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The effect of pH and buffer concentration on anode biofilms of *Thermincola ferriacetica*

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ABSTRACT

We assessed the effects of pH and buffer concentration on current production and growth of biofilms of *Thermincola ferriacetica* – a thermophilic, Gram-positive, anode-respiring bacterium (ARB) – grown on anodes poised at a potential of −0.06 V vs. SHE in microbial electrolysis cells (MECs) at 60 °C. *T. ferriacetica* generated current in the pH range of 5.2 to 8.3 with acetate as the electron donor and 50 mM bicarbonate buffer. Maximum current density was reduced by ~80% at pH 5.2 and ~14% at 7.0 compared to pH 8.3. Increasing bicarbonate buffer concentrations from 10 mM to 100 mM resulted in an increase in the current density by 40 ± 6%, from 6.8 ± 1.1 to 11.2 ± 2.7 A m^{-2}, supporting that more buffer alleviated pH depression within *T. ferriacetica* biofilms. Confocal laser scanning microscopy (CLSM) images indicated that higher bicarbonate buffer concentrations resulted in larger live biofilm thicknesses: from 68 ± 20 μm at 10 mM bicarbonate to >150 μm at 100 mM, supporting that buffer availability was a strong influence on biofilm thickness. In comparison to mesophilic *Geobacter sulfurreducens* biofilms, the faster transport rates at higher temperature and the ability to grow at relatively lower pH allowed *T. ferriacetica* to produce higher current densities with lower buffer concentrations.

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1. Introduction

The factors that limit the rate of microbial respiration on the anode of microbial electrochemical cells (MxCs) have been studied extensively with Gram-negative anode-respiring bacteria (ARB), including thermophilic biofilms of *Calditerrivibrio nitroreducens* [1] and mesophilic biofilms of *Geobacter sulfurreducens* [2–7], *Shewanella oneidensis* [8,9], and mixed communities [10–14]. In addition, although Gram-positive, thermophilic ARB – including *Brevibacillus* sp. PTH1 [15], *Thermincola potens* strain JR [16], *Thermincola ferriacetica* [17], and *Thermoanaerobacter pseudethanolicus* [18] – have previously been identified and characterized, the limitations for these ARB have not been studied in detail. Broadening understanding for thermophilic ARB can reveal the differences in kinetic limitations encountered in thermophilic Gram-positive biofilms versus their mesophilic Gram-negative counterparts [19], possibly enhancing the feasibility of using MxC technology to produce valuable products from diverse waste streams [20–24]. In this study, our aim was to determine if proton (H^+) transport was important in biofilms of the Gram-positive, thermophilic ARB *T. ferriacetica*, a previously characterized [25] and genetically sequenced [26] microorganism that has been reported to perform anode respiration using a non-shuttling, direct long-range extracellular electron transfer (EET) mechanism [17,27].

As ARB biofilms grow and mature, bacteria accumulate in biofilms with thicknesses up to >100 μm [5,6,17,18]. As bacteria in the biofilm consume the electron donor, they produce electrons, which result in current production, and H^+, which need to diffuse out of the biofilm into the bulk solution [13]. In mature biofilms, the accumulation of active bacteria producing H^+ can create a pH gradient, and low pH in the interior of the biofilm can become a major limiting factor for biofilm growth and current production by ARB [4,11,13,14]. Adding a buffer aids the transport of H^+ out of the anode biofilm and is a means to alleviate the pH gradient and low-pH inhibition [11,13,14]. For example, H^+ can be carried out of the biofilm bound to carbonic acid (H_2CO_3) formed with bicarbonate (HCO_3^-) that is transported as:

$$HCO_3^- + H^+ \leftrightarrow H_2CO_3 \quad (1)$$

H^+ transport on H_2CO_3 is much faster than transport of H^+ alone due to the large concentration difference at neutral pH, which is required for growth of most known ARB that produce high current densities [11,13,14].

Previous investigations implied that pH gradients limit biofilm growth and play a major role in the energetics of anode respiration by ARB [13,14,28,29]. However, all previous studies analyzing low-pH inhibition in anode biofilms have been performed with mesophilic Gram-

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negative bacteria. These studies confirmed that current density by ARB increases in a semi-linear manner with increasing buffer concentrations [11,13]. For example, current densities were 4-fold higher at 100 mM bicarbonate compared to 10 mM bicarbonate in mesophilic MxCs [11,13]. Here, we investigate the role bicarbonate buffer plays in influencing current production in thermophilic MxCs, since, based on the Einstein-Stokes equation [30], the rate of buffer transport via diffusion will be ~2-fold higher at 60 °C compared to 30 °C. Correspondingly, previous studies have indicated that *T. ferriacetica* biofilms produce higher current densities at lower buffer concentrations when compared to mesophilic anode biofilms composed of mixed or pure cultures [13,17].

For this study, we used chronoamperometry (CA) with thermophilic biofilms of *T. ferriacetica* grown in microbial electrolysis cells (MECs) to assess the limitations in current production caused by low-pH inhibition resulting from H^+ transport and buffer diffusion limitations in the biofilms. First, we adjusted pH with the addition of either NaOH or HCl to evaluate its effect on current production (j). Second, we analyzed the effect of bicarbonate buffer concentration (10, 25, 50, and 100 mM) on current production and the corresponding biofilm thickness (L_f). Lastly, we confirmed that the ionic conductivity and electron donor (acetate) concentration of the growth media were not limiting under the conditions used and, thus, had minimal impact on current production. This study is the first to analyze H^+ transport limitations for anode respiration in biofilms of thermophilic Gram-positive ARB, and it reveals that *T. ferriacetica* biofilms are not as limited by buffer concentration and transport of H^+ as mesophilic biofilms under low buffer and low pH conditions.

2. Materials and methods

2.1. Growth media and culture conditions

We used a modified DSMZ Medium 962: *Thermovenabulum* medium to grow *T. ferriacetica* strain 14005 (DSMZ, Braunschweig, Germany). The modified medium contained: $NaAc \cdot 3H_2O$ (3.4 g l^{-1}), NH_4Cl (0.33 g l^{-1}), K_2HPO_4 (0.33 g l^{-1}), $MgCl_2 \cdot 6H_2O$ (0.33 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (0.1 g l^{-1}), KCl (0.33 g l^{-1}), yeast extract (0.05 g l^{-1}), 1 ml selenite–tungstate stock solution (prepared by dissolving 3 mg $Na_2SeO_3 \cdot 5H_2O$, 4 mg $Na_2WO_4 \cdot 2H_2O$, and 0.5 g NaOH in 1 l distilled water), ATCC vitamin solution (10 ml l^{-1}), and trace elements solution (10 ml l^{-1}). The trace elements solution was composed of the following ingredients in 1 l deionized water: 1.5 g nitrilotriacetic acid, 3 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.18 g $COSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.18 g $ZnSO_4 \cdot 7H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.02 g $KAl(SO_4)_2 \cdot 12H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.03 g $NiCl_2 \cdot 6H_2O$, and 0.3 mg $Na_2SeO_3 \cdot 5H_2O$. We prepared media in a condenser apparatus under N_2:CO_2 (80:20) gas conditions. Media was brought to boil and allowed to boil for 15 min per liter. We stored media in 100-ml serum bottles and autoclaved the media in the bottles for 15 min at 121 °C. We added ATCC Vitamin Solution and Na_2CO_3 added after autoclaving; Na_2CO_3 concentrations were adjusted for each experimental condition. Initial *T. ferriacetica* stock cultures were grown in 100 ml serum bottles containing 10 mM $Fe(OH)_3$ on an Excella E24 Incubator Shaker (New Brunswick Scientific) at 60 °C and 150 RPM. Subsequent cultures were inoculated using culture grown in the laboratory.

2.2. H-type MEC construction

We used H-type MECs for all experiments. Prior to construction, all glassware was cleaned and sterilized. First, glassware was soaked overnight in a 10% nitric acid bath and then rinsed with DI water. Then, glassware was stored in a 200 °C oven overnight. Reactor materials including anode, cathode, gaskets, tubing, and clamps were autoclaved for 30 min at 121 °C. Reactor materials and glassware were then transferred to a clean-hood and placed under UV sterilization for 30 min. After UV sterilization, reference electrodes and anion exchange membranes were introduced to the clean hood and applied with a 70% ethanol solution. MECs were then constructed in the clean hood.

Each MEC consisted of two 350-ml compartments separated by an anion exchange membrane (AMI 7001, Membranes International, Glen Rock, NJ). For all MECs, the operating temperature was 60 °C. All MECs contained two cylindrical graphite anodes with varying surface areas and an Ag/AgCl reference electrode (BASi MF-2052). Reference potential conversion to a standard hydrogen electrode (SHE) was conducted by constructing a two chambered cell with one chamber containing modified DSMZ Medium 962 and the other containing 1 M KCl [17,31]. Anode potential was poised at −0.06 V vs. SHE and the current continuously monitored every 2 min using a potentiostat (Bio-Logic, Model VMP3, Oak Ridge, TN). For all MECs, the anode chambers were mixed via agitation with a magnetic stir bar. The cathode consisted of a single cylindrical graphite rod (0.3 cm diameter and a total area of 6.67 cm^2). Cathode was filled with 100 mM NaOH and pH was 13. Gas collection bags were placed on the anode compartments to collect volatile products (CO_2), and on the cathode to collect hydrogen [17,18].

2.3. Experiments to determine effect of pH on current density

To determine the effect of pH on the current density (j), we used two replicate MECs operated in batch mode with 50 mM bicarbonate buffer and 25 mM acetate as the electron donor. Each MEC had an anode surface area of 3.89 cm^2. MECs were inoculated with 3 ml *T. ferriacetica* stock cultures and allowed to establish mature biofilms. After a sustained current was observed, we altered the pH by the addition of HCl or NaOH. Results were also used to obtain a pH range for current production for *T. ferriacetica* when used during operation in MECs. Low Scan Cyclic Voltammetry (LSCV) analysis (1 mV s^{-1}) was conducted at low pH (5.2), neutral pH (6.9), and high pH (8.3) conditions, and the scans are included in Supplementary Information as Fig. S1.

2.4. Experiments to determine the effect of bicarbonate buffer concentrations on current density

To determine the effect of bicarbonate buffer concentrations on j, we used four replicate MECs with varying bicarbonate concentrations of 10, 25, 50, or 100 mM, and 25 mM acetate as the electron donor. The anode surface areas for each MEC were: 6.78, 3.26, 3.02, and 3.64 cm^2 respectively. First, MECs containing medium with 10-mM bicarbonate were inoculated with 3 ml *T. ferriacetica* stock culture and allowed to establish mature biofilms. Then, we continuously added new medium containing increasing buffer concentrations into each MEC at a rate of ~4.5 ml s^{-1} until the old medium was completely replaced (~1.3 min hydraulic retention time). After the new medium was added, MECs were switched into batch mode until a sustained current was observed. Once a sustained current was observed, the medium was replaced by continuously flowing in 1 l containing an increased bicarbonate concentration.

2.5. Experiments to determine the effect of live L_f on current density

To determine L_f as a function of the bicarbonate buffer concentration, two MEC biofilms were analyzed. The anode surface areas for each MEC were: 3.02, and 3.64 cm^2 respectively. First, the MECs were inoculated with 3 ml *T. ferriacetica* stock culture and allowed to establish mature biofilms at 10 mM bicarbonate. Then, bicarbonate was added sequentially at increasing bicarbonate concentrations (10 mM, then 25 mM, then 50 mM, then 100 mM) as in the previous section. After the MECs had achieved a steady current density at 100 mM bicarbonate, where they achieved maximum biofilm thickness, they were then fed with media containing sequentially decreasing bicarbonate buffer concentrations (50 mM, then 25 mM, then 10 mM). Current density results from the increasing bicarbonate buffer concentration experiments were compared to current density results from the decreasing bicarbonate buffer concentration experiments at each condition using a paired two tailed Student *t*-