples were collected for HPLC analysis every 24 hours. Lines and error bars represent averages and standard error (n=3).

We tested the amended protocol using a strain expressing PR, with and without the essential cofactor all-*trans*-retinal. Cells with active PR (holo-PR) produced more 2,3-BDO than those with inactive PR (apo-PR). Interestingly, cells with active PR produced approximately the same amount of 2,3-BDO as cells not expressing PR, while cells with inactive PR showed a decrease in 2,3-BDO production (**Figure 2**). This finding suggests that when all 2,3-BDO production is electrode-dependent, PMF generation by PR supports an increase in 2,3-BDO production but the metabolic burden or membrane occupancy constraints of expressing PR outweigh the benefits. Moreover, this result suggests that there is an unaccounted-for source of PMF in the absence of PR. Another source of PMF appears more likely than the possibility that PMF is unnecessary, based on experimental evidence and thermodynamic calculations. Our prior work demonstrated that 2,3-BDO production is halted when PMF is dissipated by CCCP(34). Additionally, electron transfer from quinols to form NADH cannot occur at an appreciable rate without the energy gained from proton translocation across the membrane. To sustain the NADH dehydrogenase-catalyzed reaction, which utilizes PMF, there must be a mechanism to replenish the proton gradient. We considered formate dehydrogenase and F_oF₁ ATP synthase as possible PMF sources but found them unlikely due to the lack of a formate or ATP source after 40 hours in the BES with no carbon source. Therefore, we speculated that trace amounts of oxygen entering the BES could be sufficient to enable bidirectional electron transfer.

Bidirectional Electron Transfer to Oxygen and NAD⁺

We investigated the possibility of bidirectional electron transfer as the unknown source of PMF because this reaction could be powered by the electrode, and the

substrate (O_2) is readily accessible. Although the working chamber was continuously degassed by N_2 bubbling (99.999% N_2), we suspected that the environment was microaerobic. The BESs may not be completely airtight due to the use of neoprene tubing and plastic connectors, and the possibility of oxygen diffusion from the anode through the ion exchange membrane (**Supp. Figure S1**). Additionally, the N_2 tank used can contain up to 1 ppm O_2 contamination, per the product specifications (Airgas). To ascertain if oxygen was present, we inserted an optical dissolved oxygen (DO) probe into the BES and conducted an experiment as normal. The DO in the working chamber was at ~100% saturation before inoculation, decreased to ~60% saturation upon cell addition, and dropped to ~1% upon N_2 bubbling (**Figure 3A**). This single experiment produced 0.046 mM 2,3-BDO over 3 days, which is consistent with previous experiments (Figure 2.3C).

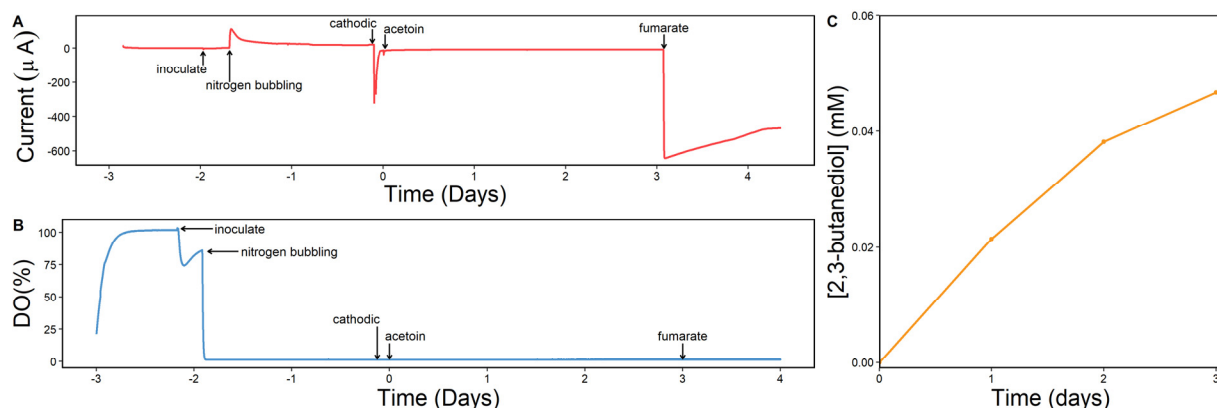


Figure 3 Presence of Oxygen in BES. Time course of the experiment from setup (Day -3) to final timepoint (Day 4), showing the current (A) and dissolved oxygen (B). Samples were taken daily from Day 0 to Day 3 to quantify 2,3-BDO production (C) (n=1).

Production of one 2,3-BDO molecule from acetoin requires oxidation of one NADH, which in turn depletes four H^+ from available PMF via Nuo(35, 37). One molecule of oxygen (O_2) allows translocation of four H^+ across the membrane if it is

reduced by either of the proton-pumping terminal oxidases, Cco and Cox(42–44). Therefore, the reduction of one O₂ molecule can sustain production of one molecule of 2,3-BDO from the perspective of PMF balance. The 1% saturation DO concentration observed is equivalent to 0.073 mg/L (30°C, 856' elevation), or ~2 μM of oxygen available throughout the experiment(45). This concentration of DO is more than enough oxygen available to support 0.046 mM 2,3-BDO production over 72 hours (0.638 μM 2,3-BDO/hour) as the sole source of PMF.

To verify the contribution of terminal oxidases in generating PMF during electron uptake, we compared current and 2,3-BDO production between WT MR-1 and a strain lacking all 3 terminal oxidases ($\Delta cyd\Delta cco\Delta cox$, here named $\Delta oxidase$)(33). This strain cannot use O₂ as a terminal electron acceptor meaning that even with trace amounts of oxygen, the mutant cells would not be able to use it.(46) This mutant cannot grow in aerobic conditions, so further comparisons were performed using anaerobic pre-growth of all strains. To ensure consistency, we compared anaerobic growth of WT versus $\Delta oxidase$ cells in M5 minimal medium (20 mM lactate, 40 mM fumarate). We observed similar growth rates (**Supp. Figure S2**), so for MES experiments, these strains were pre-grown anaerobically. WT cells pre-grown in an anoxic environment produced 0.047 ± 0.002 mM butanediol by day 3, consistent with previous work, and exhibited similar current profiles and magnitudes (**Figure 4**). Conversely, the $\Delta oxidase$ strain produced minimal butanediol, with only a small amount accumulating by day 4, and less than half the current of WT cells. This result highlights that in the absence of aerobic terminal oxidases, cells cannot maintain electrode-dependent acetoin reduction as effectively as WT, leading to an 82.8% decrease in production. We hypothesize that the $\Delta oxidase$

pBDH strain's production of a limited amount of 2,3-BDO can be attributed to the NADH generated through the oxidation of organic carbon arising from cell death.

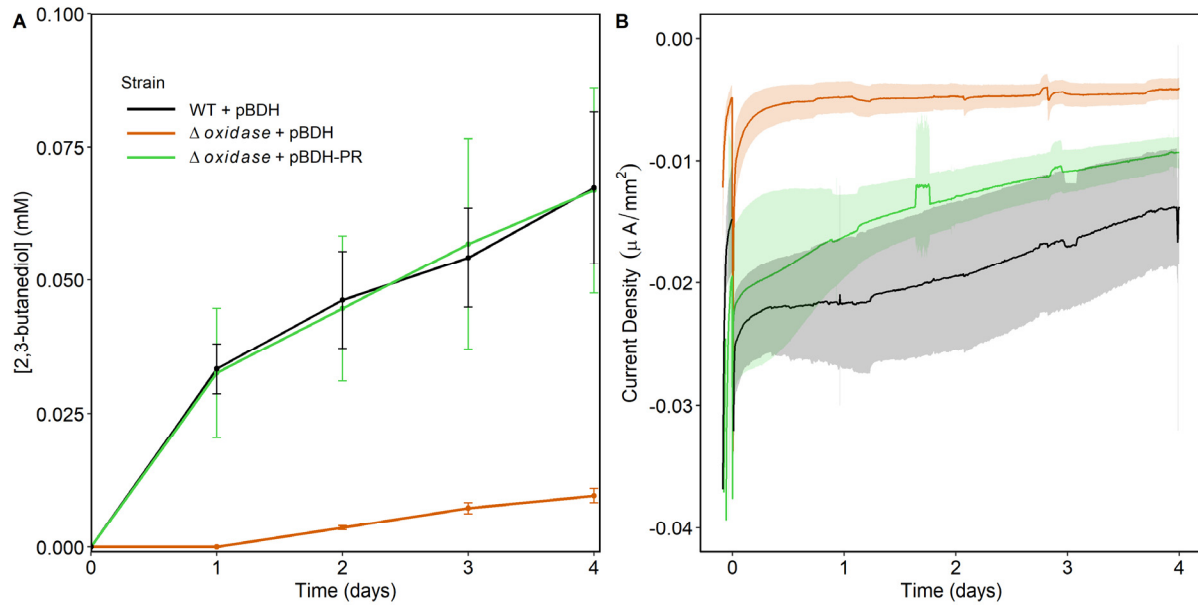


Figure 4 2,3-BDO and Current Production in Microaerobic BES. (A) 2,3-Butanediol accumulation in BES with WT pBDH, $\Delta oxidase$ pBDH, and $\Delta oxidase$ pBDH-PR with nitrogen bubbling. (B) Cathodic current from BES. Points (A) and lines (B) represent averages with standard error bars, n=3.

To confirm that the observed phenotype was due to the loss of PMF from proton-pumping oxidase activity, the $\Delta oxidase$ strain was functionally complemented by PR expression. If 2,3-BDO production is rescued by PR, it indicates that the loss of 2,3-BDO generation in $\Delta oxidase$ is caused by a loss of PMF as opposed to off target effects, such as changes in gene regulation. When PR was expressed in $\Delta oxidase$, we observed a restoration of 2,3-BDO production and partial rescue of current (**Figure 4**). In this instance, electron transfer to form NADH but not O_2 is restored, and as one O_2 is required to produce one 2,3-BDO molecule, a 50% rescue of current is consistent with our model. This is supported by the calculated coulombic efficiency for each strain (WT pBDH=14.3%, $\Delta oxidase$ pBDH=8.31%, $\Delta oxidase$ pBDH-PR = 21.7%, Supp. Figure S4).

Taken together, these results support the hypothesis that PMF is a limiting resource, and the proton pumping activity of oxidases in this microaerobic environment is essential to continuous electron transfer to form NADH.

Reactive Oxygen Species Formation

We next investigated whether increasing DO in the BES would result in an increase in 2,3-BDO. To do this, BESs were not sparged with N₂ to allow passive aeration. DO measurements indicated a highly oxygenated environment (300 μ M) in the BES (**Supp. Figure S3**). This condition resulted in a severe decrease in 2,3-BDO production relative to the N₂-bubbling microaerobic condition (**Figure 5**). Current was greatly inflated by O₂ intrusion (data not shown).

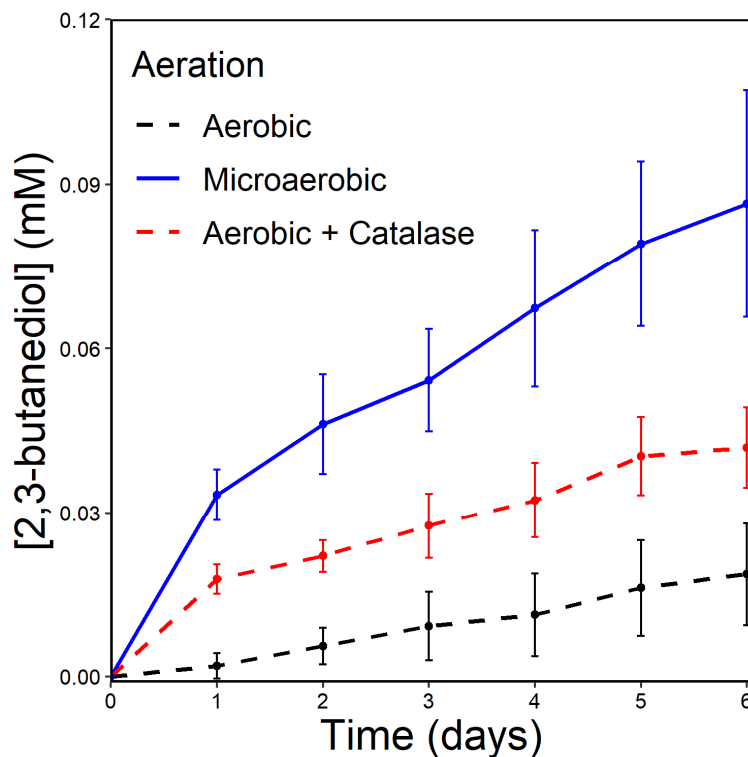


Figure 5 2,3-BDO Production in Aerobic and Microaerobic BES. (A) 2,3-Butanediol accumulation in BES with WT pBDH with N₂ bubbling (Microaerobic), and passive aeration (Aerobic) with or without the addition of 0.3 U/mL catalase. Points represent averages with standard error bars, n=3.

289 The failure of increased DO to translate to an increase in 2,3-BDO accumulation
290 could be attributed to three factors: decreased *mtrCAB* expression, formation of ROS,
291 or a shift in electron flow to favor oxygen reduction over NADH generation. In the
292 presence of oxygen, *S. oneidensis* MR-1 decreases expression of anaerobic respiration
293 pathways such as Mtr in favor of aerobic respiration and a decrease in Mtr expression
294 will likely result in a decrease in inward ET(47). However, the cells are not actively
295 growing under the experimental conditions (e.g., no carbon source), and it is improbable
296 that a significant shift in the proteome occurred under the carbon starvation conditions
297 of the experiment. Similarly, while loss of all electron flux in favor of oxygen reduction is
298 possible, the small amount of 2,3-BDO produced during passive aeration suggests
299 there are still electrons going towards NADH formation. Additionally, it has been shown
300 that bidirectional electron transfer to NAD⁺ and oxygen occurs under active aeration; if
301 all electrons were being lost to oxygen, the effect would likely have been more
302 pronounced under those conditions(33). Further research done to optimize DO
303 concentration should explore this possibility.

304 To assess the possibility of ROS formation, we measured hydrogen peroxide
305 (H₂O₂) in the BESs. In the presence of oxygen and a strong reductant, such as a
306 cathode or reduced flavin, O₂ can be reduced to form H₂O₂(47–50). H₂O₂ accumulation
307 in the BESs may result in cell death and a decrease in the ability to produce 2,3-BDO.
308 H₂O₂ can also react directly with 2,3-BDO, possibly leading to reduced accumulation
309 because of abiotic degradation(51, 52). To investigate if ROS accumulated in the
310 passive aerobic condition, experiments with WT pBDH were performed with and without
311 N₂ bubbling, with and without potential, and samples were taken for colony forming unit

(CFU) and H₂O₂ measurements. When the potential was swapped from anodic to cathodic, we observed an immediate drop in CFUs/mL and generation of H₂O₂ in BES with passive aeration, while the microaerobic BES maintained the same levels of both (**Figure 6**). There was no detectable peroxide formation in the no potential controls (data not shown). This result demonstrates that the formation of H₂O₂ is dependent on the presence of oxygen and a cathode. The formation of H₂O₂ was correlated with ~2.5 log₁₀ cell death in the first 3 hours. Notably, the initial concentration of H₂O₂ generated after the change in potential was not maintained over time in the aerobic BES. Additionally, the concentration of H₂O₂ that was generated in the working chamber (20 µM) killed a large portion of cells within the first hour, but not all. We hypothesize that this is due to a higher local concentration of oxygen on the surface on the carbon felt electrode; after the initial reaction with the electrode, the oxygen levels remain low and rate of cell death decreases. Cell attachment to the electrode surface, which promotes the removal of oxygen through both its consumption and physical exclusion, is low(53). Therefore, one potential future direction is to increase biofilm coverage on the electrode surface to further decrease the local O₂ concentration and decrease ROS formation.

We next explored whether addition of catalase (an H₂O₂ degrading enzyme) could reduce H₂O₂ accumulation. This approach has the potential to harness the benefits of oxygen inclusion, such as PMF generation, while minimizing the production of harmful by-products(47). Aerobic BESs were run with the addition of 0.3 U/mL catalase added immediately before the potential was switched to -0.5 V_{Ag/AgCl}. Aerobic BESs with catalase did not result in the same rapid decrease in CFUs/mL or increase in H₂O₂ as those without, as well as a less prominent spike in peroxide formation (**Figure**

6). Catalase addition also resulted in a partial rescue of 2,3-BDO production (Figure 5).

These results show that one of the challenges with oxygen inclusion in a BES is the cytotoxic formation of H_2O_2 , resulting in cell death and decrease in product yield.

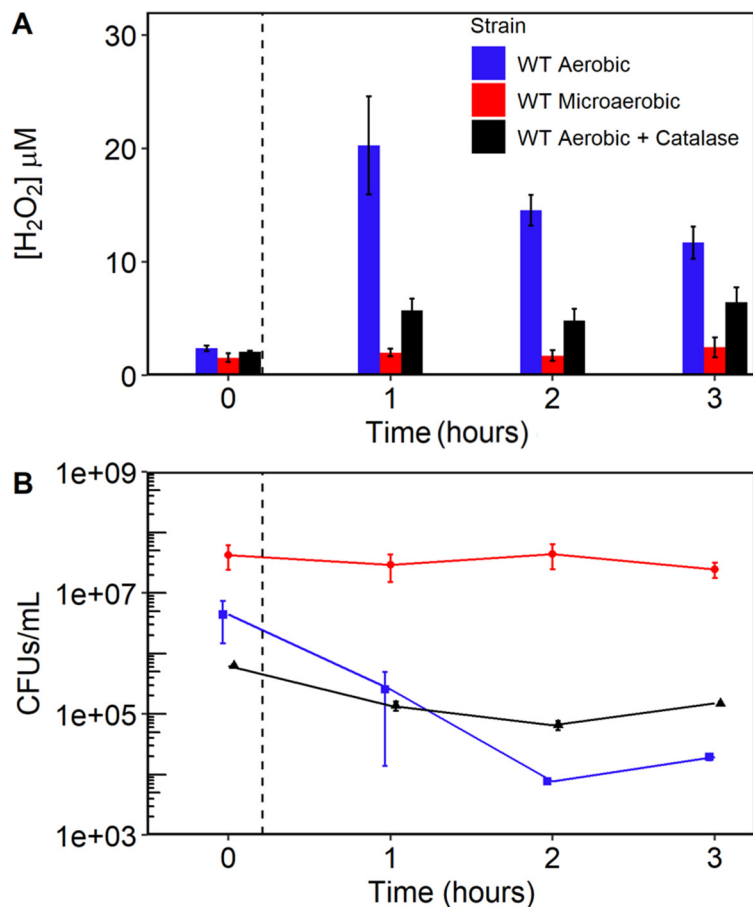


Figure 6 CFUs and $[H_2O_2]$ in Aerobic and Microaerobic BES. (A) CFUs/mL and (B) $[H_2O_2]$ μM in bulk medium of working chamber. Dashed line represents the potential change from 0.2 $V_{Ag/AgCl}$ to -0.5 $V_{Ag/AgCl}$. Points represent averages of $n=3$ with standard error bars. Lines are included in CFU/mL data to guide the eye. CFUs/mL at inoculation (~46 hrs. prior to $T=0$) for all conditions were $\sim 1.8 \times 10^8$.

Conclusion

Effective microbial electrosynthesis requires attention to detail in both BES design and bacterial physiology. In the system discussed here, understanding the thermodynamic factors involved in driving inward electron transfer is crucial. The

348 reversible nature of the electron transport pathways, which enables cells to use
349 electrodes as electron acceptors and donors, depends on the reduction potential of
350 each step(23, 54–58). Electron transfer reactions from electrode to quinol pool are
351 freely reversible, but the final transfer from menaquinol to NADH formation has a much
352 larger shift in potential between donor (-80 mV) and acceptor (-330 mV). This barrier is
353 overcome by NADH dehydrogenases catalyzing the reaction and coupling the reduction
354 to PMF utilization. In this work, we show that during electron transfer from an electrode
355 to NADH, PMF can be regenerated by bidirectional electron transfer. Importantly, *S.*
356 *oneidensis*' native aerobic terminal oxidases (Cco, Cox, Cyd) can sustain PMF via
357 oxygen reduction without fully redirecting the flow of electrons away from NADH. This
358 ability was best demonstrated in microaerobic conditions, where the DO concentration
359 struck the balance between electron flow to oxygen and NAD⁺. Higher levels of oxygen
360 had the off-target effect of generating H₂O₂ that resulted in cell death. While the
361 conditions tested here were limited to microaerobic and passively aerobic, future work
362 should focus on fine tuning the DO in BES to fully restore 2,3-BDO production in
363 aerated BES. This could be done through a combination of oxygen scavengers, gas
364 mixing/modulating inflow, higher concentrations of catalase and inclusion of other ROS
365 neutralizing enzymes such as superoxide dismutase, or selective deletion of native
366 oxidases as they have varying oxygen affinities and proton pumping efficiencies. The
367 goal should be to balance the redox state of the quinone pool to maximize the flow of
368 electrons 'uphill' to NAD⁺ relative to the energetically favorable reduction of oxygen.
369 Taken together, this work shows the strong influence even trace oxygen has on the
370 energetics of inward electron transport.

Materials and Methods

Strain or Plasmid	Description	Source
<i>S. oneidensis</i>		
MR-1	Wild type <i>S. oneidensis</i>	Meyers and Nealson, 1988
Δ oxidase	Mutant with gene deletion of <i>cco</i> , <i>cyd</i> , <i>cox</i> , (SO2361–SO2364, SO3285–SO3286, SO4606–SO4609)	Rowe et al. 2018
Plasmids		
pBDH	pBBR1MCS2 bearing butanediol dehydrogenase gene from <i>Enterobacter cloacae</i> , kan ^R	Tefft and TerAvest, 2019
pBDH-PR	pBBR1MCS2 bearing butanediol dehydrogenase from <i>Enterobacter cloacae</i> and proteorhodopsin (uncultured marine gamma proteobacterium EBAC31A08), kan ^R	Tefft and TerAvest, 2019

Strains and Plasmids

Strains and plasmids used are listed in Table 2.1. *S. oneidensis* MR-1 strains were grown at 30 °C and shaking at 275 rpm for aerobic growth, and no shaking for anaerobic growth (~5% H₂, balanced with N₂). For BES experiments, MR-1 was pre-grown aerobically in 5 mL of lysogeny broth (LB) supplemented with 50 µg/mL kanamycin for strains with pBBR1-BDH, for inoculating minimal medium. For pre-growth, cells were grown in M5 minimal medium containing: 1.29 mM K₂HPO₄, 1.65 mM KH₂PO₄, 7.87 mM NaCl, 1.70 mM NH₄SO₄, 475 µM MgSO₄·7 H₂O, 10 mM HEPES, 0.01% (w/v) casamino acids, 1× Wolfe's vitamin solution, and 1× Wolfe's mineral solution, then the pH adjusted to 7.2 with 5 M NaOH. After autoclaving, D,L-lactate was added to a final concentration of 20 mM. During anaerobic pre-growth, fumarate was added to a final concentration of 40 mM and 400 mL of medium was used per repeat.

During bioelectrochemical experiments, the M5 medium recipe was amended to 100 mM HEPES, 0.2 μ M riboflavin, and no D,L-lactate, fumarate, or casamino acids.

Growth Curves

For anaerobic growth experiments, cells were pre-grown in 5 mL LB supplemented with 40 mM fumarate and 20 mM D,L-lactate. Cells from the overnight culture were washed with M5 medium and resuspended to an OD₆₀₀ of 0.05 in 2 mL M5 medium in a 24-well plate. OD₆₀₀ was measured every 15 minutes for 35 hours in an anaerobic plate reader (BioTek, HTX). This protocol was repeated 3 times for replication.

Bioelectrochemical System Experiments

BES experiments were conducted in custom made two-chamber bioreactors kept at 30 °C as described in previous work (Tefft and TerAvest 2019)(29), and a similar set up to work described in (Tefft et al. 2022)(30). The working chamber was filled with 144 mL amended M5 medium, with 0.2 μ M riboflavin being added an hour before inoculation, and the counter chamber contained ~150 mL of 1x PBS. For experiments run with PR, green LED lights were attached to the reactors. Bioreactors were autoclaved for 45 minutes, then connected to a potentiostat (VMP, BioLogic USA) and current data was collected every 1 s for the course of the experiment. After the initial setup, the working electrode poised at an anodic potential of +0.2 V_{Ag/AgCl} for ~16 hours. For aerobic pre-growth experiments, cells were grown in two 50-mL cultures of M5 in 250-mL flasks for each bioreactor (6 total for 3 replicates) for 18 hours. For anaerobic pre-growth experiments, cells were grown in 400-mL cultures of M5 in 1-L flasks for each bioreactor (3 total for 3 replicates) for 18 hours. For experiments with PR, 400 μ L 20 mM all-*trans*-retinal was added after 17 hours of growth as the essential cofactor for PR. Cultures

were transferred to a 50-mL conical tube and centrifuged at 8000 rpm (Thermo Scientific ST8R; Rotor: 75005709) for 5 minutes. Pellets were washed twice in 30 mL M5 (100 mM HEPES, no carbon) and then resuspended in M5 (100 mM HEPES, no carbon), to a final OD₆₀₀ of 3.6 in 10 mL. Then, 9 mL of this normalized resuspension was inoculated into the working chamber of the bioreactor using a sterile 10 mL syringe with an 18 g needle. Six hours after inoculation, N₂ gas (99.999%, AirGas) was bubbled into reactors through a 0.2 µM filter, and a bubbler attached to a 0.2 µM filter connected to the gas outlet. For 40 hours after N₂ bubbling, reactors were maintained at an anodic potential of +0.2 V_{Ag/AgCl}, before being changed to a cathodic potential of -0.5 V_{Ag/AgCl}. After three hours at cathodic potential, 17 mL of a sterile, de-gassed 10 mM acetoin solution was added to a final concentration of 1 mM in the bioreactor (Final volume in working chamber = 170 mL). The bioreactors were sampled (2 mL) immediately after acetoin addition for OD₆₀₀ and HPLC analysis every 24 hours for 144 hours.

DO Measurements

DO measurements shown in Figure 3 were collected using a Hamilton VisiFerm DO sensor and ArcAir Software. The probe was calibrated before each experiment as described in the manual. The probe was inserted into the BES prior to autoclaving and secured with a rubber gasket. DO measurements were recorded every 5 s during the experiment. To ensure that the inclusion of the DO probe did not interfere with oxygen intrusion into the system, we also utilized a smaller fiber optic DO probe and collected data every 30 s using a NeoFox Fluorimeter and Software (Ocean Insight). The probe consists of a patch made from 5% mixture of polymer (poly(2,2,2-trifluoroethyl methacrylate), Scientific Polymer Products Inc.) and 5 mM porphyrin (Pt(II) meso-

tetra(pentafluorophenyl)porphine, Frontier Scientific) dissolved in a 50/50 mixture of 1,4-dioxane and 1,2-dichloroethane (Sigma Aldrich). This patch is deposited onto the end of a fiber optic probe(59). Results from this probe corroborated observations made with the Hamilton Probe (Supp. Figure 2.3A). Data from passively aerobic BES (Supp. Figure 3B) was collected using the smaller fiber optic probe.

CFU Plating

During BES experiments, samples were taken every ~24 hours starting at inoculation, with additional time points in the three hours following potential change from anodic to cathodic. These samples were used for CFU plating, H₂O₂ measurements, and HPLC analysis. Samples were serially diluted in a 96-well plate and 10 µL of each of 8 dilutions (10⁰-10⁻⁷) was plated on LB + Kan. Dilutions with between ~10¹-10² CFUs were counted and back calculated to determine CFUs/mL in bulk solution. Mean and standard error were calculated for biological replicates (n=3).

H₂O₂ Measurements

At each sampled time point, H₂O₂ formation was measured using the Pierce™ Quantitative Peroxide Assay Kit (ThermoFisher, Cat: 23280) according to the kit instructions. In brief, 20 µL of sample was mixed with 200 µL of reagent mixture in a 96-well plate, and absorbance was read at 595 nm. Sample values were compared to a standard curve with background subtraction of cell-only controls in 1xPBS to exclude any interference from cell OD₆₀₀. Mean and standard error were calculated for biological replicates (n=3).

HPLC analysis

HPLC analysis was performed as previously described (Tefft and TerAvest, 2019) with the amendments described in (Tefft et al., 2022)(29, 30). Sample analysis was performed on a Shimadzu 20A HPLC, using an Aminex HPX-87H (BioRad, Hercules, CA) column with a Microguard Cation H⁺ guard column (BioRad, Hercules, CA) at 65 °C with a 0.5 ml/min flow rate. 2,3-butanediol concentration in samples was calculated by comparing sample value to an external standard curve.

Coulombic Efficiency Calculation

Coulombic efficiency was calculated by dividing the moles of 2,3-BDO produced by the moles of electrons measured by the current and multiplying by 100 to get a percentage.

$$CE \% = \frac{(\text{BDO}_{[\text{final}]}) \times 0.17 \text{ L}}{Q \times \frac{1 \text{ M}}{96,485 \text{ C}}} \times 100 = \frac{\text{moles BDO}}{\text{moles e}^-} \times 100$$

Data analysis

Analysis of HPLC data, DO %, OD, current data, and growth curve data was done using RStudio using the following packages: ggplot2, dplyr, ggpubr, plyr, data.table, stringr, and growthcurver(60–66).

Author Contributions

K.C.F. and M.T. conceptualized the project. K.C.F. lead the investigation and data visualization under the supervision of M.T. K.C.F. wrote the original draft of the manuscript, with review and edits by M.T.

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