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Use of Electrochemistry to Monitor the Growth and Activity of Clostridium phytofermentans

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ABSTRACT

Microbes have a wide range of metabolic capabilities available that make them industrially useful organisms. Monitoring these metabolic processes is a crucial component in efficient industrial application. Unfortunately, monitoring these metabolic processes is often invasive and time consuming especially within an anaerobic environment. Electrochemical techniques, such as cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) offer a non-invasive approach to monitor microbial activity and growth. We hypothesized that EIS and CV could be used to monitor *Clostridium phytofermentans*, an anaerobic and endospore-forming bacterium. *C. phytofermentans* ferments a wide range of sugars into hydrogen, acetate, and ethanol as fermentation by-products and is a good candidate for consolidated bioprocesses. For this study, both traditional microbiological and electrochemical techniques were used to monitor the growth of *C. phytofermentans* and the formation of fermentation products. An irreversible reduction peak was observed using CV beginning at mid-logarithmic phase of growth. This peak was associated with *C. phytofermentans* and not the spent medium. Additionally, EIS parameters, phase shift and imaginary admittance generally followed the growth of *C. phytofermentans*. Results suggest that CV and EIS are useful tools in the monitoring of *C. phytofermentans* and could possibly be used to monitor other anaerobic microbial processes.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>SECTIONS</td>
<td></td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. MATERIALS AND METHODS</td>
<td>13</td>
</tr>
<tr>
<td>a. Growth analysis</td>
<td>13</td>
</tr>
<tr>
<td>b. Electrochemical analysis</td>
<td>17</td>
</tr>
<tr>
<td>III. RESULTS</td>
<td>23</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>35</td>
</tr>
<tr>
<td>WORKS CITED</td>
<td>42</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

2. Generalized diagram of an electrochemical set-up ........................................5
3. Diagram of alternating current.................................................................8
4. Polarization of a microbial cell...............................................................9
5. OCP experimental set-up.......................................................................18
6. Reference electrode experimental set-up..............................................19
7. Selection method of frequency to be used in EIS.................................21
8. Growth curve of \( C. \) phytofermentans..................................................24
9. Fermentation products of \( C. \) phytofermentans....................................25
10. DAPI staining of \( C. \) phytofermentans..................................................26
11. Voltammograms of \( C. \) phytofermentans..............................................27
12. Peak area over time of \( C. \) phytofermentans........................................28
13. Starting potential over time of \( C. \) phytofermentans..........................29
14. Reduction peak of \( C. \) phytofermentans versus reference electrode......30
15. Linear regression of peak current versus scan rate.............................31
16. Reduction peak confirmed to be associated with \( C. \) phytofermentans........32
17. Phase shift (\( \Delta \phi \)) during growth of \( C. \) phytofermentans..................33
18. Imaginary admittance (\( Y'' \)) during growth of \( C. \) phytofermentans........34
INTRODUCTION

*Monitoring microbial activity during bioprocessing*

A variety of industrial processes rely on the astounding diversity of microbial metabolic capabilities (1). Recent technological advances have led to an increase in biotechnological bioprocesses. Bioprocesses are becoming increasingly useful and are seen in a wide range of industries such as pharmaceutical, energy production, and food and beverage industries (2). Monitoring the growth and activity of the various microbes involved is an essential component of these bioprocesses. Even small changes in the growth environment, such as pH, temperature and pressure can affect metabolic processes carried out by these microbes (2, 3). Thus, there is a need for rapid and active analysis methods for monitoring microbial growth and activity (2, 4, 5).

Bioprocesses can be conducted as either batch, fed-batch or continuous culture, with fed-batch being the most common (6). Bioprocess monitoring takes place either off-line or on-line to assess parameters such as cell density, carbon source utilization, product formation and the growth conditions previously mentioned. Off-line monitoring techniques, such as gas chromatography (GC) or high performance liquid chromatography (HPLC), are normally used for high precision measurements of components such as, carbohydrate utilization and subsequent product formation. However, this type of off-line monitoring requires collection of samples and is unable to provide real-time results and thus, does not allow for early problem detection (7-9). Consequently, critical decisions are made based on time-delayed data. Although, real-
time monitoring with the use of on-line sensors is ideal, maintenance of the monitoring probes is required (10).

Monitoring and sampling must be conducted so as to not contaminate or negatively affect the culture (2, 10-12). The technology used to monitor the bioprocess must also survive harsh conditions, such as sterilization and pressure changes. (10-12). These activities can be especially difficult when working with an anaerobic culture such as a fermentation bioprocess because the system must remain sealed as not to introduce oxygen.

Many of the in situ monitoring technologies presently being used often have issues of measurement drift from precipitation of biological materials, changes in medium composition and the growth of the organisms, which can result in thick biofilms (10, 11). Drift results in inaccurate measurements and/or the need for frequent recalibration (10). Parameters such as temperature, pH and dissolved oxygen have commonly used monitoring technologies available and research into improvements is not as crucial. The critical research is technology that can measure biomass and product concentration as well as the metabolic state of the cell (13). For example, under batch fermentation conditions, knowledge of fermentation batch completion could result in more efficient time management and therefore cost savings for the industry concerned. Additionally, an indication of appropriate sampling time is also needed for efficient bioprocessing. In summary, in situ monitoring may be of value to improve the efficiency of bioprocesses, but further research is needed. The purpose of this work therefore, is to evaluate the use of electrochemical methods for on-line, in-situ bioprocess monitoring.
Electrochemistry

Electrochemistry provides a noninvasive way to monitor microbial activity. In short, electrochemistry is the study of how electrical and chemical energy relate as well as the interconversion of the energy between the two forms (14). Within an electrochemical system, electrons are transferred between the electronic conductor, the electrode, and the ionic converter, the electrolyte (14). This is referred to as the electrode/electrolyte interface. The reactions, or events that occur at the interface, provide valuable insight into the electroactive components present within the system.

Electrochemistry allows for the monitoring of electron flow within a microbial community. Advantages of electrochemistry include real-time or near real-time results and little to no sample collection. This method of monitoring is possible because microbial cells are composed of charged components. For example, the microbial cell surface is negatively charged resulting from the membrane glycoproteins (15). This charge attracts positive ions to the microbial cell membrane forming a double layer (15). Moreover, the lipid bilayer of the cell and the ions contained within the cytoplasm allow the cell to interact electrochemically (15). For this interaction to take place a potential must be applied to the electrical system. Potential is the amount of energy available for electron flow and is measured in volts (V) (16). The resulting electron flow, or current is measured in coulombs (16). A coulomb is equivalent to $6.242 \times 10^{18}$ electrons (16). Two commonly used electrochemical methods are cyclic voltammetry (CV) and
electrochemical impedance spectroscopy (EIS). In order to perform these techniques, a potentiostat is needed (Fig. 1).

A potentiostat is an electronic instrument that is able to apply and control the potential while measuring the resulting current (14). Potentiostats can be operated in two different configurations: 1) a three-electrode system in which potential is controlled versus a reference electrode; and, 2) a two-electrode system in which potential is controlled against the measured open circuit potential (OCP) of the system (17, 18). The reference electrode is an electrode with a fixed potential and is used as a reference for the application of voltage (14).
**Figure 1:** Generalized diagram of an electrochemical set-up. The electrodes are in an electrolyte solution and connected to a potentiostat as follows: a working electrode (green), the reference electrode (gray) and a counter electrode (red).

Several types of reference electrodes with known potentials are utilized. Standard hydrogen electrode (SHE) is the internationally recognized reference potential with a neutral potential (E°) of 0.000 V (14, 19). The type of reference electrode chosen is dependent on the system (20). For example, although a standard calomel electrode (SCE) is commonly used, its E° is temperature dependent and thus is unsuitable when temperature is varied or is unstable (21). Additionally, one does not want incompatible species from the electrolyte to interact with the materials present within the reference electrode as this could lead to clogging of the reference electrode (20). An easily available option is to use a double junction, such as in a Ag/AgCl reference electrode.
The double junction functions by separating the working electrode from the sampling solution via an intermediate solution. Conversion between reference electrodes makes it possible to optimize for the electrochemical system. For example, 0.197 V must be added to convert a potential from saturated Ag/AgCl to SHE (21).

Cyclic voltammetry measures redox conditions in the system and thus, the electrochemical activities of bacteria present (22, 23). CV is quickly gaining popularity for its ability to obtain information on complex electrode reactions and as such is often used for initial electrochemical studies (14). In cyclic voltammetry, the current is measured as a range of potentials is applied (Fig. 1) (24). The speed at which the potential is changed is referred to as the scan rate (17). The potentiostat controls the potential (voltage) between the working electrode and the reference electrode while measuring the electron flow (current) between the working electrode and the counter electrode (17). The potential applied between the two electrodes is also known as the excitation signal with the response signal being the measured current (24). The working electrode is where reactions of interest occur. The working electrode’s potential is controlled versus either the reference electrode or the measured open circuit potential (17, 25). The counter electrode allows the electrochemistry to take place by conducting the reverse reaction of the working electrode and maintaining the overall voltage of the system. For example, if an oxidation occurs at the working electrode, a reduction will occur at the counter electrode. Furthermore, each individual analyte has a critical point of potential (E°) at which electrons will transfer either to the electrode or from the electrode (14). CV results are visualized using a voltammogram, which is a plot of applied
potential versus measured current. When an oxidation or reduction reaction occurs, a peak can be seen on the voltammogram. The maximum current on this peak is referred to as the peak current. The potential of this current is known as the peak potential. The area of this peak is proportional to the concentration of the electrochemically active analyte responsible for the peak (14, 25).

Electrochemical impedance spectroscopy (EIS) is a powerful and rapid method that takes advantage of both the resistive and capacitance parameters of a cell (26, 27). Overall, it is a measure of the ability of a system to impede the flow of electrons (28). EIS functions by producing an alternating current (AC) and measuring the current response as voltage changes (25, 29). The AC produces a sinusoidal wave which cycles at a given amplitude (Fig. 2). The number of cycles within a given amount of time is referred to as frequency, measured in hertz (Hz) (14). The potential and current are viewed as a rotating vector or phasor with the same frequency. Most often, the potential and current are not in phase, creating a phase shift. This difference in phase is referred to as phase angle ($\phi$) (14). The phase angle is created from the combination of capacitive and resistive components in the electrochemical system.
Figure 2: Diagram of alternating current. Shown are two cycles. (Created with the aid of Matlab).

Impedance (Z) can be thought of as the total resistance and also the inverse of admittance (Y), a kind of conductance (14). The impedance is typically illustrated using either a Bode plot (log |Z| versus the frequency) or a Nyquist plot (Z’ versus Z") (30, 31). Phase shift is the result of a change in the phase angle between current and voltage which is produced as a result of the medium (32). Additionally, impedance is usually measured at different frequencies allowing a spectrum to be created (27, 30). The impedance spectrum provides information regarding cell membranes and also exchange and diffusion processes within the microbe depending on the frequency (30). This resulting spectrum relates to the interaction between the different components of the microbial cell and the range of frequencies applied (33, 34). For instance, at low frequencies (10 MHz or less), impedance relates to diffusion/mass transport (34, 35). At higher frequencies, impedance relates to membrane resistance (36). Thus, cell
concentration and other parameters of cellular activity can be estimated using impedance (37, 38). An estimated $1 \times 10^3$ to $3 \times 10^7$ cells/mL are needed to influence the electrochemistry enough to be detected in an impedance curve (27). This influence is possible because viable microbial cells have an intact plasma membrane that can be polarized as opposed to nonviable microbial cells (Fig. 3) (37). Intact plasma membranes have a transmembrane potential and are able to build up a charge at their cell surface, similar to a capacitor (2, 11, 39). Capacitance/permittivity is thought to relate to the fluid within the polarized membrane (10). It is also possible to detect metabolic products excreted by the cells since these products can change the ionic content, and thus the conductivity of the culture medium (27, 38, 40).

![Counter electrode][Working electrode]

**Figure 3:** Polarization of a microbial cell. The print patterned electrode has both a counter (red) and a working electrode (green), that work to polarize living microbes.

EIS research in the field of microbiology has been intermittent even though it has been used for over a century within the electrochemistry community (38, 41). As early as
the 1970’s impedance was proposed as a way to monitor the activity of bacteria within a clinical setting (42). Impedance was shown to correlate to growth, but to be affected by factors such as type of bacteria and growth media (42). In 1987, Harris et al., first proposed using capacitance, a component of impedance spectroscopy, to monitor biomass of *Saccharomyces cerevisiae* (43). Since then, other experiments have shown that both impedance and capacitance are directly proportional to biomass (43-46).

In 1984, Matsunaga and Namba used CV to detect and enumerate *S. cerevisiae* (47). They found that coenzyme A (CoA) concentrations in the cell wall changed as cell counts increased. These changes altered the electrochemistry of the medium, creating an oxidation peak in the voltammogram, and allowed the authors to indirectly calculate cell density. Additionally, the peak current changed and appeared to be closely related to cell viability and metabolism. In 1997, this technology was again used with *S. cerevisiae* finding similar results (48). Furthermore, Matsunaga and Nakajima, found that they could both enumerate bacteria and determine whether they were Gram positive or Gram negative (49). This finding was possible because of changes in both peak area and peak potential. For the Gram-positive bacteria tested, peak potential was shown to be between 0.65 and 0.68 V vs. SCE. The Gram-negative bacteria tested had an oxidation peak between 0.71 and 0.72 V vs. SCE. For example, *Lactobacillus fermentum*, a Gram positive bacterium had a peak potential of 0.66 ±0.02 V vs. SCE and *Escherichia coli*, a Gram negative bacterium had a peak potential of 0.71 ±0.01 V vs. SCE. The authors found the peak to be associated with CoA in the cell membrane. Once again, similar results were observed more than a decade later and determination of Gram stain
classification using electrochemistry was studied and confirmed (50). Vieira et al. found that biofilms could also be detected using cyclic voltammetry (51). Their results found this approach promising and it was suggested that CV could be used as a way to detect biofilm growth in its early stages of development in areas such as a wastewater treatment system or heat exchanger, both of which are challenging to monitor (51, 52).

Alternatively, uptake of electrochemical molecules can be measured using electrochemical technologies as microbes grow. For example, oxygen is an electroactive molecule. Thus, its consumption can, in theory, be measured. Ruan et al., found that oxygen was very quickly consumed during growth of Salmonella typhimurium and that detection time and concentration were inversely related (53). Since this study, other bacteria species have been detected using EIS, including, but not limited to Micrococcus luteus, Bacillus subtilis, B. thuringensis, B. stearothermophilus, Lactobacillus casei, Staphylococcus aureus, and Pseudomonas aeruginosa (38, 39, 54).

Clostridium phytofermentans

Clostridium phytofermentans was chosen as the bacterium for this project to evaluate the use of electrochemistry as a possible method to measure bioprocess reactions. C. phytofermentans is a rod shaped (0.5-0.8 x 3-15 µm), endospore-forming obligate anaerobe. Endospores are round and terminal, usually 0.9-1.5 µm. C. phytofermentans is motile with one or two flagella present and has been shown to grow on a large variety of organic substrates including those found in plant biomass, such as cellulose (55). Fermentation end products include carbon dioxide, hydrogen, acetate, and ethanol (55-
C. phytofermentans was first described in 2002, after being enriched from forest soil, as a novel cellulolytic species (55). Its genome was later shown to contain the highest number of cellulases and hemicellulases of any sequenced clostridial genome (57, 58). Although not yet used at the industrial level, advances in genetic tools have the ability to increase its cellulytic and hemicellulytic potential making it a possible candidate for large-scale bioprocesses (57).

This study aims to use the electrochemical techniques of CV and EIS during the growth of C. phytofermentans. We hypothesize that there will be changes in the electrochemistry that can aid in the detection of growth and physiological status of C. phytofermentans. The results obtained will be evaluated as to whether these techniques can be useful in bioprocess monitoring. These techniques not only offer the advantage of monitoring cell density, but also the overall health and status of the cells being monitored. To our knowledge, this is the first investigation using electrochemical techniques with an anaerobic microorganism.
MATERIALS AND METHODS

Growth analysis

Strain and growth conditions

*C. phytofermentans* (ATCC 700394) was grown in GS-2C medium, which was prepared as follows per liter distilled water: 6.0 g yeast extract, 2.1 g urea, 2.9 g K$_2$HPO$_4$, 1.5 g KH$_2$PO$_4$, 10.0 g 3-(N-morpholino)propanesulfonic acid (MOPS), 3.0 g trisodium citrate dehydrate, and 2.0 g L-cysteine HCl (55). Final pH was adjusted to 7 with 5 N NaOH. Media were brought to a boil and sparged using high purity N$_2$ following the Hungate method (59) After cooling, the medium was aliquoted into 125 mL Wheaton serum bottles previously degassed with high purity nitrogen, each sealed with a black butyl rubber stopper (Geo-Microbial Technologies, Inc.) and crimped with an aluminum seal (Wheaton). The butyl rubber stopper had a screen printed electrode inserted through it (Pine Instrumentation #RRPE1001C). Aliquots were autoclaved at 121°C for 15 minutes. Prior to performing experiments, the sealed serum bottle was evaluated for gas tightness by submerging under water. Additionally, serum bottles were kept upside down over night to evaluate for leakage. Once cooled, the aliquot was amended with a filter sterilized, deoxygenated cellobiose aqueous solution to a final concentration of 1g L$^{-1}$. A 2% inoculum of a stationary phase culture of *C. phytofermentans* was aseptically added using anoxic techniques.
**Growth Curves**

Liquid samples of 1 mL were collected at 0, 9, 15, 24, 33, 39, 48, 57, 63, 72, and 87 hours of growth based on a previously developed growth curve to collect samples at lag, log and stationary phase of growth. Samples were then placed into a disposable plastic cuvette for optical density measurements at 550 nm using a UV/Vis spectrophotometer (Shimadzu UV-2401PC). Liquid samples were stored in a 1.5 mL microcentrifuge tube at 4 °C for subsequent analysis of cellobiose, ethanol, acetate and protein concentrations. Generation time (G) was calculated using the following equation: \( G = t \cdot 3.3 \log(bB) \) where \( t \) is time in hours, \( B \) is the number of bacteria at the beginning of the time interval and \( b \) is the number of bacteria at the end of the time interval.

**Protein Concentrations**

Protein concentrations were used as a measurement of cell biomass. 800 µL of liquid sample were first centrifuged at 13,200 rpm for 10 minutes at 4 °C. The resulting supernatant was separated and stored at 4 °C for high performance liquid chromatography (HPLC) for later analysis. The pelleted cells were re-suspended in 800 µL of phosphate buffered saline (PBS) for protein measurement with DC Protein Assay (Bio-Rad Cat. # 500-0112). Reagents were prepared as described by kit manual. A 0.2 mL microcentrifuge tube was used to boil sample (10 µL) and 1 M NaOH (10 µL) for 10 minutes. The following standards were made: 0.0, 0.1, 0.2, 0.3, 0.5, 1.0 mg/mL. In a microplate, reagent A (25 µL) and reagent B (200 µL) were added to the boiled sample (5 µL). Colorimetric changes were measured using a Synergy H4 Multi-Mode Reader.
(BioTek). Data were analyzed using Gen5 Data Analysis Software (Biotek) and plotted in Microsoft Excel.

Cell Counts

Cell counts were performed using DAPI (4', 6-diamidino-2-phenylindole) staining. To do this, optical density was measured on a 1 ml sample. Of this sample, 900 µL was saved and 100 µL of 20% paraformaldehyde (final concentration, 2%) was added to preserve the sample for up to 48 hours at 4 °C. After at least 20 minutes (but no more than 48 hours), dilutions were made using sterile sodium buffered saline (pH 7.2). Cells were filtered and stained for 5 minutes with 2 µg mL⁻¹ DAPI. Imaging was conducted using an epifluorescence microscope (Nikon Eclipse E600) with cells in at least five fields per filter (GVS Life Sciences, poretics polycarbonate track etched black 25 mm 0.2 µm) counted with the aid of ImageJ software.

Hydrogen Production

Hydrogen production was measured using gas chromatography. Headspace samples were collected at the previously stated times. Headspace pressure was measured using a digital manometer (Dwyer Series 477). Headspace samples were collected using a gastight 250 µL glass syringe. The samples were injected into a gas chromatograph (Agilent 7890A) with a HP Plot Molesieve column (Agilent J&W, 30 m, 0.32 mm, 25 µm). The following operating conditions were used: inlet at 105°C splitless, 5.75 psi, total flow 3.5 mL/min with the oven temperature held at 85°C for 10 minutes. The
detector was a thermal conductivity detector (TCD) set at 275 °C with a reference flow at 17.0 mL/min and makeup flow at 5.0 mL/min. Argon gas was used as the carrier gas at a flow rate of 0.4 mL min⁻¹. A standard curve was created using varying volumes of a 2% hydrogen standard. Peak analysis was performed using Agilent Chemstation, Enhanced Data Analysis G1701DA ver D.00.00.38 and Microsoft Excel.

**Cellobiose utilization, ethanol and acetate production**

High performance liquid chromatography (HPLC) analysis using the supernatant collected during the preparation of the stored liquid samples for protein analysis was performed to measure acetate and ethanol concentration as well as cellobiose utilization. To do this, 500 µL of the supernatant was placed in a 2.0 mL screw-top glass vial and capped with a septum. Samples were analyzed using HPLC (Agilent 1200 series) equipped with a refractive index detector with an Aminex HPX-87H column (Bio-Rad #125-0140) with the following specifications: 300 x 7.8 mm, 9 µm particle size with an attached Cation H micro-guard, 30 x 4.6 mm (Biorad, # 125-0129) column. The samples were run using 5 mM H₂SO₄, as the mobile phase with a flow rate of 0.6 mL min⁻¹, and a sample volume of 5 µL. Standards of cellobiose, acetate, and ethanol were also run. A standard curve was created and concentrations were determined using the Agilent Chemstation software.
Electrochemical analysis

*Cyclic voltammetry vs. open circuit potential*

Open circuit cyclic voltammetry (CV) was used to monitor changes in the electrochemistry of the system (Fig. 4) during the growth of *C. phytofermentans* and uninoculated medium. Additionally, an inoculated control lacking the addition of celllobiose was also tested. The electrode was first electrochemically cleaned to remove any analytes that had possibly precipitated on the electrode. To do this, the inoculated serum bottles were placed upside down in the Innova 4430 (New Brunswick) incubator (Fig. 4). The four-channel potentiostat, VersaSTAT MC (Princeton Applied Research) was used to cycle the potential between -2.0 and 2.0 V at a scan rate of 1000 mV/s from an initial and final potential of the measured OCP for electrochemical cleaning with three cleaning scans before each analysis. Next, a more precise, analytical CV was conducted by cycling the electrode between -0.85 and 0.85 mV at 25 mV/s from an initial and final potential of the measured open circuit potential. Once again, three sweeping scans were performed. Data was recorded with VersaStudio software version 2.42.3.
Figure 4: OCP experimental set-up. *C. phytofermentans* was grown in an upside down 125mL serum bottle with an electrode forced through a black butyl stopper, crimped with an aluminum seal. The bottles were incubated at 37° with both a working and a counter electrode attached to them.

*Cyclic voltammetry vs. Ag/AgCl reference electrode*

Cyclic voltammetry was conducted using the same settings as above with the exception of using the Ag/AgCl electrode as a point of reference to calibrate the initial potential of 0.0 V vs. the Ag/AgCl reference electrode (Fig. 5). A 125 mL borosilicate glass serum bottle was modified to contain two 20 mm Balch-style ports, a 5 mm threaded O-ring sealed port for the reference electrode, and a 15 mm horizontal O-ring junction. The same screen-printed electrode was used; however it was inserted into a 15 x
30 mm gray bromobutyl stopper (Wheaton #224100-331). Silicon glue was used to seal the electrode into the stopper and maintain a gas-tight system. The stopper was clamped to ensure it would not come out as pressure rose in the system from microbial gas production. The electrode was placed sideways as to prevent particles from coating it and/or gas bubbles from getting trapped.

**Figure 5:** Reference electrode experimental set-up. A modified serum bottle with the print patterned electrode used as the counter and working electrode and a double junction Ag/AgCl reference electrode.
Electrochemical impedance spectroscopy

Electrochemical impedance spectroscopy (EIS) was performed immediately following the completion of the CV study and was thus conducted using the same equipment and culture. An alternating current (AC) potential starting at a frequency of 100,000 Hz and ending at 0.01 Hz with amplitude set to 50 mV root mean square (RMS) was used. Ten measurements were recorded per decade (order of magnitude) of frequency with a measurement delay of two seconds. This measurement delay is a delay between every current range change and frequency measurements that allow the electrochemical cell to stabilize between measurements. EIS was performed approximately every three hours.

Electrochemical data analysis

Data were collected using Versastudio (Princeton Applied Research). CV analysis was conducted by first importing the data from Versastudio into Microsoft Excel to develop graphical representations of the changes in the measured current. For voltammogram graphics, the potential was plotted against the measured current. Peak current and starting potential were both determined in Microsoft Excel with the aid of this graphical process. CView Version 3.4 was used to determine the peak area. Briefly, this measurement involved the creation of a baseline and integrating the area from the baseline.

EIS analysis was performed by first importing the data from Versastudio into Microsoft Excel. Analysis for the parameters measured (phase shift and admittance) first
involved subtracting the initial time point from the subsequent time points. Next, the desired parameter was graphed against frequency. This creates a frequency spectrum and allowed the determination of the optimal frequency since the optimal frequency is where the parameter peaks on the spectrum (Fig. 6). The parameter was then plotted against time at the chosen frequency.

Figure 6: Selection method of frequency to be used in EIS. When frequency is plotted against the desired parameter, a peak or multiple peaks can be observed. Each peak represents a potential frequency of interest and can be plotted over time.
Peak characterization

In order to determine if the observed peak(s) from the CV data were associated with the cell surface or the spent medium, two technical approaches were used. The first was an electrochemical approach where the CV scan rates were varied using the following scan rates: 10, 25, 50, 100, 500, and 1000 mV/s. Peak current was measured using the VersaStudio software and plotted versus scan rate in Microsoft Excel. For this analysis, a linear fit indicates the peak is associated with the cell surface whereas a logarithmic fit indicates the peak is associated with the spent medium (60, 61).

The second approach, involved the physical separation of the cells from the spent medium. *C. phytofermentans* was grown to the completion of logarithmic growth during which time a CV peak was observed. At the time at which this peak occurred (approximately 40 hours after inoculation), 10 mL of culture was anaerobically collected using a sterile needle and syringe and injected into a 30 mL sterile syringe and passed through a 0.22 µm filter into a sterile Balch tube filled with nitrogen gas. CV was conducted on the cell free medium. Additionally, uninoculated medium was measured to ensure the peak was not associated with medium components.
RESULTS

Microbiological analysis of *C. phytofermentans* growth

*C. phytofermentans* had approximately a three day growth cycle with a calculated doubling time of 2.3 hours per generation. Cell counts, (as well as protein concentration and optical density) indicated that lag phase of growth lasted 24 hours, followed by logarithmic growth for an additional 16 hours (Fig. 7). Upon the completion of logarithmic growth, the concentration of *C. phytofermentans* decreased until around 60 hours where concentration remained roughly constant. Chromatography results corroborated this growth with cellobiose utilization ($R^2=0.85; p=0.0029$) as well as acetate ($R^2=0.96; p=<0.001$), ethanol ($R^2=0.98; p=<0.001$), and hydrogen production ($R^2=0.97; p=<0.001$) until stationary growth phase of growth (Fig. 8). The remaining cellobiose was utilized by the end of logarithmic growth (40 hours). Acetate concentration peaked at 8.3 mM, ethanol at 2.2 mM, and headspace hydrogen at 4.8% 40 hours post inoculation.
**Figure 7:** Growth curve of *C. phytofermentans*. Concentration of *C. phytofermentans* using DAPI staining is shown over an 87 hour time period. Values are the mean ± S.D. of two staggered duplicate growth studies.
**Figure 8:** Conversion of cellobiose (blue diamond) to acetate (orange square), ethanol (gray triangle), and hydrogen (yellow x) by *C. phytofermentans* during an 84 hour growth evaluation. Values are the mean ± S.D. of two staggered duplicate growth studies.

DAPI staining also showed changes in cell morphology (Fig. 9). Initially, upon inoculation, only small dots, were observed. Roughly 24 hours later, rod shaped microorganisms were seen.
Figure 9: DAPI staining of *C. phytofermentans*. DAPI stained images at inoculation (a), lag (b), log (c), and stationary (d) phases of growth.

Electrochemical analysis of *C. phytofermentans*

*Open circuit potential (OCP) Cyclic voltammetry*

OCP Cyclic voltammetry (CV) is a useful technique for identifying electron deficiencies or excesses. When electrons are taken up, a reduction peak occurs. When the reaction is reversible, there is both an oxidation and a reduction peak. During mid-log phase (t=36 h) of *C. phytofermentans*, an irreversible reduction peak occurred. The peak was largest at the end of log phase (t=42 h) and slowly disappeared during stationary phase (t=87). This reduction peak was especially noticeable when peak area was plotted with cell concentration over time and was positively correlated with cell numbers during part of its growth ($R^2 = 0.92$; p<0.001 when t= 33-63 hours) (Fig. 11). This peak was not observed in uninoculated medium nor inoculated medium lacking cellobiose.
Figure 10: Voltammograms of *C. phytofermentans*. The voltammogram shows three cycles of potential sweep at four time periods: upon inoculation, at 36 hours, at 42 hours and at 87 hours (termination) from one of the cultures.
**Figure 11:** Peak area over time of *C. phytofermentans*. Reduction peak area (blue dots) over time during growth, of *C. phytofermentans* (orange line). Reduction peak values are the mean ± S.D. of four replicates.

At open circuit potential (OCP), the starting potential is not set for the duration of the experiment, but rather potential is measured at the start of each time point. Thus, the OCP voltammogram, as a whole, is able to shift if the open circuit potential does not remain constant. The starting potentials decreased over time, showing a change in the overall potential of the electrochemical system as the experiment continued.
Figure 12: Starting potential over time of *C. phytofermentans*. These findings are the result of four replicates at OCP. Values are the mean ± S.D of two staggered duplicate growth studies.

**Peak characterization**

Results of the CV revealed that the peak potential is -0.35 V vs. the Ag/AgCl electrode (Fig. 13). The CVs were conducted at 10, 40, 42.5 and 47.5 hours of growth with the reduction peak becoming evident in the CVs after 40 hours.
**Figure 13:** Reduction peak of *C. phytofermentans* versus reference electrode. A reduction peak can be seen at -0.35V vs. Ag/AgCl.

Plotting the peak current of the voltammogram at varying scan rates, formed a linear fit with an average $R^2$ value of $0.97 \pm 0.04$ (p=$<0.001$). Thus, the observed reduction peak is most likely associated with the surface of the bacteria and not the medium.
Figure 14: Linear regression of peak current versus scan rate. The peak current of each scan rate was plotted. Colors from the voltammogram correspond to the colors of the points plotted in the linear regression. Results from a single study are illustrated.

The location of the reduction peak was further evaluated by removing the bacterial cells from the medium and evaluating only the spent medium by CV. A reduction peak is not observed in the medium after filtration to remove the bacterial cells (Fig. 15).
**Figure 15:** Reduction peak confirmed to be associated with *C. phytofermentans*. Voltammograms of medium with bacterial cells (blue) and without bacterial cells (red) are shown. Results from a single study are illustrated.

*Electrochemical Impedance Spectroscopy*

The phase shift (Δφ) had an optimal frequency at 200 Hz and was observed to increase as time passed (Fig. 16). The rate of increase was much faster during lag phase of growth (t=0-27 h). Afterwards, the rate of increase began to decrease as *C. phytofermentans* entered logarithmic and stationary growth phase.
Figure 16: Phase shift (Δφ) during growth of *C. phytofermentans*. The Δφ at 200 Hz is (blue) is shown with protein concentration (red) over time during the growth of *C. phytofermentans*. Values are the mean ± S.D. of two growth studies.

Moreover, at an optimal frequency of 50 Hz, imaginary admittance also rose during lag phase (Fig. 17). However, unlike the Δφ, the rate began to stabilize halfway through lag phase of *C. phytofermentans* growth and decreased during stationary phase of growth.
Figure 17: Imaginary admittance ($\Delta Y''$) during growth of *C. phytofermentans*. The imaginary admittance at 50 (red) is shown along with optical density (blue) during the growth of *C. phytofermentans* growth. Values are the mean ± S.D. of two growth studies.
DISCUSSION

Electrochemical techniques offer an opportunity as a monitoring technology for bioprocesses because the growth and metabolism of microbes depends on a series of oxidation/reduction reactions (1). Hence, the movement of electrons via metabolic pathways and transport chains can be measured by these electrochemical technologies. The work presented here demonstrated that electrochemistry, specifically CV and EIS, may be used to provide key information regarding microbial metabolism and physiologic state in a bioprocess, which would allow for bioprocess optimization. The growth of the possibly industrially important *C. phytofermentans* was followed by traditional growth assays as well as CV and EIS. These electrochemical techniques were correlated with different phases of *C. phytofermentans* growth. These findings show that CV and EIS provide a tool for non-invasive *in-situ* monitoring of *C. phytofermentans*.

In this study, *C. phytofermentans* was grown on the carbohydrate cellobiose, a glucose disaccharide, a repeat unit found in cellulose (62). Growth of *C. phytofermentans* as measured by traditional microbiological techniques was similar to previous studies with an approximate three-day growth cycle (55). Growth was initially evaluated using optical density, protein concentration and DAPI-staining cell counts. Protein concentration and optical density data show similar measures for the three-day growth curves; however, the protein concentration had substantial error (data not shown). The medium components were found to interfere with the optical tests used for protein concentration analysis. We assessed this interference using a medium blank which
resulted in a false positive. A wash step was used to reduce the interference, which added to the potential for error as some protein could have been lost. Additionally, since the medium leads to a false positive, some carry over could add to the absorbance measured, increasing the calculated protein concentration. Furthermore, there are limitations to these quantification methods. As such, protein concentration within a microbial cell will vary depending on its growth phase (63).

Optical density, although more commonly used, faces similar challenges because of the changes in cell size during the growth curve as seen during DAPI staining (Fig. 9) (64). Moreover, neither optical density nor protein concentration provide a viable cell count. DAPI stains DNA and aids in visualization of C. phytofermentans while providing a more direct measure of the number of cells in a sample.

Intact membranes can easily be seen and cells counted (Fig. 9). However, non-living cells will maintain their membrane for a short time after cell death (65). Thus, DAPI also fails to produce a truly viable cell count. Also, endospores, unless compromised, do not allow DAPI to permeate, thus the blurry dots observed may not have been viable endospores (66). Hansen et al. showed that DAPI staining had a three-fold higher count than viable cell counts using plate culturing (65). In this study, C. phytofermentans was seen to congregate together on the filter rather than being evenly spread out further adding to error in the count. Consequently, it is necessary to increase the number of fields counted in subsequent studies or utilize others methods of for quantification. These errors in commonly used measurements of microbial growth further emphasize the need for more accurate bioprocess monitoring techniques.
Production of fermentation products coincided with the three-day growth pattern observed for *C. phytofermentans*. However, in contrast to other studies where ethanol production is higher than acetate, our HPLC data showed that acetate production was much higher than ethanol (56). A recent study found that ethanol and acetate production are pH dependent (67). When conditions were more acidic, acetate production was greater than ethanol production. These results show the importance of monitoring pH as changes result in different product concentrations. Once cellobiose was depleted from the medium (day two), production of acetate, ethanol and hydrogen ended. As an industrial bioprocess, information on the completion of fermentation is of tremendous value.

This study evaluated whether electrochemical methods can be used to replace or complement typically used microbiology monitoring techniques. Our results suggest the electrochemistry methods may be able to provide some information typically obtained from more traditional methods, however additional research is needed. The CV voltammogram showed changes in the electrochemistry of the microbial culture during growth; most notably, a reduction peak was observed starting around 36 hours of growth at the potential of -0.35 V vs. Ag/AgCl). This reduction peak indicated an electron deficiency in the electrochemical system. The reduction peak area, first observed at mid-log phase of growth declined once *C. phytofermentans* reached stationary phase at approximately 45 hours of growth. As an opposite oxidation peak was not observed this peak is said to be an irreversible peak (68). This occurrence indicates that the electrons are taken from the electrode and very quickly transferred away rather than transferred.
back to the electrode during the oxidative sweep portion of the CV (61). C. phytofermentans is the most likely component in the system able to permanently transfer electrons away from the electrode. We hypothesized that the reduction peak was associated with the C. phytofermentans bacteria rather than changes occurring in the liquid medium.

Varying the scan rates electrochemically confirmed that the peak was associated with the surface phase (cell membrane) rather than the bulk phase (spent medium) (61). A filtration study was completed whereby the bacteria were separated from the spent medium. After filtration, the peak was no longer visible further confirming that it was the C. phytofermentans that were taking electrons, not the medium (Fig. 15). In studies conducted by Matsunaga and Nakajima, the irreversible oxidation peak observed in their voltammograms correlated to CoA in the membrane of both Gram negative and Gram positive bacteria, grown under aerobic conditions (49). One main difference between the Matsunaga and Nakajima (1985) studies and those done in this study was the presence of oxygen. Oxygen is an important regulator and under anaerobic conditions, metabolic pathways are very different (69). During anaerobic metabolism protons (H+) and electrons (e-) are released from the various metabolic pathways occurring in the microbe (70). The protons are transferred out of the cell to maintain membrane potential (70). One of the main enzymes responsible for the transfer of protons and electrons is dehydrogenase. The separation of protons and electrons from growth substrate often results in the production of hydrogen under anaerobic conditions. For this reaction to occur, two protons must be reduced. Typically, this occurs with electrons donated by
ferredoxin, a co-factor, and the enzyme hydrogenase. It is likely that the observed reduction peak is associated with this process. Thus, one possibility for the peak is that of the NAD+ / NADH couple which has a potential of -0.32 ± 0.03 V vs. Ag/AgCl (70).

Additional studies testing the facultative anaerobes previously studied by Matsunaga and Nakajima under anaerobic conditions would be of great interest. Preliminary results produced by our lab observed a similar reduction peak in *E. coli* when grown under anaerobic conditions, similar to those *C. phytofermentans* (data not shown). These results indicate that the reduction peak may not be specific to *C. phytofermentans* and may instead be common in other fermentations. This would mean that these results could relate to other fermentation bioprocesses increasing the significance of this study.

Additional studies need to be performed to further evaluate whether the reduction peak correlates to NAD+ / NADH or some other component present in the membrane of *C. phytofermentans*. The appearance of the reduction peak, is an important indication that the culture will soon enter into stationary phase of growth. This information would be especially valuable in an industrial setting where a culture is grown under batch or fed batch conditions and real-time knowledge of growth phase could improve bioprocess efficiency. For example, L-glutamic acid and L-lysine are both produced using fermentation technology (1). Upon completion of fermentation, the products, such as L-glutamic acid and L-lysine, are ready to be extracted and there is no longer any need to continue the bioprocess.

During the use of OCP, the starting potential did not remain constant during the growth of *C. phytofermentans*, but rather was seen to decrease over time. This decrease
indicates that the system became more chemically reduced as *C. phytofermentans* grew. As *C. phytofermentans* grows, the chemically reduced products, acetate and ethanol, build up in the liquid phase. Subsequently, the starting potential coincided with this phenomenon. Monitoring the overall reduction of the system may also prove useful in an industrial process when determining appropriate sampling and/or harvesting times.

EIS is used to evaluate the $\Delta \phi$ in a system by measuring the change of the angle between current and voltage (32). This change will depend on whether impedance results from either reactance or resistance (32). The closer the shift is to 90°, the more the impedance is due to reactance and the less it is due to resistance. It has been hypothesized that the $\Delta \phi$ relates to the amount of lipid bilayer present in the system (60). Our results show the $\Delta \phi$ increasing throughout the growth of *C. phytofermentans* and slowing down as the cells reach stationary phase. Thus, as the cells are growing, the change in phase shift indicates an increase in reactance and/or a decrease in resistance within the electrochemical cell. This increase in $\Delta \phi$ may be the result of an increase in lipid bilayers. However, during this study, the lipid composition and concentrations were not measured. DAPI staining indicated the increase in cell numbers and mass during growth. The increase in cell mass would also result in the increase of lipid components of the membrane. As the cells elongate, their surface area to volume ratio changes (71). Based on the increased cell mass during growth of *C. phytofermentans*, it seems likely that the observed increase in $\Delta \phi$ throughout the growth period is related to an increased lipid content of the cells. Further studies to model the changes in surface area to volume ratio could help answer this question.
Admittance, the inverse of impedance is thought to relate to the activity of the cell as well as the energy storage and possibly adhesion to surfaces (60, 72). The results obtained in this study are consistent with this hypothesis. The admittance is shown to quickly increase during lag phase. During this time, although not dividing, cells are increasing their activity in preparation for division (63). The admittance slows down during exponential growth and begins to decrease during stationary and death. Lag phase is difficult to study as microbial cell numbers are low. Although our study does not determine what exactly is producing the observed admittance results, it is clear that changes in the electrochemistry are occurring during lag phase of growth. This is valuable information as microbial activity is not easily detectable. For example, if no change in admittance occurred in an industrial fermentation bioprocess, it would be an early warning sign that growth is inhibited in the bioreactor. This information would be very useful to the bioprocess operator and potentially avoiding bioprocess failure.

In summary, the results of these studies show that CV and EIS may be useful as a real-time measure of bacterial growth. These results are significant in that an industrial process can be monitored without the need to collect samples, which can disrupt or even contaminate a process. Furthermore, this methodology can potentially provide additional insight into various cellular metabolic processes such as those present in batch fermentations. Finally, the use of electrochemical methods as described in this study may lead to methods able to assess the growth of microbes in environmental settings such as during biorestoration of soils and groundwater. (49)


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