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 $40 \pm 6\%$, 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.

32 Keywords: Anode respiring bacteria, thermophilic, biofilm, microbial electrochemical cell

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* [2,3,4,5,6,7], *Shewanella oneidensis* [8,9], and mixed communities [10,11,12,13,14]. 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 [20,21,22,23,24]. 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 $>100\ \mu m$ [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:

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-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

78 limitations in the biofilms. First, we adjusted pH with the addition of either NaOH or HCl to
79 evaluate its effect on current production (j). Second, we analyzed the effect of bicarbonate
80 buffer concentration (10, 25, 50, and 100 mM) on current production and the corresponding
81 biofilm thickness (L_f). Lastly, we confirmed that the ionic conductivity and electron donor
82 (acetate) concentration of the growth media were not limiting under the conditions used and,
83 thus, had minimal impact on current production. This study is the first to analyze H^+ transport
84 limitations for anode respiration in biofilms of thermophilic Gram-positive ARB, and it reveals
85 that *T. ferriacetica* biofilms are not as limited by buffer concentration and transport of H^+ as
86 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•3H₂O (3.4 g l⁻¹), NH₄Cl (0.33 g l⁻¹), K₂HPO₄ (0.33 g l⁻¹), MgCl₂•6H₂O (0.33 g l⁻¹), CaCl₂•2H₂O (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 Na₂SeO₃•5H₂O, 4 mg Na₂WO₄•2H₂O, and 0.5g 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 MgSO₄•7H₂O, 0.5 g MnSO₄•H₂O, 1 g NaCl, 0.1g FeSO₄•7H₂O, 0.18 g COSO₄•7H₂O, 0.1 g CaCl₂•2H₂O, 0.18 g ZnSO₄•7H₂O, 0.01 g CuSO₄•5H₂O, 0.02 g KAl(SO₄)₂•12H₂O, 0.01 g H₃BO₃, 0.01 g Na₂MoO₄•2H₂O, 0.03 g NiCl₂•6H₂O, and 0.3 mg Na₂SeO₃•5H₂O. We prepared media in a condenser apparatus under N₂:CO₂ (80:20) gas conditions. Media was brought to boil and allowed to boil for 15 minutes per liter. We stored media in 100-ml serum bottles and autoclaved the media in the bottles for 15 minutes at 121 °C. We added ATCC Vitamin Solution and Na₂CO₃ added after autoclaving; Na₂CO₃ 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 minutes at 121 °C. Reactor materials and glassware were then transferred to a clean-hood and placed under UV sterilization for 30 minutes. 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 1M KCl [17,31]. Anode potential was poised at -0.06 V vs. SHE and the current continuously monitored every two minutes 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 cm²). Cathode was filled with 100 mM NaOH and pH was 13. Gas collection bags were placed on the anode compartments to collect volatile products (CO₂), 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 cm². 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 sec⁻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 Figure 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 cm² 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 cm² 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 (10mM, 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-test with an $\alpha = 0.05$ (95% confidence interval). Representative LSCVs (1 mV s⁻¹) for each condition are in Supplemental Information Figure S3 a-d.

2.6 Thickness Measurements and Microscopic Analysis.

Confocal laser scanning microscopy (CSLM) was used to measure biofilm thickness (L_f) per the protocol from [17], except that the images were acquired using an upright Leica SP5 CSLM after applying a LIVE/DEAD stain (BacLight Cell vitality kit, Invitrogen, USA). For measuring L_f at various bicarbonate concentrations, we used an H-type MEC with six anodes with a total surface area of 23.52 cm². The medium used initially consisted of 10 mM bicarbonate and the inoculum was transferred from a biofilm of a previous MEC. After a sustained current was observed, we replaced the medium with new media containing 25, 50, and

100 mM bicarbonate. After a constant j was observed at each condition, one of the six anodes was successively excised from the anode compartment for CSLM, and immediately subjected to the LIVE/DEAD protocol. For imaging purposes, we placed the stained anode biofilms in open petri dishes containing fresh media and observed them using an HCX PL APO CS 20x/0.70NA immersion objective with a working distance of 260 μm (for SCSLM images, see Figure S2 A-D in Supplementary Information). All thickness measurements are reported in μm of live biomass as assayed by LIVE/DEAD staining. We also performed scanning electron microscopy (SEM) on mature intact biofilms from two MECs under 50 mM bicarbonate buffer and 25 mM acetate conditions (from the MECs in the experiments described earlier). In addition, we prepared SEM samples using scrapped biofilm on glass slides (for SEM images, see Figure S4 A-D in Supplementary Information). Preparation for SEM followed the protocol from [17]. We used an FEI XL-30 environmental SEM (Philips) and a JSM6300 (JOEL) with an accelerating voltage of 5–20 kV and a working distance of 8–10 mm.

2.7 Experiments to Determine the Effect of Salt and Acetate Concentrations on Current Density.

To evaluate whether j was affected by the buffer concentration and not ionic strength, we grew mature biofilms on anodes with medium containing 50 mM bicarbonate and 25 mM acetate, and then additional salt (NaCl) was added. Once a constant j was observed, 0 (control), 10, 25, or 50 mM NaCl was added to the medium. To determine if j was not limited by the standard 25 mM acetate concentration used in our experiments, mature biofilms were grown on anodes with media containing 50 mM bicarbonate and 25 mM acetate. Acetate was then added to the reactor up to 25 (control), 50, 75, or 100 mM. Studies were performed in duplicate reactors, and the anode surface area for all NaCl and acetate experiments was 1.47 cm^2 .

3. Results and Discussion

3.1 Effect of pH on Current Density Produced by *T. ferriacetica* Biofilms

The current generation capabilities of mesophilic Gram-negative ARB biofilms are strongly correlated with the pH conditions of the biofilm [4,11,13]. To understand the role pH plays in current production for thermophilic Gram-positive biofilms, we manipulated the bulk-liquid pH with the addition of HCl or NaOH in the anode chamber of MECs containing *T. ferriacetica* biofilm anodes. Current density (j) in *T. ferriacetica* biofilms declined significantly for pH values below 7.5. Current density data presented in Figure 1 were averaged from two biofilms, and current density was normalized vs. j at ~pH 8.0 ($j_{pH\ 8}$), since this pH had the highest current density (14.3 A m⁻²). *G. sulfurreducens* biofilms also reached their highest current density value at pH 8.0 (14.0 A m⁻²) in similar experiments [11]. Current production was sustained from pH 5.2-8.3; however, pH lower than 5.2 and higher than pH 8.3 resulted in $j = \sim 0$ A m⁻². The functioning pH range for *T. ferriacetica* was larger than the ~6-8 pH range reported for mesophilic *G. sulfurreducens* biofilms [4,13]. This difference shows that *T. ferriacetica* biofilms can produce appreciable j at pH < 6, which is likely the result of increased rates of diffusion at 60°C compared 30°C based on the Einstein-Stokes equation [30].

Figure 1 also indicates that j increased with increasing pH, an observation similar to that for mesophilic Gram-negative biofilms [3,4,13]. j at ~pH 5.2 was only ~20% (2.8 A m⁻²) of $j_{pH\ 8}$. The biofilms produced $\frac{1}{2} j_{pH\ 8}$ at pH ~6.0 (6.5 A m⁻²), and j increased consistently up to ~pH 7.4, where it reached ~97% ($j_{pH\ 7.4} = 13.9$ A m⁻²) of the $j_{pH\ 8}$ value. Given that the pK_{a1} of bicarbonate is ~6.3 at 60°C, the increase in H⁺ carrying capacity for pH > 7.4 is marginal since it was about 90% of its maximum at pH 7.4. LSCV analysis (1 mV sec⁻¹) was conducted at low

pH (5.2), neutral pH (6.9), and high pH (8.3) conditions and is included in Supplementary Information as Figure S1.

3.2 Effect of Bicarbonate Concentration on Current Density Produced by *T. ferriacetica* Biofilms

In addition to pH, previous reports in mesophilic Gram-negative ARB have indicated that j depends upon the buffering conditions of the bulk media due to enhanced H^+ transport from the biofilm [11,13]. To investigate how bicarbonate buffer concentrations affected j in biofilm anodes of thermophilic Gram-positive ARB, biofilms were tested with media containing 25 mM acetate and increasing bicarbonate buffer concentrations from 10 to 100 mM. Figure 2 summarizes the results. For all conditions, j was normalized to $j_{100\text{ mM bicarbonate}}$ – the current density of each biofilm at 100 mM bicarbonate ($11.0 \pm 3.1\text{ A m}^{-2}$, see Table S1 for j values). The j observed at 10 mM bicarbonate was $61 \pm 6\%$ ($6.6 \pm 1.6\text{ A m}^{-2}$ at $\text{pH} = 6.9 \pm 0.05$), 25 mM was $80 \pm 6\%$ ($8.5 \pm 1.6\text{ A m}^{-2}$ at $\text{pH} 7.1 \pm 0.06$), and 50 mM was $92 \pm 6\%$ ($9.9 \pm 2.2\text{ A m}^{-2}$ at $\text{pH} 7.3 \pm 0.03$), all normalized to j at 100 mM bicarbonate ($11.0 \pm 3.1\text{ A m}^{-2}$ at $\text{pH} 7.8 \pm 0.06$). A bicarbonate buffer concentration of 150 mM resulted in decreased j . This was probably was due to development of CO_2 bubbles within the biofilm (illustrated in Figure 3), which resulted in biofilm shearing. Thus, it was not possible to assess the effect of increasing the bicarbonate buffer beyond 100 mM.

To determine significance of j with increasing bicarbonate buffer concentrations, a one-way analysis of variance (ANOVA) with a post-hoc Tukey honest significant difference (HSD) test was conducted [32]. For these results, a p-value lower than 0.01 indicates that one or more condition is significantly different from the other at the 99% confidence level, while a p-value of

0.05 indicates 95% confidence. The ANOVA test revealed that at least one bicarbonate buffer condition was significantly different from the others ($p < 0.01$). The Tukey HSD test results indicated that j increases with buffer were most significant ($p < 0.01$) for 10 to 25 mM bicarbonate concentrations, but the increase becomes less significant ($p < 0.05$) for 25 to 50mM, and insignificant ($p > 0.13$) for 50 to 100 mM. Statistical tables for Figure 2 are in Supplementary Information Table S2.

All studies were conducted in a bicarbonate/ CO_2 buffered system; thus, increasing buffer concentration resulted in a slightly higher pH for each condition. However, these data show that the increase in j with higher bicarbonate buffer was primarily due to greater H^+ transport from the biofilm, and the higher bulk-liquid pH was a secondary factor that correlated with greater H^+ transport capacity. Previous studies investigating biofilm anodes of MxCs have shown that the carrying capacity of the buffer to transport H^+ from the biofilm to the bulk-liquid is the primary limitation of j [11,13]. In parallel to previous studies [13,17], *T. ferriacetica* biofilms produced higher j ($6.6 \pm 1.6 \text{ A m}^{-2}$) at 10 mM bicarbonate buffer concentrations than mesophilic ARB ($\sim 3\text{-}4 \text{ A m}^{-2}$) with the same buffer [11]. This gives thermophilic ARB an important advantage when treating wastewaters that have low alkalinity. Moreover, transport of H^+ could be overcome using a lower buffer concentration with *T. ferriacetica* biofilms at 60 °C (50 mM bicarbonate), compared to mesophilic *G. sulfurreducens*, which had increasing current density up to 100 mM bicarbonate [11,13].

3.3 Effect of Bicarbonate Concentration on *T. ferriacetica* Live Biofilm Thickness

Confocal laser scanning microscopy (CLSM) was employed to quantify the impact of bicarbonate buffer concentrations on live biofilm thickness (L_f) to determine if j showed a correlation with L_f . L_f values for live portions of the biofilm were 68 ± 20 μm at 10 mM, 57 ± 11 μm at 25 mM, 124 ± 58 μm at 50 mM, and 180 ± 47 μm at 100 mM- the results are shown in Figure 4. Tukey-Kramer HSD [32] results indicate that L_f did not change with buffer within 10 to 25 mM bicarbonate which suggests that transport of H^+ out of the biofilm, not L_f , was the major limitation on j (Figure 2). Significant increases in L_f ($p < 0.05$) were observed for 25 to 50 mM and 50 mM to 100 mM. This increase in L_f with buffer concentration was predicted for mesophilic biofilms based on enhanced transport of H^+ from the biofilm [11,33]. Raw confocal images are in Supplementary Information Figure S2 a-d, and statistical tables for Figure 4 are in Supplementary Information Table S3.

As shown in Figure 4, L_f increases significantly ($p < 0.05$) from 50 mM bicarbonate to 100 mM bicarbonate. However, as shown in Figure 2, this increase in L_f did not correlate to a significant increase in j , a similar phenomenon to that observed in biofilms of *G. sulfurreducens* [5,6]. (It is important to mention that L_f measurements at 100 mM bicarbonate buffer concentration may have been close to the limits of the microscope since some were > 200 μm [34].) However, these results suggest that j is not limited by L_f from 50 to 100 mM bicarbonate and that increased live L_f did not correlate with increased j .

3.4 Bicarbonate Buffer is Primary Determinant for Current Density in *T. ferriacetica* Biofilms

Next, we confirmed that bicarbonate buffer, not L_f , was governing j for the results at all buffer concentrations shown in Figure 2 except 25 mM bicarbonate. To accomplish this,

biofilms were fed with increasing bicarbonate buffer (10 mM, then 25 mM, then 50 mM, and finally 100 mM) to reach maximum L_f values. Results shown with the black line in Figure 5 represent biofilms that were fed with media containing 25 mM acetate as an electron donor and increasing bicarbonate buffer which results in both increased L_f and increased j . Next, we evaluated that the biofilms from higher bicarbonate concentrations were producing more j as a result of increased H^+ transport and not producing more j as a result of increased L_f . After a biofilm had reached a steady current at 100 mM bicarbonate, it was fed new medium with gradually decreasing bicarbonate buffer concentrations (sequentially 100 mM, 50 mM, 25 mM, and 10 mM). Results shown with the grey line in Figure 5 represent the j value for a biofilm that has reached its maximum L_f value and is then fed decreasing bicarbonate buffer concentration- thus eliminating the influence of increasing L_f on j . Representative LSCVs (1 mV s^{-1}) for each condition are in Supplemental Information Figure S3 a-d.

Results in Figure 5 indicate that L_f had no significant ($p > 0.05$) impact on j at low or high buffer, but showed a significant difference ($p < 0.05$) at 25 mM bicarbonate buffer (Student t-test statistics shown in Supplementary Information Table S3). Similar to mesophilic *G. sulfurreducens* biofilms, *T. ferriacetica* biofilms at high buffer concentrations appear to not be limited by L_f after reaching a steady j value [3,4,5,6]. However, unlike *G. sulfurreducens* biofilms, *T. ferriacetica* biofilms did not experience a loss in j after ~60-80 μm [6]. This suggests that j does not increase with $L_f > 68 \pm 20 \mu m$ or bicarbonate buffer > 50 mM indicating that j does not correlate to live biomass thickness in *T. ferriacetica* biofilms at these conditions.

Results also indicate that H^+ transport is not a major limitation in current production for mature biofilms until the buffer concentration is as low as 10 mM bicarbonate ($pH < 7.3$), compared to mesophilic ARB biofilms, where 100 mM bicarbonate concentrations seem

insufficient to alleviate H^+ transport limitations resulting in lower current production [11,13]. This experiment also showed that limitations in j caused by H^+ transport do not appear at high buffer concentrations in mature *T. ferriacetica* biofilms in which $pH > 7.3$. This corroborates with the data set from Figure 1, suggesting that j in *T. ferriacetica* biofilms does not become limited by H^+ transport until pH is lower than ~ 7.4 .

3.5 Effect of NaCl and Acetate Concentration on Current Density in *T. ferriacetica* Biofilms.

In addition to pH and bicarbonate buffer concentration experiments, we conducted studies to assess the importance of ionic strength and migration in the biofilm via adding NaCl (results in Figure 6a shown in percentage $j/j_{0\text{ mM NaCl}}$) and to determine if 25 mM acetate was limiting (results in Figure 6b shown in percentage $j/j_{25\text{ mM acetate}}$). Adding NaCl at concentrations from 0 mM to 50 mM to biofilms grown with medium containing 50 mM bicarbonate and 25 mM acetate had no impact on j values ($100 \pm 0.4\%$ vs $j_{0\text{ mM NaCl}}$ at 0 mM NaCl, $101 \pm 0.2\%$ vs $j_{0\text{ mM NaCl}}$ at 10 mM NaCl, $101 \pm 0.2\%$ vs $j_{0\text{ mM NaCl}}$ at 25 mM NaCl, and $100 \pm 0.3\%$ vs $j_{0\text{ mM NaCl}}$ at 50 mM NaCl), indicating that ionic strength was not a factor in determining j . This outcome was expected because we placed the reference electrode adjacent to the anode to make Ohmic losses negligible, even at the lower ionic strength. [13] saw a similar result with mesophilic Gram-negative biofilms. Although ARB biofilms may become limited by a low electron donor concentration [7], we observed no significant change in j when we increased the acetate concentration from 25 mM to 100 mM in biofilms grown with 50 mM bicarbonate. (Current density values were: $100 \pm 0.5\%$ vs $j_{25\text{ mM acetate}}$ for 25 mM, $97.5 \pm 0.6\%$ vs $j_{25\text{ mM acetate}}$ for 50

mM, 96.1 ± 0.1 % vs $j_{25 \text{ mM acetate}}$ for 75 mM, and 94.8 ± 0.4 % vs $j_{25 \text{ mM acetate}}$ for 100 mM.) Thus, 25 mM acetate was non-limiting in thermophilic Gram-positive biofilms.

4. Conclusions

Similar to biofilm anodes composed of mesophilic Gram-negative ARB, H^+ transport and low-pH inhibition were significant limiting factors in current production for *T. ferriacetica* at 60°C. However, compared to mesophilic *G. sulfurreducens*, *T. ferriacetica* could generate current at lower pH (down to 5.2), which may provide an advantage for treating low-alkalinity waste streams. Increasing the concentration of bicarbonate buffer led to greater j , but the effect was important only to 50 mM bicarbonate and ~pH 7.3. Bicarbonate up to 100 mM allowed larger L_f , but it did not lead to a larger j at steady state.

These observations suggest that H^+ transport is a major limitation in current production and biofilm thickness, and also that elevated temperatures enhance H^+ transport from the biofilm. The addition of bicarbonate buffer had no influence on current production after current reached $\sim 9.9 \pm 2.2 \text{ A m}^{-2}$ at $\text{pH} > 7.3$, partially due to the limited H^+ carrying capacity of bicarbonate buffer ($\text{pK}_{a1} = 6.3$). However, live biofilm thickness continued to increase with bicarbonate buffer up to 100 mM ($11.0 \pm 3.1 \text{ A m}^{-2}$). As a biofilm develops, H^+ accumulates within its inner layers, creating a pH gradient. The increase in bicarbonate concentration facilitates H^+ transport within the biofilm, allowing for the biofilm to grow thicker. However, the activity of these cell layers was not directly measured in this study, and it may be that cells measured as live during CLSM were not all producing current in equal quantities or may have been surviving via direct transfer of electrons to adjacent cells rather than to the anode; both phenomena that have been

reported for biofilms anodes of *G. sulfurreducens* [5,35,36]. Future research will be required to quantify the activity of *T. ferriacetica* in biofilm anodes in a way that can determine the contribution of active layers to current production and can further enhance our understanding of pH inhibition within thermophilic Gram-positive biofilms.

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517

518 **Figure 1:** Effect of pH on current density (j) normalized to percent of that produced at pH 8.0

519 ($j_{pH8} = 14.3 \text{ A m}^{-2}$). The current density was ~ 0 for $\text{pH} < 5.2$ and > 8.3 .

520

521 **Figure 2:** Effect of bicarbonate buffer concentration on current density (j) normalized to that
 522 produced with 100-mM bicarbonate buffer ($11.0 \pm 3.1 \text{ A m}^{-2}$). Normalized values shown are the
 523 average of four MECs. Black bars represent standard deviation. Double-plus symbol indicates a
 524 significant difference ($p < 0.01$) for 10 mM vs 25 mM. Single-plus symbol indicates a
 525 significant difference ($p < 0.05$) for 25 mM vs 50 mM.

526

527 **Figure 3:** CO₂ bubbles resulting from 150-mM bicarbonate buffer.

528

529 **Figure 4:** Effect of bicarbonate buffer concentration on live biofilm thickness (L_f). Black bars
 530 represent standard deviation. Single-plus symbol indicates a significant difference ($p < 0.05$) for
 531 L_f at 25 mM vs 50 mM and for L_f at 50 mM vs 100 mM.

Figure 5: Effect of buffer concentration on current density (j) normalized to 100-mM bicarbonate for adding sequentially increasing bicarbonate buffer from 10 mM bicarbonate to 100 mM (Increasing, represented by black line) and adding sequentially decreasing 100 mM bicarbonate to 10 mM bicarbonate buffer (Decreasing, represented by grey line). The two anode biofilms in MECs were first grown at increasing bicarbonate concentrations: 10 mM, 25 mM, 50 mM, and then 100 mM where they achieved their highest current density and thickness. These same biofilms were then fed media containing decreasing bicarbonate concentrations: 100 mM, 50 mM, 25 mM, and 10 mM. Single-plus symbol indicates a significant difference ($p < 0.05$) for 25 mM increasing vs 25 mM decreasing.

Figure 6: (a) NaCl concentration had no effect on j . (b) Increasing the acetate concentration above 25 mM had minimal effect on j .