12-2015

ELECTROCHEMICAL ENHANCEMENT OF BUTANOL PRODUCTION AND XYLOSE CONSUMPTION IN PURE AND MIXED CULTURE FERMENTATIONS

Jovan Popovic
Clemson University, jpopovi@clemson.edu

ELECTROCHEMICAL ENHANCEMENT OF BUTANOL PRODUCTION AND XYLOSE CONSUMPTION IN PURE AND MIXED CULTURE FERMENTATIONS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Engineering and Science

---

by
Jovan Popovic
December 2015

Accepted by:
Dr. Kevin T. Finneran, Committee Chair
Dr. David L. Freedman
Dr. J. Michael Henson
Dr. Terry H. Walker

ABSTRACT

The volatility of foreign and domestic petroleum markets has prompted initiatives for the development of alternative liquid energy carriers which have the capacity to accommodate our current transportation infrastructure. Butanol, which has an energy density similar to that of gasoline and can be produced through the fermentation of carbohydrates by solventogenic *Clostridia*, has been investigated as a supplement or direct replacement to gasoline. However, most butanol fermentations rely on glucose as a feedstock which is in direct competition with our food supply, and this requires the exploration of alternative fermentable substrates.

This study investigates the use of electrochemical fermentation modifications as "drop-in" strategies to stimulate bacterial solventogenesis (butanol) and the consumption of xylose, which is the second most abundant sugar contained within lignocellulosic biomass. Monoculture, solventogenic *Clostridia* and mixed consortia were challenged with electron shuttling compounds and/or a terminal electron acceptor in the form of ferric iron or solid state graphite electrodes. Results from this study indicate that electron shuttling to ferrihydrite stimulates concomitant increases in metabolite production and xylose consumption for both pure and mixed culture fermentations in which xylose was utilized as the sole fermentable substrate. Mediatorless electrochemical stimulation of solventogenesis and xylose consumption was observed in fermentations challenged with an economical, solid-state graphite electrode system. Data presented within this study indicate that extracellular electron transport to terminal electron acceptors has a global, favorable effect on fermentative bacterial metabolism.

DEDICATION

The following dissertation is dedicated to family, both present and departed, for their unwavering support throughout the course of my journey. The stories of your triumph over obstacles and struggle to exist has provided and will continue to provide the necessary inspiration for my work.

TABLE OF CONTENTS

Table of Contents (Continued)

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

List of Figures (Continued)

Figure Page

List of Figures (Continued)

Figure Page

CHAPTER 1
INTRODUCTION

Introduction

Butanol is a four carbon alcohol with an energy density of 29.2 MJ/L, which is nearly equivalent to the energy density of 87 octane gasoline used in internal combustion engines {1}. Butanol has excellent buffering properties for engine longevity, and it has low miscibility with water in comparison to ethanol {3-4}. Recent studies have indicated that butanol fuel can be blended with gasoline at high ratios, or it can be used to directly replace gasoline {2-3}. Butanol is most widely used in the plastics, resin, paint, pharmaceutical, and automotive industries {1}. Its use as an industrial solvent is ubiquitous due to its many favorable chemical properties. The market for butanol in the chemical sector has been projected to reach $19 billion by the year 2020, making less costly processes more ideal to stay abreast with the demand {5}.

The acetone-butanol-ethanol (ABE) fermentation process rose to prominence during the First World War when Chaim Weizmann isolated the "Weizmann Bacillus (*C. acetobutylicum*)" and developed a process for the British to produce Cordite for artillery and ammunition during the great shell shortage in 1916, creating a favorable outcome for the Entente {6-7}. ABE fermentation processes declined as petroleum prices tumbled during the 1950s, and petrochemical synthesis of these chemicals prevailed until recent spikes in the price of petroleum which are attributed to global political unrest and price fixing.

Biological butanol synthesis is limited to select wild-type strains of *Clostridium* {8}, most of which are endospore-forming obligate anaerobes. These Bacteria are

classified under the *Firmicutes*, and are Gram positive (or Gram variable) organisms with a low G+C mol%. Substrates, such as glucose or xylose, are chemically transformed into secondary metabolites such as volatile acids, hydrogen, ketones, and alcohols (butanol and ethanol) through a cascade of central and solventogenic metabolic reactions (Figure 1-1).

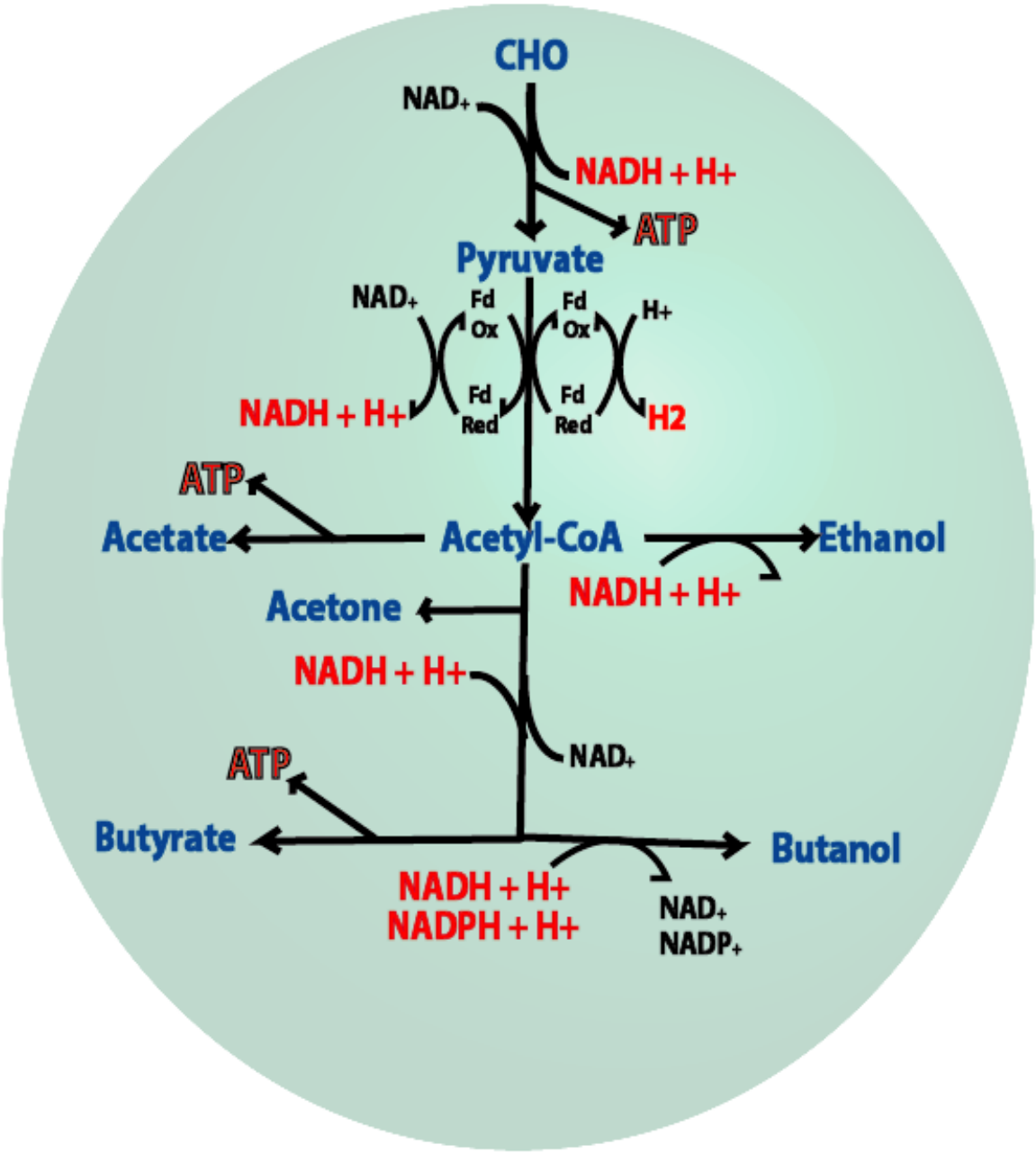

Figure 1-1: Simplified pathways of central and solventogenic metabolism in ABE producing *Clostrdium* species. CHO represents carbohydrate (glucose or xylose) entry into central metabolism. Ratios of pyruvate, acetyl-CoA, ATP, NADH, NADPH, and metabolites per mol carbohydrate have not been included in this diagram.

Substrate Utilization

Substrate cost and competition with food sources has favored the replacement of glucose as a feedstock for future butanol fermentations {9-11}. Recently, many have been investigating the use of hemicellulose as a feedstock due to natural abundance. Hemicellulose is a polymer which composes nearly 30-40% of the earth's carbon {12-14}, and xylose and arabinose are pentose monomers which compose hemicellulose. Although terrestrially abundant, pentose catabolism is limited or non-existent in many industrially relevant organisms, and although xylose flux is poorly understood in solventogenic organisms, many efforts are being made to elucidate the processes surrounding its uptake and metabolism for manipulation.

The favorability of hexose over pentose sugars is apparent from the rate of substrate co-fermentation within *Clostridia* {15-16}. Xylose can be metabolized in bacterial cells following entry catalyzed by proton motive force (symport) {17-19}. Following entry into the cell, xylose is metabolized via the pentose phosphate pathway (PPP) or the phosphoketolase pathway in organisms such as *C. acetobutylicum*, accounting for the slower rate of utilization attributed to the requirement of additional metabolic steps prior to entry into central and/or solventogenic downstream pathways.

In the PPP, xylose is phosphorylated to xylulose -5-phosphate, and from this position, xylulose-5-phosphate is converted to fructose-6-phosphate or glyceraldehyde-3-phosphate, allowing for entry into glycolysis {19}. Recently, evidence has shown that under high xylose concentrations (20 g/L), the phosphoketolase pathway in *C.*

acetobutylicum is upregulated, indicating that some solventogenic *Clostridia* possess the ability to metabolize xylose in parallel pathways under high substrate stress {20}. This allows for xylose to be converted to glyceraldeyhyde-3-phosphate or acetyl-phosphate, which can be shunted to acetyl-CoA, thus bypassing glycolysis if the phosphoketolase pathway is activated.

Electron Shuttling

Natural secretion of redox mediating compounds has been observed throughout many prokaryotic genera, including but not limited to *Shewanella, Pseudomonas,* and *Clostridium*. Previous studies have indicated that select strains of *Pseudomonas* can secrete phenazine compounds to distort the typical electron flow of competing organisms {21-22}. In anaerobic environments, *Shewanella* has the ability to secrete flavins to mediate electron transfer to terminal electron acceptors, allowing for continuation of metabolic processes {23}. Flavin secretion in the *Clostridia* has not been studied in depth, and the function of flavins in *Clostridial* metabolism has yet to be elucidated.

Analogs of these natural electronophores have found their value in laboratory studies. In a study performed with *E. coli*, it was shown that the polycyclic dye, neutral red, can be used to mediate the electron transfer between *E. coli* hydrogenases and ferric iron {24}. In a separate study using *C. acetobutylicum*, neutral red stimulated butanol production in a previously acidogenic culture, and this was associated with hydrogenase regulation {25}. Metabolic alterations at the hydrogenase level were apparent in other studies which observed an increase in hydrogen evolution from *C. beijerinckii* using

AQDS as the redox mediator {26-28}. The significance of these data lie within the idea that exogenous redox mediators can adapt metabolic processes which were formerly thought to be tightly regulated to follow alternative metabolism. It has been previously shown that electron shuttling compounds have the ability to cycle metabolic cofactors, NAD/NADH {29,30}, but direct in vitro NADH oxidation has not been observed when ferric iron was used as the sole electron sink, indicating that synthetic redox mediating compounds act as conduits between cellular metabolism and iron, similar to that which has been described previously {Figure 1-2; 23-24, 31}.

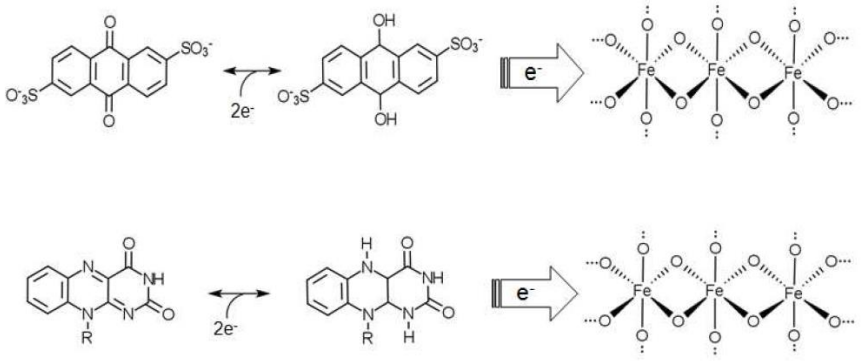

Figure 1-2. Proposed electron transfer model describing electron flow from AQDS (top) and riboflavin (bottom) to ferrihydrite

Objectives and Hypotheses

The broad objectives of the proposed dissertation research is to determine the extent to which redox mediation can alter the fermentative dynamics of butanol producing organisms to increase overall solvent titers and increase substrate consumption, with specific emphasis being placed on xylose consumption.

More specifically, redox mediation will be performed with electroactive chemicals, and the objectives for this study are aligned with removing electrons from fermentations to relieve thermodynamic constraints placed on organisms which have respiratory limitations. These objectives will be met with the following hypotheses:

1.) The presence of electron shuttles coupled to insoluble ferrihydrite will increase xylose consumption and solvent production in a wild type industrial strain.

2.) ABE producing *Clostridia* not restricted to the *beijerinckii* species will display the same phenotype as *C. beijerinckii* when challenged with electron shuttles, thus providing evidence for global regulation of ABE metabolism.

3.) Electron shuttling to ferrihydrite will influence the fermentative phenotype of mixed microbial communities to produce increased levels of butanol and exhibit increased consumption of xylose.

4.) Application of a low cost graphite electrode system will stimulate solventogenic productivity of solventogenic *Clostridia*.

References

1.) Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by Clostridia. *Biotechnology and Bioengineering*, *101*(2), 209-228.

2.) Wallner, T., Miers, S. A., & McConnell, S. (2009). A comparison of ethanol and butanol as oxygenates using a direct-injection, spark-ignition engine. *Journal of Engineering for Gas Turbines and Power*, *131*(3), 032802.

3.) Szwaja, S., & Naber, J. D. (2010). Combustion of *n*-butanol in a spark-ignition IC engine. *Fuel*, *89*(7), 1573-1582.

4.) Cooney, C., Wallner, T., McConnell, S., Gillen, J. C., Abell, C., Miers, S. A., & Naber, J. D. (2009, January). Effects of blending gasoline with ethanol and butanol on engine efficiency and emissions using a direct-injection, spark-ignition engine. In *ASME 2009 Internal Combustion Engine Division Spring Technical Conference* (pp. 157-165). American Society of Mechanical Engineers.

5.) N-Butanol Market by Applications (Butyl Acrylate, Butyl Acetate, Glycol Ethers, and Others) & Geography-Global Trends & Forecasts to 2018. *Markets and Markets*. 2013.

6.) Brown, G. I. (2011). Explosives: History with a Bang. The History Press.

7.) Patakova, P., Maxa, D., Sebor, G., Lipovsky, J., Melzoch, K., Paulova, L., & Muzikova, Z. (2011). Perspectives of biobutanol production and use. *INTECH Open Access Publisher*.

8.) Valentine, J., Clifton-Brown, J., Hastings, A., Robson, P., Allison, G., & Smith, P. (2012). Food vs. fuel: the use of land for lignocellulosic 'next generation'energy crops that minimize competition with primary food production. *GCB Bioenergy*, *4*(1), 1-19.

9.) Yang, M., Kuittinen, S., Zhang, J., Vepsäläinen, J., Keinänen, M., & Pappinen, A. (2015). Co-fermentation of hemicellulose and starch from barley straw and grain for efficient pentoses utilization in acetone–butanol–ethanol production. *Bioresource Technology*, *179*, 128-135.

10.) Xue, C., Zhao, X. Q., Liu, C. G., Chen, L. J., & Bai, F. W. (2013). Prospective and development of butanol as an advanced biofuel. *Biotechnology Advances*, *31*(8), 1575-1584.

11.) Zhang, F., Rodriguez, S., & Keasling, J. D. (2011). Metabolic engineering of microbial pathways for advanced biofuels production. *Current Opinion in Biotechnology*, *22*(6), 775-783.

12.) Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*, *48*(8), 3713-3729.

13.) Gong, C. S., Chen, L. F., Flickinger, M. C., & Tsao, G. T. (1981, January). Conversion of hemicellulose carbohydrates. *In Bioenergy* (pp. 93-118). Springer Berlin Heidelberg.

14.) Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W. J., Jiang, W., & Yang, S. (2011). Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. *Applied and Environmental Microbiology*, *77*(22), 7886-7895.

15.) Chen, Y., Zhou, T., Liu, D., Li, A., Xu, S., Liu, Q., & Ying, H. (2013). Production of butanol from glucose and xylose with immobilized cells of *Clostridium acetobutylicum*. *Biotechnology and Bioprocess Engineering*, *18*(2), 234-241.

16.) Grimmler, C., Held, C., Liebl, W., & Ehrenreich, A. (2010). Transcriptional analysis of catabolite repression in *Clostridium acetobutylicum* growing on mixtures of d-glucose and d-xylose. *Journal of Biotechnology*, *150*(3), 315-323.

17.) Jeffries, T. W. (1983). Utilization of xylose by bacteria, yeasts, and fungi (pp. 1-32). Springer Berlin Heidelberg.

18.) Walmsley, A. R., Barrett, M. P., Bringaud, F., & Gould, G. W. (1998). Sugar transporters from bacteria, parasites and mammals: structure–activity relationships. *Trends in Biochemical Sciences*, *23*(12), 476-481.

19.) MA, C. (1958). Metabolism of pentoses by clostridia. II. The fermentation of C14-labeled pentoses by *Clostridium* per fringens, *Clostridium beijerinckii*, and *Clostridium* butylicum. *Journal of Bacteriology*, *75*(3), 335-338.

20.) Liu, L., Zhang, L., Tang, W., Gu, Y., Hua, Q., Yang, S., & Yang, C. (2012). Phosphoketolase pathway for xylose catabolism in *Clostridium acetobutylicum* revealed by 13C metabolic flux analysis. *Journal of Bacteriology*, *194*(19), 5413-5422.

21.) Wang, Y., Kern, S. E., & Newman, D. K. (2010). Endogenous phenazine antibiotics promote anaerobic survival of Pseudomonas aeruginosa via extracellular electron transfer. *Journal of Bacteriology*, *192*(1), 365-369.

22.) Thomashow, L. S., & Weller, D. M. (1988). Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis var. tritici. Journal of Bacteriology*, *170*(8), 3499-3508.

23.) Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., & Bond, D. R. (2008). *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences*, *105*(10), 3968-3973.

24.) McKinlay, J. B., & Zeikus, J. G. (2004). Extracellular iron reduction is mediated in part by neutral red and hydrogenase in Escherichia coli. *Applied and Environmental Microbiology*, *70*(6), 3467-3474.

25.) Girbal, L., Vasconcelos, I., Saint-Amans, S., & Soucaille, P. (1995). How neutral red modified carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH. *FEMS Microbiology Reviews*, *16*(2), 151-162.

26.) Ye, X., Morgenroth, E., Zhang, X., & Finneran, K. T. (2011). Anthrahydroquinone-2, 6,-disulfonate (AH2QDS) increases hydrogen molar yield and xylose utilization in growing cultures of *Clostridium beijerinckii*. *Applied Microbiology and Biotechnology*, *92*(4), 855-864.

27.) Ye, X., Zhang, X., Morgenroth, E., & Finneran, K. T. (2013). Exogenous anthrahydroquinone-2, 6-disulfonate specifically increases xylose utilization during mixed sugar fermentation by *Clostridium beijerinckii* NCIMB 8052. *International Journal of Hydrogen Energy*, *38*(6), 2719-2727.

28.) Hatch, J. L., & Finneran, K. T. (2008). Influence of reduced electron shuttling compounds on biological H2 production in the fermentative pure culture *Clostridium beijerinckii*. *Current Microbiology*, *56*(3), 268-273.

29.) Park, D. H., & Zeikus, J. G. (2000). Electricity generation in microbial fuel cells using neutral red as an electronophore. *Applied and Environmental Microbiology*, *66*(4), 1292-1297.

30.) Park, D. H., Laivenieks, M., Guettler, M. V., Jain, M. K., & Zeikus, J. G. (1999). Microbial utilization of electrically reduced neutral red as the sole electron donor for growth and metabolite production. *Applied and Environmental Microbiology*, *65*(7), 2912-2917.

31.) Mehta, T., Coppi, M. V., Childers, S. E., & Lovley, D. R. (2005). Outer membrane c-type cytochromes required for Fe (III) and Mn (IV) oxide reduction in Geobacter sulfurreducens. *Applied and Environmental Microbiology*, *71*(12), 8634-8641.

CHAPTER 2

INCREASING XYLOSE CONSUMPTION AND BUTANOL PRODUCTION WITH FERRIC IRON AND EXTRACELLULAR ELECTRON SHUTTLING MOLECULES DURING FERMENTATION WITH *CLOSTRIDIUM BEIJERINCKII* NCIMB 8052

Abstract

Xylose is the second most abundant sugar derived from lignocellulosic pre-treatment, and strategies that increase xylose utilization in wild type cells are desirable goals for the biofuels industry. *Clostridium beijerinckii* NCIMB 8052 is a widely used Bacterium for producing biofuels, usually with glucose as the primary substrate. Xylose consumption, butanol production, and hydrogen production increased in both *C. beijerinckii* and a newly isolated, solventogenic bacillus (strain DC-1) when anthraquinone-2,6,-disulfonate or riboflavin were used as redox mediators to transfer electrons to ferrihydrite as an extracellular electron sink. Strain DC-1 was most closely related to *Rhizobiales* bacterium Mfc52 based on 95% 16S rRNA gene sequence similarity, which demonstrates that this response is not limited to a single genus of xylose fermenting Bacteria. Xylose utilization and butanol production (0.05 g/L) were minimal in controls containing cells plus 3% (w/v) xylose alone during a 10-day batch fermentation, for both strains tested. AQDS and riboflavin were added as electron shuttling compounds with ferrihydrite as an insoluble electron acceptor, and respective *n*-butanol titers increased to 6.35 g/L and 7.46 g/L. Increases in xylose consumption for the iron treatments were substantial; 25.98 g/L and 29.15 g/L for the AQDS and riboflavin treatments, respectively, compared to control incubations that consumed just 0.49 g/L xylose. Hydrogen production was 3.68 times greater for the AQDS treatment and 5.27 greater for the riboflavin treatment relative to controls. Strain DC-1 data were similar; again indicating that the effects are not specific to the genus *Clostridium*.

Broader Context

Butanol (*n*-butanol), a long-chain alcohol, has a higher energy density than ethanol, which is currently used to supplement gasoline supplies in the United States. Butanol is a co-product during ABE fermentation. However, typical ABE fermentations are inefficient with respect to solvent production and substrate consumption, and most of the industrial focus is on the utilization of glucose, which is commonly derived from sources that are in direct competition with human food. This has led to the investigation of xylose utilization as a feedstock since xylose composes a large percentage of non-consumable biomass. The strategy presented here addresses constraints within the characteristic flow of electrons in ABE fermentation with wild type (non-genetically modified) cells to remedy low levels of xylose consumption as well as butanol yields.

Introduction

Butanol (*n*-butanol) is used as a chemical feedstock in several industrial sectors, and has been suggested as a biofuel alternative to ethanol {1-3}. Biologically synthesized butanol is attractive as a supplement for the world's gasoline powered transportation infrastructure due its favorable combustion properties, and high energy content. However, substrate costs, poor substrate utilization, low productivity, and low solvent titers continue to impede advancements of traditional industrial fermentations.

The global market for *n*-butanol has risen sharply in the last decade and it is projected to reach $9.4 billion by 2018 {4}. Butanol is considered an alternative fuel either as an amendment to gasoline in lieu of ethanol, or as a stand-alone liquid fuel for

next generation vehicles {5}; the latter use is less likely given current production. Although it is a low molecular mass alcohol, similar to ethanol, it has a much higher energy content. Complete butanol combustion releases 29.2 MJ/L while ethanol releases 19.6 MJ/L {6}. Butanol is stable and has good blending characteristics for use with traditional gasoline. It has become a significant target for alternative energy platforms in the U.S. and abroad. However, butanol derived from petroleum is undesirable due to uncertainty in the petroleum market and because petroleum-based fuels are regarded as less sustainable than operationally defined renewable fuels. Synthetic biological processes, such as ABE fermentations, are being investigated to supplement these approaches {7-10}.

ABE fermentation is promising but does have several limitations, including, but not restricted to low solvent yields, poor substrate utilization, and low biomass conversion rates {11}. These limitations have been addressed through genetic modifications and/or reactor engineering design alterations {12-14}. Both of these approaches have been successful, but these strategies are typically proprietary, and they often cannot be inserted into existing reactor infrastructure due to economic infeasibility. Emerging biofuels markets are seeking "drop-in" technologies that will work with current reactor designs with few modifications {15-16}. In addition, genetically modified organisms (GMOs) are often stigmatized by a skeptical public that views the term as negative, despite the strong progress made in the field.

Hemicellulose monomers such as xylose are desirable feedstocks for industrial ABE production since they are not in direct competition with animal feed {17}. Xylose, a

pentose sugar, composes nearly 30% of all plant-derived biomass {18}. Many organisms cannot efficiently ferment xylose, or they lack the necessary machinery to transport and assimilate it into central metabolism {19}. This has made glucose the preferential sugar substrate; it is readily fermented by most industrial solvent producing strains {20}. Certain members of the genus *Clostridium* rely either on xylose proton symporters or ATP-dependent xylose transport mechanisms to move the sugar molecules across the cell membrane {21}. Furthermore, xylose must be converted prior to glycolysis, with steps that require ATP hydrolysis and regeneration of NAD^+ and $NADP^+$ cofactors {22}. While glucose is usually 100% fermented by the cells of interest, xylose utilization in ABE fermentation is typically 20% or less {23}, and any strategies that increase xylose utilization will be beneficial to mixed sugar fermentations that rely on a variety of plant feedstocks.

Previous reports suggest that electron mediators such as neutral red alter substrate utilization, solventogenesis, and most recently, hydrogen production {24-27}. Butanol production was increased in comparison to controls in pH-controlled, fed batch anode (electrode) reactors amended with methyl viologen; the sole substrate was glucose {28}. More recently the redox potential of a fed batch reactor was modified using a potentiostat to mimic "near oxic" conditions, and data indicated that solvent yield increased relative to controls {29}. Influencing NAD^+/NADH ratios was reported to increase production of certain fermentation end products {30-31}. However, previous studies utilized glucose as the sole substrate, and did not directly address improved substrate utilization due to electron stripping {25-26, 28-30}.

The following data demonstrate that *Clostridium beijerinckii* and non-*Clostridia* fermentative cells can utilize extracellular electron transfer molecules and ferric iron to alter the normal flow of carbon and electrons during xylose fermentation, thereby substantially increasing both solventogenesis and xylose utilization in ABE producing organisms. Both of these are desirable outcomes for eventual use in reactors with wild type organisms used in biofuel production.

Materials and Methods

Culture Maintenance

Clostridium beijerinckii strain 8052 was re-vegetated from frozen spores to start each experiment. A 50 µL volume of the spore suspension was used to inoculate 10 mL of fresh tryptone-yeast-glucose (TYG) media, and this was incubated at 37 ^{o}C for 28 hours. Fresh TYG media was inoculated with 3% (v/v) of a dormant culture of strain DC-1. This was incubated for 28 h at 37 ^{o}C. TYG medium consisted of 30 g/L tryptone, 20 g/L glucose, and 10 g/L yeast extract in nanopure water. TYG medium was prepared by dispensing 10 mL of media into anoxic Balch pressure tubes, and each was sparged with nitrogen for 10 minutes in the liquid phase, followed by a 1-minute degassing of the headspace. Tubes were capped with blue butyl stoppers and sealed with aluminum crimps. The tubes were autoclaved for 15 min at 121^{o}C and cooled prior to inoculation.

Experimental Conditions

Experiments using xylose as the fermentation feedstock were run in defined P2 medium, which consisted of 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 2.0 g/L $(NH_4)_2SO_4$, 10 mL/L mineral mix (20 g $MgSO_4$, 1 g $MnSO_4$, 1 g NaCl, and 1 g $FeSO_4$), and 10 mL/L vitamin mix (100 g p-aminobenzoic acid, 1 g biotin, and 10 g thiamine). P2 media for the batch fermentation containing *Clostridium beijerinckii* NCIMB 8052 was prepared in 160 mL serum bottles; the media was sparged with nitrogen for 15 min, and each headspace was degassed for 5 min. P2 media for DC-1 batch fermentation was prepared in anaerobic Balch tubes; the media was sparged with nitrogen for 10 min in the liquid phase, followed by a 1-min degassing of the headspace. Bottles and tubes were capped, crimped and autoclaved at 121 ^oC for 20 min. Final liquid volumes at the onset of experimentation for *Clostridium beijerinckii* NCIMB 8052 and DC-1 were 100 mL and 10 mL, respectively.

C. beijerinckii cells (6% v/v) were transferred from a culture grown on TYG for 28 h into P2 media containing 6 g/L xylose; this was incubated for an additional 28 h. A 6% (v/v) transfer of this culture was made to experimental 160 mL bottles. Strain DC-1 was transferred from a dormant culture to TYG media and allowed to incubate at 37 ^oC for 28 h. Following the 28-h incubation, 6% (v/v) of the culture grown on TYG was transferred to P2 media containing 6 g/L xylose, this incubated for 28 h at 37 ^oC. A 6% (v/v) transfer of this was made to experimental tubes, bringing the final volume to 10 mL at the start of each experiment.

The experiment in which glucose was used as the fermentation substrate was prepared as described previously {32}. This media recipe contained supplementary acetate in the form of ammonium acetate. The fermentation broth was prepared under anoxic conditions in Balch tubes, and the final glucose concentration was 6% (w/v). An additional replicate was prepared in 160 mL serum bottles for pH monitoring throughout the fermentation. Prior to autoclaving, bottles were sparged and degassed with nitrogen, capped with blue butyl stoppers, and crimped. Final liquid volume at the onset of experimentation was 100 mL.

Stock solutions of 20 mM AQDS (Sigma, 98%), 20 mM riboflavin (Sigma, 98+%), and 1 M poorly crystalline Fe(III) oxy(hydroxide) (ferrihydrite) {33} were prepared in Nanopure nanopure H_2O, sparged for 15 min in the liquid phase, and degassed for 5 min in the headspace with nitrogen which was passed over a heated copper column to remove trace oxygen. The bottles were then sealed with blue butyl stoppers and crimped with aluminum caps. Stocks were autoclaved at 121 ^{o}C for 20 min, and upon cooling, they were transferred to respective experimental bottles prior to inoculation. Chemical structures of the oxidized versus reduced forms are illustrated in Figure 1-1.

Stock solutions of 600 g/L xylose and 600 g/L glucose were prepared in Nanopure H_2O, and they were degassed following the same procedures as listed above. Anoxic sugar stock solutions were filter sterilized into experimental triplicates using sterile 0.2 μm filters (Pall Supor Membrane) prior to starting the experiments.

Samples were collected periodically over a 240-h period. For each experimental analysis, 0.3 mL was withdrawn from experimental triplicates using sterile, anoxic syringes, and samples were filtered through 0.2 μm filters into autosampler vials with 250 μL glass inserts (Lab Supply Distributors). Screw top PFTE caps were used to seal the vials, and the samples were stored at 4 ^{o}C until GC and HPLC analyses. For the 100 mL batch fermentations, an additional 3 mL of culture broth was removed from experimental bottles at each time point, and they were eluted into clean 15 mL screw top conical tubes for pH analysis. The pH was analyzed immediately after sample withdrawal. Headspace H_2 was analyzed following liquid sampling. Headspace gas (0.5 mL) was withdrawn from each sample and analyzed as described below.

Analytical Techniques

Solvents were analyzed using a Shimadzu 2014 gas chromatograph with a flame ionization detector (GC-FID) equipped with an autosampler and an Agilent DB-FFAP column (30-m x 0.250-mm; 0.25 μm film thickness), using helium as the carrier gas at a constant pressure of 125 kPa. Injector and detector temperatures were 200 ^{o}C and 250 ^{o}C, respectively. Liquid injections (1 μL) were used for sample analysis, followed by a methanol wash step and two Nanopure H_2O rinse steps prior to each successive injection. The temperature program included a 40 ^{o}C initial dwell for 4 min, followed by a temperature ramp at a rate of 50 ^{o}C/min until the column oven reached 220 ^{o}C. The column was held at this temperature for 4 min until cool down.

Xylose, glucose and organic acids were analyzed using a Dionex high performance liquid chromatograph (HPLC) equipped with a Bio-Rad HP-Aminex column. The mobile phase consisted of 5 mM degassed H_2SO_4, and the flow rate was 0.6 mL/min. Temperatures of the column oven and the RI detector were 60 ^oC and 50 ^oC, respectively. Organic acids were quantified using a UV-Visible wavelength detector set at 210 nm. Xylose and glucose were quantified using a Shodex RI-101 refractive index detector.

Headspace hydrogen was analyzed using a Shimadzu GC-8A equipped with an internal TCD and a 100/120 Carbosieve SII column (10' length x 1/8" outer diameter). Ultra high-purity nitrogen was the carrier gas at a constant pressure of 400 kPa. The TCD voltage was set at 60V; the column temperature was set at 50 ^oC, and the injector and detector temperatures were set at 150 ^oC. A VICI gas-tight syringe was used to deliver a 0.5 mL injection volume for each sample analyzed. Headspace hydrogen values were used to calculate total hydrogen present within the liquid and headspace using the dimensionless Henry's constant for hydrogen (0.01907 at 25 ^oC). The gas transfer calculation for closed systems has been described previously {34}.

pH was measured with a Thermo Scientific OrionStar A111 pH meter equipped with an Orion 9107BNMD probe. The meter was calibrated prior to each use with a reference standard buffer kit.

Total HCl-extractable ferrous iron accumulation was measured spectrophotometrically throughout the time course of each insoluble ferrihydrite amended

fermentation using the Ferrozine assay {35}. Samples (0.1 mL) were directly filtered into acid-washed vials containing 0.5 N HCl (4.9 mL) using 0.2 μm syringe filters. The ferrous iron extract (0.1 mL) was added directly to acid washed vials containing room temperature Ferrozine reagent (4.9 mL) which is composed of 11.62 g/L HEPES buffer and 1 g/L Ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid; Sigma, 97%) in Nanopure water (final pH 7). Samples were aliquoted into polystyrene cuvettes and immediately analyzed at 562 nm using a Thermo Fisher GENESYS 10S UV-Vis spectrophotometer.

Biomass was quantified by measuring total dry weight of cells. Aluminum weigh boats were baked overnight at 100 ^{o}C and were cooled in a desiccation chamber. Weigh boats containing cells were baked overnight at 100 ^{o}C and subsequently weighed after cooling to determine the total dry weight in grams per liter. Residual xylose was quantified and subtracted from the total weight, thus providing a more accurate biomass reading.

Kinetic Modeling

A modified Gompertz equation (Equation 1) was used to fit the cumulative metabolite production curve to obtain the metabolite production rate, $R_{Metabolite}$ {36-40} for butanol. Volumetric production rate (in units of g butanol/L/h) were calculated by normalizing the $R_{Butanol}$ by the volume of the medium in the batch experiments. The same equation was also used to fit the substrate utilization, plotted as substrate utilized versus time. Substrate utilization rates were expressed as g/L/h. λi is in units of hours.

$$P = P_{max} * \exp\left\{-\exp\left[\frac{\exp(1)*R}{P_{max}(\lambda - t)} + 1\right]\right\}$$ (Equation 1)

Electron Balances

Electron flow modeling was performed as described previously {41-42}. Xylose oxidation was assumed to yield 20 electrons per mol fermented; biomass, butanol, ethanol, acetone, hydrogen, acetate, and butyrate production were assumed to consume 28, 24, 12, 16, 2, 8, 20 electron equivalents per mol, respectively. Electron equivalents consumed in biomass formation was substracted from total available electron equivalents from acetyl-CoA. Electron equivalents consumed in the reduction of iron were accounted for by subtracting observed values from the total electrons liberated as a result of xylose oxidation. Since electron flow to solvents has not been previously modeled using this method, the following assumptions were made on a per mol basis:

- Σe-acetyl-CoA = [½ meq Acetoacetyl-CoA + meq Acetate +meq Ethanol] – [meq NADH + meq NADPH] (Equation 2)
- Σe-acetoacetyl-CoA = meq Butyryl-CoA + meq Acetone (Equation 3)
- Σe-butyryl-CoA = meq Butanol + meq Butyrate (Equation 4)
- 1 mol butyryl-CoA consumes 2 mol NADH for its formation from Acetyl-CoA
- From butyryl-CoA, butyrate and butanol formation share one third and two thirds mol NADH consumed from butyryl-CoA formation, respectively. This is intended

to account for NADH sharing and the extra NADH consumed in butanol formation.

- 1 mol NADPH are consumed per mol butanol produced
- 2 mol NADH is consumed per mol ethanol produced

Results and Discussion

Electron shuttle-enhanced xylose utilization and solventogenesis in C. beijerinckii NCIMB *8052*

Ten-day batch studies were performed to demonstrate that electron shuttling to ferric iron increased xylose consumption and butanol production with 3% xylose as the sole fermentable substrate. Acetate was not supplemented in any experiment. Xylose consumption was limited in the controls containing cells and xylose alone (0.49 g/L), along with the incubation containing ferrihydrite alone (3.76 g/L; Figure 2-1); though xylose consumption and butanol production in the ferrihydrite alone incubations were higher than xylose only controls. However, cells amended with riboflavin or AQDS plus ferrihydrite consumed 29.15 g/L and 25.98 g/L xylose, respectively. These levels of xylose consumption have thus far only been reported for genetically modified *Clostridium* strains, where the xylose utilization genes had been altered to increase uptake and metabolism {41-42}; to the best of our knowledge this is the first report of nearly 100% xylose utilization in wild type *C. beijerinckii* NCIMB 8052.

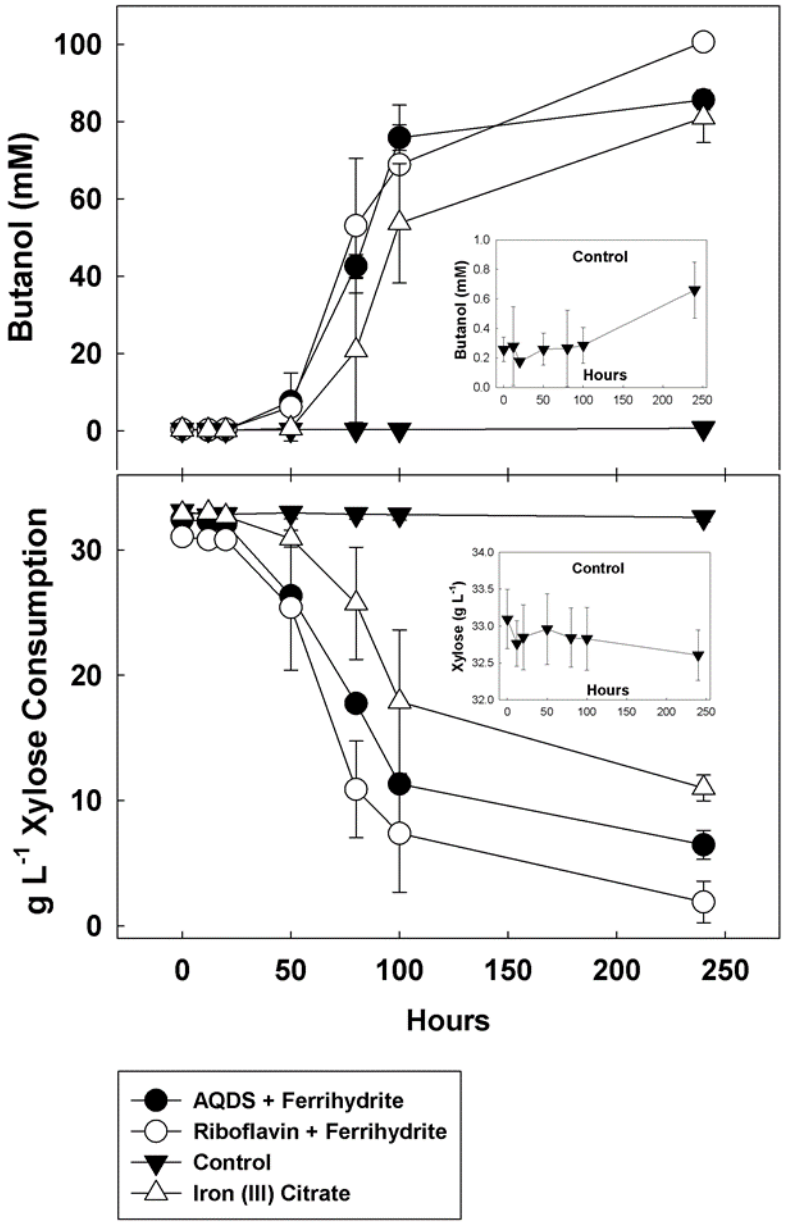

Figure 2-1: Butanol production and xylose consumption using *C. beijerinckii* NCIMB 8052 and 3% xylose as the sole fermentable substrate. Error bars represent standard deviation between triplicates.

Butanol titers were monitored throughout the study to demonstrate the effects of electron shuttling on solventogenesis. Butanol production in controls containing xylose alone (0.05 g/L; Figure 2-1) or ferrihydrite plus xylose (0.26 g/L; Figure 2-1) was relatively limited after ten days of incubation. Cells amended with ferrihydrite plus AQDS or riboflavin produced butanol at much higher concentrations (Figure 2-1), the respective butanol titers for AQDS and riboflavin amended batches were 6.34 g/L and 7.45 g/L at the end of the batch fermentation. The rates of butanol production were also significantly higher ($p < 0.05$) in the electron shuttle amended incubations (Table 2-1).

The solubility of the specific electron sink influenced xylose consumption and butanol production in ABE fermentations. Soluble iron in the form of Fe^{3+} citrate was also utilized as an electron sink in fermentations containing 3% xylose to which AQDS or riboflavin were not added. After ten days of growth, respective xylose consumption and butanol production levels were 21.88 g/L and 6.01 g/L (Figure 2-1), which are similar to those with AQDS plus insoluble ferrihydrite and riboflavin plus ferrihydrite. Citrate alone was not fermented. However, xylose fermentations amended with 20 mM citrate produced 4.1 times less butanol than the Fe^{3+} citrate amended triplicate (data not shown). The increase in solvent production in the citrate amended treatment compared to the 3% xylose control can be attributed to an elevated buffering capacity which prevents a rapid decrease in pH {43}.

Experimental data were fitted using a modified Gompertz equation as previously reported {34-38}. Xylose consumption rates for the treatments containing ferrihydrite supplemented with AQDS and riboflavin were 104 times and 148 times greater than the

control and 6.7 times and 9.6 times greater than the treatment amended with ferrihydrite plus xylose, respectively (Table 2-1; Figure 2-2). The Gompertz function was unable to fit the data generated for butanol productivity in the batch that was amended with cells plus 3% xylose (alone) due to the low levels of butanol production in those controls (see inset within Figure 2-1). However, data fitting was possible for the triplicate containing cells and 3% xylose in 50mM KH_2PO_4 buffered P2 media (Table 2-1). Increasing the phosphate buffering capacity within Clostridial batch fermentations resulted in a greater extent of substrate utilization and solvent production possibly through decreasing undissociated organic acids to levels which are less cytotoxic{44}. This is reflected in the batch containing the elevated phosphate salt concentration.

	Butanol Productivity (g /L/h)	λ (h)	Butanol Pmax (g/L)	Xylose Consumption Rate (g/L/h)	Butanol Yield (g/g)
Cells + Xylose Control	ND*	ND*	ND*	0.0033 ± 0.00	0.100
Overbuffered Control**	0.0005 ± 0.00	17.4983 ± 23.83	0.1177 ± 0.06	ND*	ND*
20 mM FeGel	0.0025 ± 0.00	2.7043 ± 10.71	0.2641 ± 0.02	0.0510 ± 0.01	0.072
20 mM Iron(III) Citrate	0.1631 ± 0.04	65.8961 ± 4.88	6.5280 ± 0.31	0.3355 ± 0.06	0.275
500 µM AQDS + 20 mM FeGel	0.1915 ± 0.04	63.4988 ± 5.63	6.7022 ± 0.20	0.3420 ± 0.02	0.244
500 µM Riboflavin + 20 mM FeGel	0.1252 ± 0.02	56.5378 ± 4.56	6.7705 ± 0.28	0.4884 ± 0.03	0.256

Table 2-1: *C. beijerinckii* NCIMB 8052 butanol productivities and xylose consumption in 100mL batch fermentations containing 3% xylose

* ND = Not Determined

** Cells + 30 g L^{-1} xylose control in 50 mM KH_2PO_4 overbuffered P2 media (excluding the effects of acid crash)

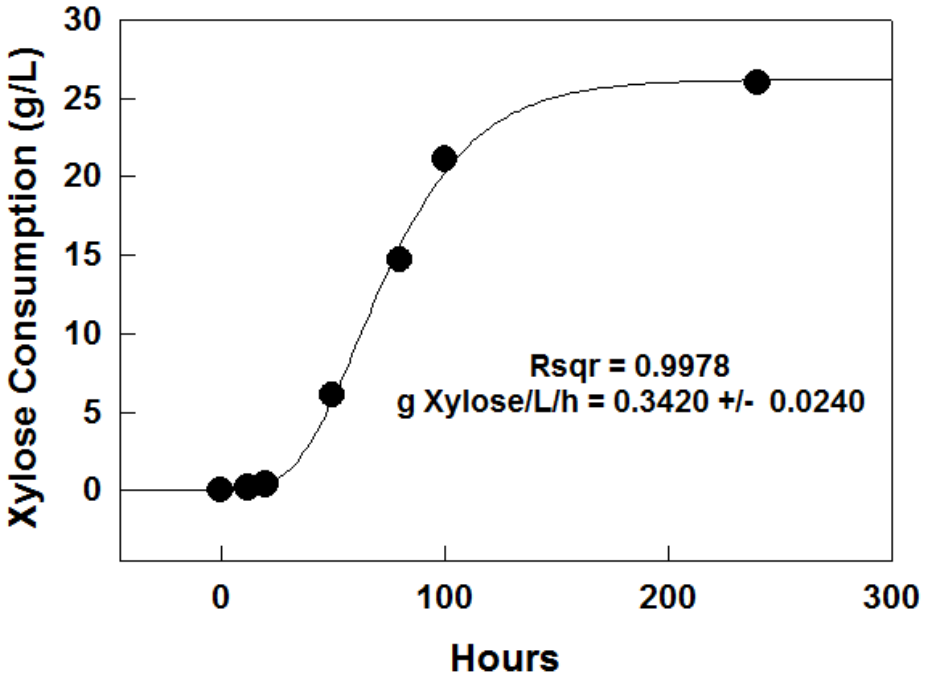

Figure 2-2: Xylose consumption data fitting with modified Gompertz equation in treatments containing 500 uM AQDS and 20 mM ferrihydrite, using 3% xylose as the sole fermentable substrate.

Glucose fermentation by C. beijerinckii NCIMB 8052

Ten-day batch fermentations using *C. beijerinckii* NCIMB 8052 with 6% glucose as the sole fermentable substrate were conducted to demonstrate that the wild type cells used in these experiments functioned identically to strain 8052 reported in other studies, to negate the possibility that the xylose data were an artifact of a degenerate strain of *C. beijerinckii* NCIMB 8052. The fermentations were conducted using conditions identical to past reports and contained acetate amended P2 media with 6% glucose {44}. Butanol

was produced in all treatments (Figure 2-3), and the glucose only control incubations had similar solvent titers relative to previously reported studies {45-46}. Ferrihydrite, with or without electron shuttle amendment, increased butanol production in a manner similar to the xylose fermenting cultures, but the variations amongst treatments was not as large (Figure 2-3). This is consistent with previous data suggesting the electron stripping effect is minimal with glucose as the sole fermentable substrate, suggesting that it is better as a strategy for targeting less effectively utilized carbon molecules such as xylose {40}.

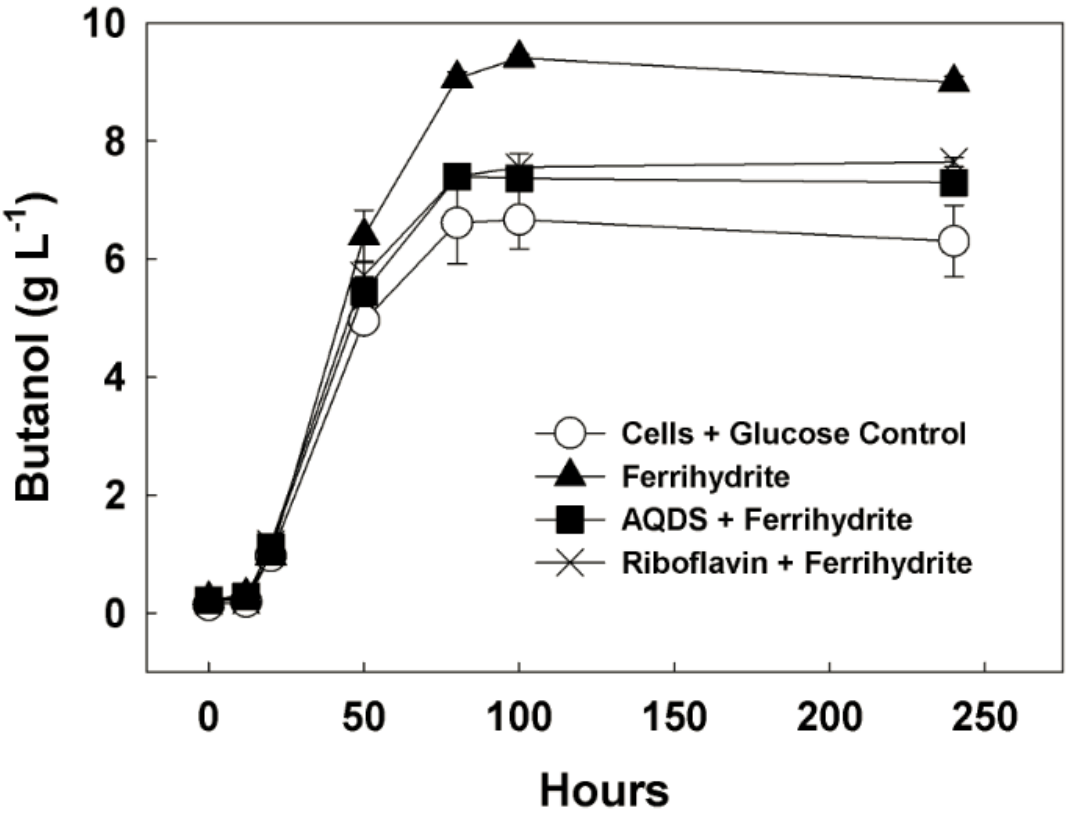

Figure 2-3: Butanol production in acetate amended fermentations using *C. beijerinckii* NCIMB 8052 with 6% glucose as the sole fermentable substrate. Error bars represent standard deviation among triplicates.

Hydrogen production with xylose and glucose

Hydrogen concentrations increased in the presence of electron shuttles plus ferric iron (Figures 2-4, 2-5, and 2-6), while the control containing cells alone and 6% glucose was 1.7-1.9 times less than these treatments after 240 h. These data are consistent with previous results suggesting that hydrogen production increased as a response to electron shuttling {38, 40}. Experiments with glucose were conducted with acetate added to the growth medium, which is the standard culturing condition for this strain {43, 45-46}. Previous studies have indicated that ABE fermentations amended with acetate exhibit increased cellular bioenergentics and possibly a direct substrate for acetoacetyl-CoA, thus increasing solvent production {46}. However, acetate was withheld from all xylose fermentation experiments in an effort to simplify the culture conditions and isolate the effects of Fe^{3+} plus electron shuttles, by eliminating another carbon/electron donating molecule from the reactions.

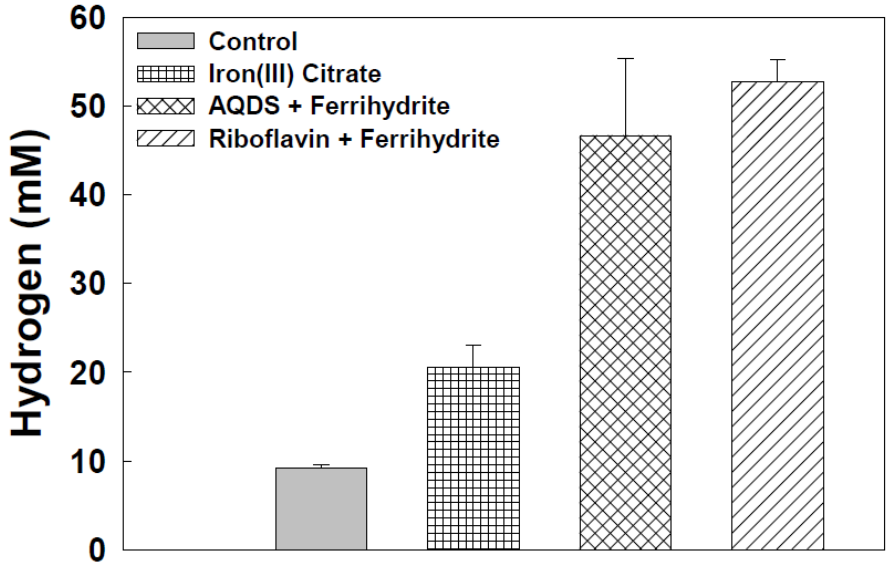

Figure 2-4: Hydrogen production in the presence and absence of electron shuttling compounds and ferrihydrite using *Clostridium beijerinckii* NCIMB 8052 and 3% xylose as the sole fermentable substrate. Error bars represent standard deviation.

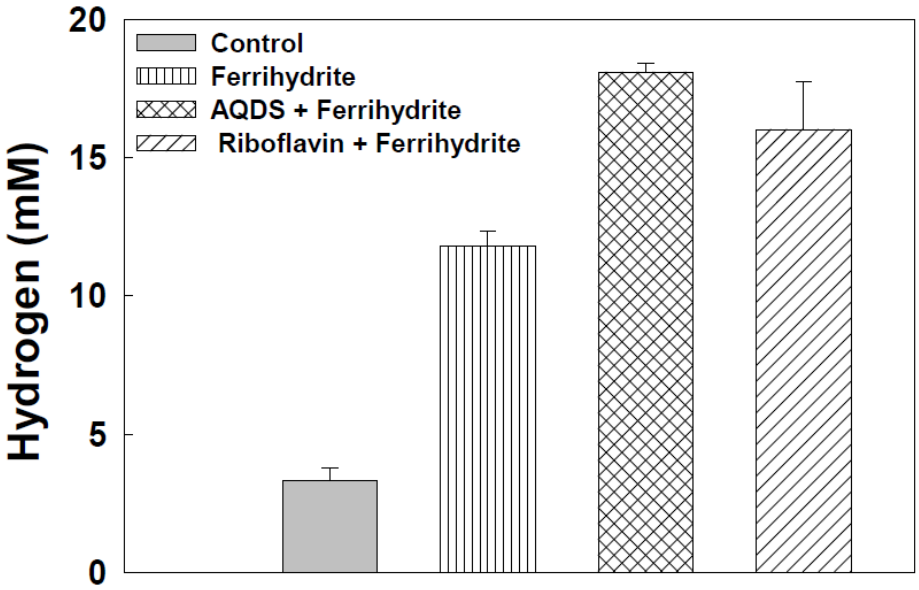

Figure 2-5: Hydrogen production (DC-1) in the presence and absence of electron shuttles and ferrihydrite using 3% xylose as the sole fermentable substrate. Error bars represent standard deviation between triplicates.

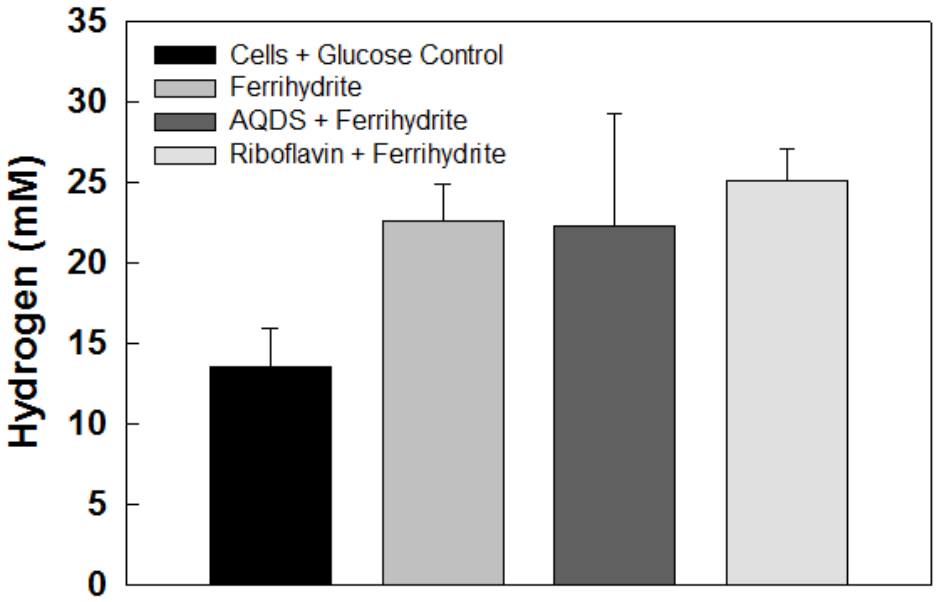

Figure 2-6: Hydrogen production in acetate amended *C. beijerinckii* NCIMB 8052 fermentations using 6% glucose as the sole fermentable substrate. Error bars represent standard deviation between triplicates.

Electron shuttling-enhanced xylose utilization and solventogenesis in strain DC-1

Strain DC-1, a novel solventogenic bacillus was isolated from crystalline cellulose fed enrichment culture containing woodland marsh sediment collected at Clemson University {Manuscript In Progress}. Partial 16S rDNA sequence analysis indicated that DC-1 shares a 95% sequence similarity to *Rhizomicrobium electricum* Mfc52 (*Rhizobiales* bacterium Mfc52, AB365487.1), but all other data indicate that this is a novel species or genus of solvent generating Bacteria. Strain DC-1 is phylogenetically distinct from most ABE producing organisms, but it possesses similar metabolic characteristics, including the ability to carry out ABE fermentation. Furthermore, the bacterium forms central endospores, and terminal inclusion bodies were visible in both

sporulated and non-sporulated cells suggesting morphological differences between it and other ABE generating genera or species.

Using nearly identical fermentation conditions as for *C. beijerinckii* NCIMB 8052, strain DC-1 was tested to determine if electron shuttles plus ferric iron influenced xylose consumption and butanol production in a similar manner. The only difference was that strain DC-1 fermentations were performed in 10 mL Balch tubes, rather than 100 mL bottles. Cells incubated with 3% xylose alone produced little butanol (0.11 g/L) relative to cells incubated with ferrihydrite (7.58 g/L) or AQDS or riboflavin (both approximately 8 g/L) (Table 2-2). Xylose consumption for the 3% xylose-alone control and the 20 mM ferrihydrite amended incubations were 3.07 g/L and 8.41 g/L (Figure 2-7), respectively. In comparison, the ferrihydrite amended treatments that contained AQDS or riboflavin had respective xylose consumption values of 29.2 g/L and 29.6 g/L (Figure 2-7). Kinetic data for xylose consumption and butanol production by treatment, quantified using the modified Gompertz equation, are listed in Table 2-2.

	Butanol Productivity (g/L/h)	λ (h)	Butanol Pmax (g/L)	Xylose Consumption Rate (g/L/h)	Butanol Yield (g/g)
Control	ND*	ND*	ND*	0.2194 ± 0.09	0.04
20mM FeGel	0.0184 ± 0.00	23.3311 ± 1.41	1.8279 ± 0.04	0.0779 ± 0.01	0.18
20 mM FeGel + 500 µM AQDS	0.2175 ± 0.04	37.1125 ± 2.63	7.7182 ± 0.26	0.7424 ± 0.15	0.26
20 mM FeGel + 500 µM Riboflavin	0.1614 ± 0.02	38.4339 ± 2.75	8.2424 ± 0.30	0.6054 ± 0.09	0.27

Table 2-2: Butanol productivities and xylose consumption rates for DC-1 batch fermentation. Fermentations were carried out in 10 mL tubes with 3% xylose as the feedstock.

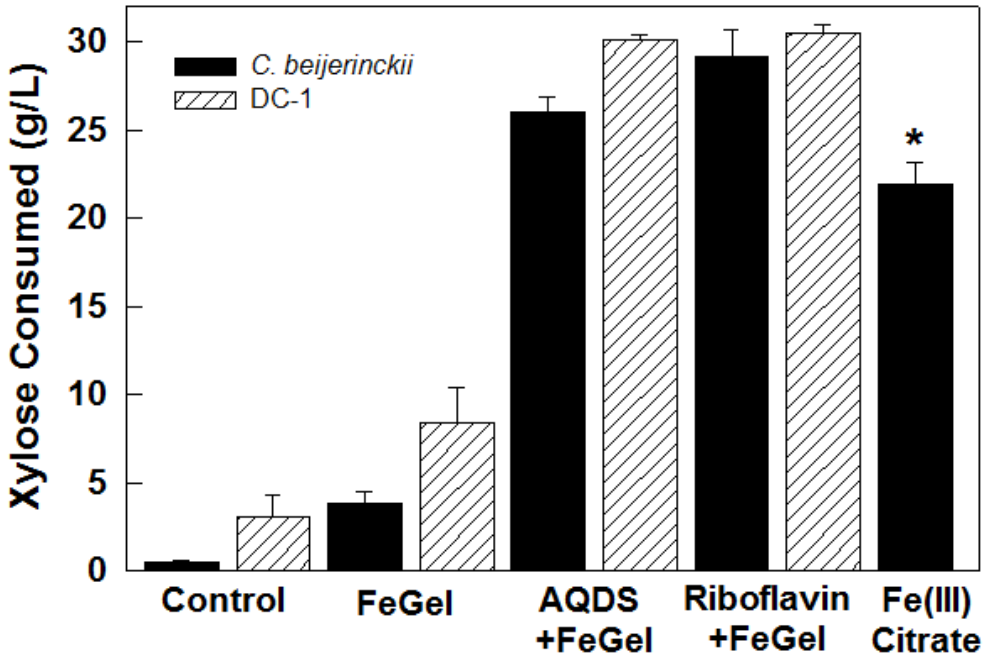

Figure 2-7: Xylose consumption after 10-days of fermentation using *C. beijerinckii* NCIMB 8052 or strain DC-1. Error bars represent standard deviation between triplicates. Asterisk (*****) indicates treatment was tested on *C. beijerinckii* only.

These data demonstrate that altering electron flow influences solventogenic organisms other than *C. beijerinckii* in a similar manner. This is critical because it demonstrates that this influence is not limited to a single prokaryotic species or genus, but rather its influence is exerted on the specific metabolic pathway, namely xylose consumption and butanol production. The data suggest that xylose consumption may be influenced by iron and electron shuttles equally, irrespective of the specific microbe being used for biofuel production. This would allow engineers to retrofit existing bioreactors, without the need to alter other conditions already conducive to the organisms

utilized. The next steps were to establish possible pathways influenced by the electron stripping, using a previously reported electron distribution technique {39-40}.

Electron Distribution Analysis

Of the total electrons liberated from pyruvate, roughly 69-81% were consumed by the butanol pathway, and only 10-11% of the reducing equivalents contributed to the acidogenic pathways in the shuttle amended treatments and the treatment amended with ferric citrate (Table 2-3). The acetate and butyrate pathways accounted for the largest end product electron distribution (32%) in the cells plus xylose control. The amount of electrons consumed with respect to xylose oxidized by the control in the hydrogen pathway was 2-3.5 times greater than in the iron and shuttle amended triplicates, indicating that overall electron flow is shunted away from the hydrogenase towards longer chain alcohols and ketones in these treatments. ATP generation and reducing equivalents consumed by ferredoxin remained consistent throughout the control and the experimental triplicates with respect to the amount of xylose consumed (Figure 2-1).

	3% Xylose Control	Iron(III) Citrate	AQDS + Ferrihydrite	Riboflavin + Ferrihydrite
Butanol (meq e^-)	0.69	128.93	180.00	240.36
Acetone (meq e^-)	0.00	15.38	25.60	23.06
Ethanol (meq e^-)	0.00	3.19	2.81	2.80
Acetate + Butyrate (meq e^-)	7.47	17.39	25.98	33.91
Butanol/Butyrate Ratio	0.10	8.99	10.00	13.72
Fd_{Red} (meq e^-)	6.53	1.96	12.12	13.60
H_2 (meq e^-)	1.85	4.12	9.40	10.54
Fe^{2+} (meq e^-)	N/A	20.00*	19.63	18.71
NAD^+_{-ox} (meq e^-)	1.70	14.24	21.50	26.59
$NADP^+_{-ox}$ (meq e^-)	0.03	5.37	7.50	10.02
ATP (mmol)	2.31	17.63	25.45	29.27

Table 2-3: Electron distribution analysis from *C. beijerinckii* xylose oxidation after 100 h of growth. Electron equivalents from xylose consumption, metabolite production, and iron reduction are based off observed values, while the remainining intermediates (Fd, NAD^+, $NADP^+$, and ATP) were calculated from the observed data. Units for electron distribution are in milli-equivalent electrons. NAD^+_{-ox} and $NADP^+_{-ox}$ represents milli-equivalent electrons liberated through nicotinamide cofactor oxidation. ATP represented in the table accounts for calculated ATP generation from glycolytic and acidogenic pathways.
* Assumed complete iron reduction for soluble iron(III) citrate amended fermentations

Solvent Yields

Total butanol yields for treatments containing the terminal electron acceptor ferrihydrite and amended AQDS were 2.4 and 6.5 times greater than controls containing xylose alone for *C. beijerinckii* NCIMB 8052 and strain DC-1, respectively. Treatments amended with ferrihydrite and riboflavin had respective butanol yields of 2.6 and 6.75 times higher than that of the cells plus xylose control for *C. beijerinckii* and strain DC-1 (Table 2-1 and 2-2). In comparison, strain BA101, a butanol hyperproducing mutant *of C.*

beijerinckii NCIMB 8052, was able to achieve roughly 1.2 times higher butanol yield than the wild type when xylose was used as the fermentation substrate {49}.

Engineering processes to provide the highest metabolite yield is paramount in industrial fermentation design. Ideal processes have the highest stoichiometric ratios of metabolite produced-to-substrate consumed. As mentioned previously, the fermentations in this study were not performed in the presence of exogenous acetate, a supplementary carbon source which increases ABE fermentation efficiency. Supplementation of this value added product would contribute to an overall decrease in the ratio of carbon transformed into butanol, thus rendering the process economically infeasible if performed on the scale required to meet current alternative energy demands.

Conclusion

Xylose fermentation is inefficient with most wild type prokaryotic cells; this fact limits the utility of certain lignocellulosic pre-treatment strategies that result in large xylose fractions for use in biofuel applications. Results from this study appear to provide the first report of complete xylose consumption by *Clostridium beijerinckii* NCIMB 8052 with concomitant increases in butanol production. Results were identical using a xylose-fermenting novel strain most closely related to members of the Rhizobiales, indicating the physiological response is not limited to a specific Order of microorganisms, but rather influences the xylose fermentation pathway irrespective of taxonomic affiliation.

Xylose utilization increased in cells amended with electron shuttles plus ferric iron, indicating that extracellular electron transfer disrupts metabolism in a manner

beneficial to biofuel production. Electron shuttling was a major driver in substantially increasing both productivities and yields from xylose, making batch fermentation kinetics more favorable, notably in that these cultures were incubated without supplemental acetate (to increase butanol yield). Glucose grown cultures always utilized 100% of the added glucose, although extracellular electron transfer did slightly increase butanol production. These data demonstrate a strategy for specifically targeting xylose uptake and utilization in biofuel-producing wild type cells, which is easily retrofitted to existing fermentation systems without needing to modify reactor infrastructure. Any technologies that increase xylose fermentation make pre-treated lignocellulose more feasible as a feedstock.

The electron flow model described above estimates the fate of reducing equivalents in ABE fermentations that were subjected to electron stripping, and how carbon and electron flow amongst the various pathways responsible resulted in increased butanol production. Data suggest that increased ATP yield and NAD^+/NADH ratios are critical to both phenomena described here, and more work is required to elucidate the exact mechanisms by which electron shuttles and/or ferric iron increase xylose utilization and increased butanol production. As research in this area continues to grow, models predicting the dynamics of electron flow will be essential in determining favorable pathway alterations in wild type fermentations of both mono- and mixed cultures.

References

1.) Szwaja, S. & Naber, J. D. (2010). Combustion of *n*-butanol in a spark-ignition IC engine. *Fuel*, *89*(7), 1573-1582.

2.) Cooney, C., Wallner, T., McConnell, S., Gillen, J. C., Abell, C., Miers, S. A., & Naber, J. D. (2009, January). Effects of blending gasoline with ethanol and butanol on engine efficiency and emissions using a direct-injection, spark-ignition engine. In ASME 2009 Internal Combustion Engine Division Spring Technical Conference (pp. 157-165). *American Society of Mechanical Engineers*.

3.) Masum, B. M., Kalam, M. A., Masjuki, H. H., Palash, S. M., & Fattah, I. R. (2014). Performance and emission analysis of a multi cylinder gasoline engine operating at different alcohol–gasoline blends. *Rsc Advances*, *4*(53), 27898-27904.

4.) *N*-Butanol Market by Applications (Butyl Acrylate, Butyl Acetate, Glycol Ethers, and Others) & Geography-Global Trends & Forecasts to 2018. *Markets and Markets*. 2013.

5.) Harvey, B. G., & Meylemans, H. A. (2011). The role of butanol in the development of sustainable fuel technologies. *Journal of Chemical Technology and Biotechnology*, *86*(1), 2-9.

6.) Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by *Clostridia*. *Biotechnology and Bioengineering*, *101*(2), 209-228.

7.) Jiang, Y., Xu, C., Dong, F., Yang, Y., Jiang, W., & Yang, S. (2009). Disruption of the acetoacetate decarboxylase gene in solvent-producing *Clostridium acetobutylicum* increases the butanol ratio. *Metabolic Engineering*, *11*(4), 284-291.

8.) Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., & Liao, J. C. (2008). Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metabolic Engineering*, *10*(6), 305-311.

9.) Connor, M. R., & Liao, J. C. (2009). Microbial production of advanced transportation fuels in non-natural hosts. *Current Opinion in Biotechnology*, *20*(3), 307-315.

10.) Lee, J., Jang, Y. S., Choi, S. J., Im, J. A., Song, H., Cho, J. H., & Lee, S. Y. (2012). Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for isopropanol-butanol-ethanol fermentation. *Applied and Environmental Microbiology*, *78*(5), 1416-1423.

11.) Green, E. M. (2011). Fermentative production of butanol—the industrial perspective. *Current Opinion in Biotechnology*, *22*(3), 337-343.

12.) Harris, L. M., Blank, L., Desai, R. P., Welker, N. E., & Papoutsakis, E. T. (2001). Fermentation characterization and flux analysis of recombinant strains of *Clostridium acetobutylicum* with an inactivated solR gene. *Journal of Industrial Microbiology and Biotechnology*, *27*(5), 322-328.

13.) Qureshi, N., & Maddox, I. S. (1988). Reactor design for the ABE fermentation using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. *Bioprocess Engineering*, *3*(2), 69-72.

14.) Roffler, S., Blanch, H. W., & Wilke, C. R. (1987). Extractive fermentation of acetone and butanol: process design and economic evaluation. *Biotechnology Progress*, *3*(3), 131-140.

15.) Liang, T. M., Cheng, S. S., & Wu, K. L. (2002). Behavioral study on hydrogen fermentation reactor installed with silicone rubber membrane. *International Journal of Hydrogen Energy*, *27*(11), 1157-1165.

16.) Alkasrawi, M., Eriksson, T., Börjesson, J., Wingren, A., Galbe, M., Tjerneld, F., & Zacchi, G. (2003). The effect of Tween-20 on simultaneous saccharification and fermentation of softwood to ethanol. *Enzyme and Microbial Technology*, *33*(1), 71-78.

17.) Valentine, J., Clifton-Brown, J., Hastings, A., Robson, P., Allison, G., & Smith, P. (2012). Food vs. fuel: the use of land for lignocellulosic 'next generation'energy crops that minimize competition with primary food production. *GCB Bioenergy*, *4*(1), 1-19.

18.) Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*, *48*(8), 3713-3729.

19.) Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., & Bogel-Łukasik, R. (2010). Hemicelluloses for fuel ethanol: a review. *Bioresource Technology*, *101*(13), 4775-4800.

20.) El Kanouni, A., Zerdani, I., Zaafa, S., Znassni, M., Loutfi, M., & Boudouma, M. (1998). The improvement of glucose/xylose fermentation by *Clostridium acetobutylicum* using calcium carbonate. *World Journal of Microbiology and Biotechnology*, *14*(3), 431-435.

21.) Servinsky, M. D., Kiel, J. T., Dupuy, N. F., & Sund, C. J. (2010). Transcriptional analysis of differential carbohydrate utilization by *Clostridium acetobutylicum*. *Microbiology*, *156*(11), 3478-3491.

22.) Jeffries, T. W. (1983). *Utilization of xylose by bacteria, yeasts, and fungi* (pp. 1-32). Springer Berlin Heidelberg.

23.) Robinson, G. C. (1922). A study of the acetone and butyl alcohol fermentation of various carbohydrates. *Journal of Biological Chemistry*, *53*(1), 125-154.

24.) Park, D. H., Laivenieks, M., Guettler, M. V., Jain, M. K., & Zeikus, J. G. (1999). Microbial utilization of electrically reduced neutral red as the sole electron donor for growth and metabolite production. *Applied and Environmental Microbiology*, *65*(7), 2912-2917.

25.) Peguin, S., & Soucaille, P. (1995). Modulation of carbon and electron flow in *Clostridium acetobutylicum* by iron limitation and methyl viologen addition. *Applied and Environmental Microbiology*, *61*(1), 403-405.

26.) Rao, G., & Mutharasan, R. (1987). Altered electron flow in continuous cultures of *Clostridium acetobutylicum* induced by viologen dyes. *Applied and Environmental Microbiology*, *53*(6), 1232-1235.

27.) Ye, X., Zhang, X., Morgenroth, E., & Finneran, K. T. (2012). Anthrahydroquinone-2, 6-disulfonate increases the rate of hydrogen production during *Clostridium beijerinckii* fermentation with glucose, xylose, and cellobiose. *International Journal of Hydrogen Energy*, *37*(16), 11701-11709.

28.) Peguin, S., & Soucaille, P. (1996). Modulation of metabolism of *Clostridium acetobutylicum* grown in chemostat culture in a three-electrode potentiostatic system with methyl viologen as electron carrier. *Biotechnology and Bioengineering*, *51*(3), 342-348.

29.) Shin, H., Zeikus, J., & Jain, M. (2002). Electrically enhanced ethanol fermentation by *Clostridium thermocellum* and *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *58*(4), 476-481.

30.) Meyer, C. L., & Papoutsakis, E. T. (1989). Increased levels of ATP and NADH are associated with increased solvent production in continuous cultures of *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology*, *30*(5), 450-459.

31.) Singh, A., Lynch, M. D., & Gill, R. T. (2009). Genes restoring redox balance in fermentation-deficient *E. coli* NZN111. *Metabolic Engineering*, *11*(6), 347-354.

32.) Baer, S. H., Blaschek, H. P., & Smith, T. L. (1987). Effect of butanol challenge and temperature on lipid composition and membrane fluidity of butanol-tolerant *Clostridium acetobutylicum*. *Applied and Environmental Microbiology*, *53*(12), 2854-2861.

33.) Lovley, D. R., & Phillips, E. J. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology*, *51*(4), 683-689.

34.) Lincoff, A. H., & Gossett, J. M. (1984). The determination of Henry's constant for volatile organics by equilibrium partitioning in closed systems. In *Gas Transfer at Water Surfaces* (pp. 17-25). Springer Netherlands.

35.) Stookey, L. L. (1970). Ferrozine---a new spectrophotometric reagent for iron. *Analytical Chemistry*, *42*(7), 779-781.

36.) Lin, C. Y., & Lay, C. H. (2004). Effects of carbonate and phosphate concentrations on hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy*, *29*(3), 275-281.

37.) Mu, Y., Zheng, X. J., Yu, H. Q., & Zhu, R. F. (2006). Biological hydrogen production by anaerobic sludge at various temperatures. *International Journal of Hydrogen Energy*, *31*(6), 780-785.

38.) Mu, Y., Yu, H. Q., & Wang, G. (2007). A kinetic approach to anaerobic hydrogen-producing process. *Water Research*, *41*(5), 1152-1160.

39.) Zwietering, M. H., Jongenburger, I., Rombouts, F. M., & Van't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, *56*(6), 1875-1881.

40.) Zhang, X., Ye, X., Guo, B., Finneran, K. T., Zilles, J. L., & Morgenroth, E. (2013). Lignocellulosic hydrolysates and extracellular electron shuttles for H 2 production using co-culture fermentation with *Clostridium beijerinckii* and *Geobacter metallireducens*. *Bioresource Technology*, *147*, 89-95.

41.) Lee, H. S., Krajmalinik-Brown, R., Zhang, H., & Rittmann, B. E. (2009). An electron-flow model can predict complex redox reactions in mixed-culture fermentative BioH2: Microbial ecology evidence. *Biotechnology and Bioengineering*, *104*(4), 687-697.

42.) Ye, X., Morgenroth, E., Zhang, X., & Finneran, K. T. (2011). Anthrahydroquinone-2, 6,-disulfonate (AH2QDS) increases hydrogen molar yield and xylose utilization in growing cultures of *Clostridium beijerinckii*. *Applied Microbiology and Biotechnology*, *92*(4), 855-864.

43.) Li, Z., Xiao, H., Jiang, W., Jiang, Y., & Yang, S. (2013). Improvement of solvent production from xylose mother liquor by engineering the xylose metabolic pathway in *Clostridium acetobutylicum* EA 2018. *Applied Biochemistry and Biotechnology*, *171*(3), 555-568.

44.) Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W. J., Jiang, W., & Yang, S. (2011). Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. *Applied and Environmental Microbiology*, *77*(22), 7886-7895.

45.) Bryant, D. L., & Blaschek, H. P. (1988). Buffering as a means for increasing growth and butanol production by *Clostridium acetobutylicum*. *Journal of Industrial Microbiology*, *3*(1), 49-55.

46.) Formanek, J., Mackie, R., & Blaschek, H. P. (1997). Enhanced Butanol Production by *Clostridium beijerinckii* BA101 Grown in Semidefined P2 Medium Containing 6 Percent Maltodextrin or Glucose. *Applied and Environmental Microbiology*, *63*(6), 2306-2310.

47.) Zhang, Y., & Ezeji, T. C. (2013). Transcriptional analysis of *Clostridium beijerinckii* NCIMB 8052 to elucidate role of furfural stress during acetone butanol ethanol fermentation. *Biotechnol Biofuels*, *6*(1), 66.

48.) Chen, C. K., & Blaschek, H. P. (1999). Effect of acetate on molecular and physiological aspects of *Clostridium beijerinckii* NCIMB 8052 solvent production and strain degeneration. *Applied and Environmental Microbiology*, *65*(2), 499-505.

49.) Qureshi, N., Ezeji, T. C., Ebener, J., Dien, B. S., Cotta, M. A., & Blaschek, H. P. (2008). Butanol production by *Clostridium beijerinckii*. Part I: use of acid and enzyme hydrolyzed corn fiber. *Bioresource Technology*, *99*(13), 5915-5922.

CHAPTER 3

A TALE OF THREE ECOSYSTEMS: ELECTRON SHUTLLING TO FERRIHYDRITE INFLUENCES XYLOSE-FED MIXED CULTURE FERMENTATIONS TO INCREASE SOLVENT, VOLATILE ACID, AND BIO-HYDROGEN PRODUCTION

Abstract

The influence of the redox mediators, AQDS and riboflavin, on mixed culture fermentations was investigated using xylose as the sole fermentation substrate. Electron shuttling to insoluble ferrihydrite enhanced solventogenesis, acidogenesis, hydrogen production, and xylose consumption, relative to the cells plus xylose controls in fermentations inoculated with woodland marsh sediment, wetwood disease, or raw septic liquid. This behavior was observed over multiple transfers in 15-day batch fermentations. Partial 16S rDNA community screening revealed that either ferrihydrite alone or AQDS or riboflavin coupled to ferrihydrite immediately shifted native heterogeneous populations to those predominantly belonging to the *Clostdridiales*. Stimulation was observed in other fermentative populations belonging to the *Lactobacillaceae* and *Sporolactobacilaceae* to a lesser extent, rather than stimulating iron respiring populations contained within each consortia.

Introduction

Iron is one of the most abundant transition metals in the earth's crust and is an essential element required to catalyze vital metabolic reactions in nearly every living organism {1-4}. Microorganisms have adapted mechanisms to utilize ferric iron as an electron sink through dissimilatory reduction reactions in order to promote metabolism of organic compounds and gain energy for microbial processes {5-8}. Microbial dissimilatory iron reduction processes have a profound influence on the biogeochemical dynamics of oxygen-depleted environments, especially in regards to decomposition reactions and nutrient cycling.

Several strategies exist for extracellular electron transport from bacteria. It has been previously reported that secretion of reduced redox mediators contribute to the transport of electrons to ferric iron in order to augment cellular metabolism through the creation of novel respiratory networks outside of the cell {9-14}. Soluble redox active compounds, such as quinones and flavins, are secreted by numerous bacterial genera to catalyze electron transport to terminal electron acceptors. Quinone and flavin molecules can undergo repeated reduction and oxidation, and this cyclic nature allows for multiple respiration events to occur extracellularly {15-18}.

The addition of exogenous electron shuttling compounds to engineered systems increases interactions between cellular electron transport networks and insoluble terminal electron acceptors {19-20}. This characteristic proves to be important in applications including, but not limited to microbial fuel cells (MFC), the bioremediation industry, and the production of energy carriers such as hydrogen and alcohols {20-27}. It has been

previously reported that the addition of electron shuttling compounds enhances both fermentative end product formation as well as xylose consumption in pure culture *Clostridial* fermentations, and this is, in part, due to increasing the availability of oxidized nicotinamide co-factors {26-27}. This provides evidence that electron shuttling compounds may provide a competitive advantage for microorganisms capable of transferring electrons to redox active molecules, allowing them to augment their capability to utilize xylose as an energy source.

It is estimated that xylose, a pentose sugar, composes nearly 30% of the world's terrestrial biomass {28}. However, major metabolic bottlenecks exist which limits xylose consumption in most organisms {29-30}. Animals cannot utilize xylose as an energy source, and its non-competetive nature with food supplies makes it an ideal feedstock for chemical production. Thus, increasing the capacity for xylose consumption in wild-type organisms has great significance in the bioprocess industry, especially with respect to the production of commodity solvents which can be used as alternative energy carriers, as well as chemical precursors for polymer synthesis {31-33}.

Results presented in this chapter demonstrate that electron shuttling to iron increases levels of xylose metabolism as well as augments the production of fermentative end products, such as butanol, hydrogen, and organic acids, in mixed consortia from three disparate environmental samples (marsh sediment, wetwood disease, and raw septic liquid). Through the use of next-generation microbial community screening, the results indicatethat the presence of iron and electron shuttles contribute to shifts in microbial populations towards predominately fermentative organisms, rather than iron respiring

populations. This appears to be the first report of the presence of ferric iron and/or electron shuttles coupled to ferric iron providing a complete competitive advantage for xylose-utilizing fermentative populations in mixed consortia, as iron reducing, fatty acid oxidizing bacteria went undetected in the presence of any ferric iron challenged experimental treatments.

Materials and Methods

Inoculation sources

Wetwood disease was collected from a residential suburb outside of Charlotte, North Carolina from a rotting wooden post (Figure 3-1). Samples containing diseased portions were placed in Balch tubes containing P2 media {34} filled nearly to the top to limit oxygen exposure. Tubes were capped and crimped in the field, and the remaining headspace was degassed with nitrogen upon returning to a laboratory setting. Following a 5 min degassing period, tubes containing the wetwood disease samples were stored at 37 ^{o}C until experimental set up or downstream enrichment applications.

Woodland marsh sediment was collected in a forested area near Clemson, SC, and samples were stored in plastic Nalgene bottles at 4 ^{o}C until enrichment setup. Roughly 10 g of solids were dispensed into Balch tubes and these were filled with an additional 5 mL tap water in an anaerobic glove chamber containing an atmosphere of 100% nitrogen. Tubes were capped with blue butyl stoppers and crimped in the glove bag.

Raw, homogenized septic liquid was collected from a septic handler in the upstate South Carolina area, and this was stored in 4 L Nalgene bottles in the dark at 18 ^{o}C until use. Septic sludge remained unagitated until it was used for downstream inoculation.

Figure 3-1: Source of wetwood disease inoculum collected from a suburb outside of Charlotte, NC.

Fermentation Conditions

Fermentation studies were constructed to address the mixed consortia response of electron shuttling to ferrihydrite using three separate inoculation sources. Fermentations were conducted in anaerobic Balch tubes containing P2 media. P2 media was prepared by adding 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 2.0 g/L $(NH_4)_2SO_4$, 10 mL/L mineral mix (20 g $MgSO_4$, 1 g $MnSO_4$, 1 g NaCl, and 1 g $FeSO_4$), and 10 mL/L vitamin mix (100 g p-aminobenzoic acid, 1 g biotin, and 10 g thiamine) to Nanopure water. Media was aliquoted into tubes and degassed for 10 min in the liquid phase using nitrogen passed

over a heated copper column. The headspace of the tubes were degassed for an additional minute, and following this, the tubes were capped with thick blue butyl stopper and crimped with aluminum crimp tops. Media was sterilized at 121 ^{o}C for 15 min to eliminate potential contamination unassociated with the mixed culture inocula.
Stock solutions of AQDS (Sigma 98%) and riboflavin (Sigma 98+%) were prepared in Nanopure water at concentrations of 20 mM each, and pH was adjusted to 7. These were aliquoted into 160 mL anaerobic serum bottles at a final volume of 100 mL. Poorly crystalline ferrihydrite (FeGel) was prepared at a concentration of 1 M and aliquoted into 160 mL anaerobic serum bottles with a final volume of 100 mL. The recipe for FeGel has been described previously {35}. All stock solutions were degassed with nitrogen passed over a heated copper column for 15 min in the liquid phase and 5 min in the headspace. These were capped with thick blue butyl stoppers and crimped with aluminum crimp tops. Stock solutions were autoclaved at 121 ^{o}C for 15 min. After cooling, each stock solution was stored in the dark until the addition to their corresponding experimental tubes prior to inoculation.

Stock solutions of D-xylose (Alfa Aesar) were prepared in Nanopure water at a concentration of 600 g/L. Liquid xylose stock solution were dispensed into 160 mL anaerobic serum bottles at a final volume of 100 mL. Xylose was degassed with nitrogen passed over a heated copper column for 15 min in the liquid phase and 5 min in the headspace. Serum bottles were capped with blue butyl stoppers and crimped with aluminum crimp tops. This was filter-sterilized (sterile 0.2 μm syringe filters) into all

experimental tubes, bringing the final fermentation feedstock concentration to 30 g/L xylose prior to inoculation.

Experimental tubes were inoculated with 5% (v/v) of their respective inoculation sources, and these were incubated at 37 ºC in the dark. The final fermentation volume at the start of each experiment was 10 mL following the addition of all amendments. Experimental tubes were sampled periodically over a 15 day period for metabolite production. Changes in metabolite profiles and xylose consumption were monitored in experimental triplicates containing cells plus 30 g/L xylose alone, cells plus xylose plus 30 mM ferrihydrite, cells plus xylose plus ferrihydrite plus 500 μM AQDS, and cells plus xylose plus ferrihydrite plus 500 μM riboflavin. Cells from each tube were transferred (6% v/v) to fresh experimental tubes corresponding to their treatment after 10 days of growth, and this was repeated over a period of three transfers.

Analytical

All liquid samples were filtered through 0.2 μm syringe filters into screw-top autosampler vials containing 250 μL glass inserts (Lab Supply Distributors). Vials were capped with PFTE lined screw top septa, and sugars/metabolites were measured using GC-FID and HPLC UV/RI.

Solvent (acetone and butanol) concentrations were measured with a Shimadzu GC-2014 equipped with an AOC-20s autosampler. Analytes were separated on a DB-FFAP column (J&W Scientific; 30-m x 0.250-mm; 0.25-μm film thickness) and were detected with a flame ionization detector. Liquid samples (1 μL) were withdrawn using a 10 μL syringe (SGE Analytical) and injected into the instrument. The temperature

program is as follows: 40 ^{o}C dwell for 2 min, followed by a temperature ramp of 50^{o}C/min until the oven reached 220 ^{o}C. This was held for 1 min until restarting the temperature program. The injector and detector temperatures were set to 200 ^{o}C and 300 ^{o}C, respectively. Helium was used as the carrier gas at a linear velocity of 80.3 cm/sec.

Volatile fatty acids and xylose were monitored using a Dionex HPLC equipped with a Biorad HP-Aminex column, using sonicated 5 mM H_2SO_4 as the mobile phase. Samples were injected in volumes of 25 µL. The mobile phase was set at a constant flow of 0.6 mL/min, and the column oven was set to 60 ^{o}C. Sugars were analyzed using a refractive index detector, while organic acids were analyzed with a variable wavelength detector set at 210 nm.

Hydrogen was periodically measured using a Shimadzu GC-8A equipped with an internal TCD. Headspace gases were separated on a 100/120 Carbosieve SII column (10' length x 1/8" outer diameter) using ultra high-purity nitrogen as the carrier gas at a constant pressure of 400 kPa. The TCD voltage and the column oven were set to 60 V and 50 ^{o}C, respectively. The injector and detector temperatures were both set to 150 ^{o}C. Sample volumes of 0.5 mL were injected into the GC with a nitrogen degassed, VICI gas-tight syringe. Hydrogen partitioning in a closed system was calculated as described previously using the dimensionless Henry's constant for hydrogen (0.01907 at 25 ^{o}C){36}.

DNA extraction, Amplification, and 16S rDNA Community Analysis

Biomass from respective experimental triplicates were pooled and homogenized, and total community DNA from each triplicate was extracted using Fast Spin DNA kit

for soils (MP Biomedical). Microbial community DNA concentration and purity were determined using a NanoDrop spectrophotometer. DNA extracts were used for downstream 16S PCR amplification specific for the Illumina MiSeq platform. One universal Eubacterial primer pair was used to amplify the partial 16S region of template DNA: 338F (5'- [TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG]ACTCCTACGGG AGGCAGC -3') and 907R (5'[GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAG]CCGTCAATTCCTTTGAGTTT -3'). Illumina tags are denoted by square brackets. The thermocycler program used for amplification was 10 min at 94 ^{o}C (initial denaturation), 0.45 min at 94 ^{o}C (denaturation), 1 min at 55^{o}C (annealing), 0.50 min at 72 ^{o}C (extension), repeat for 35 cycles, final extension at 72 ^{o}C for 5 min, infinite 4 ^{o}C dwell temperature. Amplicon length was confirmed using 1% (w/v) agarose gel electrophoresis, with ethidium bromide as the DNA intercalating agent. This was performed at an initial 15V for 10 min, followed by 80V for an additional 50 min. Bands on the gel were visualized on a UV transilluminator (312 nm) to confirm amplicon presence. PCR products were purified using a Qiaquick PCR purification kit (Qiagen). Purified amplicon concentrations were determined using the Qubit high sensitivity double stranded DNA assay (Thermo). Concentrations were further normalized in TE buffer to 30 ng/µL, and these were submitted to Clemson University Genomics Institute (Clemson, SC) for Illumina MiSeq analysis.

Illumina MiSeq samples were computed in BaseSpace (Illumina) cloud service. The 16S Metagenomics application was used to output raw taxonomic classification for

each sample, and data was generated in tabular form. Tabular data was further normalized to provide readings in units of percent frequency.

Results and Discussion

Wetwood Disease

Fifteen-day metabolite production and xylose consumption profiles are listed in Figure 3-2A. Solventogenesis, acidogenesis, hydrogen production, and xylose consumption were minimal in controls containing cells plus 30 g/L xylose alone for reach consecutive transfer. The addition of 30 mM ferrihydrite increased hydrogen production by 2.6, 4.1, and 5.0 times and butyric acid production by 2.1, 3.7, and 4.3 times, respectively, in comparison to the controls over a period of three transfers (Figure 3-2; F; T1-3). Solvent production and xylose consumption was minimal in the ferrihydrite alone treatment. Elevated levels of metabolite production and xylose consumption were observed when electron shuttling compounds were coupled to ferrihydrite in fermentations. Treatments containing 500 μM AQDS plus ferrihydrite showed an increase in butanol production and xylose consumption over a period of three transfers, where 18.0 mM (T1), 42.9 mM (T2), and 46.5 mM butanol were produced and 16.5 g/L (T1), 24.6 g/L (T2), and 30.9 g/L (T3; complete oxidation) xylose were consumed (A + F; T1-3). Butyrate and hydrogen production remained relatively stable over the course of each transfer. However, an increase in acetate production was observed from the initial transfer to the final transfer. Xylose consumption and metabolite production were enhanced in fermentations containing 500 μM riboflavin plus ferrihydrite, however, these levels were not as pronounced as those observed in treatments containing AQDS plus

ferrihydrite (R + F; T1-3). Xylose consumption in this treatment was observed to be 21.6 g/L (T1), 21.7 g/L (T2), and 25.5 g/L (T3). Treatments containing riboflavin favored butyrate production over butanol, acetate, and hydrogen, and observed butyrate titers were 50.7 mM (T1), 66.3 mM (T2), and 58.3 mM (T3).

Family-level 16S community analysis revealed that the wetwood disease parent inoculum was composed of predominantly *Clostridiaceae* (76.2%), with lesser populations belonging to the *Lactobacillaceae* (10.7%), *Oxalobacteraceae* (2.5%), *Xanthomondaceae* (1.2%), and *Phyllobacteraceae* (1.0%) (Figure 3-2; B; "I"). Shifts in the populations towards *Clostridiaceae* and *Lactobacillaceae* were observed throughout all treatments, including the cells plus 30 g/L xylose control, over multiple transfers. However, a higher occurrence of *Lactobacillaceae* were observed in experimental treatments containing either AQDS or Riboflavin.

Lactobacillaceae composed nearly 42.7% and 50.5% of the total population in AQDS plus ferrihydrite amended treatments for the second and third transfer. This is 2 and 2.5 times greater than that was observed in the corresponding transfers from the cells plus xylose control, respectively, and 3.9 and 4.6 times greater than the initial inoculation source, indicating that lactic acid producing bacteria may benefit from the presence of electron shuttling compounds. The second and third transfers of the AQDS plus ferrihydrite treatment exhibited the highest level of butanol production out of any treatment within this experiment, although the frequency of *Clostridiaceae* in these treatments, which would likely contribute to increased levels of solventogenesis, were lower than that of the controls. Similar community data and levels of metabolite

stimulation were observed in the treatment containing riboflavin plus ferrihydrite. Lactate titers were enhanced in shuttle amended treatments (data not shown), and previous evidence suggests that lactate, as well as butyrate and acetate, may have additive effects on solventogenesis {37-39}.

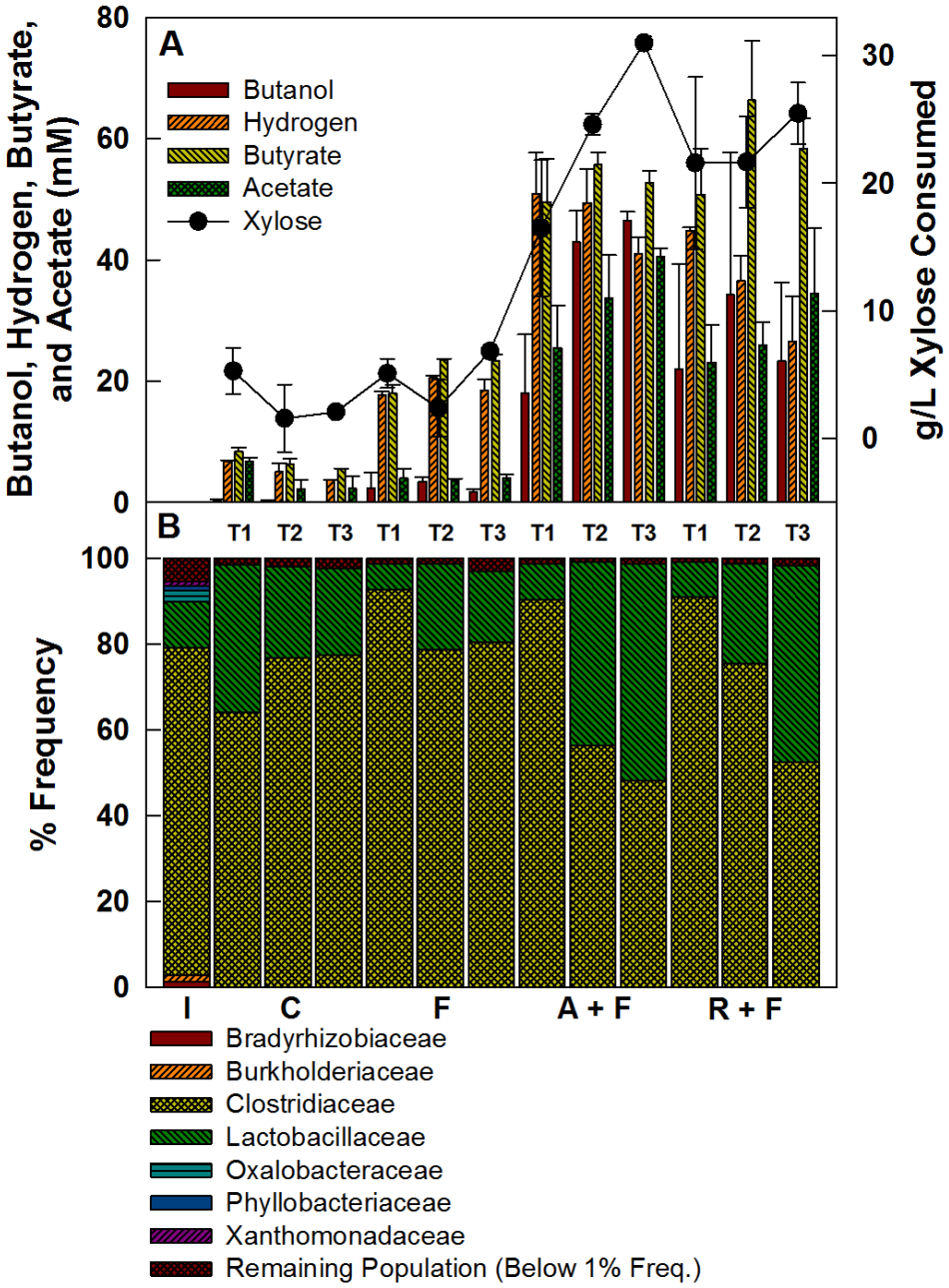

Figure 3-2: Metabolite production, xylose consumption, and 16S rDNA community screening for fermentations inoculated with bacterial wetwood disease in the presence and absence of electron shuttles and ferrihydrite after 15 days of growth. Inoculation source, cells plus 3% xylose alone, 30 mM ferrihydrite, 500 µM AQDS plus ferrihydrite, and 500 µM riboflavin plus ferrihydrite are denoted by I, C, F, A+F, and R+F, respectively. Error bars represent standard deviation between triplicates.

Septic Liquid

Similar metabolite production and xylose consumption responses for each corresponding treatment were observed in fermentations inoculated with septic liquid, as those reported in the previous section containing wetwood disease as the parent inoculum (Figure 3-3; A). Metabolite production and xylose consumption were minimal in controls containing 30 g/L xylose alone at the end of 15-day batch fermentations. Values for xylose consumption are reported to be 4.7 g/L, 3.0 g/L, and 3.2 g/L for transfers 1, 2, and 3, respectively. Ferrihydrite alone (F) increased xylose consumption by 4.0-fold (T1), 1.8-fold (T2), and 4.4-fold (T3). Xylose consumption was further elevated in treatments amended with 500 μM AQDS or 500 μM riboflavin plus ferrihydrite. Final xylose consumption values of 20.8 g/L (T1), 23.3 g/L (T2), and 29.9 g/L (T3) were observed in fermentations containing AQDS plus ferrihydrite (A+F). Similar xylose oxidation values for riboflavin amended fermentations were observed (R+F), and these values were 22.3 g/L (T1), 23.9 g/L (T2), and 28.6 g/L (T3).

The addition of AQDS or riboflavin coupled to ferrihydrite stimulated butanol production over the course of three transfers (Figure 3-3; A). However, 15-day butanol titers were less pronounced than those observed in fermentations inoculated with the wetwood parent culture. Respective butanol titers for AQDS amended fermentations were 15.4 mM, 15.8 mM, and 14.7 mM for transfers 1-3. The addition of AQDS increased butanol titers by 4.6-fold (T1), 9.3-fold (T2), and 1.4-fold (T3), in comparison to the fermentations amended with 30 mM ferrihydrite alone. Butanol titers in the cells plus xylose controls were below 1 mM for all transfers. Final butanol titers in treatments

amended with riboflavin plus ferrihydrite were 3.7 mM (T1), 10.8, mM (T2), and 23.5 mM (T3), and the observed increased in butanol titers over the course of three transfers may be attributed to the community adaptation to the presence of exogenous flavins.

Cumulative hydrogen production in fermentation containing 30 mM ferrihydrite were observed to be 51.1 mM (T1), 33.4 mM (T2), and 34.6 mM (T3), and these values were 4.8, 4.4, and 6.7 times greater than that was observed for the controls in their corresponding transfers (Figure 3-3; A). Hydrogen levels were increased by 1.3-fold (T1), 1.7-fold (T2), and 1.7-fold (T3) in comparison to fermentations amended with ferrihydrite alone when AQDS was supplied as an exogenous electron shuttle, indicating that electron shuttling to iron increases levels of hydrogen production in mixed consortia, which was observed in pure culture *Clostridium* studies performed in Chapter 2. This was confirmed in riboflavin plus ferrihydrite amended fermentations. Final hydrogen titers for riboflavin amended treatments were 1.1 (T1), 1.5 (T2), and 1.5 (T3) times greater than those observed in treatments containing ferrihydrite alone.

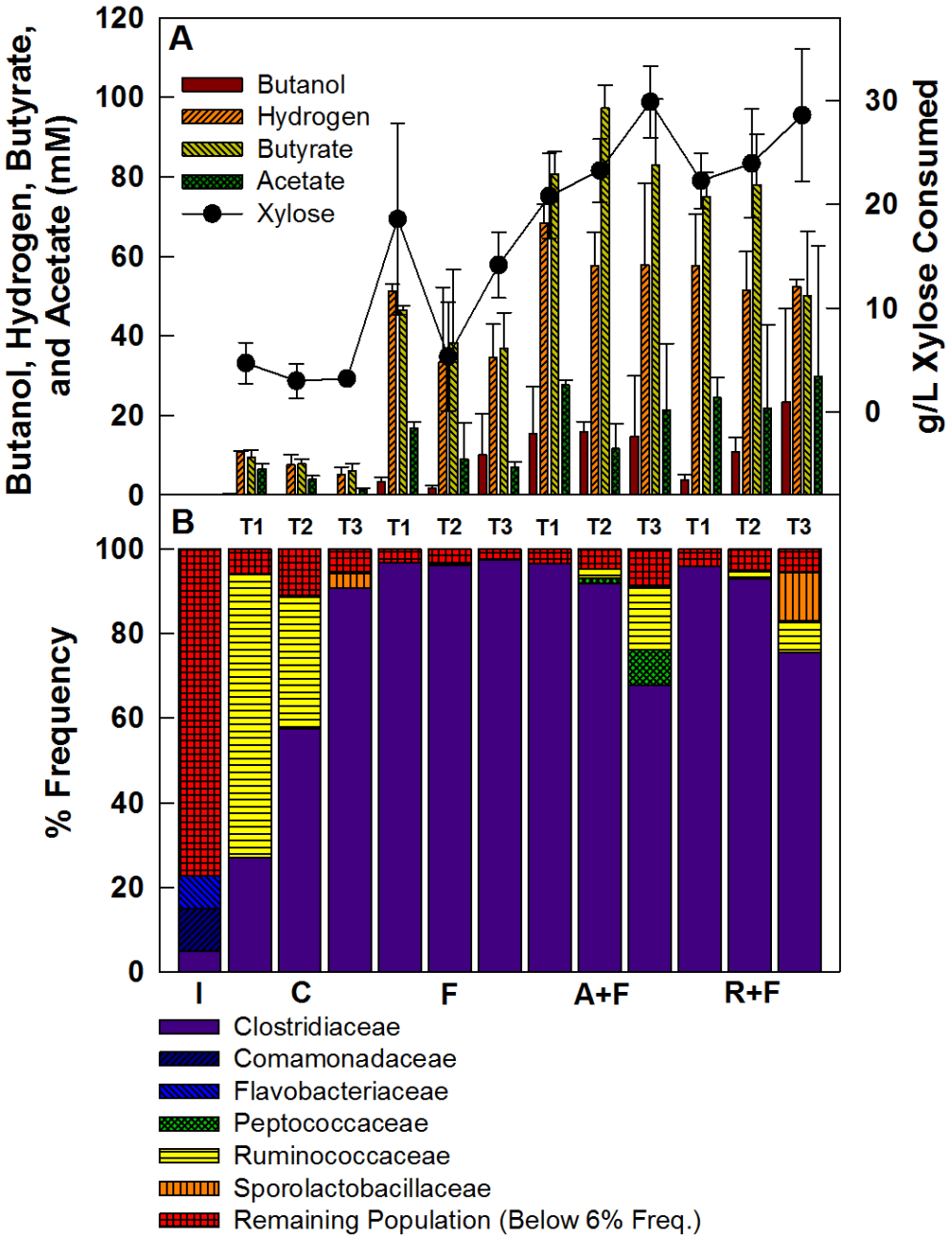

Figure 3-3: Metabolite production, xylose consumption, and 16S rDNA community screening for fermentations inoculated with raw septic liquid in the presence and absence of electron shuttles and ferrihydrite after 15 days of growth. Inoculation source, cells plus 3% xylose alone, 30 mM ferrihydrite, 500 µM AQDS plus ferrihydrite, and 500 µM riboflavin plus ferrihydrite are denoted by I, C, F, A+F, and R+F, respectively. Error bars represent standard deviation between triplicates.

Family level 16S community analysis revealed that the raw septic parent inoculation source was composed of highly heterogeneous bacterial populations. Major populations composing the inoculum belonged to the *Comamonadaceae* (10.1%), *Flavobacteraceae* (7.7%), and *Clostridiaceae* (5.0%) Families (Figure 3-2; B; "I"). Lesser populations (> 1% frequency) are listed in Table 3-1.

The presence of 30 g/L xylose alone shifted populations towards those predominantly belonging to the *Clostridiaceae* and *Ruminococcaceae* over the course of two transfers, and at the end of the third transfer, the community composition was a nearly homogenous population of *Clostridiaceae* (Figure 3-3; B; C; T1-3). The initial transfer of the control containing 30 g/L xylose alone were composed of 67.1% *Ruminococcaceae* and 27.1% *Clostridiaceae*, and these values shifted to 57.5% *Clostridiaceae* and 31.5% *Ruminococcaceae* of the total frequency for the cells plus xylose control in the second transfer. The addition of 30 mM ferrihydrite (F; T1-3) immediately shifted the Family-level community makeup towards the *Clostridiaceae*, and this was maintained for all transfers containing this treatment. AQDS and riboflavin amended fermentations were observed to be composed primarily of *Clostridiaceae* throughout the course of this experiment. However, populations belonging to the *Ruminococcaceae* and *Peptococcaceae* (AQDS amended) and *Sporolactobacillaceae* and *Ruminococcaceae* (riboflavin amended) began to emerge at the last transfer.

It has been previously reported that fermenters can use iron as a minor electron sink under glucose-oxidizing conditions, distributing most of their electrons to fermentative end products such as organic acids, alcohols, and hydrogen {40-41}. The

presence of these metabolites, more specifically organic acids, would ultimately contribute to interspecies electron transfer towards iron reducing organisms, which should be responsible for catalyzing any complete reduction of iron(III) contained within a microbial consortium {40}. Many iron respiring, fatty acid oxidizing populations were detected within the septic parent inoculum (Table 3-1). However, these populations were suppressed in the presence of ferric iron (Table 3-2), while fermentative populations belonging to the *Clostridiaceae* were amplified in every iron(III)-containing treatment.

Family	% Frequency of Total Population
Comamonadaceae	10.1
Flavobacteriaceae	7.7
Sphingobacteriaceae	6.1
Rhodocyclaceae	6.0
Desulfobacteraceae	5.5
Flexibacteraceae	5.3
Clostridiaceae	5.0
Spirochaetaceae	4.3
Helicobacteraceae	4.2
Sphingomonadaceae	3.8
Desulfovibrionaceae	3.6
Porphyromonadaceae	3.3
Caulobacteraceae	2.8
Mycobacteriaceae	2.2
Geobacteraceae	1.6
Ruminococcaceae	1.5
Campylobacteraceae	1.2
Synergistaceae	1.2
Chitinophagaceae	1.2
Populations Below 1% Composition	23.4

Table 3-1: Representative 16S Family-level composition of septic inoculum. Family aggregates were normalized to 100% of the total population. Populations scoring below 1% were omitted from the table due to the high heterogeneity of the microbial community.

		% *Geobacteraceae* Frequency		
Parent Inoculum	Woodland Marsh		0.051	
	Septic Liquid		1.575	
	Wetwood Disease		0.047	
	Experimental Treatments	T1	T2	T3
Marsh	Cells + Xylose Control	0.021	0.024	0.046
	30 mM Ferrihydrite	0.054	0.034	0.035
	500 μM AQDS + Ferrihydrite	0.019	0.048	0.012
	500 μM Riboflavin + Ferrihydrite	0.016	0.040	0.013
Septic	Cells + Xylose Control	0.020	0.044	0.034
	30 mM Ferrihydrite	0.018	0.016	0.028
	500 μM AQDS + Ferrihydrite	0.016	0.018	0.023
	500 μM Riboflavin + Ferrihydrite	0.037	0.019	0.035
Wetwood	Cells + Xylose Control	0.013	0.018	0.033
	30 mM Ferrihydrite	0.010	0.010	0.063
	500 μM AQDS + Ferrihydrite	0.015	0.005	0.018
	500 μM Riboflavin + Ferrihydrite	0.014	0.007	0.011

Table 3-2: Percent frequency of *Geobacteraceae* contained within parent inocula and experimental treatments after 15 days of growth on 30 g/L xylose as the sole fermentable substrate.

Woodland Marsh Sediment

Limited xylose consumption was observed for all controls containing cells plus xylose alone in fermentations inoculated with woodland marsh sediment native to Upstate South Carolina (Figure 3-4; A; C; T1-3). These values were observed to be 1.8 g/L (T1), 1.8 g/L (T2), and 0.5 g/L (T3) after 15 days of growth. The addition of 30 mM ferrihydrite (F) increased substrate utilization levels by 7.8-fold (T1), 3.9-fold (T2), and 24.2-fold (T3). The coupling of electron shuttles to ferrihydrite further increased 15-day xylose utilization levels relative to the controls containing cells plus xylose alone and

ferrihydrite plus xylose ameneded fermentations, and this is consistent with the previous sections using wetwood disease or septic liquid as the inoculation source. Xylose utilization in fermentations challenged with 500 μM AQDS plus ferrihydrite (A + F; T1-3) was observed to be 19.6 g/L (T1), 20.9 g/L, (T2), and 24.5 g/L (T3), and this was 10.9, 11.6, and 49.0 times greater than that of the controls containing cells plus xylose alone for each corresponding transfer after 15 days of growth. Fermentations challenged with 500 μM riboflavin plus ferrihyrite (R + F; T1-3) showed the most consistent results with respect to maintaining elevated levels of substrate utilization. Riboflavin amended treatments were observed to consume 22.4 g/L, 22.4 g/, and 23.4 g/L xylose over the course of three transfers.

Butanol production was enhanced in the first transfer of ferrihydrite alone (Figure 3-4; F; T1). However, butanol was limited or not detected in subsequent transfers on ferrihydrite, while volatile fatty acids and hydrogen were the favored metabolites. The addition of AQDS or riboflavin to ferrihydrite stimulated butanol production over the course of three transfers. Conversely, levels of solventogenesis were not as pronounced as those observed in the previous sections.

Levels of cumulative hydrogen were elevated in all treatments containing ferrihydrite. Increases in total hydrogen production of 6.5-9.1-fold were observed in fermentations containing ferrihydrite plus xylose alone, relative to the cells plus xylose controls, over the course of three transfers. The presence of AQDS coupled to ferrihydrite increased hydrogen relative to the controls by 10.0-16.4-fold. Riboflavin amended

treatments also stimulated high levels of hydrogen production, and these values were 6.1-10.6-fold greater than that was observed in the controls.

The marsh sediment parent inoculum (Figure 3-4; B; I) was composed primarily of bacterial populations belonging to the *Clostridiaceae* (40.3%), *Spicrochaetaceae* (18.3%), *Anaerolinaceae* (8.0%), *Solibacteraceae* (6.1%), *Veillonellaceae* (5.7%), *Methylobacteriaceae* (4.6%), and *Rhodospirilliaceae* (4.0%). The community makeup was observed to shift towards *Clostridiaceae* (66.8%) and *Ruminococcaceae* (28.9%) after 15 days of growth on 30 g/L xylose alone (C; T1). Subsequent transfers on xylose alone increased the frequency of *Clostridiaceae* to 86.5% and 93.0% after the second and third transfers, respectively. Populations were shifted towards a nearly homogenous composition of *Clostridiaceae* in treatments containing 30 mM ferrihydrite plus xylose alone (F; T1-3), similar to the results observed in the previous sections.

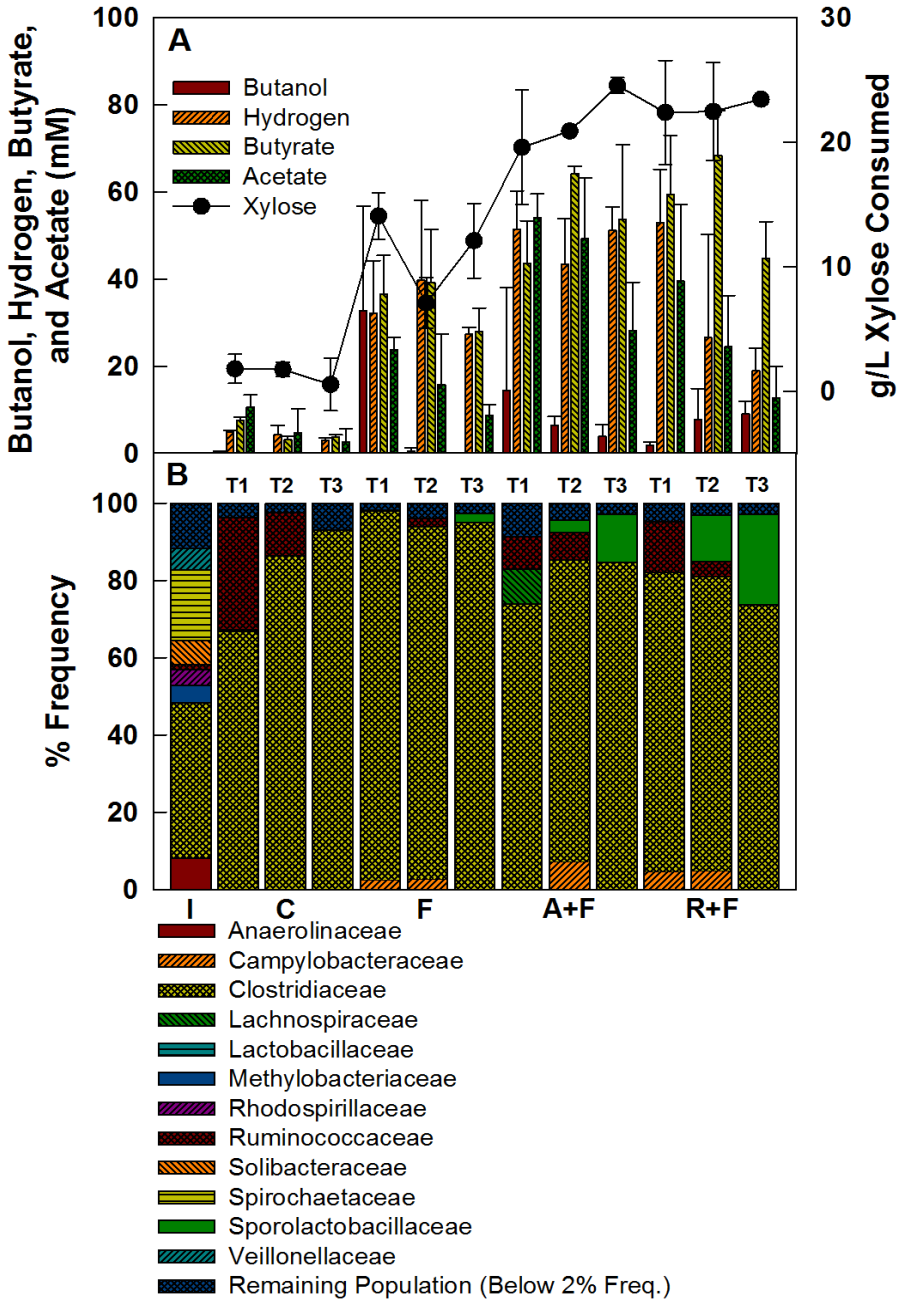

Figure 3-4: Metabolite production, xylose consumption, and 16S rDNA community screening for fermentations inoculated with marsh sediment in the presence and absence of electron shuttles and ferrihydrite after 15 days of growth. Inoculation source, cells plus 3% xylose alone, 30 mM ferrihydrite, 500 µM AQDS plus ferrihydrite, and 500 µM riboflavin plus ferrihydrite are denoted by I, C, F, A+F, and R+F, respectively. Error bars represent standard deviation among triplicates.

Electron shuttles decreased the frequency of observed *Clostridiaceae* in comparison to treatments containing ferrihydrite alone, and they stimulated the growth of alternate fermentative populations. The first 15-day AQDS amended fermentations (A+F; T1) were composed of 73.9% *Clostridiaceae*, 8.5% *Ruminococcaceae*, and 9.0% *Lachnospiraceae* as the dominant populations. Shifts towards 77.9% *Clostridiaceae*, 7.3% *Camplyobacteraceae*, 7.1% *Ruminococcaceae*, and 3.4% *Sporolactobacillaceae* were observed for the subsequent transfer (A+F; T2) after 15 days of growth. The final transfer on AQDS plus ferrihydrite amended fermentations (A+F; T3) revealed that *Clostridiaceae* (84.7%) and *Sporolactobacillaceae* (12.4%) were the dominant populations at the end of the study. Similar results, with respect to the emergence of *Sporolactobacillaceae* following subsequent transfers, were observed in treatments containing riboflavin plus ferrihydrite. Members belonging to the *Clostridiaceae* (77.4%), *Ruminococcaceae* (13.0%), and *Camplybacteraceae* (4.7%) were observed to constitute the majority of the microbial community for the first transfer under these conditions (R+F; T1). *Camplyobacteraceae* and *Ruminococcaceae* populations were present within this treatment until the final transfer, where *Clostridiaceae* (73.8%) and *Sporolactobacillaceae* (23.4%) were the predominant members.

Conclusion

The results from experiments with disparate environmental inocula demonstrated that electron shuttling to ferrihydrite enhanced xylose utilization concomitantly with fermentation end product formation. Furthermore, this study demonstrated that the presence of ferrihydrite alone evolves microbial consortia to stimulate the growth of

populations belonging to the *Clostridiaceae*, while suppressing iron reducing populations in xylose-fed fermentations, even in the presence of high-titer volatile acids, alcohols, and hydrogen. These data suggest that iron(III) alone or iron(III) coupled to electron shuttling compounds may play an important role in providing fermenters, which have adapted approaches to engage in dissimilatory iron reduction, a metabolic advantage over less processive organisms contained in oxygen-depleted environments containing high organic loading.

References

1.) Sandy, M., & Butler, A. (2009). Microbial iron acquisition: marine and terrestrial siderophores. *Chemical Reviews*, *109*(10), 4580-4595.

2.) Miethke, M., & Marahiel, M. A. (2007). Siderophore-based iron acquisition and pathogen control. *Microbiology and Molecular Biology Reviews*, *71*(3), 413-451.

3.) Braun, V., Hantke, K., & Koester, W. (1998). Bacterial iron transport: mechanisms, genetics, and regulation. *Metal Ions in Biological Systems*, *35*, 67-146.

4.) Anderson, G. J., & Vulpe, C. D. (2009). Mammalian iron transport. *Cellular and Molecular Life Sciences*, *66*(20), 3241-3261.

5.) Lovley, D. R., & Phillips, E. J. (1988). Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Applied and Environmental Microbiology*, *54*(6), 1472-1480.

6.) Reguera, G., McCarthy, K. D., Mehta, T., Nicoll, J. S., Tuominen, M. T., & Lovley, D. R. (2005). Extracellular electron transfer via microbial nanowires. *Nature*, *435*(7045), 1098-1101.

7.) Lovley, D. R., & Lonergan, D. J. (1990). Anaerobic oxidation of toluene, phenol, and p-cresol by the dissimilatory iron-reducing organism, GS-15. *Applied and Environmental Microbiology*, *56*(6), 1858-1864.

8.) Lovley, D. R. (1993). Dissimilatory metal reduction. *Annual Reviews in Microbiology*, *47*(1), 263-290.

9.) Marsili, E., Baron, D. B., Shikhare, I. D., Coursolle, D., Gralnick, J. A., & Bond, D. R. (2008). *Shewanella* secretes flavins that mediate extracellular electron transfer. *Proceedings of the National Academy of Sciences*, *105*(10), 3968-3973.

10.) Lovley, D. R. (2008). The microbe electric: conversion of organic matter to electricity. *Current Opinion in Biotechnology*, *19*(6), 564-571.

11.) Von Canstein, H., Ogawa, J., Shimizu, S., & Lloyd, J. R. (2008). Secretion of flavins by *Shewanella* species and their role in extracellular electron transfer. *Applied and Environmental Microbiology*, *74*(3), 615-623.

12.) Rabaey, K., Boon, N., Höfte, M., & Verstraete, W. (2005). Microbial phenazine production enhances electron transfer in biofuel cells. *Environmental Science & Technology*, *39*(9), 3401-3408.

13.) Hernandez, M. E., & Newman, D. K. (2001). Extracellular electron transfer. *Cellular and Molecular Life Sciences CMLS*, *58*(11), 1562-1571.

14.) Newman, D. K., & Kolter, R. (2000). A role for excreted quinones in extracellular electron transfer. *Nature*, *405*(6782), 94-97.

15.) Misra, H. P., & Fridovich, I. (1972). The univalent reduction of oxygen by reduced flavins and quinones. *Journal of Biological Chemistry*, *247*(1), 188-192.

16.) Luijten, M. L., Weelink, S. A., Godschalk, B., Langenhoff, A. A., van Eekert, M. H., Schraa, G., & Stams, A. J. (2004). Anaerobic reduction and oxidation of quinone moieties and the reduction of oxidized metals by halorespiring and related organisms. *FEMS Microbiology Ecology*, *49*(1), 145-150.

17.) Jones, J. B., & Taylor, K. E. (1976). Nicotinamide coenzyme regeneration. Flavin mononucleotide (riboflavin phosphate) as an efficient, economical, and enzyme-compatible recycling agent. *Canadian Journal of Chemistry*, *54*(19), 2969-2973.

18.) Field, J., Cervantes, F., Van der Zee, F., & Lettinga, G. (2000). Role of quinones in the biodegradation of priority pollutants: a review. *Water Science & Technology*, *42*(5-6), 215-222.

19.) Schröder, U. (2007). Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. *Physical Chemistry Chemical Physics*, *9*(21), 2619-2629.

20.) Rabaey, K., & Rozendal, R. A. (2010). Microbial electrosynthesis—revisiting the electrical route for microbial production. *Nature Reviews Microbiology*, *8*(10), 706-716.

21.) Torres, C. I., Marcus, A. K., Lee, H. S., Parameswaran, P., Krajmalnik-Brown, R., & Rittmann, B. E. (2010). A kinetic perspective on extracellular electron transfer by anode-respiring bacteria. *FEMS Microbiology Reviews*, *34*(1), 3-17.

22.) Logan, B. E. (2010). Scaling up microbial fuel cells and other bioelectrochemical systems. *Applied microbiology and biotechnology*, *85*(6), 1665-1671.

23.) Franks, A. E., & Nevin, K. P. (2010). Microbial fuel cells, a current review. *Energies*, *3*(5), 899-919.

24.) Wei, J., Liang, P., & Huang, X. (2011). Recent progress in electrodes for microbial fuel cells. *Bioresource Technology*, *102*(20), 9335-9344.

25.) Hatch, J. L., & Finneran, K. T. (2008). Influence of reduced electron shuttling compounds on biological H2 production in the fermentative pure culture *Clostridium beijerinckii*. *Current Microbiology*, *56*(3), 268-273.

26.) Ye, X., Zhang, X., Morgenroth, E., & Finneran, K. T. (2012). Anthrahydroquinone-2, 6-disulfonate increases the rate of hydrogen production during *Clostridium beijerinckii* fermentation with glucose, xylose, and cellobiose. *International Journal of Hydrogen Energy*, *37*(16), 11701-11709.

27.) Ye, X., Morgenroth, E., Zhang, X., & Finneran, K. T. (2011). Anthrahydroquinone-2, 6,-disulfonate (AH2QDS) increases hydrogen molar yield and xylose utilization in growing cultures of *Clostridium beijerinckii*. *Applied Microbiology and Biotechnology*, *92*(4), 855-864.

28.) Kumar, P., Barrett, D. M., Delwiche, M. J., & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial & Engineering Chemistry Research*, *48*(8), 3713-3729.

29.) Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W. J., Jiang, W., & Yang, S. (2011). Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. *Applied and Environmental Microbiology*, *77*(22), 7886-7895.

30.) Jeffries, T. W. (1983). *Utilization of xylose by bacteria, yeasts, and fungi* (pp. 1-32). Springer Berlin Heidelberg.

31.) Lindblad, M. S., Liu, Y., Albertsson, A. C., Ranucci, E., & Karlsson, S. (2002). Polymers from renewable resources. In *Degradable Aliphatic Polyesters* (pp. 139-161). Springer Berlin Heidelberg.

32.) Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by *Clostridia*. *Biotechnology and Bioengineering*, *101*(2), 209-228.

33.) Harvey, B. G., & Meylemans, H. A. (2011). The role of butanol in the development of sustainable fuel technologies. *Journal of Chemical Technology and Biotechnology*, *86*(1), 2-9.

34.) Robinson, G. C. (1922). A study of the acetone and butyl alcohol fermentation of various carbohydrates. *Journal of Biological Chemistry*, *53*(1), 125-154.

35.) Lovley, D. R., & Phillips, E. J. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology*, *51*(4), 683-689.

36.) Lincoff, A. H., & Gossett, J. M. (1984). The determination of Henry's constant for volatile organics by equilibrium partitioning in closed systems. In *Gas Transfer at Water Surfaces* (pp. 17-25). Springer Netherlands.

37.) Gu, Y., Hu, S., Chen, J., Shao, L., He, H., Yang, Y., & Jiang, W. (2009). Ammonium acetate enhances solvent production by *Clostridium acetobutylicum* EA 2018 using cassava as a fermentation medium. *Journal of Industrial Microbiology & Biotechnology*, *36*(9), 1225-1232.

38.) Wang, Y., Li, X., & Blaschek, H. P. (2013). Effects of supplementary butyrate on butanol production and the metabolic switch in *Clostridium beijerinckii* NCIMB 8052: genome-wide transcriptional analysis with RNA-Seq. *Biotechnology for Biofuels*, *6*(1), 138.

39.) Kwon, G. S., & Kim, B. H. (1991). Electron Flow Shift in *Clostridium acetobutylicum* Fermentation by Lactate. *Journal of Microbiology and Biotechnology*, *1*(4), 261-265.

40.) Lovley, D. R., & Phillips, E. J. (1989). Requirement for a microbial consortium to completely oxidize glucose in Fe (III)-reducing sediments. *Applied and Environmental Microbiology*, *55*(12), 3234-3236.

41.) Jones, J. G., Davison, W., & Gardener, S. (1984). Iron reduction by bacteria: range of organisms involved and metals reduced. *FEMS Microbiology Letters*, *21*(1), 133-136.

CHAPTER 4

OPTIMIZING OXYGEN EXPOSED BUTANOL FERMENTATIONS USING THE AEROTOLERANT *CLOSTRIDIUM* SP. C10

Abstract

Clostridium sp. C10, a cellulose degrading bacterium, was isolated from marsh sediment. Strain C10 grew and produced solvents in the presence of 4.5 mg/L or less dissolved oxygen and completely consumed dissolved oxygen in the presence of ammonium sulfate, which is atypical for solventogenic *Clostridia*. Oxygen-stressed fermentations were optimized around the strain to determine conditions fostering the highest butanol titers, volumetric productivities, and yields. Strain C10 produced 4.59 g/L butanol in oxygen-exposed, agitated batch fermentations containing 2 g/L ammonium acetate and 60 g/L glucose. Butanol titers were increased 1.35-fold relative to the optimized cells plus glucose control when ferrihydrite was amended in a subsequent study. Butanol production was not compromised in oxygen exposed fermentations relative to anoxic preparations of each treatment. Similar results, with respect to solvent production, were observed in oxygen-exposed fermentations containing xylose as the sole fermentable substrate.

Introduction

Bio-butanol has great potential to supplement or directly replace gasoline for internal combustion engines {1-3}. Industrial acetone-butanol-ethanol (ABE) production is performed primarily with obligately anaerobic strains of *Clostridium beijerinckii*, and *Clostridium acetobutylicum* {4}. However, limited reports exist in which the ABE fermentation process is carried out in environments which rigorous anoxia is not maintained.

Oxygen sensitivity in strict anaerobes can be attributed to the formation of destructive oxygen species which may damage DNA or enzymes involved in energy metabolism, ultimately causing cell death {5-7}.The mechanism by which oxygen inhibits downstream anaerobic metabolism is attributed to oxidation of iron-sulfur clusters located within pyruvate-ferredoxin oxidoreductase, precipitating ferric iron and destroying the central catalyst accountable for electron transport to solventogenic and hydrogen-forming pathways {8}.

It has been previously indicated that C. *acetobutylicum* has the ability to grow in moderately aerated environments (<1 μM O_2), but ceases growth and butyrate production under short exposure to aerobic environments of up to 40-50 μM O_2 {9}. Growth of *C. acetobutylicum* in microoxic environments has been attributed to upregulation of oxygen consuming and scavenging enzymes such as NADPH-dependent peroxide reductase as well as O_2 induced polypeptides {10-11}. Furthermore, the deletion of the PerR peroxide repressor in C. *acetobutylicum* has been attributed to increased levels of oxygen tolerance

and oxygen consumption {12}. These observations indicate that certain solventogenic species of *Clostridium* may possess mechanisms responsible for regulating growth and secondary metabolism in response to oxygen exposure. Recently, TU-103, a cellulose degrading *Clostridium*, was isolated from animal feces {13}. This strain has the ability to degrade cellulose, and it is also touted to be the only solvent producing strain of *Clostridium* capable of producing butanol in the presence of oxygen. However, little is known about the characteristics of this microbe.

In an effort to make the implementation of next-generation biofuels more plausible, robust organisms, such as those that can tolerate oxygen exposure, must be implemented in order to lower production costs, thereby making the supply price increasingly attractive for the consumer. The use of oxygen-tolerant solventogenic organisms allows for greater freedom in fermentation engineering design, and this includes the use of continuous, or steady-state, reactors. Strict bioreactor constraints limit biofuel processing capabilities by restricting most fermentation processes to traditional batch or fed-batch reactors, which are better suited for small volume operations {14}. Thus, the purpose of this study was to establish a framework for carrying out oxygen-exposed ABE fermentation processes using aerotolerant *Clostridium* species due to the novelty pertaining to both the physiology and the process. This appears to be the first study that demonstrates the potential for industrial butanol production under oxygen stress without decreasing or inhibiting process performance.

Materials and Methods

Culture Description and Maintenance

Clostridium sp. C10 was isolated from crystalline cellulose-fed woodland marsh sediment. Phylogenetic analysis revealed that strain C10 shared high full 16S rDNA sequence similarity with other solventogenic *Clostridia* (Figure 4-1). Aerotolerance was confirmed in three separate physiological tests during strain characterization.

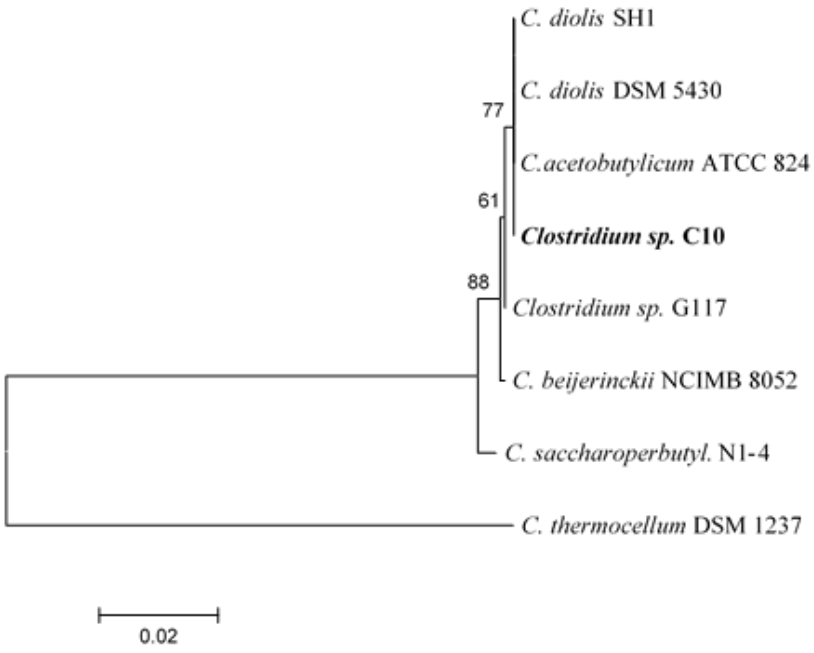

Figure 4-1: *Clostridium sp.* C10 molecular phylogeny. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model {15}. There were a total of 1345 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 {16}.

Spore suspensions of *Clostridium sp.* C10 were prepared and stored at -20^{o}C in Nanopure water. Aerobic preparations of 100 mL TYG media in screw top Pyrex bottles were inoculated with 0.1 mL spore suspensions for all oxygen-exposed studies. TYG

media consisted of 30 g/L tryptone, 20 g/L glucose, and 10 g/L yeast extract in Nanopure water. Anaerobic preparations of TYG media were prepared for parallel studies comparing performance under strictly anoxic conditions. TYG media was boiled for one min and aliquoted into 160 mL anaerobic serum bottles at a volume of 100 mL for anaerobic studies. Media was cooled under nitrogen passed over a heated copper column for 15 min in the liquid phase and 5 min in the headspace. Serum bottles were capped with thick blue butyl stoppers and crimped with aluminum crimp tops. Aerobic and anoxic preparations were sterilized for 30 min at 121^{o}C. Upon inoculation with spores, pre-culture preparations were allowed to incubate without agitation for 24 hours at 37^{o}C prior to the start of each experiment.

pH and nitrogen source optimization

Bench scale experiments were performed to assess optimal fermentation media conditions for aerotolerant butanol production for strain C10. Experiments which tested optimal pH for solvent production was prepared with 2 g/L tryptone, 1 g/L yeast extract, 20 mM KH_2PO_4 in nanopure water. The pH was adjusted with either concentrated HCl or NaOH to bring final values to pH 3, 4, 5, 6, and 7. Media was aliquoted into 250 mL screw top Pyrex bottles and was autoclaved for 30 min at 121 ^{o}C. Bottles were allowed to reach room temperature prior to glucose addition. Aerobic glucose stock (600 g/L) was prepared in NanoPure water and was filter sterilized into fermentation bottles using 0.2 µm Pall Supor syringe filters to bring the final fermentation concentration to 30 g/L. Cells grown in TYG media were added (6% v/v) to duplicate bottles to start the

experiment. Initial dissolved oxygen levels were assessed using single uninoculated controls corresponding to the various pH values in order to prevent against the introduction of contaminating organisms within experimental bottles. Active fermentation volumes were 200 mL. Fermentations were run at 37^{o}C with loosened caps and low agitation (80 rpm) in a VWR shaker incubator. Agitation was fixed in all studies to provide oxygen stress throughout the duration of the fermentations. Dissolved oxygen readings from experimental bottles were taken at the end of the fermentation period to assess residual DO levels from the oxygen-exposed fermentations.

Subsequent experiments were performed using the initial pH value that generated the highest butanol titer and volumetric butanol productivity. Experiments comparing optimal nitrogen sources for aerotolerant butanol production were performed with ammonium acetate, ammonium chloride, ammonium sulfate, and urea to examine whether butanol titers can be further increased with constituents less costly and more defined than tryptone. Duplicate experimental bottles and single uninoculated controls containing 2 g/L of each respective N-source, 1 g/L yeast extract, and 20 mM KH_2PO_4 were prepared in nanopure water. Media was sterilized as described previously. Glucose was filter sterilized into the media upon cooling to room temperature, and cells grown on TYG were used to inoculate experimental bottles. Fermentations were incubated at 37 ^{o}C, and agitation was set to 80 rpm.

Effects of redox mediation

Oxygen-exposed fermentations containing excess feedstock concentration (60 g/L) and optimal media conditions were prepared as previously described in this text. Ferrihydrite and AQDS was aseptically amended to respective fermentations prior to inoculation. Ferrihydrite stock solutions were prepared as described previously {17}. AQDS stock solutions were prepared in Nanopure water. The pH was adjusted to 7, and the stock solution was autoclaved for 15 min at 121 ^{o}C. AQDS stock solutions were stored in the dark at room temperature. Agitation was set to 80 rpm, and fermentations were incubated at 37 ^{o}C.

Comparison with strictly anoxic conditions

Duplicate experimental treatments were prepared to compare solventogenic performance under strictly anoxic conditions using optimal fermentation conditions garnered previously. Media was prepared with 2 g/L ammonium acetate, 1 g/L yeast extract, and 20 mM KH_2PO_4 in Nanopure water. The pH was adjusted to 7 with sodium hydroxide, and the media preparation was boiled for 1 min immediately following pH measurement. Media was immediately aliquoted into 160 mL anaerobic serum bottles and was cooled for 15 min in the liquid phase and 5 min in the headspace with nitrogen gas which was passed over a heated copper column. This was sterilized at 121 ^{o}C for 30 min, and anoxic glucose stock was filter sterilized into each preparation after reaching room temperature. Sterile, anaerobic stock solutions of ferrihydrite and AQDS were added aseptically to respective serum bottles prior to inoculation. Final fermentation

volumes were 100 mL after receiving 6% v/v inoculum from an anaerobic culture grown for 24 h on TYG media. These were incubated at 37 ºC with 80 rpm agitation.

Sample Collection and Analytical Techniques

Liquid samples (3 mL) were periodically withdrawn from fermentations over a 120-h period using sterile syringes. Samples for chromatographic analysis were filter sterilized (0.2 μm) into autosampler vials which contained 250 μL glass inserts (Lab Supply Distributors). These were sealed with screw top PFTE caps, and stored at 4 ºC until analysis. The remaining sample volume was distributed into either new polystyrene cuvettes (optical density) or clean falcon tubes for pH analysis.

Butanol and acetone evolution was monitored with a Shimadzu GC-2014 equipped with a flame ionization detector and a DB-FFAP capillary column (30-m x 0.250-mm; 0.25-μm film thickness). Helium was used as the carrier gas. Linear velocity was set to 80.7 cm/s. Liquid sample volumes of 1μL were injected using an AOC20i+S autosampler. Following sample injection, the syringe was automatically rinsed three times in NanoPure water. The temperature program included an initial 2 minute dwell at 40 ºC, followed by a 50 ºC/min temperature ramp until the oven reached 220 ºC. The oven temperature was held at 220ºC for 1 min. Injector and detector temperatures were set to 200 ºC and 300 ºC respectively.

Sugars were separated using a high performance liquid chromatograph (Dionex) equipped with a Bio-Rad HP-Aminex column, and they were analyzed with a Refractomax 520 refractive index detector (Thermo Scientific). Mobile phase flow rates

were set to 0.6 mL/min., and the column compartment temperature was set to 60 ^{o}C. The mobile phase was prepared by adding concentrated sulfuric acid (chromatography grade) to NanoPure water. The final concentration of this preparation was 5 mM H_2SO_4. All eluent was degassed for 10 min in a sonicating bath prior to use.

Optical density was measured spectrophotometrically using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer and disposable VWR polystyrene cuvettes. Measurements for pH were taken using a Thermo Scientific Orion Star A111 pH meter equipped with an Orion 9107BNMD probe. All dissolved oxygen measurements were performed at room temperature using a Thermo Scientific OrionSTAR 5 with an Orion 081010MD probe, and this was calibrated in 100% water saturated air.

Kinetic Analysis

Volumetric butanol productivity was modeled using a modified Gompertz equation {18-20}. Butanol data were plotted as g/L and were plotted against a 120 h sampling period. Rates (R; in units of g butanol/L/h) were calculated by normalizing the $R_{Butanol}$ by the volume of the medium in the batch experiments. Lag (λ) is described in units of hours, maximum butanol production (P_{max}) is in units of g/L, and time (t) is in units of hours. All statistical analysis was performed with SigmaPlot statistical software. P values and correlation coefficients were generated alongside of the generated constants.

$$P = P_{max} * \exp\left\{-\exp\left[\frac{\exp(1)*R}{P_{max}(\lambda - t)} + 1\right]\right\} \qquad \text{(Equation 1)}$$

Results and Discussion

Effect of pH on Aerotolerant Butanol Fermentation

Five-day batch studies using 30 g/L glucose as the sole fermentable substrate were performed to determine the influence of pH on aerotolerant butanol production. All oxygen exposed fermentations were agitated at 80 rpm to provide continuous oxygen stress, and tryptone was used as the nitrogen source. The initial fermentation pH influenced both five-day solvent titers, volumetric productivity of butanol production, and butanol yield from glucose. Fermentations with a starting pH of 7 had a final butanol titer of 3.13 g/L, which was 9-31% higher than those in the starting pH range of 5-6 (Figure 4-2). Furthermore, the volumetric butanol productivity in the fermentation (0.055 g/L/h) was 25-37% greater than those with lower starting pH values (Figure 4-3). Net glucose utilization was higher in the duplicate containing media with initial pH of 7, and this treatment had the highest observed butanol yield which was 19% (Table 4-1). No growth or solvent production was observed in starting pH values of 3 and 4. During strain characterization, strain C10 possessed the ability to grow at decreased initial pH levels in tri-buffered, strictly anoxic TYG media (data not shown). Hence, oxygen exposure may have inhibited growth at these pH levels.

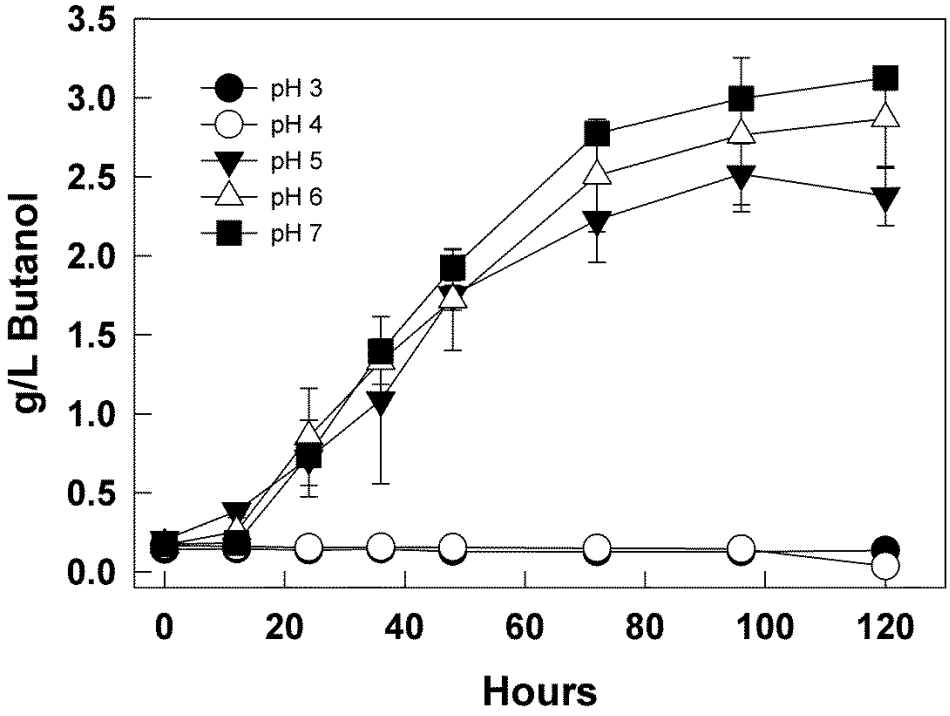

Figure 4-2: Butanol comparison at starting pH values of 5-7 using 2 g/L tryptone as the initial nitrogen source for fermentations.

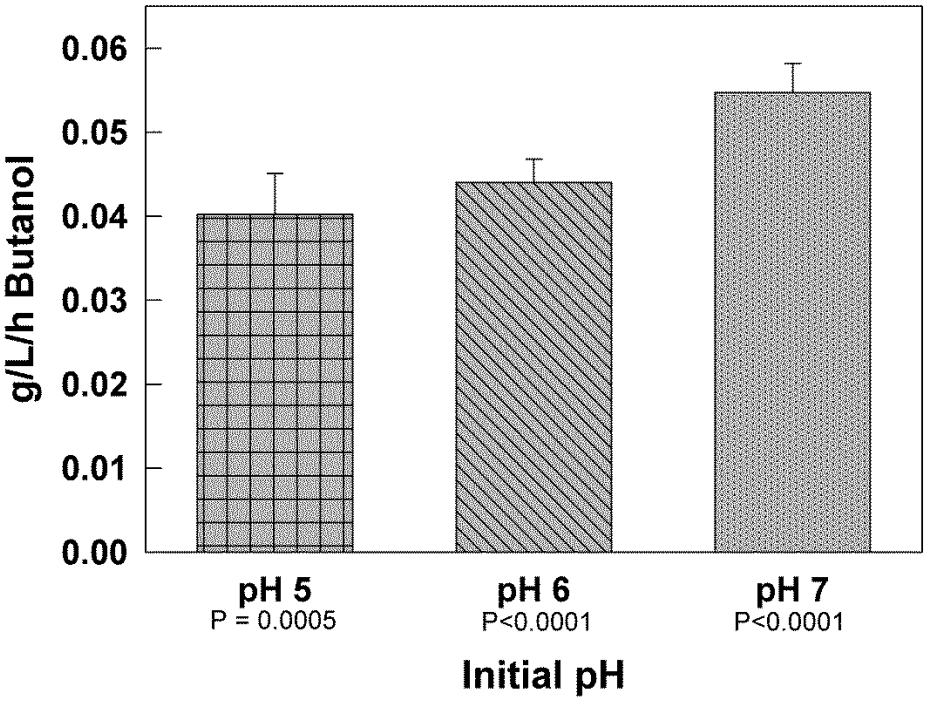

Figure 4-3: Volumetric butanol productivities at starting pH values of 5, 6, and 7 as modeled by the modified Gompertz function. Error bars represent standard deviation.

Starting pH	Butanol Yield from Substrate (g/g)	g/L Substrate Consumed
5	0.18	12.9
6	0.17	16.5
7	0.19	16.7

Table 4-1: Butanol yields and substrate consumption corresponding to starting fermentation pH values.

Nitrogen Sources

Although tryptone promotes fastidious growth of microbes, its usage as a nitrogen source in fermentations is not economically viable {21}. Thus, alternate nitrogen sources were sought. The influence of the source of nitrogen on oxygen-exposed butanol fermentations is illustrated in Figure 4-4. Glucose (30 g/L) was used as the fermentation feedstock. The optimal initial fermentation pH value (pH 7) was established in the previous section. Ammonium acetate (2 g/L) addition stimulated butanol production 1.51-1.70-fold, relative to fermentations containing ammonium chloride and ammonium sulfate. Greater net substrate utilization was observed in media preparations containing ammonium acetate (Table 4-2). Duplicate fermentations containing ammonium acetate resulted in a 48.1-61.3% increase in glucose utilization in comparison to fermentations supplemented with ammonium sulfate or ammonium chloride. These results are consistent with a previous study performed with *C. acetobutylicum* ATCC 824, in which acetate supplementation had an additive effect on solventogenesis in fermentations carried out at pH 7 {22}. Acetate addition was also confirmed to promote solvent evolution and substrate utilization in fermentations using the strict anaerobe *C. beijerinckii* NCIMB 8052 {23}.

Figure 4-5 illustrates the effect of nitrogen source on volumetric butanol productivity. Butanol productivity in media preparations containing ammonium acetate was 0.063 g/L/h, which is 1.51-1.60 times greater than preparations containing ammonium sulfate or ammonium chloride. Limited variations were observed in butanol yields throughout each treatment (Table 4-2). However, the increased substrate utilization

observed in the ammonium acetate treatment ultimately lowered the fermentative butanol yield. All subsequent studies were performed with media preparations containing ammonium acetate and an initial pH value of 7 since these conditions provided the most robust results for butanol production.

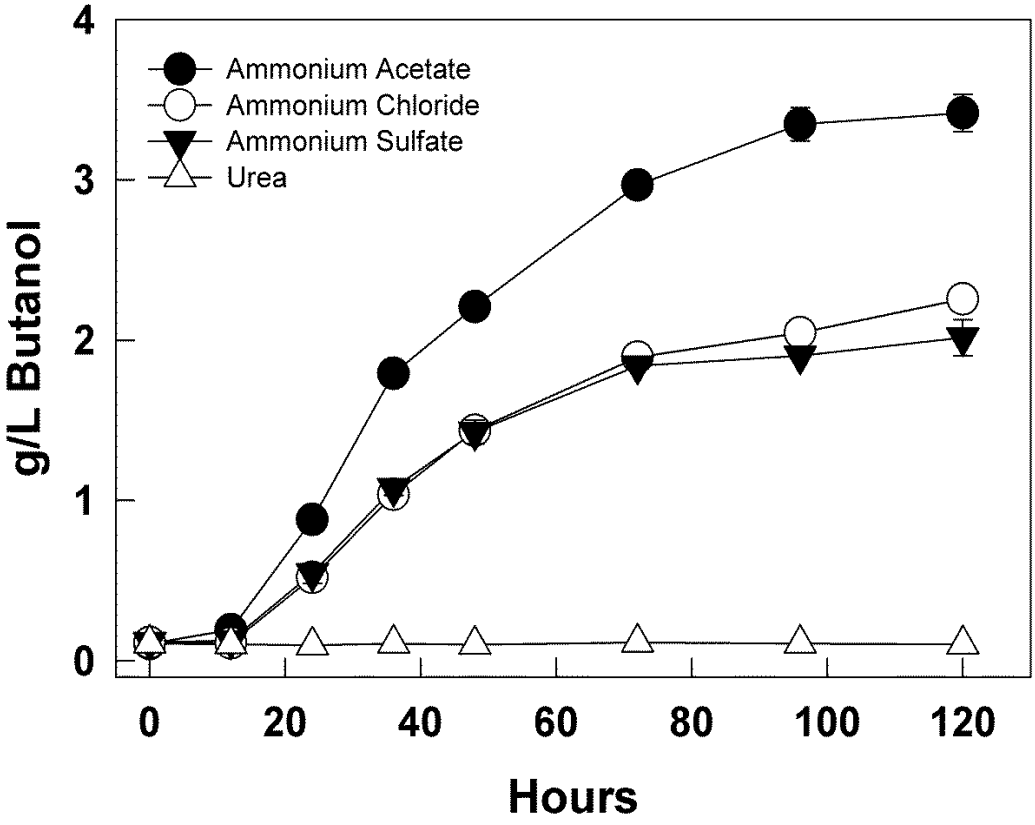

Figure 4-4: Effect of nitrogen sources on butanol production. Error bars represent standard deviation.

N-Source	Butanol Yield from Substrate (g/g)	g/L Substrate Consumed
Ammonium Acetate	0.17	20.0
Ammonium Chloride	0.17	13.5
Ammonium Sulfate	0.16	12.4

Table 4-2: Butanol yield and glucose consumption in the presence of various nitrogen sources. Fermentations were carried out at a starting pH of 7.

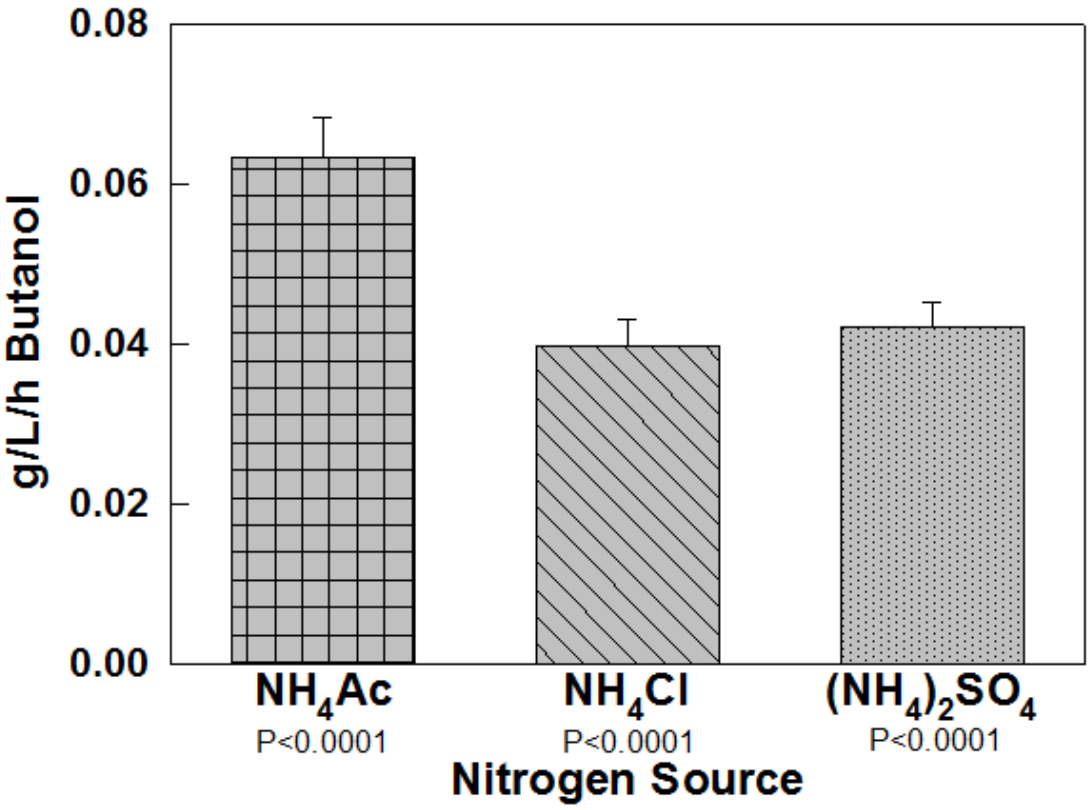

Figure 4-5: Volumetric butanol productivities comparing various nitrogen sources. Error bars indicate standard error generated during non-linear regression analysis.

Effect of Redox Mediators on Oxygen Exposed Fermentations

Subsequent studies were performed to further enhance oxygen exposed butanol production. Ferric iron alone had stimulatory effects on *Clostridial* butanol production, while decreases relative to the ferric iron alone treatment were observed in fermentations challenged with electron shuttling compounds and ferric iron. Five day, oxygen-exposed batch fermentations containing optimized media conditions were performed in the presence of electron shuttles (AQDS) alone, ferrihydrite alone, or ferrihydrite plus AQDS using 60 g/L glucose as the sole fermentable substrate to investigate whether or not the supplementation of electron sinks in oxygen exposed fermentations positively affects solventogenesis. Butanol production was stimulated in the presence of insoluble 20 mM ferrihydrite (FeGel), relative to the cells plus glucose control (Figure 4-6). Five-day butanol titers for these treatments were 6.18 g/L and 4.59 g/L, respectively. AQDS addition to the fermentation media had no effect on the overall butanol titer. However, the observed butanol yield for this treatment (0.22 g/g) was slightly higher than the cells plus glucose control (0.19 g/g), on account of the observed decrease of substrate consumption (Table 4-3). Coupling AQDS to ferrihydrite had an antagonistic effect on solvent production relative to the treatment containing ferrihydrite plus glucose alone. Ammonium acetate alone did not contribute to solvent production, indicating that strain C10 cannot utilize acetate as a fermentation substrate (Figure 4-6).

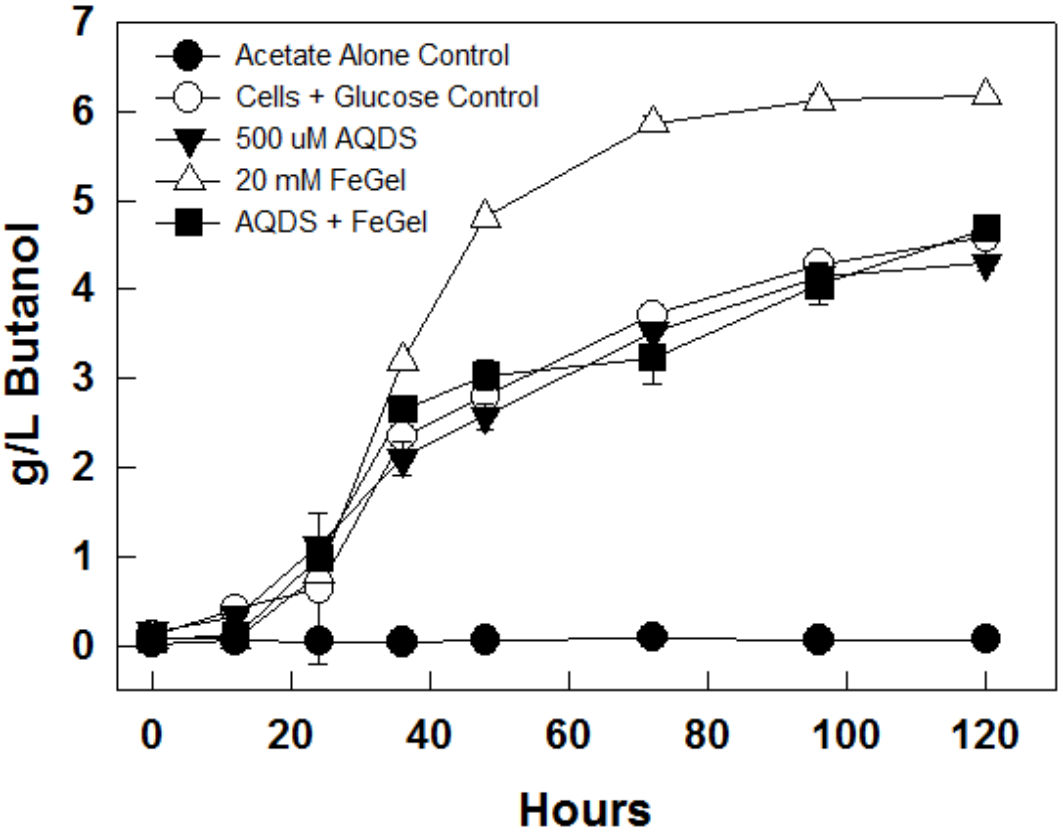

Figure 4-6: Oxygen exposed butanol fermentations in the presence and absence of redox mediators. Error bars represent standard deviation.

A parallel study was performed under strictly anoxic conditions to investigate the effects of oxygen exposure on solventogenic performance. Fermentations were agitated at 80 rpm to simulate similar mixing conditions as the previous studies, and glucose was present in a concentration of 60 g/L. The final butanol titer in the treatment containing 20 mM ferrihydrite was 81% greater than the cells plus glucose control (Figure 4-7). In comparison to the oxygen-exposed study, butanol titers were only increased by 35% in the presence of ferrihydrite due to the elevated butanol titer which was observed in the cells plus glucose control. These data indicate that oxygen exposure may increase solvent

titers in unamended fermentations. The five-day butanol titer of the anoxic treatment containing AQDS plus ferrihydrite increased by 13% in comparison to the same oxygen-exposed treatment, indicating that the presence of oxygen coupled to the shuttle/acceptor pair, AQDS and ferrihydrite, has an inhibitory effect on butanol production for strain C10.

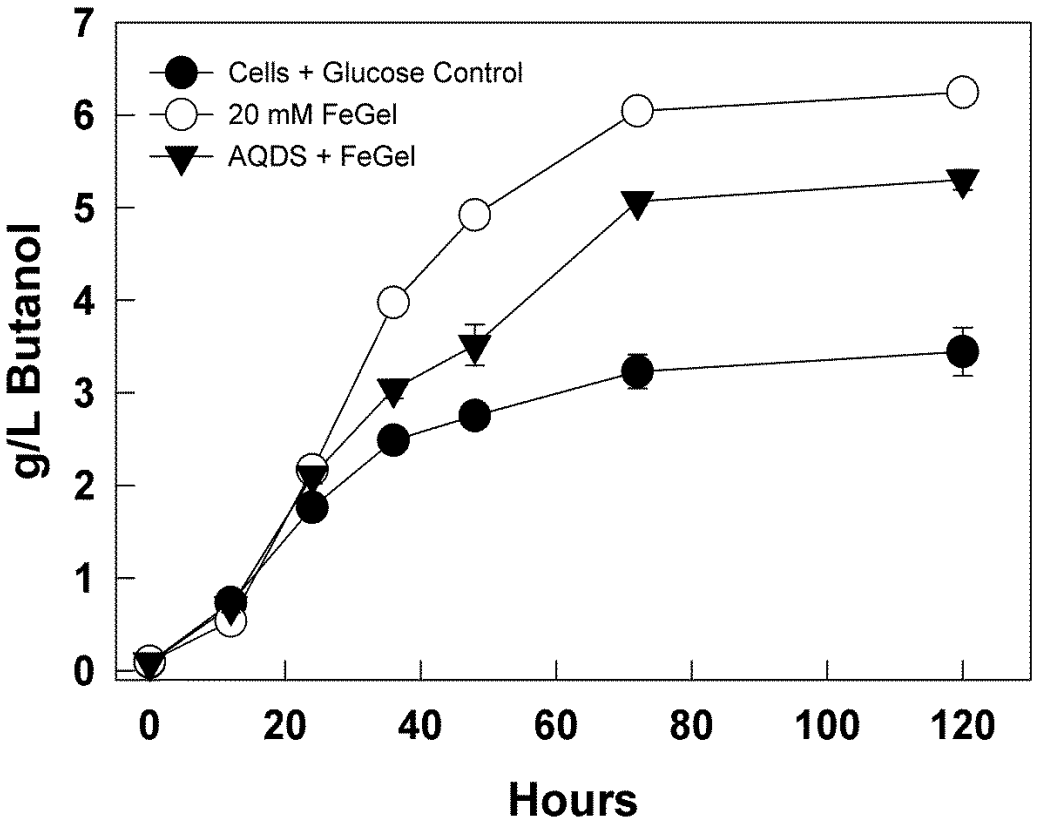

Figure 4-7: Anaerobic comparison of optimized conditions for strain C10. Error bars represent standard deviation.

Table 4-3 illustrates the effect of ferrihydrite and AQDS on volumetric productivity, butanol yield, and substrate consumption in oxygen exposed and strictly anoxic butanol fermentations. The observed volumetric butanol productivity was the highest in the oxygen-exposed fermentation containing 20 mM ferrihydrite (0.201 g/L/h), and this value was 2.48 times higher than the cells plus glucose control (oxygen exposed) and 1.31 times higher than the anoxic treatment containing 20 mM ferrihydrite. Additionally, glucose consumption increased in the aerobic preparation of the treatment containing ferrihydrite without compromising the butanol yield (0.23 g/g). In the cells plus glucose control, respective increases in butanol productivity, yield, and substrate consumption were elevated in the presence of oxygen in comparison to the strictly anoxic control, indicating that oxygen may increase glucose metabolism and butanol evolution in strain C10.

	Treatment	g/L/h Butanol (P<0.05)	Std. Error	Butanol Yield from Substrate (g/g)	g/L Substrate Consumed
Oxygen Exposed	Cells + 60 g/L Glucose Control	0.081	0.013	0.19	24.2
	500 μM AQDS	0.067	0.005	0.22	19.7
	20 mM FeGel	0.201	0.008	0.23	27.3
	AQDS + FeGel	0.090	0.025	0.18	25.5
Strictly Anoxic	Cells + 60 g/L Glucose Control	0.078	0.006	0.16	21.9
	20 mM FeGel	0.153	0.006	0.25	25.3
	AQDS + FeGel	0.095	0.011	0.21	25.4

Table 4-3: Oxygen-exposed versus anoxic fermentations

Xylose as the Sole Fermentable Substrate in Oxygen Exposed Fermentations

Hemicellulose polysaccharides constitute nearly 30% of all lignocellulosic biomass, with a composition of nearly 90% xylose and 10% arabinose {24}. The major driver for xylose utilization as a feedstock in bioprocess applications is its non-competitive nature with our own food sources. Conversely, microbial xylose utilization is highly inefficient in wild-type strains, and no reports exist which couples xylose consumption to oxygen exposed butanol fermentations {25-26}. Hence, a separate study was constructed in an effort to determine whether or not *Clostridium* sp. C10 could utilize xylose as a fermentation feedstock in the presence of oxygen. Experimental conditions which were previously optimized in this study were replicated in oxygen exposed fermentations using 60 g/L xylose as the sole fermentable substrate. The results from Table 4-4 indicate that oxygen exposure did not inhibit growth on xylose, nor did it inhibit solvent evolution when xylose was used as the sole fermentable substrate (control). Butanol productivity, butanol yield, and substrate consumption were increased in the presence of ferrihydrite, and these data are consistent with the ferrihydrite-challenged parent study which used glucose as a feedstock.

Treatment	g/L/h Butanol (P<0.05)	Std. Error	Yield (g/g)	g/L Substrate Consumed
Cells + 60 g/L Xylose Control	0.059	0.006	0.16	22.1
20 mM FeGel	0.153	0.013	0.18	35.4

Table 4-4: Results from oxygen-exposed, 60 g/L xylose-fed fermentations run with *Clostridium* sp. C10. Volumetric productivities are in units of g/L/h. Substrate consumption is indicated by the average of two experiments.

Dissolved oxygen monitoring

Dissolved oxygen (DO) readings were taken at the end of the 5-days for pH and nitrogen source optimization studies. Measurements were taken in an effort to assess the oxygen concentration contained within the media and to determine the extent to which oxygen consumption occurs. Uninoculated controls were analyzed at the beginning of the experiment to assess initial DO conditions without introducing contaminating organisms into the fermentations (Figures 4-8 & 4-9). Oxygen-exposed growth was apparent in agitated (80 rpm) 250 mL Erlenmeyer flasks containing 100 mL fermentation broth using 30 g/L glucose as the sole fermentable substrate (Supplementary Figure 4-1). Cells did not grow at atmospheric concentrations of oxygen, and this was confirmed with streak plating.

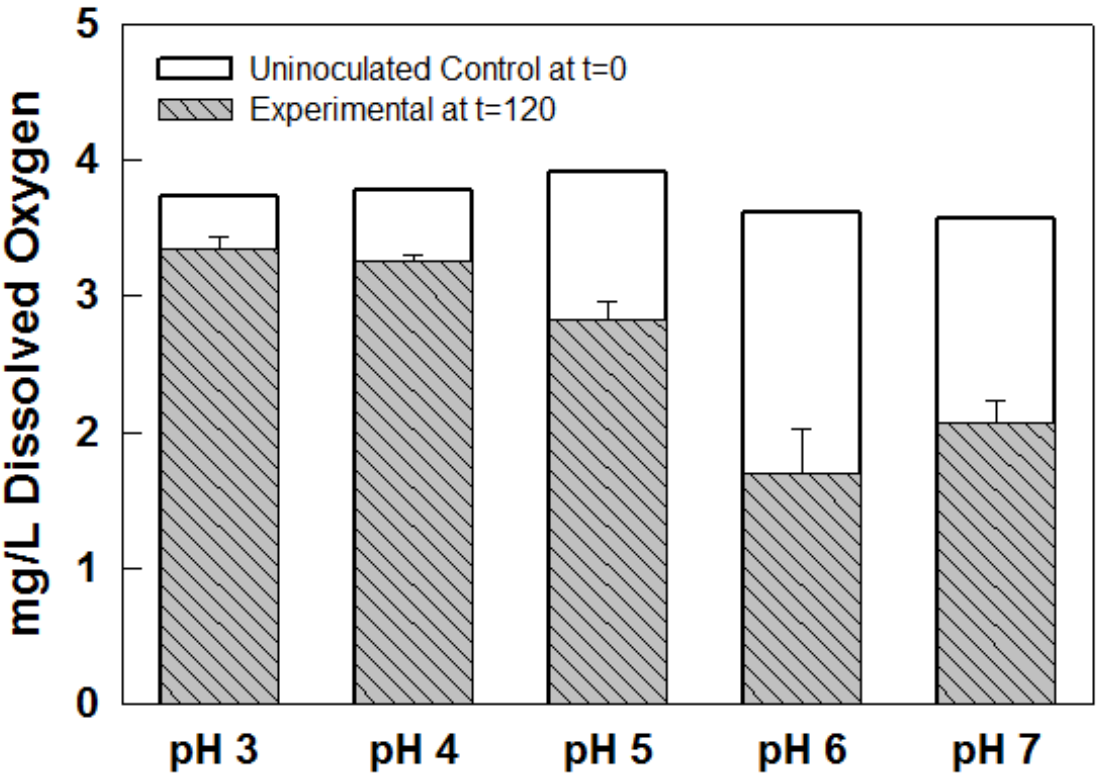

Figure 4-8: Dissolved oxygen readings from initial conditions at the onset of inoculation (uninoculated controls; solid white bars) and at the end of the fermentation (t = 120; gray striped bars). No growth was observed in fermentations with starting pH values of 3 and 4.

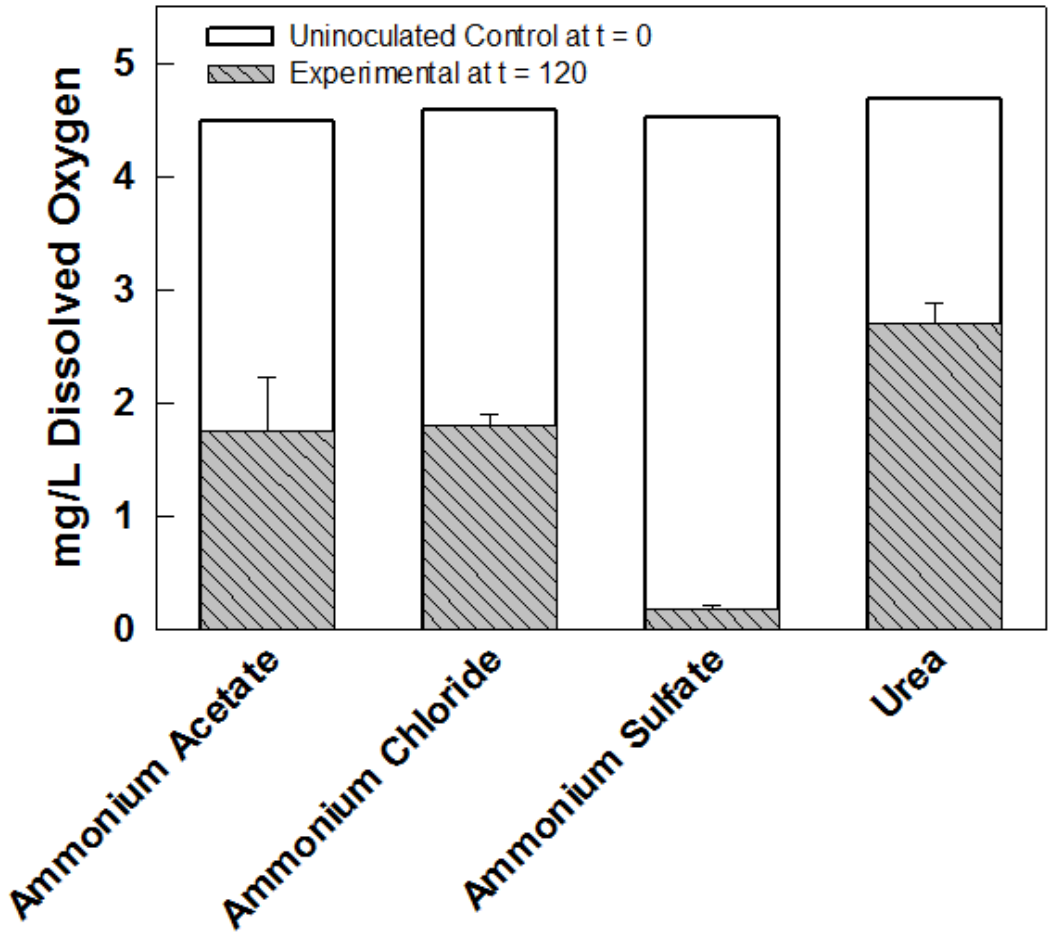

Figure 4-9: Dissolved oxygen readings from initial conditions at the onset of inoculation (uninoculated controls; solid white bars) and at the end of the fermentation (t = 120; gray striped bars)

Conclusions

This study investigated the effects of oxygen exposure on butanol production using *Clostridium sp.* C10. The results demonstrated that strain C10 produced optimal butanol titers (4.59 g/L), yield (0.19 g/g), and productivity (0.081 g/L/h) in fermentation media containing ammonium acetate with an initial pH of 7 and 60 g/L glucose.

Ferrihydrite addition to oxygen-exposed fermentations increased butanol titer (6.18 g/L), yield (0.23 g/g), and productivity (0.201 g/L/h). A parallel study performed under strictly anoxic conditions revealed that oxygen exposure did not compromise butanol production for both the cells plus glucose control and the ferrihydrite amended treatment. Butanol production and xylose consumption were enhanced under optimized fermentation conditions in a separate study, indicating that *Clostridium sp.* C10 can utilize disparate carbohydrate sources to promote solvent evolution in the presence of oxygen. These data are significant from not only the perspective of process economics associated with feedstock utilization and bioreactor operating costs, but also the physiological novelty of *Clostridium sp. C10*.

References

1.) Szwaja, S., & Naber, J. D. (2010). Combustion of *n*-butanol in a spark-ignition IC engine. *Fuel*, *89*(7), 1573-1582.

2.) Cooney, C., Wallner, T., McConnell, S., Gillen, J. C., Abell, C., Miers, S. A., & Naber, J. D. (2009, January). Effects of blending gasoline with ethanol and butanol on engine efficiency and emissions using a direct-injection, spark-ignition engine. In ASME 2009 Internal Combustion Engine Division Spring Technical Conference (pp. 157-165). *American Society of Mechanical Engineers*.

3.) Masum, B. M., Kalam, M. A., Masjuki, H. H., Palash, S. M., & Fattah, I. R. (2014). Performance and emission analysis of a multi cylinder gasoline engine operating at different alcohol–gasoline blends. *Rsc Advances*, *4*(53), 27898-27904.

4.) Jones, D. T., & Woods, D. R. (1986). Acetone-butanol fermentation revisited. Microbiological Reviews, *50*(4), 484.

5.) Woods, D. R., & Jones, D. T. (1986). Physiological Responses of *Bacteroides* and *Clostridium* Strains to Environmental. *Adv in Microbial Physiology APL*, *28*, 1.

6.) Cabiscol, E., Tamarit, J., & Ros, J. (2010). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology*, *3*(1), 3-8.

7.) Imlay, J. A. (2002). How oxygen damages microbes: oxygen tolerance and obligate anaerobiosis. *Advances in microbial physiology*, *46*, 111-153.

8.) Imlay, J. A. (2003). Pathways of oxidative damage. *Annual Reviews in Microbiology*, *57*(1), 395-418.

9.) O'brien, R. W., & Morris, J. G. (1971). Oxygen and the growth and metabolism of *Clostridium acetobutylicum*. *Journal of General Microbiology*, *68*(3), 307-318.

10.) Kawasaki, S., Ishikura, J., Watamura, Y., Ono, M., and Niimura, Y. (2004) Identification of O2-induced peptides in the obligatory anaerobe *Clostridium aceobutylicum*. FEBS Letters, *571*, 21–25.

11.) Kawasaki, S., Watamura, Y., Ono, M., Watanabe, T., Takeda, K., & Niimura, Y. (2005). Adaptive responses to oxygen stress in obligatory anaerobes *Clostridium acetobutylicum* and *Clostridium aminovalericum*. *Applied and Environmental Microbiology*, *71*(12), 8442-8450.

12.) Hillmann, F., Fischer, R. J., Saint-Prix, F., Girbal, L., & Bahl, H. (2008). PerR acts as a switch for oxygen tolerance in the strict anaerobe *Clostridium acetobutylicum*. *Molecular Microbiology*, *68*(4), 848-860.

13.) Mullin, D. A., & Velankar, H. R. (2011). U.S. Patent Application 13/816,231.

14.) Maddox, I. S. (1989). The acetone-butanol-ethanol fermentation: recent progress in technology. *Biotechnology and Genetic Engineering Reviews*, *7*(1), 189-220.

15.) Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111-120.

16.) Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*(10), 2731-2739.

17.) Lovley, D. R., & Phillips, E. J. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology*, *51*(4), 683-689.

18.) Lin, C. Y., & Lay, C. H. (2004). Effects of carbonate and phosphate concentrations on hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy*, *29*(3), 275-281.

19.) Mu, Y., Zheng, X. J., Yu, H. Q., & Zhu, R. F. (2006). Biological hydrogen production by anaerobic sludge at various temperatures. *International Journal of Hydrogen Energy*, *31*(6), 780-785.

20.) Mu, Y., Yu, H. Q., & Wang, G. (2007). A kinetic approach to anaerobic hydrogen-producing process. *Water Research*, *41*(5), 1152-1160.

21.) Parekh, M., Formanek, J., & Blaschek, H. P. (1999). Pilot-scale production of butanol by *Clostridium beijerinckii* BA101 using a low-cost fermentation medium based on corn steep water. *Applied Microbiology and Biotechnology*, *51*(2), 152-157.

22.) Holt, R. A., Stephens, G. M., & Morris, J. G. (1984). Production of solvents by *Clostridium acetobutylicum* cultures maintained at neutral pH. *Applied and Environmental Microbiology*, *48*(6), 1166-1170.

23.) Chen, C. K., & Blaschek, H. P. (1999). Acetate enhances solvent production and prevents degeneration in *Clostridium beijerinckii* BA101. *Applied Microbiology and Biotechnology*, *52*(2), 170-173.

24.) Limayem, A., & Ricke, S. C. (2012). Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. *Progress in Energy and Combustion Science*, *38*(4), 449-467.

25.) Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., & Bogel-Łukasik, R. (2010). Hemicelluloses for fuel ethanol: a review. *Bioresource Technology*, *101*(13), 4775-4800.

25.) Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W. J., Jiang, W., & Yang, S. (2011). Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. *Applied and Environmental Microbiology*, *77*(22), 7886-7895.

26.) Li, Z., Xiao, H., Jiang, W., Jiang, Y., & Yang, S. (2013). Improvement of solvent production from xylose mother liquor by engineering the xylose metabolic pathway in *Clostridium acetobutylicum* EA 2018. *Applied Biochemistry and Biotechnology*, *171*(3), 555-568.

CHAPTER 5

ENHANCING SUBSTRATE (XYLOSE AND GLUCOSE) UTILIZATION AND SOLVENT EVOLUTION USING AN OPEN-SOURCE ELECTRODE SYSTEM IN MEDIATORLESS *CLOSTRIDIUM* FERMENTATIONS

Abstract

An open source electrode system was constructed as a strategy to enhance wild-type solvent formation in non-sterilized, mediatorless, and oxygen exposed fermentations inoculated with *Clostridium* sp. C10. Elevated *n*-butanol and acetone titers were observed in all fermentations containing either glucose or xylose in the presence of electrodes poised at 500 mV relative to cells plus substrate only controls. Respective butanol titers and volumetric butanol productivities in studies performed with 30 g/L glucose or 30 g/L xylose were 1.67 and 2.27 times and 1.90 and 6.13 times greater in the presence of electrodes compared to controls. Elevated solventogenic activity was related to increased substrate consumption, since butanol yields were unaffected in modified fermentations. Xylose and glucose utilization in the presence of electrodes was observed to be 125% and 61% greater than the controls. Increasing substrate concentrations to 60 g/L resulted in a decrease of butanol yields relative to the studies performed at 30 g/L.

Introduction

Select groups have pioneered the concept of creating open source or low-cost electrode systems which are beneficial for resource-limited laboratories performing electrochemical research {1-2}. These systems are practical for executing cyclic voltammetry studies, anodic electron stripping, as well as monitoring microbial respiration. However, the application of these systems to promote bacterial fermentative metabolism has not been addressed.

Previous evidence suggests that anodic electron stripping enhances cellular bioenergetics in both Prokaryotic and Eukaryotic organisms, possibly through the creation of more thermodynamically favorable metabolic intermediates by acting as a solid-state surrogate for terminal electron accepting processes {3-6}. Electron mediated enhancement of metabolite production has been studied extensively {4, 7-10}. However, mediatorless or direct electrode stimulation is still burgeoning as a strategy for stimulating wild-type metabolism {11-14}.

A three electrode potentiostat system was developed from low cost material in response to the need for drop-in treatments capable of enhancing butanol production in wild-type acetone-butanol-ethanol fermentations using either glucose or xylose, a poorly utilized pentose sugar, as growth substrates {15-18}. Butanol is employed as a C4 solvent in myriad commercial applications for the plastic, resin, pharmaceutical, automotive, and paint industries {19-20}. Recently, butanol produced through bacterial fermentation (acetone-butanol-ethanol or ABE) has been investigated as a viable alternative to directly replace gasoline and/or replace current gasoline oxygenates, such as ethanol, since it can

be utilized in internal combustion engines without engine modification {21}. Additionally, researchers have been investigating its use as a precursor for military-grade JP-5 jet fuel, further proving the utility of the solvent across multiple platforms {22}. While biologically produced butanol can be easily adapted as an energy carrier into our current transportation infrastructure and continue to carry the operational definition of "renewable," butanol fermentations have many limitations which hinge upon the physiology of the microorganism catalyzing the conversion of feedstock to solvent. These limitations include low batch solvent titer, rates of production, feedstock consumption, and the maintenance of strict reactor conditions, such as anoxia and sterility.

Herein, the focus of this communication is to demonstrate the ability of an open source electrode system to stimulate solventogenesis in liberally controlled fermentations using the aerotolerant, solventogenic *Clostridium* sp. C10 without the use of electron mediators, which are typically used to enhance electron transfer interactions between cells and electrodes {23-24}. The considerations addressed within the study are paramount to lowering overall process economics surrounding industrial *n*-butanol production.

Materials and Methods

Electrode System Construction

A three electrode analog potentiostat prototype was constructed to control select *Clostridium* sp. C10 batch fermentations. The potentiostat circuit was modeled after a simple potentiostat circuit, and this was created on a prototyping shield which used an

LM324 quadruple operational amplifier to supply power to each electrode (SI Figure 5-1). Voltage was regulated using a 10 kΩ dial potentiometer (SI Figure 5-2). The parts-list for the circuit construction can be found in SI Table 5-1. Steady voltage was supplied to the circuit board using the 5V output voltage pin from an Arduino Uno powered by a D-link multi-port USB {25}.

Graphite rods for the working and counter electrodes were retrieved from a 6V battery cell, and these were cut into smaller pieces with a Dremel tool. The pieces were washed in 1N HCl, followed by rinsing with NanoPure water and subjecting the graphite to flaming by a butane hand torch. Holes (5/64") were drilled into the top of each electrode, and 22 AWG exposed copper wire was inserted into each hole. Solder was applied to the cavity, and the graphite was flamed until the solder melted. Epoxy was applied to the remaining exposed wire within the electrode after cooling. Heat shrink tubing was used to further encapsulate the wire and increase the strength of the joint after the epoxy hardened. The exposed surface areas for the counter and working electrodes were 780.7 mm^2 and 472.8 mm^2, respectively (SI Figure 5-3). Opposing ends of the wires were stripped and soldered to jumper wires to facilitate more seamless integration with the prototyping board.

The reference electrode was constructed by scoring and cracking the tip from a 1 mL Pasteur pipette using a ceramic GC column cutter, followed by adding a 4A molecular sieve to the tip of the electrode. The 4A molecular sieve was bonded to the glass pipette tip using silicone waterproofing sealant. Glass beads (0.1 mm) were added over the top of the molecular sieve after the silicone sealant dried. The liquid-tight

electrode was filled with a 50 mM copper sulfate solution. Three inches of insulation was stripped from the copper wire, and the exposed portion was inserted into a rubber plug using an 18 Ga needle as a guide. The plug containing the wire was inserted into the Pasteur pipette containing the copper solution. A portion of insulation on the opposing side of the wire was stripped and soldered to a jumper wire to provide more seamless integration into the circuit board. Heat shrink tubing was used to cover this joint. New wire and copper sulfate solution were prepared prior to the start of each experiment since wire oxidation and copper deposition within the electrode were visible at the end of each fermentation.

The total energy used by the electrode system over the course of 5-day fermentations was determined by calculating the energy required to supply the LM324 operational amplifier alone, using characteristics described for +5.0V input, at which the typical input bias current was 45 nA {26} (Equations 1 &2). The number of Coulombs (C) were determined by dividing input current (A; amperes) over the time course of the fermentation. Units were converted to Joules (J) and subtracted from the total energy content of butanol generated (29.2 MJ/L) at the end of each fermentation.

$$J = V\ x\ C \quad \text{(Equation 1)}$$

$$C = \frac{A}{seconds} \quad \text{(Equation 2)}$$

Culture Maintenance and Fermentation Conditions

Spores from aerotolerant *Clostridium sp.* C10 were stored at -20 ^{o}C in NanoPure water. A 0.1 mL volume of the thawed spore suspension was used to inoculate an oxygen

exposed, 100 mL screw top Pyrex bottle containing TYG media at room temperature for each batch study. TYG media was prepared by adding 30 g/L tryptone, 20 g/L glucose, and 10 g/L yeast extract to NanoPure water. Liquid volume was brought up to 100 mL, and this was autoclaved on a liquid cycle at 121 ^{o}C for 30 min. Cells growing in TYG were incubated at 37 ^{o}C in the dark for 26 h prior to the start of each batch study.

Experimental media was prepared immediately prior to inoculation with vegetative cultures of strain C10. Strain C10 fermentation broth was prepared by mixing 2 g/L ammonium acetate, 1 g/L yeast extract, 20 mM KH_2PO_4, and either glucose or xylose in NanoPure water. Media was aliquoted into 200 mL screw top Pyrex bottles without degassing, sterilization, or pH adjustment. This was inoculated with a 6% (v/v) volume of an actively growing culture of strain C10 to start the experiment. Final fermentation volumes were 200 mL. All controls were run in duplicate. The electrode system was added to a single bottle which contained the same media constituents as that of the controls. The exposed graphite portions of both working and counter electrodes were flamed with a hand torch for five seconds each, and after cooling, the electrodes were grouped together with a plastic zip tie and placed in the fermentation vessel. Proper care was ensured that there was no contact between any of the electroconductive portions of the electrodes to prevent short-circuiting. Pyrex bottles containing the electrode bundle were covered with a layer of Parafilm, while controls were run with loosened caps. Voltage between the working and counter electrode was fixed at an initial voltage of 500 mV for all electrode studies using a 10 kΩ dial potentiometer. Initial voltage differences between the working and counter electrodes in the bulk fermentation liquid were

measured using a digital multimeter. Fermentations were incubated in the dark at 37 ^{o}C without agitation. Samples (5 mL) were withdrawn periodically over a 120 h period.

Sample Collection and Analytical Methods

Liquid samples for metabolite analysis were filtered (0.2 μm) into autosampler vials containing 250 μL glass inserts (Lab Supply Distributors). Vials were sealed with screw top PFTE caps and stored at 4 ^{o}C until chromatographic analysis. Remaining sample volumes were distributed into polystyrene cuvettes (VWR) for optical density analysis and clean falcon tubes for pH analysis.

Acetone and butanol were analyzed on a Shimadzu GC-2014 equipped with a flame ionization detector and a DB-FFAP column (30-m x 0.250-mm; 0.25-μm film thickness). Helium was used as the carrier gas, and the linear velocity was set to 80.3 cm/s. Sample volumes of 1 μL were withdrawn from the autosampler vials using an AOC20i+S. Following injection, the syringe was washed three times with NanoPure water. The oven temperature program included a 40 ^{o}C dwell (2 min) and a temperature ramp of 50 ^{o}C/min until the oven reached 220 ^{o}C. The oven temperature was held at 220 ^{o}C for 1 min until returning back to the initial dwell temperature. The injector and detector temperatures were set to 200 ^{o}C and 300 ^{o}C, respectively.

Glucose and xylose were separated with a Dionex HPLC equipped with a Biorad HP-Aminex column and were analyzed using a Refractomax 521 refractive index detector (Thermo Scientific). The mobile phase (degassed 5 mM H_2SO_4 in NanoPure

water) was set at a constant flow of 0.6 mL/min, and the column oven was set to 60 ^{o}C. Sample volumes of 0.25 µL were used for the analysis.

Optical density was measured using a Genesys 10S UV-Vis spectrophotometer (Thermo). Measurements for pH were performed with a Thermo Scientific Orion Star A111 pH meter equipped with an Orion 9107BNMD probe. This was calibrated prior to each measurement using BDH general pH buffer solutions, ranging from pH 4-10.

Kinetic Analysis

Time-course butanol data were fitted using a modified Gompertz equation. Rate constants were generated using non-linear regression. This model's utility has been described in previously {27-30}. Volumetric butanol productivities of the batch fermentations were calculated in SigmaPlot statistical software, and units are listed in g/L/h.

$$P = P_{max} * \exp\left\{-\exp\left[\frac{\exp(1)*R}{P_{max}(\lambda - t)} + 1\right]\right\} \text{ (Equation 3)}$$

Specific butanol production was determined using the Luedeking-Pieret equation for mixed growth associated metabolite production, where α is the growth associated coefficient for product formation (g butanol/g biomass), X denotes biomass concentration (g/L), t denotes time (h), and β is non-growth associated production (g butanol/g biomass) (Equation 4) {31}. Biomass was quantified spectrophotometrically (OD600), and these values were correlated to g/L dry weight cells. Units are described in g butanol/g biomass/h.

$$q_{Butanol} = \alpha \frac{dX}{dt} + \beta X \quad \text{(Equation 4)}$$

Results and Discussion

Electrode System Influence on Pentose Metabolism and *Solvent Evolution*

Batch fermentations were constructed without sterilization and were inoculated with a vegetative culture of the oxygen tolerant *Clostridium sp.* C10. Non-sterile electrodes were inserted into experimental treatments after inoculation, and voltage was adjusted to 500 mV at the start of the experiment. Controls were run in the absence of electrodes and contained cells plus substrate (glucose or xylose) alone. The emphasis of this study was to demonstrate that an open source, solid state electrode system can increase levels of solventogenesis and substrate consumption in liberally-controlled ABE fermentations.

Evidence for electrode-stimulated solvent production in fermentations is presented in Figure 5-1. Butanol titers in the presence of the electrode system were increased by 2.3-fold (5.0 g/L) relative to the cells plus xylose controls (2.2 g/L) after 5 days of growth in fermentations containing 30 g/L xylose. Acetone concentrations improved by 59.7% in electrode challenged fermentations (1.1 g/L). In a subsequent study using 60 g/L xylose as the sole fermentable substrate, electrode-challenged butanol titers (5.8 g/L) were 1.9-fold greater than that was observed in the cells plus xylose controls (3.1 g/L) while acetone concentrations were 1.5-fold greater than that in the absence of electrodes.

Butanol productivity (volumetric and specific) and xylose consumption were enhanced in electrode-challenged fermentations (Table 5-1). Specific and volumetric butanol production was increased by 4.4-fold and 6.1-fold, respectively, in the presence of the electrodes for fermentations containing 30 g/L xylose. A similar profile was observed in fermentations containing 60 g/L xylose. Specific and volumetric butanol productivities were 3.3-fold and 3.8-fold greater than that of the control, respectively.

Pentose metabolism was elevated in the presence of electrodes. However, increasing the concentration of xylose alone enhanced substrate consumption in the controls, where only 11.0 g/L xylose was consumed in 30 g/L xylose fed fermentations and 24.1 g/L xylose consumed in controls containing 60 g/L xylose. It has been previously indicated that the solventogenic *C. acetobutylicum* has the capability of altering xylose consuming pathways under high xylose concentrations through enabling the phosphoketolase pathway to supplement pentose metabolism {32}. However, induction of this pathway under high xylose stress has not been confirmed with *Clostridium* sp. C10. Butanol yields were decreased in all fermentations containing 60 g/L xylose, on account of the observed increase in substrate utilization for these treatments.

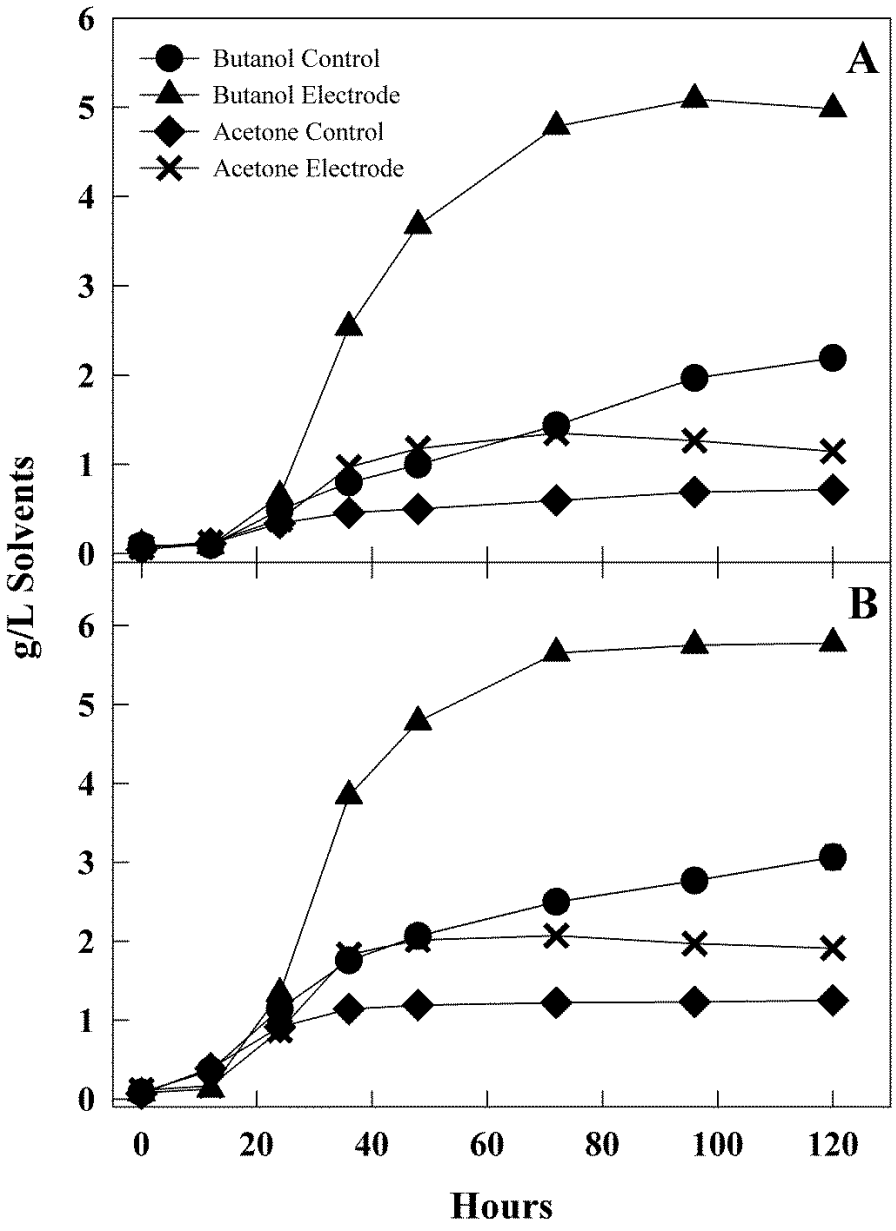

Figure 5-1: Solvent evolution in batch fermentations containing 30 g/L (A) and 60 g/L (B) xylose in the presence and absence of the electrode system. Results for the control treatment are presented as the average of duplicates, while the electrode treatment was performed in single fermentations. Error bars represent standard deviation between samples.

Treatment	g/L/h Butanol (P<0.05)	Specific Butanol Production (g/g/h)	Butanol yield (g/g)	g/L Substrate Consumed
30 g/L Xylose	0.024 ± 0.002	0.08	0.20	11.0
30 g/L Xylose + Electrode	0.147 ± 0.010	0.35	0.20	24.8
60 g/L Xylose	0.052 ± 0.006	0.14	0.13	24.1
60 g/L Xylose + Electrode	0.200 ± 0.014	0.46	0.17	34.1

Table 5-1: Fermentation productivity and substrate consumption in fermentations containing 30 g/L or 60 g/L xylose. Butanol yields were determined by calculating g butanol produced per g xylose consumed.

Glucose as the Sole Fermentable Substrate

The electrode system increased respective five-day butanol titers by 66.6% relative to the control (4.12 g/L) in 30 g/L glucose-fed fermentations. Increasing glucose concentrations had inhibitory effects on solventogenesis. Five-day butanol and acetone titers were inhibited by 6.1% and 20.7%, respectively, at 60 g/L glucose. Furthermore, butanol productivities decreased at elevated glucose concentrations in fermentations treated with the electrode system (Table 5-2). Acetone titers increased minimally in the presence of the electrode system at 30 g/L and 60 g/L glucose concentrations, and these data are consistent with the previous study which showed that the presence of the electrode system had the most pronounced effect on butanol producing pathways.

Initial and final substrate concentrations were quantified to determine the extent of glucose utilization in the presence and absence of the electrode system (Table 5-2). Glucose consumption in the absence of electrodes was greater than that was observed in the controls containing xylose, and glucose consumption reached a limit of 28.9-30.4 g/L

in the presence of electrodes, while consumption was similar (18 g/L vs. 19.9 g/L) between the 30 g/L and 60 g/L glucose controls.

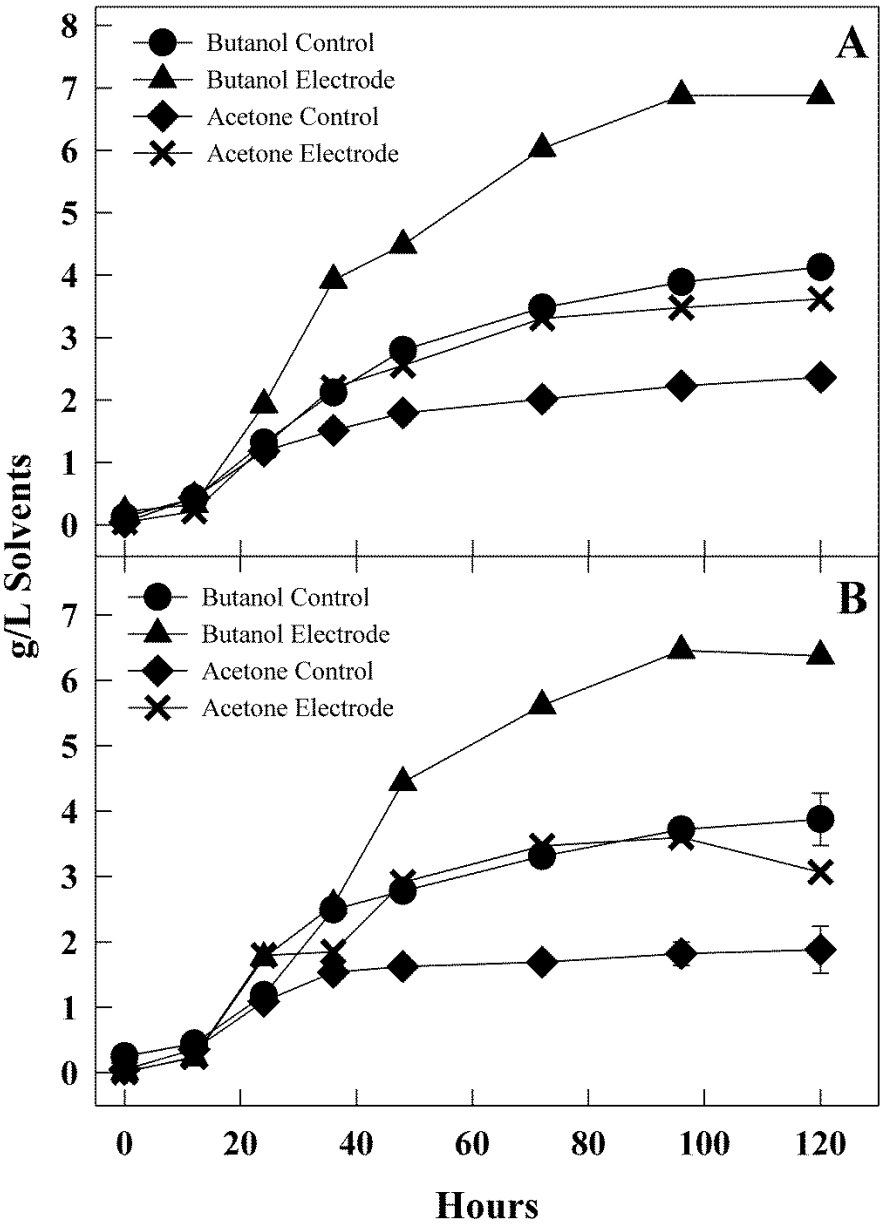

Figure 5-2: Solvent evolution in batch fermentations containing 30 g/L (A) and 60 g/L (B) glucose in the presence and absence of the electrode system. Results for the control treatment are presented as the average of duplicates, while the electrode treatment was performed in single fermentations. Error bars represent standard deviation.

Treatment	g/L/h Butanol (P<0.05)	Specific Butanol Production (g/g/h)	Butanol yield (g/g)	g/L Substrate Consumed
30 g/L Glucose	0.070 ± 0.004	0.18	0.23	18.0
30 g/L Glucose + Electrode	0.133 ± 0.015	0.62	0.24	28.9
60 g/L Glucose	0.076 ± 0.010	0.25	0.19	19.9
60 g/L Glucose + Electrode	0.123 ± 0.013	0.38	0.21	30.4

Table 5-2: Fermentation productivity and substrate consumption in fermentations containing 30 g/L or 60 g/L glucose as the sole fermentable substrate.

Energy Output

Total energy output per fermentation was calculated from the perspective of butanol produced and the energy required to deliver voltage through the electrode system, meaning that electrical energy input required to supply volage to the electrodes was subtracted from each fermentation run in the presence of electrodes. Xylose-fed fermentations challenged with the electrode system were able to extract 179.5-208.0 kJ/L from the provided reducing sugars, which equates to a 1.9-2.3-fold increase in butanol energy output than that was observed in non-challenged fermentations (Table 5-3). Similar results were observed for glucose-fed fermentations. Observed increases in the total energy output for glucose-fed, electrode-challenged fermentations was 1.6-1.7-fold greater than that of the controls.

	Butanol Energy Yield from Fermentations (kJ/L)
30 g/L Xylose + Electrode	179.5
60 g/L Xylose + Electrode	208.0
30 g/L Glucose + Electrode	248.0
60 g/L Glucose + Electrode	230.0
30 g/L Xylose	79.0
60 g/L Xylose	110.5
30 g/L Glucose	149.0
60 g/L Glucose	140.0

Table 5-3: Comparison of the total energy output from butanol produced per fermentation. Energy required to supply power to the electrode system was subtracted from each electrode challenged treatment.

Conclusions

A low-cost electrode system was constructed to modify the native redox environment of liberally-controlled ABE fermentations containing either xylose or glucose as the sole fermentable substrates. Solventogenic enhancement for fermentations in the presence and absence of the electrode system was examined from the perspective of overall solvent titers, volumetric and specific butanol productivity, substrate utilization, butanol yield from substrate, and total energy content produced per fermentation in the form of butanol. Increases in each of the previously listed metrics, excluding butanol yield, were readily apparent in fermentations challenged with the

electrode system. These data indicate that this system may be deployed as an economical, drop-in strategy to lower processing costs associated with ABE fermentations.

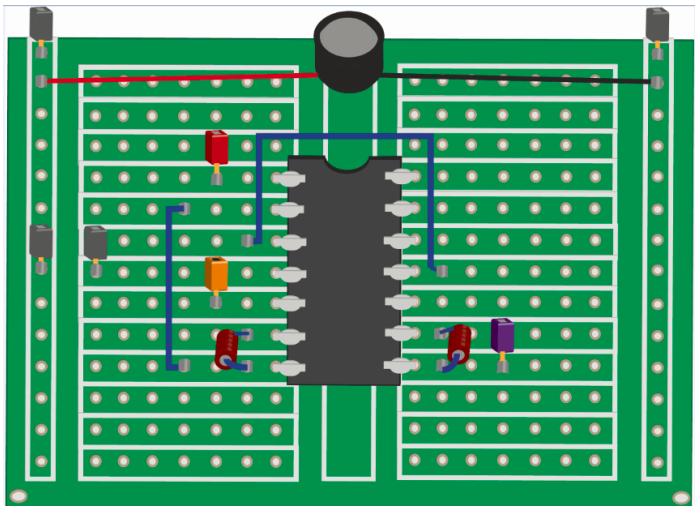

Figure 5-3: Pinout Diagram for electrode system. Working electrode input (WE); Counter electrode input (CE); Reference electrode input (REF).

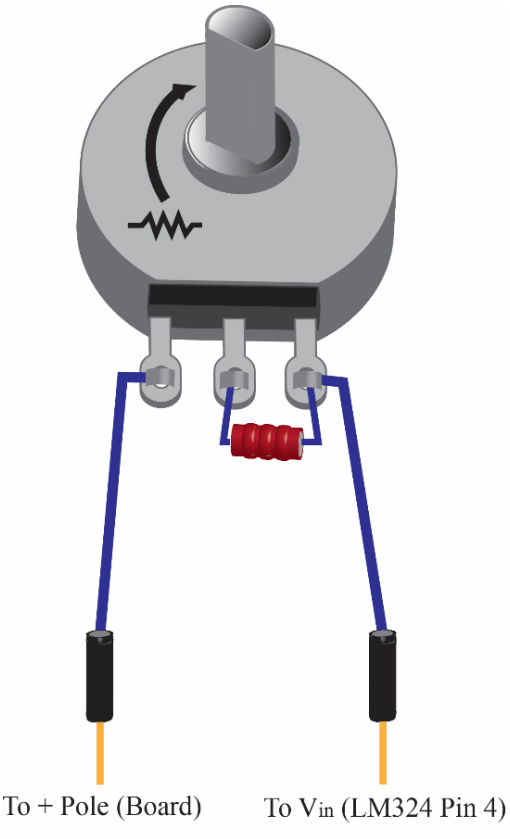

Figure 5-4: Dial potentiometer

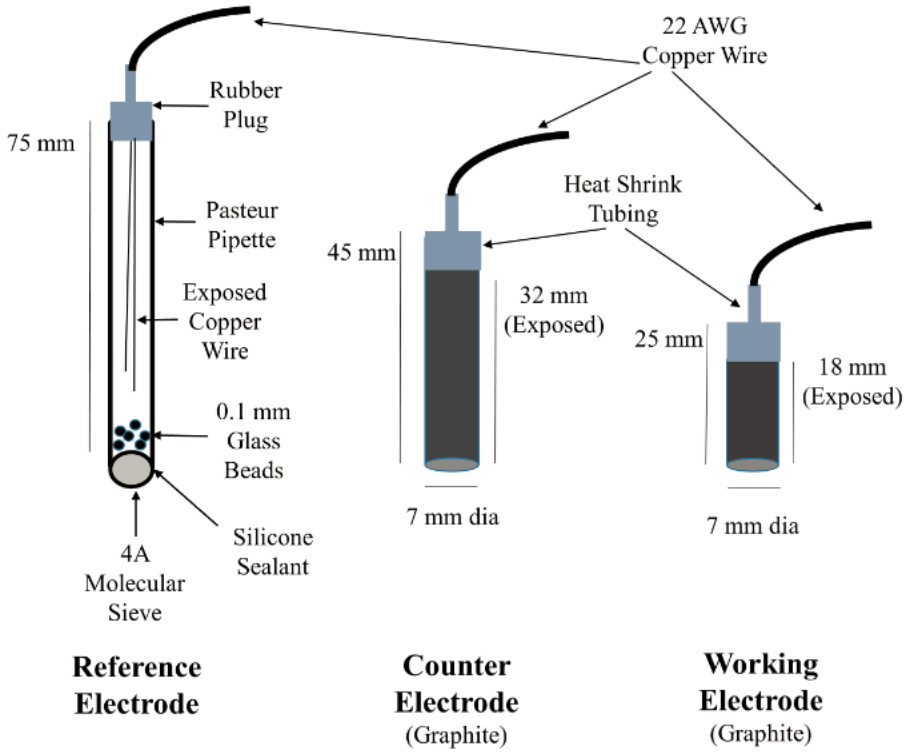

Figure 5-5: Electrode Construction

Description	Supplier	Cat. Number	Qty	Unit Price (USD)
mikroElektronika PCB	Mouser	932-MIKROE-767	1	6.90
10 kΩ Dial Potentiometer	Mouser	858-P270SF21R10K	1	10.27
LM324N Quad Operational Amplifier	Mouser	512-LM324N	1	0.33
1 kΩ Carbon Composite Resistor	Mouser	588-OA102KE	3	2.62
220 uF 16V Capacitor	Mouser	667-EEU-FC1C221	1	0.49
Headers and Wire Housing	Mouser	517-929984-01-26-RK	2	2.46
Alpha Wire Hook-up Wire	Mouser	602-7057-100-09	1	37.21
22 Ga AWG Copper Wire (100 ft.)	Amazon	N/A	1	11.97
Solderless Breadboard Jumper Wires	Amazon	N/A	1	6.59
Arduino Uno (Opt.)	Amazon	N/A	1	23.11
D-Link USB 2.0 Powered Hub (Opt.)	Amazon	N/A	1	17.36
Weller WPS18MP Soldering Iron (Opt.)	Amazon	N/A	1	27.97
NTE Heat Shrink Tubing	Amazon	N/A	1	11.17
BernzOmatic SRC050 Electrical Solder	Amazon	N/A	1	9.21
Data Logging Shield for Arduino (Opt.)	Adafruit	1141	1	19.95
Stacking Headers for Arduino (Opt.)	Adafruit	85	1	1.95

Table 5-3: Electrode system parts list and prices

References

1.) Rowe, A. A., Bonham, A. J., White, R. J., Zimmer, M. P., Yadgar, R. J., Hobza, T. M., ... & Plaxco, K. W. (2011). CheapStat: An open-source,"do-it-yourself" potentiostat for analytical and educational applications. *PloS One*, *6*(9), e23783.

2.) Friedman, E. S., Rosenbaum, M. A., Lee, A. W., Lipson, D. A., Land, B. R., & Angenent, L. T. (2012). A cost-effective and field-ready potentiostat that poises subsurface electrodes to monitor bacterial respiration. *Biosensors and Bioelectronics*, *32*(1), 309-313.

3.) Lovley, D. R. (2006). Microbial fuel cells: novel microbial physiologies and engineering approaches. *Current Opinion in Biotechnology*, *17*(3), 327-332.

4.) Shin, H., Zeikus, J., & Jain, M. (2002). Electrically enhanced ethanol fermentation by *Clostridium thermocellum* and *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology*, *58*(4), 476-481.

5.) Thrash, J. C., & Coates, J. D. (2008). Review: direct and indirect electrical stimulation of microbial metabolism. *Environmental Science & Technology*, *42*(11), 3921-3931.

6.) Lovley, D. R. (2006). Bug juice: harvesting electricity with microorganisms. *Nature Reviews Microbiology*, *4*(7), 497-508.

7.) Price-Whelan, A., Dietrich, L. E., & Newman, D. K. (2006). Rethinking 'secondary' metabolism: physiological roles for phenazine antibiotics. *Nature Chemical Biology*, *2*(2), 71-78.

8.) Popovic, J**.**, X. Ye, A. Haluska, K.T. Finneran. Increasing xylose consumption and butanol production with ferric iron and extracellular electron shuttling molecules during fermentation with *Clostridium beijerinckii* NCIMB 8052, Article in preparation

9.) Kim, T. S., & Kim, B. H. (1988). Electron flow shift in *Clostridium acetobutylicum* fermentation by electrochemically introduced reducing equivalent. *Biotechnology Letters*, *10*(2), 123-128.

10.) Rao, G., & Mutharasan, R. (1986). Alcohol production by *Clostridium acetobutylicum* induced by methyl viologen. *Biotechnology letters*, *8*(12), 893-896.

11.) Chaudhuri, S. K., & Lovley, D. R. (2003). Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells. *Nature Biotechnology*, *21*(10), 1229-1232.

12.) Zebda, A., Gondran, C., Le Goff, A., Holzinger, M., Cinquin, P., & Cosnier, S. (2011). Mediatorless high-power glucose biofuel cells based on compressed carbon nanotube-enzyme electrodes. *Nature Communications*, *2*, 370.

13.) Kim, H. J., Park, H. S., Hyun, M. S., Chang, I. S., Kim, M., & Kim, B. H. (2002). A mediator-less microbial fuel cell using a metal reducing bacterium, *Shewanella putrefaciens*. *Enzyme and Microbial Technology*, *30*(2), 145-152.

14.) Liu, Y., Wang, M., Zhao, F., Xu, Z., & Dong, S. (2005). The direct electron transfer of glucose oxidase and glucose biosensor based on carbon nanotubes/chitosan matrix. *Biosensors and Bioelectronics*, *21*(6), 984-988.

15.) Servinsky, M. D., Kiel, J. T., Dupuy, N. F., & Sund, C. J. (2010). Transcriptional analysis of differential carbohydrate utilization by *Clostridium acetobutylicum*. *Microbiology*, *156*(11), 3478-3491.

16.) Xiao, H., Gu, Y., Ning, Y., Yang, Y., Mitchell, W. J., Jiang, W., & Yang, S. (2011). Confirmation and elimination of xylose metabolism bottlenecks in glucose phosphoenolpyruvate-dependent phosphotransferase system-deficient *Clostridium acetobutylicum* for simultaneous utilization of glucose, xylose, and arabinose. *Applied and Environmental Microbiology*, *77*(22), 7886-7895.

17.) Jeffries, T. W. (1983). *Utilization of xylose by bacteria, yeasts, and fungi* (pp. 1-32). Springer Berlin Heidelberg.

18.) Jojima, T., Omumasaba, C. A., Inui, M., & Yukawa, H. (2010). Sugar transporters in efficient utilization of mixed sugar substrates: current knowledge and outlook. *Applied Microbiology and Biotechnology*, *85*(3), 471-480.

19.) Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by *Clostridia*. *Biotechnology and Bioengineering*, *101*(2), 209-228.

20.) Harvey, B. G., & Meylemans, H. A. (2011). The role of butanol in the development of sustainable fuel technologies. *Journal of Chemical Technology and Biotechnology*, *86*(1), 2-9.

21.) Szulczyk, K. R. (2010). Which is a better transportation fuel–butanol or ethanol?. *Int Journal of Energy and Environmental Science*, *1*(3), 501-512.

22.) Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., & Keasling, J. D. (2008). Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Current Opinion in Biotechnology*, *19*(6), 556-563.

23.) Schröder, U. (2007). Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. *Physical Chemistry Chemical Physics*, *9*(21), 2619-2629.

24.) Rabaey, K., & Rozendal, R. A. (2010). Microbial electrosynthesis—revisiting the electrical route for microbial production. *Nature Reviews Microbiology*, *8*(10), 706-716.

25.) Arduino. http://arduino.cc/ (accessed 2015)

26.) LMx24-N, LM2902-N Low-Power, Quad-Operational Amplifiers. http://www.ti.com/lit/ds/symlink/lm124-n.pdf (accessed 2015)

27.) Lin, C. Y., & Lay, C. H. (2004). Effects of carbonate and phosphate concentrations on hydrogen production using anaerobic sewage sludge microflora. *International Journal of Hydrogen Energy*, *29*(3), 275-281.

28.) Mu, Y., Zheng, X. J., Yu, H. Q., & Zhu, R. F. (2006). Biological hydrogen production by anaerobic sludge at various temperatures. *International Journal of Hydrogen Energy*, *31*(6), 780-785.

29.) Mu, Y., Yu, H. Q., & Wang, G. (2007). A kinetic approach to anaerobic hydrogen-producing process. *Water Research*, *41*(5), 1152-1160.

30.) Zwietering, M. H., Jongenburger, I., Rombouts, F. M., & Van't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology*, *56*(6), 1875-1881.

31.) Pazouki, M., Najafpour, G., & Hosseini, M. R. (2008). Kinetic models of cell growth, substrate utilization and bio-decolorization of distillery wastewater by *Aspergillus fumigatus* U B2 60. *African Journal of Biotechnology*, *7*(9).

32.) Liu, L., Zhang, L., Tang, W., Gu, Y., Hua, Q., Yang, S., Jiang, W., and Yang, C. (2012). Phosphoketolase pathway for xylose catabolism in *Clostridium acetobutylicum* revealed by 13C metabolic flux analysis. *Journal of Bacteriology*, *194*(19), 5413-5422.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE RECOMMENDATIONS

Importance of Research/Relevance

Energy security is paramount for flourishing modern economies, and national investment in alternative energy carriers will aid in uncoupling our nation from the volatility of foreign oil markets. Biologically produced butanol fuel has gained much interest as a supplement or direct replacement for gasoline, owing to many of its favorable properties as a solvent and its potential for large scale production. In the past, the acetone-butanol-ethanol (ABE) fermentation process played an indirect role in shaping many of the current borders across Europe and the Middle East, and its utility is experiencing a reemergence as the demand for energy increases.

Fermentations which use wild type microorganisms to produce butanol are limited with respect to solvent productivity and substrate utilization. Successful efforts have been made to genetically alter microorganisms to stimulate fermentative metabolism, however, many "designer" butanol producing microorganisms are proprietary and/or are subjected to considerable regulations with respect to usage and disposal, which is unfavorable for the large scale operations necessary to meet the production demand. High costs associated with biocatalysis, stemming from inefficient processing attributed to the organism/catalyst used, must be lowered in order for butanol fuel to remain a strong competitor with petroleum based fuels. In an effort to remedy shortcomings of industrial ABE fermentations while circumventing the need for genetically modified organisms, physiological pressures can be applied to fermentations to overstimulate cellular metabolic activity without genetic manipulation.

The purpose of this work was to offer a "drop-in" adaptation to existing ABE fermentation processes to increase overall solvent titers and substrate utilization in wild

type, solventogenic organisms. As stated previously, most advancements in ABE fermentation processes rely on the use of genetic engineering, and the proposed study focused on using exogenous compounds, both soluble and solid-state, to mediate the redox environment in bench-scale batch fermentations, ultimately providing an artificial stimulus to drive butanol production in industrially relevant pure cultures. Additionally, the data presented within this dissertation suggests that the application of redox mediators to mixed culture fermentations has the ability to establish a steady phenotype of solvent overproduction. This ultimately has the potential to lead to more streamlined integration of biofuel processes into current mixed culture systems, and it also would limit the need for bioreactor sterilization. Broader implications for these benefits would be reflected in more efficient and economically feasible large-scale solvent production processes.

This work focused on the use of hemicellulose-derived xylose as a fermentation feedstock, rather than glucose, which is currently the most widely used substrate for industrial ABE fermentations. Presently, the glucose utilized in industrial fermentations is derived from food crops such as corn, creating competition between human consumption and solvent production, thus increasing the processing costs. Alternative feedstocks, which are in abundance and do not compete with food, need to be used in order for large scale ABE fermentation operations to become more attractive to the consumer.

Summary of Key Findings

The main objective of this research was to determine the extent to which altering the redox environment in ABE fermentations can increase wild-type solvent production and xylose consumption. Pure culture studies demonstrated that electron shuttling to iron

distorts native fermentative metabolism in solventogenic *Clostridium* species, thus enhancing solvent output and pentose metabolism without genetic modification. Subsequent studies using mixed culture inocula demonstrated that electronophore challenged fermentations shifts populations towards those that are fermentative while simultaneously eliciting a similar physiological response to the previously studied pure culture fermentations with respect to xylose metabolism and fermentative end product evolution. Furthermore, this study has demonstrated that the use of an inexpensive, solid-state device can provide the necessary physiological pressure on *Clostridium* fermentations to deliver similar results for xylose consumption and butanol production, acting as a substitute for iron or electron mediators.

Dissertation Chapter	Inoculum	Treatment	Total Xylose Consumed (g/L)	Final Butanol Titer (g/L)	Butanol Yield (g/g)
Chapter 2	*C. beijerinckii* NCIMB 8052	C + 30 g/L X	0.49 ± 0.06	0.05 ± 0.01	0.10
		C + X + 20 mM F	3.76 ± 0.76	0.27 ± 0.13	0.07
		C + X + F + 500 μM A	25.98 ± 0.88	6.35 ± 0.19	0.24
		C + X + F + 500 μM R	29.15 ± 1.52	7.46 ± 0.09	0.26
Chapter 3	Wetwood Transfer 1	C + 30 g/L X	5.30 ± 1.81	0.02 ± 0.02	0.00
		C + X + 30 mM F	5.10 ± 1.15	0.17 ± 0.19	0.03
		C + X + F + 500 μM A	16.48 ± 5.32	1.33 ± 0.72	0.08
		C + X + F + 500 μM R	21.58 ± 6.74	1.63 ± 1.28	0.08
	Wetwood Transfer 2	C + 30 g/L X	1.57 ± 2.67	0.01 ± 0.02	0.01
		C + X + 30 mM F	2.38 ± 2.23	0.26 ± 0.05	0.11
		C + X + F + 500 μM A	24.60 ± 0.82	3.18 ± 0.38	0.13
		C + X + F + 500 μM R	21.66 ± 3.56	2.54 ± 1.74	0.12
	Wetwood Transfer 3	C + 30 g/L X	2.09 ± 0.19	0.00 ± 0.00	0.00
		C + X + 30 mM F	6.84 ± 0.16	0.13 ± 0.03	0.02
		C + X + F + 500 μM A	31.00 ± 0.50	3.45 ± 0.10	0.11
		C + X + F + 500 μM R	25.47 ± 2.40	1.73 ± 0.96	0.07
	Septic Transfer 1	C + 30 g/L X	4.70 ± 1.97	0.02 ± 0.00	0.00
		C + X + 30 mM F	18.58 ± 9.21	0.25 ± 0.07	0.01
		C + X + F + 500 μM A	20.81 ± 4.13	1.14 ± 0.88	0.05
		C + X + F + 500 μM R	22.28 ± 2.68	0.27 ± 0.11	0.01
	Septic Transfer 2	C + 30 g/L X	2.99 ± 1.67	0.00 ± 0.00	0.00
		C + X + 30 mM F	5.34 ± 5.26	0.13 ± 0.05	0.02
		C + X + F + 500 μM A	23.26 ± 3.05	1.17 ± 0.19	0.05
		C + X + F + 500 μM R	23.94 ± 5.23	0.80 ± 0.27	0.03
	Septic Transfer 3	C + 30 g/L X	3.20 ± 0.32	0.00 ± 0.01	0.00
		C + X + 30 mM F	14.16 ± 3.18	0.75 ± 0.75	0.05
		C + X + F + 500 μM A	29.87 ± 3.46	1.09 ± 1.12	0.04
		C + X + F + 500 μM R	28.59 ± 6.38	1.74 ± 1.73	0.06

Table 6-1: Summary of total xylose consumption, final butanol titers, and butanol yields from each chapter. Cells, xylose, ferrihydrite, AQDS, and riboflavin are denoted by C, X, F, A, and R, respectively.

Dissertation Chapter	Inoculum	Treatment	Total Xylose Consumed (g/L)	Final Butanol Titer (g/L)	Butanol Yield (g/g)
Chapter 3	Marsh Transfer 1	C + 30 g/L X	1.81 ± 1.16	0.04 ± 0.01	0.02
		C + X + 30 mM F	14.06 ± 1.87	2.43 ± 1.78	0.17
		C + X + F + 500 µM A	19.57 ± 4.60	1.07 ± 1.74	0.05
		C + X + F + 500 µM R	22.36 ± 4.18	0.14 ± 0.06	0.01
	Marsh Transfer 2	C + 30 g/L X	1.75 ± 0.57	0.00 ± 0.01	0.00
		C + X + 30 mM F	7.09 ± 2.05	0.04 ± 0.06	0.01
		C + X + F + 500 µM A	20.87 ± 0.12	0.48 ± 0.15	0.02
		C + X + F + 500 µM R	22.43 ± 3.94	0.58 ± 0.52	0.03
	Marsh Transfer 3	C + 30 g/L X	0.55 ± 2.10	0.00 ± 0.00	0.00
		C + X + 30 mM F	12.08 ± 3.00	0.01 ± 0.01	0.00
		C + X + F + 500 µM A	24.52 ± 0.64	0.29 ± 0.20	0.01
		C + X + F + 500 µM R	23.42 ± 0.36	0.68 ± 0.21	0.03
Chapter 4	*Clostridium* sp. C10 (Oxic)	C + 60 g/L X	22.14 ± 1.00	3.47 ± 0.14	0.16
		C + X + 20 mM F	35.43 ± 1.10	6.27 ± 0.21	0.18
Chapter 5	*Clostridium* sp. C10 (Electrode)	C + 30 g/L X	11.01 ± 0.17	2.19 ± 0.06	0.20
		C + 30 g/L X + E	24.8	4.98	0.20
		C + 60 g/L X	24.08 ± 1.22	3.06 ± 0.15	0.13
		C + 60 g/L X + E	34.11	5.78	0.17

Table 6-1 (continued): Summary of total xylose consumption, final butanol titers, and butanol yields from each chapter. Cells, xylose, ferrihydrite, AQDS, and riboflavin are denoted by C, X, F, A, and R, respectively.

Future Recommendations

Addressing Fermentative Populations in Bioremediation

While the essence of this dissertation addressed the electrochemical augmentation of solventogenic and pentose metabolism in ABE fermentations, it is vital to investigate fields to which this work can be applied outside the realm of alternative energy. Common strategies for the in situ bioremediation of various contaminants including, but not limited to, chlorinated hydrocarbons, perchlorate, and radionuclides, focus on stimulating iron and/or sulfate reducing bacterial populations, with inadequate emphasis placed on

enhancing fermentative populations, such as the *Clostridiaceae*, to catalyze similar reduction reactions {1-2}. The adopted dogma for dissimilatory mineral oxide bioreduction in mixed consortia is primarily attributed to respiring bacterial populations, while fermenters have been overlooked as minor contributors in these same reactions.

Remediation strategies should not disregard the community contributions provided by fermenters, as these populations may ultimately promote microbial degradation/reduction activity through enhanced dissimilatory mineral oxide reduction and interspecies electron transfer. The work presented in this dissertation has demonstrated that the addition of riboflavin or AQDS and insoluble ferrihydrite drives hydrogen and organic acid production to levels which are substantially greater than that of controls containing cells plus substrate alone and treatments amended with electron shuttles or iron alone when xylose or glucose are supplied as a feedstock. Furthermore, these data reveal that the addition electron mediators facilitates complete iron reduction in *Clostridium* fermentations (Figure A-2). Given that proper redox conditions and growth substrate are provided for fermenters in various remediation sites, increased performance with respect to degradation rates may be observed, as fermentative organisms typically have have short doubling times.

Iron Metabolism: Competition Between Fermenters and Iron Respiring Organisms

To demonstrate the extent to which fermenters can overshadow respiring organisms' role in dissimilatory mineral oxide reduction, rates of iron/mineral reduction for pure culture *Clostridium* species in the presence and absence of electron shuttling compounds should be compared to rates of pure culture iron respiring species such as

Geobacter metallireducens. Both soluble and insoluble forms of iron, such as ferric citrate and ferrihydrite, should be used for this analysis. The mixed culture study from Chapter 3 showed that the presence of iron alone favors fermentative organisms belonging to the *Clostridiaceae* over iron respiring populations. It has been previously reported that iron may act as a minor supplement to the fermentative metabolism of carbohydrates for acid, alcohol, and hydrogen production, while most iron reduction is attributed to iron respiring organisms such as those belonging to the *Geobacter* genus, which has the capacity to utilize organic acids generated from fermenters as electron donor sources (Figure 6-1) {3-4}. It is not to say that iron respiring populations do not play a large role in iron/mineral cycling, however, data generated from Chapter 3 provides evidence that fermenters may play an even bigger role than initially thought. Pursuing the suggested study would provide evidence that iron respiring populations may have less of an influence on the biogeochemical processes of terrestrial iron/minerals in oxygen limited environments in comparison to fermenters.

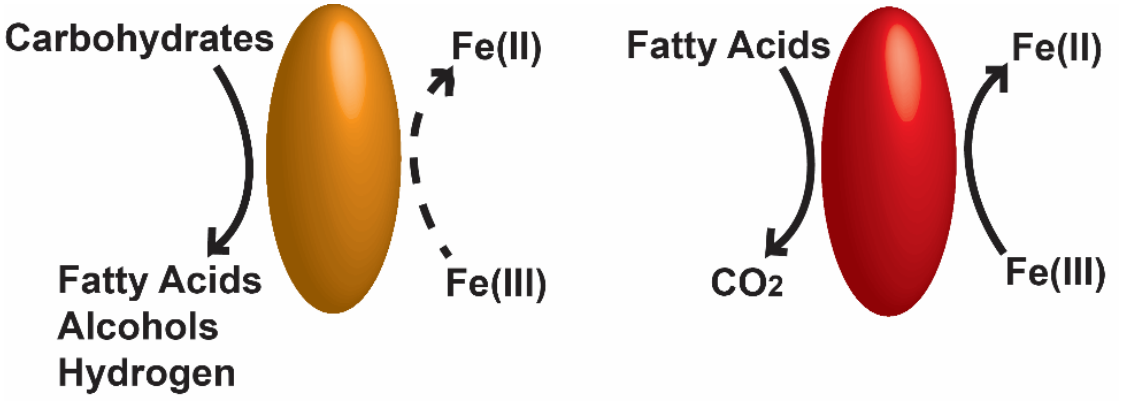

Figure 6-1: Interactions between fermenters and iron respiring bacteria proposed previously by Lovley and Phillips. Dashed lines indicate minor iron reduction.

Batch Reactors and Chemostat Studies

Most data to which we were comparing our studies used fed batch reactors for which productivity values were generated by multiplying dilution rates by solvent titers to generate values for volumetric solvent productivity {5}. Using fed batch reactors or continuous culture for future studies, along with the previously listed method for reporting solvent productivity, would be beneficial for increasing our reported values as well as facilitate a more realistic means towards the commercialization of the technology developed in the laboratory.

Potentiostat Studies

The open source potentiostat presented in Chapter 5 was an effective proof-of-concept tool for stimulating wild-type solventogenesis in *Clostridium* fermentations. However, future electrode-based experiments should be performed with a potentiostat system which has voltage regulation with less variability. The analog dial required a substantial amount of time to adjust the redox potential of the bulk fermentation liquid. A more sophisticated potentiostat would allow for better or more constant regulation as well as allowing for electrode challenged fermentations to be constructed with greater ease.

Hydroxyl Radicals Generated in Iron and/or Shuttle Amended Fermentations

Cellular production of flavins and/or addition of exogenous quinones, flavins, and iron may alleviate thermodynamic constraints by readily reoxidizing metabolic co-factors such as NADH and NADPH. However, they may also be responsible for the production

of intracellular or extracellular reactive oxygen species (ROS), and *Clostridium* cells may respond to this stress by producing alcohols or fatty acids to offset damage caused by ROS. Hydroxyl radical interactions with cellular components are non-specific, but oxidative damage to cellular fatty acids and DNA are most cited in literature. Metabolite production in response to these stresses with the trade-off being increases in membrane fluidity and/or osmotic imbalances. Increased expression of ROS genes have not been studied in *Clostridia*, but in the C. *beijerinckii* genome, Fe/Mn superoxide dismutases localized near solventogenic genes.

While there may be a lack of existing correlations linking metabolite production to ROS stress responses, it may be worthwhile to investigate intracellular and extracellular ROS in the presence and absence of electron shuttles and iron to further elucidate explanations for solventogenesis. Iron mediated hydroxyl, quinone, and paraquat radical generation have been investigated in plants and eukaryotes, especially in regards to herbicides for plants. However, studies are lacking that address the effects of ROS on solventogenic or fermentative organisms. One benefit of studying the stimulation of ROS produced from fermenters could also have applications for stimulating non-specific xenobiotic degradation in oxygen limited environments.

References

1.) Hazen, T. C., & Tabak, H. H. (2005). Developments in bioremediation of soils and sediments polluted with metals and radionuclides: 2. Field research on bioremediation of metals and radionuclides. *Reviews in Environmental Science and Bio/Technology*, *4*(3), 157-183.

2.) Watson, D. B., Wu, W. M., Mehlhorn, T., Tang, G., Earles, J., Lowe, K., Gihring, T. M., Zhang, G., Phillips, J., Boyanov, M. I., Spalding, B. P., Schadt, C., Kemner, K. M., Criddle, C. S., Jardine, P. M., & Brooks, S. C. (2013). In situ bioremediation of uranium with emulsified vegetable oil as the electron donor. *Environmental Science & Technology*, *47*(12), 6440-6448

3.) Lovley, D. R., & Phillips, E. J. (1988). Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. *Applied and Environmental Microbiology*, *54*(6), 1472-1480.

4.) Lovley, D. R., & Phillips, E. J. (1986). Organic matter mineralization with reduction of ferric iron in anaerobic sediments. *Applied and Environmental Microbiology*, *51*(4), 683-689.

5.) Ezeji, T., Qureshi, N., & Blaschek, H. P. (2007). Production of acetone–butanol–ethanol (ABE) in a continuous flow bioreactor using degermed corn and Clostridium beijerinckii. Process Biochemistry, 42(1), 34-39.

APPENDIX

LIST OF APPENDIX TABLES

List of Appendix Tables (Continued)

LIST OF APPENDIX FIGURES

Figure Page

List of Appendix Figures (Continued)

Figure Page

MISCELLANEOUS APPENDIX DATA

***Clostridium beijerinckii* NCIMB 8052 Study (Chapter 2)**

Scaled Iron Gel (AQDS Fixed at 500 μM)							
	g/L/h Butanol	SD	P		Butanol Pmax (g/L)	SD	P
5 mM	0.1052	0.0277	0.0322	5 mM	3.5337	0.1589	0.0002
20 mM	**0.2687**	**0.0086**	**<0.0001**	**20 mM**	**5.9718**	**0.0217**	**<0.0001**
50 mM	0.0071	0.0004	0.0005	50 mM	1.2771	0.0942	0.0009
100 mM	0.0090	0.0045	0.1384	100 mM	3.5519	3.2018	0.3482
Scaled AQDS (Iron Gel Fixed at 20 mM)							
	g/L/h Butanol	SD	P		Butanol Pmax (g/L)	SD	P
100 μM	0.4501	19.4246	ND	100 μM	6.9278	0.4190	<0.0005
500 μM	**0.2687**	**0.0086**	**<0.0001**	**500 μM**	**5.9718**	**0.0217**	**<0.0001**
1 mM	0.0744	0.0012	<0.0001	1 mM	4.8632	0.0258	<0.0001
2 mM	0.0594	0.0056	0.0018	2 mM	4.9827	0.0523	<0.0001

Table A-1: Butanol production from *C. beijerinckii* NCIMB 8052 under various concentrations of iron gel and AQDS. Concentrations of 20 mM iron gel and 500 uM AQDS were chosen as optimized treatments for electron shuttling to iron experiments. These conditions proved to provide the most stable results with respect to butanol productivity.

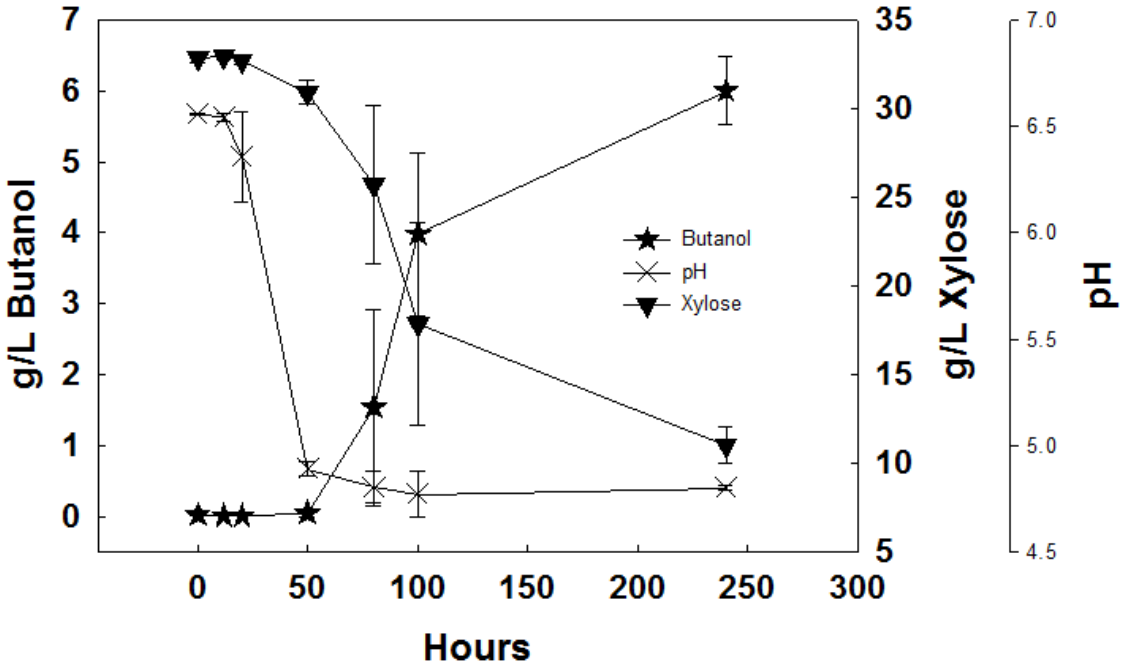

Figure A-1: Soluble ferric citrate (20 mM) challenged fermentations using *C. beijerinckii* 8052 and 3% xylose as the sole fermentable substrate. Error bars represent standard deviation between triplicates.

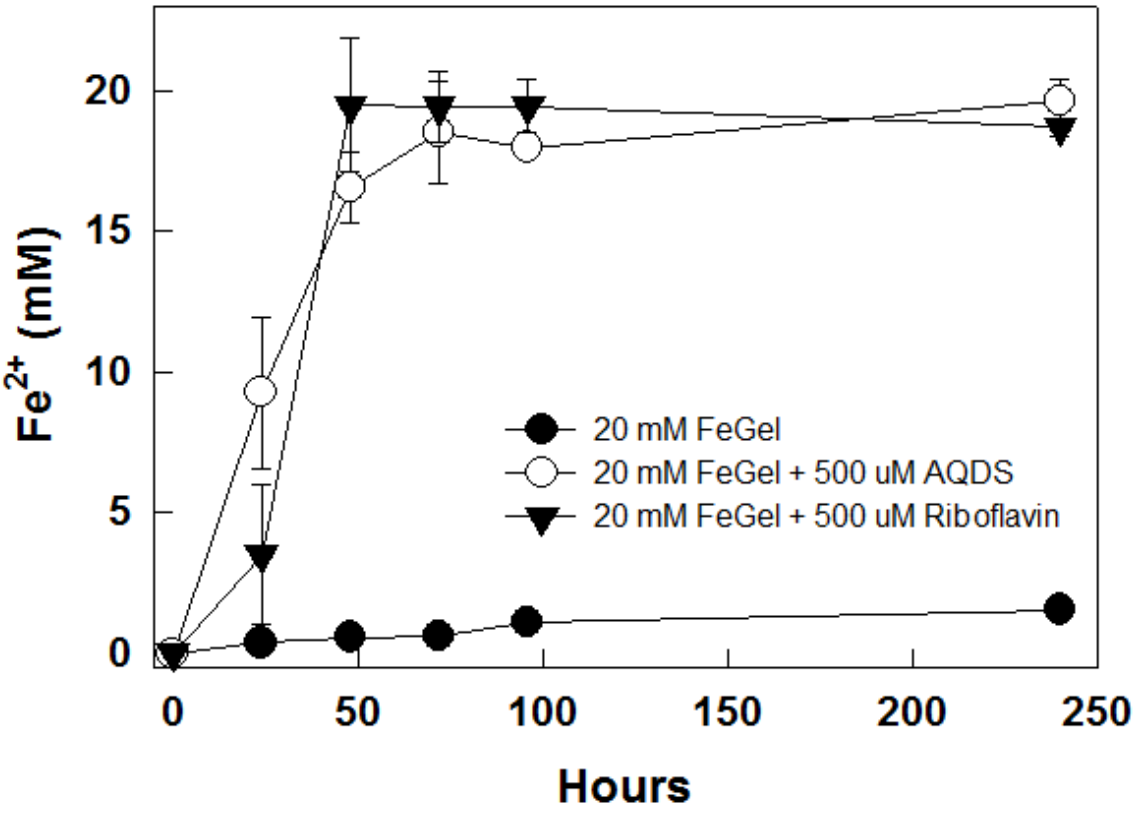

Figure A-2: Ferrous iron accumulation in ferrihydrite (FeGel) amended *C. beijerinckii* NCIMB 8052 fermentations using 30 g/L xylose as the sole fermentable substrate. Fermentations were performed in non-acetate amended P2 media. Error bars represent standard deviation between triplicates.

Cells + 30 g/L Xylose Control							#C for Standardized products																					
	g Xylose consumed	g/L Produced					4	3	2	2	4	g Xylose consumed	Carbon/Carbon Ratio						Normalized Ratio					Normalized Ratio (100%)				
Hours		BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate	x5 C	BuOH	Acetone	EtOH	Acetate	Butyrate	Sum	BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate
0	0.00	0.02	0.00	0.00	0.08	0.08	0.08	0.00	0.00	0.17	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.33	0.02	0.00	0.00	0.03	0.16	0.08	0.00	0.00	0.06	0.65	1.66	0.05	0.00	0.00	0.04	0.39	0.48	0.10	0.00	0.00	0.08	0.82	10.33	0.00	0.00	7.97	81.70
20	0.24	0.01	0.00	0.00	0.03	0.43	0.05	0.00	0.00	0.07	1.71	1.22	0.04	0.00	0.00	0.06	1.40	1.50	0.03	0.00	0.00	0.04	0.93	2.83	0.00	0.00	3.79	93.38
50	0.14	0.02	0.00	0.00	0.02	0.53	0.08	0.00	0.00	0.05	2.12	0.68	0.11	0.00	0.00	0.07	3.14	3.32	0.03	0.00	0.00	0.02	0.94	3.42	0.00	0.00	2.11	94.47
80	0.25	0.02	0.00	0.00	0.07	0.53	0.08	0.00	0.00	0.14	2.12	1.26	0.06	0.00	0.00	0.11	1.68	1.85	0.03	0.00	0.00	0.06	0.91	3.36	0.00	0.00	5.79	90.85
100	0.27	0.02	0.00	0.00	0.02	0.52	0.08	0.00	0.00	0.04	2.09	1.34	0.06	0.00	0.00	0.03	1.57	1.66	0.04	0.00	0.00	0.02	0.94	3.82	0.00	0.00	1.84	94.34
240	0.49	0.05	0.00	0.00	0.01	0.54	0.20	0.00	0.00	0.03	2.17	2.44	0.08	0.00	0.00	0.01	0.89	0.98	0.08	0.00	0.00	0.01	0.91	8.17	0.00	0.00	1.21	90.62

Cells + 30 g/L Xylose + 20 mM FeGel + 500 uM Riboflavin							#C for Standardized products																					
	g Xylose consumed	g/L Produced					4	3	2	2	4	g Xylose consumed	Carbon/Carbon Ratio						Normalized Ratio					Normalized Ratio (100%)				
Hours		BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate	x5 C	BuOH	Acetone	EtOH	Acetate	Butyrate	Sum	BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate
0	0.00	0.02	0.00	0.00	0.04	0.08	0.09	0.00	0.00	0.08	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.15	0.01	0.00	0.00	0.10	0.16	0.02	0.01	0.00	0.19	0.65	0.77	0.03	0.01	0.00	0.25	0.85	1.15	0.03	0.01	0.00	0.22	0.74	2.71	0.93	0.00	22.15	74.21
20	0.36	0.02	0.00	0.00	0.12	0.43	0.06	0.00	0.00	0.24	1.71	1.78	0.03	0.00	0.00	0.13	0.96	1.13	0.03	0.00	0.00	0.12	0.85	3.07	0.00	0.00	11.74	85.19
50	6.09	0.56	0.01	0.03	0.39	0.53	2.24	0.02	0.05	0.78	2.12	30.47	0.07	0.00	0.00	0.03	0.07	0.17	0.43	0.00	0.01	0.15	0.41	42.99	0.34	1.00	15.03	40.64
80	14.69	3.16	0.18	0.07	0.70	0.53	12.64	0.55	0.15	1.40	2.12	73.47	0.17	0.01	0.00	0.02	0.03	0.23	0.75	0.03	0.01	0.08	0.13	74.97	3.25	0.88	8.30	12.60
100	21.11	5.62	0.41	0.11	0.60	0.52	22.49	1.23	0.22	1.20	2.09	105.57	0.21	0.01	0.00	0.01	0.02	0.26	0.83	0.05	0.01	0.04	0.08	82.61	4.50	0.79	4.40	7.69
240	25.98	6.35	0.49	0.11	0.23	0.54	25.39	1.47	0.23	0.45	2.17	129.88	0.20	0.01	0.00	0.00	0.02	0.23	0.85	0.05	0.01	0.02	0.07	85.45	4.96	0.77	1.52	7.30

Table A-2: *C. beijerinckii* 8052 carbon metabolite balance data (Chapter 2)

Cells + 30 g/L Xylose + 20 mM FeCitrate						#C for Standardized products																							
g Xylose consumed	g/L Produced																												
Hours		BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate	g Xylose consumed	Carbon/Carbon Ratio						Normalized Ratio						Normalized Ratio (100%)				
							4	3	2	2	4		x5 C	BuOH	Acetone	EtOH	Acetate	Butyrate	Sum	BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate
0	0.00	0.02	0.00	0.00	0.17	0.07	0.08	0.00	0.00	0.34	0.27		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.00	0.01	0.00	0.00	0.29	0.13	0.04	0.00	0.00	0.58	0.53		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20	0.21	0.01	0.00	0.00	0.35	0.35	0.04	0.00	0.00	0.71	1.41		1.06	0.04	0.00	0.00	0.67	1.33	2.04	0.02	0.00	0.00	0.33	0.65	1.94	0.00	0.00	32.80	65.27
50	1.98	0.04	0.00	0.00	0.19	1.88	0.17	0.00	0.00	0.38	7.53		9.90	0.02	0.00	0.00	0.04	0.76	0.82	0.02	0.00	0.00	0.05	0.93	2.14	0.00	0.00	4.67	93.20
80	7.16	1.54	0.15	0.00	0.27	2.33	6.16	0.45	0.00	0.53	9.33		35.79	0.17	0.01	0.00	0.01	0.26	0.46	0.37	0.03	0.00	0.03	0.57	37.39	2.71	0.00	3.24	56.66
100	15.01	3.98	0.56	0.12	0.23	2.70	15.93	1.68	0.24	0.46	10.82		75.04	0.21	0.02	0.00	0.01	0.14	0.39	0.55	0.06	0.01	0.02	0.37	54.69	5.75	0.84	1.57	37.15
240	21.88	6.01	0.82	0.16	0.33	2.23	24.03	2.46	0.32	0.66	8.93		109.42	0.22	0.02	0.00	0.01	0.08	0.33	0.66	0.07	0.01	0.02	0.25	66.03	6.76	0.87	1.82	24.53
Cells + 30 g/L Xylose + 20 mM FeGel + 500 uM Riboflavin							#C for Standardized products																						
g Xylose consumed	g/L Produced											g Xylose consumed	Carbon/Carbon Ratio						Normalized Ratio						Normalized Ratio (100%)				
Hours		BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate	x5 C	BuOH	Acetone	EtOH	Acetate	Butyrate	Sum	BuOH	Acetone	EtOH	Acetate	Butyrate	BuOH	Acetone	EtOH	Acetate	Butyrate
							4	3	2	2	4																		
0	0.00	0.02	0.00	0.00	0.00	0.08	0.09	0.00	0.00	0.01	0.31		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	0.23	0.02	0.00	0.00	0.03	0.10	0.07	0.00	0.00	0.06	0.40		1.13	0.06	0.00	0.00	0.05	0.35	0.46	0.13	0.00	0.00	0.11	0.76	13.08	0.00	0.00	10.90	76.02
20	0.23	0.03	0.00	0.00	0.12	0.13	0.11	0.00	0.00	0.24	0.54		1.13	0.10	0.00	0.00	0.21	0.48	0.78	0.12	0.00	0.00	0.27	0.61	12.40	0.00	0.00	26.93	60.68
50	5.64	0.45	0.05	0.03	0.30	0.79	1.82	0.15	0.05	0.60	3.16		28.22	0.06	0.01	0.00	0.02	0.11	0.20	0.32	0.03	0.00	0.11	0.55	31.71	2.68	0.00	10.54	55.07
80	20.16	3.93	0.63	0.07	0.45	0.85	15.73	1.90	0.15	0.90	3.40		100.81	0.16	0.02	0.00	0.01	0.03	0.22	0.72	0.09	0.00	0.04	0.16	71.77	8.65	0.00	4.08	15.50
100	23.68	5.10	0.84	0.11	1.23	0.77	20.42	2.51	0.22	2.46	3.09		118.38	0.17	0.02	0.00	0.02	0.03	0.24	0.72	0.09	0.00	0.09	0.11	71.70	8.82	0.00	8.64	10.85
240	29.15	7.46	1.20	0.11	0.36	1.15	29.83	3.61	0.23	0.72	4.58		145.75	0.20	0.02	0.00	0.00	0.03	0.27	0.77	0.09	0.00	0.02	0.12	77.01	9.31	0.00	1.86	11.83

Table A-3: *C. beijerinckii* 8052 carbon metabolite balance data (Chapter 2; Continued)

Cells + 30gL Xylose	OD600			AVG	SD
0	0.104	0.116	0.11	0.11	0.006
24	0.491	0.521	0.493	0.501667	0.016773
48	0.481	0.53	0.494	0.501667	0.025384
96	0.477	0.535	0.49	0.500667	0.030436
120	0.479	0.529	0.492	0.5	0.025942
Cells + 20mM Citrate	OD600			AVG	SD
0	0.089	0.063	0.071	0.074333	0.013317
24	0.171	0.142	0.082	0.131667	0.045391
48	0.163	0.149	0.098	0.136667	0.03421
96	0.14	0.151	0.1	0.130333	0.026839
120	0.136	0.148	0.096	0.126667	0.027227
Cell+Xylose+ 20mM Citrate	OD600			AVG	SD
0	0.066	0.097	0.081	0.081333	0.015503
24	0.356	0.088	0.075	0.173	0.158616
48	1.228	0.475	0.318	0.673667	0.486443
96	1.262	1.297	1.278	1.279	0.017521
120	1.269	1.385	1.291	1.315	0.061612

Table A-4: *C. beijerinckii* 8052 growth in 20 mM citrate amended fermentations using 30 g/L xylose as the fermentation feedstock (non-acetate amended P2 media)

Mixed Culture Fermentations (Chapter 3)

	Wetwood (g/g)	Septic (g/g)	Marsh (g/g)
Cells + 30 g/L Xylose Control T1	0.00	0.00	0.02
Cells + 30 g/L Xylose Control T2	0.01	0.00	0.00
Cells + 30 g/L Xylose Control T3	0.00	0.00	0.00
30 mM FeGel T1	0.03	0.01	0.17
30 mM FeGel T2	0.11	0.02	0.01
30 mM FeGel T3	0.02	0.05	0.00
500 µM AQDS + FeGel T1	0.08	0.05	0.05
500 µM AQDS + FeGel T2	0.13	0.05	0.02
500 µM AQDS + FeGel T3	0.11	0.04	0.01
500 µM Riboflavin + FeGel T1	0.08	0.01	0.01
500 µM Riboflavin + FeGel T2	0.12	0.03	0.03
500 µM Riboflavin + FeGel T3	0.07	0.06	0.03

Table A-5: Butanol yields from xylose in mixed culture fermentations. Dimensionless values are represented in g butanol produced per g xylose consumed after 15 days of growth.

Sample Number	Treatment	Transfer Number
1	Marsh Inoculum	N/A
2	Septic Inoculum	N/A
3	Wetwood Inoculum	N/A
4	Marsh - 30 g/L Xylose Control	1
5	Marsh - 30 g/L Xylose + 30 mM FeGel	1
6	Marsh - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	1
7	Marsh - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	1
8	Septic - 30 g/L Xylose Control	1
9	Septic - 30 g/L Xylose + 30 mM FeGel	1
10	Septic - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	1
11	Septic - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	1
12	Wetwood - 30 g/L Xylose Control	1
13	Wetwood - 30 g/L Xylose + 30 mM FeGel	1
14	Wetwood - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	1
15	Wetwood - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	1
16	Marsh - 30 g/L Xylose Control	2
17	Marsh - 30 g/L Xylose + 30 mM FeGel	2
18	Marsh - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	2
19	Marsh - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	2
20	Septic - 30 g/L Xylose Control	2

Table A-6: Illumina sequencing data. Normalized frequency of bacterial populations (Family-level).

Sample Number	Treatment	Transfer Number
21	Septic - 30 g/L Xylose + 30 mM FeGel	2
22	Septic - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	2
23	Septic - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	2
24	Wetwood - 30 g/L Xylose Control	2
25	Wetwood - 30 g/L Xylose + 30 mM FeGel	2
26	Wetwood - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	2
27	Wetwood - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	2
28	Marsh - 30 g/L Xylose Control	3
29	Marsh - 30 g/L Xylose + 30 mM FeGel	3
30	Marsh - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	3
31	Marsh - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	3
32	Septic - 30 g/L Xylose Control	3
33	Septic - 30 g/L Xylose + 30 mM FeGel	3
34	Septic - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	3
35	Septic - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	3
36	Wetwood - 30 g/L Xylose Control	3
37	Wetwood - 30 g/L Xylose + 30 mM FeGel	3
38	Wetwood - 30 g/L Xylose + 30 mM FeGel + 500 uM AQDS	3
39	Wetwood - 30 g/L Xylose + 30 mM FeGel + 500 uM Riboflavin	3

Table A-6 Cont'd: Illumina sequencing data. Normalized frequency of bacterial populations (Family-level).

	Sample Number					
Family	39	38	37	36	35	34
Clostridiaceae	0.523441	0.481334	0.803092	0.774431	0.754726	0.678831
Lactobacillaceae	0.458657	0.505444	0.16586	0.201338	0.003462	0.008002
Ruminococcaceae	0.000316	0.000499	0.000337	0.00022	0.07577	0.149226
Sporolactobacillaceae	3.66E-05	0.000439	0.000276	0.000298	0.113403	0.000616
Campylobacteraceae	0.000747	0.000798	0.000995	0.000992	0.015572	0.031452
Spirochaetaceae	8.45E-06	0	0.00023	5.51E-05	3.03E-05	5.86E-05
Lachnospiraceae	0.000132	0.000179	0.000168	0.000187	0.001736	0.00044
Peptococcaceae	0.000341	0.000279	0.001317	0.000231	0.003885	0.083802
Veillonellaceae	0.000352	0.000339	0.000521	0.000342	0.000717	0.000264
Comamonadaceae	0.000378	0.000199	0.000658	0.000783	9.08E-05	0.000234
Oxalobacteraceae	0.0004	5.98E-05	0.00173	0.001146	0.000131	0.000176
Anaerolinaceae	1.41E-05	0	0.000276	0.000198	0	0
Rhodospirillaceae	0.001017	0.000917	0.000765	0.000595	0.000525	0.000703
Burkholderiaceae	0.000206	9.97E-05	0.001164	0.001146	3.03E-05	0.000147
Flavobacteriaceae	0.00013	0.000279	0.000597	0.000364	0.000172	5.86E-05
Flexibacteraceae	6.48E-05	7.98E-05	4.59E-05	5.51E-05	0.000161	0.001554
Sphingobacteriaceae	0.000186	7.98E-05	0.000306	0.000265	0.000323	0.000147
Solibacteraceae	2.82E-06	0	0	1.1E-05	0	0
Sphingomonadaceae	0.000383	0.00016	0.000857	0.000419	0.000212	0.001202
Phyllobacteriaceae	8.45E-05	0	0.001056	0.002006	0.000272	0.000381
Desulfobacteraceae	0.000223	0.000199	0.000321	0.000287	0.000192	8.79E-05
Bradyrhizobiaceae	0.000392	9.97E-05	0.000429	0.000783	0.000172	8.79E-05
Methylobacteriaceae	0.000203	5.98E-05	0.000199	0.000132	0.000101	5.86E-05
Xanthomonadaceae	0.000116	3.99E-05	0.00147	0.000871	1.01E-05	0.000117
Desulfovibrionaceae	0.000192	0.00014	0.000566	0.000595	0.000212	0.000264
Rhodocyclaceae	9.02E-05	1.99E-05	0.00023	2.2E-05	1.01E-05	8.79E-05
Chitinophagaceae	0.000406	0.00016	0.000199	0.000276	0.000999	0.027143
Caulobacteraceae	0.000406	0.000199	0.000367	0.000783	0.000394	0.000117
Paenibacillaceae	0.000527	0.000339	0.000505	0.000198	0.003219	0.000498
Thermotogaceae	0.00011	5.98E-05	0.000306	0.000353	0.000373	0.000352
Helicobacteraceae	8.45E-06	0	6.12E-05	8.82E-05	7.06E-05	2.93E-05
Bacillaceae	0.000654	0.000698	0.001026	0.000518	0.001938	0.000293
Microbacteriaceae	0.000766	0.000339	0.000337	0.000331	0.000757	0.000997
Halanaerobiaceae	0.001133	0.001077	0.000597	0.000507	0.000787	0.000498
Planococcaceae	0.000194	0.000199	0.00026	0.000165	0.009597	0.000176
Thermoanaerobacteraceae	0.000135	3.99E-05	0.000214	5.51E-05	0.000192	0.000147
Propionibacteriaceae	9.3E-05	7.98E-05	0.000475	0.00011	2.02E-05	5.86E-05
Porphyromonadaceae	2.82E-06	1.99E-05	1.53E-05	1.1E-05	0	0
Enterobacteriaceae	0.000338	0.000319	0.000674	0.000463	0.000434	0.000293
Mycobacteriaceae	7.89E-05	3.99E-05	0.000122	0.000121	7.06E-05	0.000176
Staphylococcaceae	0.000451	0.000479	0.000245	0.000419	0.00108	0.000117
Rhodobacteraceae	0.00033	0.000359	0.000184	0.000265	0.000151	5.86E-05
Actinomycetaceae	0.000341	0.00014	0.000214	0.000265	0.000383	0.001026
Hyphomicrobiaceae	0.000107	5.98E-05	0.000122	0.000143	0.000121	0.000147
Acetobacteraceae	6.48E-05	1.99E-05	0.000214	6.61E-05	2.02E-05	8.79E-05

Family	39	38	37	36	35	34
Caldicellulosiruptoraceae	7.61E-05	3.99E-05	3.06E-05	4.41E-05	0.000777	0.000117
Piscirickettsiaceae	2.82E-05	0	1.53E-05	5.51E-05	0.000363	0.000703
Xanthobacteraceae	0.000147	1.99E-05	0.000153	0.000176	0.000101	0.000117
Dethiosulfovibrionaceae	5.64E-06	1.99E-05	6.12E-05	0	0.000343	0.000176
Bdellovibrionaceae	0.000282	0.000479	0.00026	0.000298	0.000172	8.79E-05
Syntrophobacteraceae	2.25E-05	0	4.59E-05	5.51E-05	0.000111	0
Brevibacteriaceae	0.000155	0	0.000276	9.92E-05	0.000121	0.000117
Phormidiaceae	3.38E-05	1.99E-05	0.000138	5.51E-05	3.03E-05	0
Exiguobacteraceae	0.000144	0.00012	0.002143	0.000132	0.000192	0.000117
Syntrophaceae	5.64E-05	5.98E-05	0.000245	0.00022	9.08E-05	0.000117
Rhizobiaceae	0.000113	1.99E-05	0.000107	0.000143	9.08E-05	0.000293
Glycomycetaceae	0.000234	9.97E-05	0.00023	0.000176	0.000252	0.000147
Synergistaceae	1.13E-05	5.98E-05	1.53E-05	2.2E-05	2.02E-05	2.93E-05
Streptomycetaceae	0.000149	5.98E-05	0.000245	0.000121	0.000101	0.000176
Nocardioidaceae	0.000118	3.99E-05	0.000168	0.000165	0.000232	0.000322
Caldithrixaceae	5.35E-05	5.98E-05	0.000199	0.000165	6.06E-05	5.86E-05
Thermodesulfovibrionaceae	3.38E-05	3.99E-05	9.19E-05	0.000176	3.03E-05	2.93E-05
Hydrogenophilaceae	4.23E-05	0	0.000276	7.72E-05	2.02E-05	2.93E-05
Pseudomonadaceae	5.92E-05	0	9.19E-05	0.000364	3.03E-05	0.000117
Heliobacteriaceae	9.86E-05	9.97E-05	0.000107	0.000143	0.000182	5.86E-05
Acidobacteriaceae	0.000186	1.99E-05	0.000352	0.000132	8.07E-05	5.86E-05
Sphaerochaetaceae	0	0	0	0	0	0
Desulfuromonadaceae	6.48E-05	5.98E-05	1.53E-05	9.92E-05	4.04E-05	5.86E-05
Pelobacteraceae	3.38E-05	1.99E-05	7.65E-05	5.51E-05	0.000121	0
Litoricolaceae	2.25E-05	3.99E-05	1.53E-05	0	0.000272	0.000264
Mycoplasmataceae	0.0002	0.000239	6.12E-05	0.000165	0.000182	2.93E-05
Thiotrichaceae	7.61E-05	1.99E-05	0.000184	3.31E-05	0.000101	0.000352
Deinococcaceae	7.04E-05	5.98E-05	4.59E-05	2.2E-05	0.000172	0.000703
Pseudonocardiaceae	0.000158	5.98E-05	7.65E-05	0.000121	0.000182	0.000117
Bacteroidaceae	0	1.99E-05	0	1.1E-05	0	0
Streptococcaceae	0.000217	0.000219	4.59E-05	0.000165	1.01E-05	0.000117
Hyphomonadaceae	9.86E-05	7.98E-05	0.000138	2.2E-05	4.04E-05	2.93E-05
Family	39	38	37	36	35	34
Desulfonatronumaceae	6.48E-05	7.98E-05	1.53E-05	2.2E-05	2.02E-05	2.93E-05
Yaniellaceae	5.64E-06	0	1.53E-05	0	3.03E-05	0.000381
Desulfohalobiaceae	1.13E-05	0	3.06E-05	1.1E-05	1.01E-05	0
Chromatiaceae	9.02E-05	7.98E-05	0.000153	5.51E-05	5.05E-05	0.000147
Bartonellaceae	8.74E-05	3.99E-05	7.65E-05	0.000121	9.08E-05	0.000205
Symbiobacteriaceae	3.38E-05	0	6.12E-05	1.1E-05	0	8.79E-05
Eubacteriaceae	1.13E-05	0	0	0	2.02E-05	8.79E-05
Thermodesulfobacteriaceae	5.07E-05	3.99E-05	7.65E-05	4.41E-05	9.08E-05	8.79E-05
Peptostreptococcaceae	5.64E-06	0	0	0	0	0
Sinobacteraceae	8.17E-05	0	0.000122	2.2E-05	1.01E-05	8.79E-05
Alcaligenaceae	4.79E-05	1.99E-05	1.53E-05	0.00011	3.03E-05	5.86E-05
Actinosynnemataceae	0.000127	5.98E-05	0.000184	4.41E-05	8.07E-05	2.93E-05
Amoebophilaceae	1.69E-05	0	4.59E-05	3.31E-05	5.05E-05	2.93E-05

Family	39	38	37	36	35	34
Polyangiaceae	7.04E-05	0	6.12E-05	2.2E-05	3.03E-05	5.86E-05
Thermobaculaceae	2.25E-05	5.98E-05	3.06E-05	1.1E-05	5.05E-05	0
Deferribacteraceae	2.82E-06	0	0	0	3.03E-05	0
Chlorobiaceae	3.1E-05	0	3.06E-05	4.41E-05	4.04E-05	0.000264
Nannocystaceae	4.79E-05	3.99E-05	4.59E-05	4.41E-05	5.05E-05	2.93E-05
Enterococcaceae	8.17E-05	5.98E-05	0.00026	4.41E-05	8.07E-05	2.93E-05
Rickettsiaceae	2.54E-05	0	4.59E-05	7.72E-05	3.03E-05	2.93E-05
Moraxellaceae	2.25E-05	5.98E-05	3.06E-05	1.1E-05	4.04E-05	5.86E-05
Streptosporangiaceae	7.61E-05	5.98E-05	0.000122	0.000176	3.03E-05	8.79E-05
Coriobacteriaceae	5.92E-05	3.99E-05	6.12E-05	0.00011	3.03E-05	2.93E-05
Micromonosporaceae	4.23E-05	1.99E-05	7.65E-05	2.2E-05	8.07E-05	0.000176
Anaerobrancaceae	2.82E-06	0	1.53E-05	0	4.04E-05	0.000205
Cystobacteraceae	5.64E-05	0	6.12E-05	3.31E-05	1.01E-05	2.93E-05
Ectothiorhodospiraceae	3.95E-05	1.99E-05	3.06E-05	1.1E-05	1.01E-05	2.93E-05
Caldisericaceae	0	1.99E-05	0.000459	0.000728	0	0
Leuconostocaceae	0.000104	0.00012	7.65E-05	5.51E-05	5.05E-05	5.86E-05
Contubernalisaceae	5.64E-06	1.99E-05	1.53E-05	3.31E-05	3.03E-05	2.93E-05
Neisseriaceae	4.79E-05	3.99E-05	1.53E-05	2.2E-05	2.02E-05	0
Vibrionaceae	3.95E-05	1.99E-05	0.000153	7.72E-05	7.06E-05	2.93E-05
Actinopolysporaceae	1.41E-05	0	3.06E-05	1.1E-05	9.08E-05	0.001612
Brocadiaceae	1.13E-05	3.99E-05	7.65E-05	0.000132	1.01E-05	0
Corynebacteriaceae	2.25E-05	0	3.06E-05	0.00011	4.04E-05	2.93E-05
Holophagaceae	1.97E-05	0	0	0	0	0
Sulfobacillaceae	0.000149	7.98E-05	3.06E-05	5.51E-05	2.02E-05	0
Kiloniellaceae	5.35E-05	0	9.19E-05	2.2E-05	4.04E-05	0
Legionellaceae	2.82E-05	0	3.06E-05	0	3.03E-05	5.86E-05
Methylocystaceae	1.41E-05	0	0	1.1E-05	0	5.86E-05
Halomonadaceae	1.41E-05	0	4.59E-05	2.2E-05	1.01E-05	0.000117
Borreliaceae	2.82E-06	0	3.06E-05	0	3.03E-05	2.93E-05
Thermovenabulum	2.82E-06	0	0	0	3.03E-05	2.93E-05
Bifidobacteriaceae	2.82E-05	0	3.06E-05	3.31E-05	5.05E-05	0
Fusobacteriaceae	2.54E-05	1.99E-05	0	4.41E-05	4.04E-05	2.93E-05
Pasteurellaceae	1.97E-05	7.98E-05	0.000107	7.72E-05	3.03E-05	0
Psychromonadaceae	5.64E-06	0	0	0	5.05E-05	8.79E-05
Pelagicoccaceae	3.1E-05	0	3.06E-05	1.1E-05	4.04E-05	5.86E-05
Acholeplasmataceae	3.38E-05	3.99E-05	3.06E-05	1.1E-05	3.03E-05	5.86E-05
Acidimicrobiaceae	4.79E-05	0	7.65E-05	2.2E-05	4.04E-05	0
Microviridae	4.23E-05	3.99E-05	7.65E-05	7.72E-05	1.01E-05	0
Anaplasmataceae	2.25E-05	0	1.53E-05	2.2E-05	1.01E-05	0
Desulfobulbaceae	0	0	0	0	0	0
Thermomonosporaceae	1.97E-05	0	1.53E-05	1.1E-05	8.07E-05	2.93E-05
Saprospiraceae	5.35E-05	1.99E-05	3.06E-05	2.2E-05	0	0
Aerococcaceae	2.54E-05	1.99E-05	1.53E-05	2.2E-05	1.01E-05	0
Shewanellaceae	1.13E-05	1.99E-05	1.53E-05	1.1E-05	1.01E-05	0
Nostocaceae	2.82E-06	0	1.53E-05	0	1.01E-05	0
Oceanospirillaceae	8.45E-06	0	3.06E-05	4.41E-05	1.01E-05	2.93E-05

Family	39	38	37	36	35	34
Euzebyaceae	2.25E-05	0	6.12E-05	0	2.02E-05	0
Beijerinckiaceae	2.82E-06	0	0	0	0	0
Turicibacteraceae	0	0	0	0	0	0
Coxiellaceae	2.82E-06	1.99E-05	0	0	0	0
Nocardiaceae	2.25E-05	1.99E-05	1.53E-05	0	3.03E-05	0
Aminiphilaceae	5.64E-06	0	1.53E-05	2.2E-05	0	0
Caldilineaceae	1.13E-05	0	3.06E-05	0	5.05E-05	0
Alteromonadaceae	5.64E-06	0	3.06E-05	0	5.05E-05	2.93E-05
Carnobacteriaceae	1.97E-05	0	0	0	2.02E-05	0
Entomoplasmataceae	1.97E-05	0	0	2.2E-05	1.01E-05	2.93E-05
Methylophilaceae	7.04E-05	3.99E-05	1.53E-05	1.1E-05	0	0
Micrococcaceae	2.54E-05	0	0	1.1E-05	4.04E-05	0
Rhodothermaceae	5.64E-06	0	0	0	1.01E-05	0
Thermaceae	8.45E-06	0	0	0	0	0
Erythrobacteraceae	2.82E-06	1.99E-05	4.59E-05	0	0	2.93E-05
Cellulomonadaceae	1.41E-05	0	4.59E-05	0	0	2.93E-05
Pseudanabaenaceae	2.82E-06	0	1.53E-05	0	0	0
Thermogemmatisporaceae	1.13E-05	0	1.53E-05	1.1E-05	1.01E-05	2.93E-05
Carboxydocellaceae	1.97E-05	0	0	3.31E-05	2.02E-05	0
Thermoactinomycetaceae	1.41E-05	0	0	1.1E-05	4.04E-05	5.86E-05
Rivulariaceae	5.64E-06	0	0	0	0	0
Aurantimonadaceae	5.64E-06	1.99E-05	0	1.1E-05	0	0
Dehalococcoidaceae	0	0	0.000122	2.2E-05	6.06E-05	2.93E-05
Ignavibacteriaceae	8.45E-06	0	0	0	0	0
Rhabdochlamydiaceae	2.25E-05	0	0	1.1E-05	1.01E-05	0
Brachyspiraceae	8.45E-06	0	3.06E-05	0	0	0
Waddliaceae	2.82E-06	0	0	0	0	0
Cerasicoccaceae	1.13E-05	0	0	5.51E-05	0	0
Thermicanaceae	1.41E-05	0	0	1.1E-05	2.02E-05	0
Solirubrobacteraceae	2.82E-06	0	0	1.1E-05	1.01E-05	0
Francisellaceae	0	0	0	0	0	0
Chrysiogenaceae	8.45E-06	0	1.53E-05	0	0	5.86E-05
Conexibacteraceae	8.45E-06	0	0	7.72E-05	1.01E-05	2.93E-05
Odoribacteraceae	0	0	0	0	0	0
Leptotrichiaceae	0	0	1.53E-05	1.1E-05	0	0
Opitutaceae	0	0	0	0	0	0
Acidithiobacillaceae	0	0	0	0	0	0
Nautiliaceae	0	0	0	0	0	0
Erysipelotrichaceae	2.82E-06	0	0	0	0	0
Gemellaceae	2.82E-06	0	0	0	0	0
Myxococcaceae	0	0	0	0	0	0
Haliangiaceae	1.41E-05	0	0	0	0	0
Pseudoalteromonadaceae	5.64E-06	0	0	0	1.01E-05	0
Idiomarinaceae	0	0	0	0	1.01E-05	0
Chroococcaceae	2.82E-06	0	0	0	4.04E-05	0
Methylacidiphilaceae	0	0	0	3.31E-05	0	0

Family	39	38	37	36	35	34
Elusimicrobiaceae	0	0	0	0	0	0
Kineosporiaceae	1.13E-05	0	1.53E-05	1.1E-05	0	0
Microcystaceae	5.64E-06	0	0	0	0	0
Rikenellaceae	0	0	0	0	0	0
Verrucomicrobiaceae	0	0	1.53E-05	0	0	0
Prevotellaceae	0	0	0	0	0	0
Halobacteroidaceae	1.13E-05	0	0	0	2.02E-05	0
Dietziaceae	2.82E-06	0	0	0	0	0
Listeriaceae	0	0	0	2.2E-05	0	0
Flammeovirgaceae	5.64E-06	0	0	0	1.01E-05	0
Isosphaeraceae	2.82E-06	0	0	0	0	0
Kouleothrixaceae	5.64E-06	0	0	0	3.03E-05	0
Gemmatimonadaceae	0	0	0	0	0	0
Tsukamurellaceae	0	0	0	0	0	0
Dehalobacteriaceae	0	0	0	0	0	0
Thiohalorhabdaceae	0	0	0	0	1.01E-05	0
Gordoniaceae	0	0	0	0	0	2.93E-05
Leptospiraceae	0	0	0	0	0	0
Dermabacteraceae	2.82E-06	0	0	0	0	0
Intrasporangiaceae	0	0	0	0	0	0
Saccharospirillaceae	0	0	0	0	0	2.93E-05
Puniceicoccaceae	0	0	0	0	0	0
Frankiaceae	8.45E-06	0	0	0	0	0
Aeromonadaceae	0	0	0	0	0	0
Promicromonosporaceae	0	0	0	0	0	0
Moritellaceae	0	0	0	0	0	0
Dermacoccaceae	0	0	0	0	0	0
Methanocorpusculaceae	5.64E-06	0	0	0	0	0
Patulibacteraceae	0	0	1.53E-05	0	0	0
Methanosarcinaceae	0	0	0	0	1.01E-05	0
Sanguibacteraceae	0	0	0	0	0	0
Alcanivoracaceae	0	0	0	0	1.01E-05	0
Archaeoglobaceae	0	0	0	0	0	0
Bogoriellaceae	0	0	0	0	0	0
Chthonomonadaceae	0	0	0	0	0	0
Nitrospinaceae	0	0	0	0	0	0
Catenulisporaceae	0	0	0	0	0	0
Nocardiopsaceae	2.82E-06	0	0	0	0	0
Roseiflexaceae	5.64E-06	0	0	0	0	0
Anaeroplasmataceae	0	0	0	0	1.01E-05	0
Oscillochloridaceae	0	0	0	0	1.01E-05	0
Chloroflexaceae	0	0	0	0	0	0
Sporichthyaceae	0	0	0	0	0	0
Armatimonadaceae	0	0	0	0	0	0
Fibrobacteraceae	0	0	0	0	0	0
Sulfolobaceae	0	0	0	0	0	0

Family	39	38	37	36	35	34
Coprobacillaceae	0	0	0	0	0	0
Desulfomicrobiaceae	0	0	0	0	0	0
Cyanobacteriaceae	0	0	0	0	0	0
Halobacteriaceae	2.82E-06	0	0	0	0	0
Geodermatophilaceae	0	0	0	0	0	0
Family	39	38	37	36	35	34
Desulfurococcaceae	0	0	0	0	0	0
Methanobacteriaceae	0	0	0	0	0	0
Thermoproteaceae	0	0	0	0	0	0
Nitrospiraceae	2.82E-06	0	0	0	0	0

			Sample Number			
Family	33	32	31	30	29	28
Clostridiaceae	0.975009	0.907942	0.737858	0.847089	0.949242	0.930594
Lactobacillaceae	0.003178	0.002908	0.002115	0.002907	0.00182	0.002545
Ruminococcaceae	0.000508	0.000687	0.001075	0.000555	0.005002	0.006063
Sporolactobacillaceae	0.000737	0.035449	0.234131	0.124493	0.02477	0.000281
Campylobacteraceae	0.00122	0.001401	0.005281	0.00524	0.001205	0.000819
Spirochaetaceae	0.000102	0.000145	0	0	3.62E-05	5.12E-05
Lachnospiraceae	0.000153	0.00078	0.000189	0.000306	0.000133	0.00032
Peptococcaceae	0.000458	0.00152	0.000378	0.000325	0.000265	0.002264
Veillonellaceae	0.000508	0.00111	0.000402	0.000574	0.000434	0.000614
Comamonadaceae	0.000153	0.000819	0.000154	0.00021	0.000193	0.001381
Oxalobacteraceae	0.000381	0.004811	9.45E-05	0.000191	0.000603	0.003249
Anaerolinaceae	0	0.00074	0	1.91E-05	0	0.00064
Rhodospirillaceae	0.000712	0.001057	0.000437	0.000746	0.00094	0.000652
Burkholderiaceae	0.000331	0.004005	0.00013	0.000287	0.000362	0.003889
Flavobacteriaceae	0.000203	0.001705	8.27E-05	0.000115	0.000169	0.000806
Flexibacteraceae	0.000102	0.000106	5.91E-05	7.65E-05	9.64E-05	0.000192
Sphingobacteriaceae	0.000407	0.00041	0.000106	0.000229	0.000386	0.000345
Solibacteraceae	0	0	0	0	1.21E-05	0
Sphingomonadaceae	0.000559	0.000978	0.00013	0.000402	0.00053	0.001202
Phyllobacteriaceae	0.000102	0.00119	8.27E-05	0.000115	0.000301	0.002341
Desulfobacteraceae	0.000254	0.000278	0.000154	0.000382	0.000265	0.000409
Bradyrhizobiaceae	0.000178	0.00193	0.000118	0.00021	0.000796	0.001394
Methylobacteriaceae	0.000229	0.000238	5.91E-05	0.000249	0.000567	0.000499
Xanthomonadaceae	7.63E-05	0.001877	2.36E-05	1.91E-05	8.44E-05	0.00275
Desulfovibrionaceae	0.000356	0.001084	0.000236	0.000421	0.000205	0.001113
Rhodocyclaceae	5.08E-05	0.000701	1.18E-05	5.74E-05	9.64E-05	0.000269
Chitinophagaceae	0.000203	0.000211	5.91E-05	9.56E-05	0.000494	0.000563
Caulobacteraceae	0.000432	0.001441	0.000284	0.000478	0.000277	0.001727
Paenibacillaceae	0.00061	0.001546	0.006569	0.003194	0.00082	0.000281
Thermotogaceae	0.000178	0.000912	8.27E-05	9.56E-05	0.000121	0.000563
Helicobacteraceae	0	1.32E-05	4.73E-05	5.74E-05	2.41E-05	0
Bacillaceae	0.000534	0.001758	0.001808	0.001453	0.000265	0.000665

Family	33	32	31	30	29	28
Microbacteriaceae	0.000661	0.000661	0.000201	0.000688	0.000325	0.001023
Halanaerobiaceae	0.000559	0.000621	0.000437	0.000612	0.000362	0.000333
Planococcaceae	0.000127	0.000357	0.000886	0.000746	0.000265	0.000281
Thermoanaerobacteraceae	0.000356	0.000502	0.000118	0.00021	7.23E-05	0.000435
Propionibacteriaceae	7.63E-05	0.000264	5.91E-05	3.82E-05	6.03E-05	0.009299
Porphyromonadaceae	0	0.000172	0	0	0	1.28E-05
Enterobacteriaceae	0.000407	0.000634	0.000201	0.000459	0.000567	0.00064
Geobacteraceae	0.00028	0.000344	0.00013	0.000115	0.00035	0.00046
Mycobacteriaceae	0.000305	0.000238	4.73E-05	0.000153	0.000193	0.00046
Rhodobacteraceae	0.000153	0.000278	0.000213	9.56E-05	0.000241	0.000307
Actinomycetaceae	0.000254	0.000542	0.00013	0.00021	0.000145	0.000205
Hyphomicrobiaceae	0.000203	0.000753	0.000106	9.56E-05	0.000844	0.00023
Acetobacteraceae	0.000153	0.000463	7.09E-05	3.82E-05	6.03E-05	0.000115
Caldicellulosiruptoraceae	0	6.61E-05	4.73E-05	5.74E-05	0.00041	0.000205
Piscirickettsiaceae	0	9.25E-05	0.000189	9.56E-05	7.23E-05	3.84E-05
Xanthobacteraceae	0.000127	0.000344	1.18E-05	5.74E-05	0.000277	0.000217
Dethiosulfovibrionaceae	2.54E-05	6.61E-05	1.18E-05	1.91E-05	3.62E-05	5.12E-05
Bdellovibrionaceae	0.000407	0.000357	0.000165	0.000306	0.000325	0.000256
Syntrophobacteraceae	2.54E-05	3.97E-05	2.36E-05	1.91E-05	3.62E-05	7.67E-05
Brevibacteriaceae	0.000331	0.000317	7.09E-05	0.000268	0.000434	0.00142
Phormidiaceae	0.000153	0.000833	2.36E-05	1.91E-05	3.62E-05	0.000576
Exiguobacteraceae	5.08E-05	7.93E-05	4.73E-05	0.000115	4.82E-05	6.4E-05
Syntrophaceae	0.000203	0.000198	5.91E-05	9.56E-05	6.03E-05	0.000102
Rhizobiaceae	0.000102	0.000172	3.54E-05	3.82E-05	0.000253	0.001202
Glycomycetaceae	0.000381	0.00041	8.27E-05	0.000229	0.000289	0.000205
Synergistaceae	0	1.32E-05	0	0	0	0
Streptomycetaceae	0.000305	0.000251	7.09E-05	9.56E-05	0.000108	0.000153
Nocardioidaceae	0.000305	0.000264	0.000165	0.000134	7.23E-05	0.000755
Caldithrixaceae	0.000178	0.00037	5.91E-05	0.000115	6.03E-05	7.67E-05
Thermodesulfovibrionaceae	5.08E-05	0.000119	0	3.82E-05	2.41E-05	8.95E-05
Hydrogenophilaceae	0.000102	0.000172	1.18E-05	0	3.62E-05	0.000128
Pseudomonadaceae	2.54E-05	0.00037	2.36E-05	7.65E-05	0.000145	0.000524
Heliobacteriaceae	0.000203	0.000264	9.45E-05	0.000172	4.82E-05	0.000179
Acidobacteriaceae	7.63E-05	0.000489	3.54E-05	0.000134	1.21E-05	0.000256
Sphaerochaetaceae	2.54E-05	0	0	0	0	0
Desulfuromonadaceae	0.000127	0.000278	8.27E-05	0.00021	6.03E-05	8.95E-05
Pelobacteraceae	5.08E-05	7.93E-05	0.000118	7.65E-05	9.64E-05	0.000141
Litoricolaceae	7.63E-05	2.64E-05	5.91E-05	7.65E-05	3.62E-05	2.56E-05
Mycoplasmataceae	7.63E-05	3.97E-05	5.91E-05	9.56E-05	3.62E-05	8.95E-05
Thiotrichaceae	0.000102	0.000106	4.73E-05	1.91E-05	4.82E-05	5.12E-05
Deinococcaceae	0.000102	0.000225	1.18E-05	9.56E-05	4.82E-05	0.000179
Pseudonocardiaceae	0.000508	0.000278	0.000118	0.000153	4.82E-05	0.000307
Bacteroidaceae	0	6.61E-05	0	1.91E-05	1.21E-05	0.000141
Streptococcaceae	0	0.000423	3.54E-05	1.91E-05	0	0.001279
Hyphomonadaceae	0.000127	6.61E-05	2.36E-05	1.91E-05	3.62E-05	2.56E-05
Desulfonatronumaceae	2.54E-05	6.61E-05	2.36E-05	5.74E-05	3.62E-05	3.84E-05
Yaniellaceae	0	2.64E-05	1.18E-05	0	0	5.12E-05

Family	39	38	37	36	35	34
Desulfohalobiaceae	0	1.32E-05	2.36E-05	5.74E-05	2.41E-05	3.84E-05
Syntrophomonadaceae	7.63E-05	9.25E-05	2.36E-05	1.91E-05	7.23E-05	6.4E-05
Chromatiaceae	0.000102	6.61E-05	1.18E-05	0.000115	1.21E-05	2.56E-05
Symbiobacteriaceae	2.54E-05	1.32E-05	5.91E-05	0	0	1.28E-05
Eubacteriaceae	5.08E-05	1.32E-05	2.36E-05	1.91E-05	1.21E-05	5.12E-05
Thermodesulfobacteriaceae	0.000102	9.25E-05	3.54E-05	3.82E-05	6.03E-05	7.67E-05
Peptostreptococcaceae	0	1.32E-05	1.18E-05	0	0	0
Sinobacteraceae	0.000102	6.61E-05	3.54E-05	5.74E-05	2.41E-05	0.000115
Alcaligenaceae	5.08E-05	0.000225	1.18E-05	1.91E-05	7.23E-05	7.67E-05
Actinosynnemataceae	0.000178	7.93E-05	9.45E-05	0	6.03E-05	2.56E-05
Amoebophilaceae	2.54E-05	3.97E-05	3.54E-05	5.74E-05	4.82E-05	5.12E-05
Polyangiaceae	0.000127	0.00033	3.54E-05	3.82E-05	0.000121	0.000166
Thermobaculaceae	5.08E-05	3.97E-05	0	0	0	3.84E-05
Deferribacteraceae	0	1.32E-05	0	1.91E-05	0	5.12E-05
Chlorobiaceae	0	6.61E-05	0	1.91E-05	3.62E-05	8.95E-05
Nannocystaceae	7.63E-05	0.000172	2.36E-05	3.82E-05	0.000229	8.95E-05
Enterococcaceae	0	5.29E-05	0.000224	0.000172	3.62E-05	6.4E-05
Rickettsiaceae	7.63E-05	9.25E-05	1.18E-05	3.82E-05	9.64E-05	0.000102
Moraxellaceae	7.63E-05	6.61E-05	4.73E-05	5.74E-05	8.44E-05	0.000115
Streptosporangiaceae	0.000127	0.000132	7.09E-05	0	0.000108	3.84E-05
Coriobacteriaceae	0.000127	0.000238	3.54E-05	1.91E-05	0	7.67E-05
Micromonosporaceae	0.000127	0.000225	2.36E-05	0	2.41E-05	7.67E-05
Anaerobrancaceae	5.08E-05	2.64E-05	0	0	1.21E-05	0
Cystobacteraceae	2.54E-05	0.000238	3.54E-05	1.91E-05	1.21E-05	0.000179
Ectothiorhodospiraceae	5.08E-05	3.97E-05	0	3.82E-05	3.62E-05	6.4E-05
Caldisericaceae	0	0.000661	0	0	2.41E-05	7.67E-05
Leuconostocaceae	0.000102	3.97E-05	3.54E-05	5.74E-05	4.82E-05	6.4E-05
Contubernalisaceae	5.08E-05	0.000278	1.18E-05	1.91E-05	6.03E-05	0.000128
Neisseriaceae	0.000127	5.29E-05	1.18E-05	0	1.21E-05	7.67E-05
Vibrionaceae	7.63E-05	5.29E-05	2.36E-05	5.74E-05	0.000108	8.95E-05
Actinopolysporaceae	0.000102	5.29E-05	3.54E-05	0	2.41E-05	0
Family	33	32	31	30	29	28
Brocadiaceae	5.08E-05	0.000132	0	3.82E-05	1.21E-05	0.000371
Corynebacteriaceae	5.08E-05	6.61E-05	1.18E-05	0	0	0.000499
Holophagaceae	0	6.61E-05	0	0	0	3.84E-05
Sulfobacillaceae	5.08E-05	5.29E-05	1.18E-05	1.91E-05	2.41E-05	0
Kiloniellaceae	0.000153	9.25E-05	1.18E-05	0	3.62E-05	0.000115
Legionellaceae	0.000102	5.29E-05	1.18E-05	7.65E-05	7.23E-05	2.56E-05
Methylocystaceae	0	6.61E-05	0	3.82E-05	0	0.000102
Halomonadaceae	0.000102	2.64E-05	3.54E-05	3.82E-05	2.41E-05	3.84E-05
Borreliaceae	5.08E-05	2.64E-05	1.18E-05	0	2.41E-05	7.67E-05
Thermovenabulum	0	9.25E-05	0	0	0	0
Bifidobacteriaceae	5.08E-05	9.25E-05	1.18E-05	1.91E-05	0	2.56E-05
Gallionellaceae	0	2.64E-05	0	0	0	1.28E-05
Fusobacteriaceae	0	0	0	0	1.21E-05	3.84E-05
Psychromonadaceae	0	2.64E-05	1.18E-05	0	0	0
Pelagicoccaceae	5.08E-05	0.000211	0	1.91E-05	1.21E-05	0

Family	39	38	37	36	35	34
Acholeplasmataceae	0.000102	2.64E-05	4.73E-05	1.91E-05	1.21E-05	6.4E-05
Acidimicrobiaceae	7.63E-05	7.93E-05	3.54E-05	0	2.41E-05	0.000345
Microviridae	5.08E-05	7.93E-05	1.18E-05	1.91E-05	6.03E-05	0.000128
Anaplasmataceae	5.08E-05	6.61E-05	0	3.82E-05	0	1.28E-05
Desulfobulbaceae	0	0	0	0.000115	0	5.12E-05
Thermomonosporaceae	2.54E-05	0.000172	0	1.91E-05	6.03E-05	7.67E-05
Saprospiraceae	2.54E-05	2.64E-05	0	1.91E-05	0.000133	5.12E-05
Aerococcaceae	0	1.32E-05	1.18E-05	1.91E-05	0	5.12E-05
Shewanellaceae	2.54E-05	1.32E-05	2.36E-05	0	4.82E-05	5.12E-05
Nostocaceae	0	2.64E-05	0	1.91E-05	0	5.12E-05
Oceanospirillaceae	0.000102	6.61E-05	0	0	2.41E-05	8.95E-05
Euzebyaceae	5.08E-05	1.32E-05	0	0	0	1.28E-05
Beijerinckiaceae	0	0	0	0	1.21E-05	0
Turicibacteraceae	0	0	0	0	0	0
Coxiellaceae	7.63E-05	0	0	1.91E-05	0	2.56E-05
Nocardiaceae	0	5.29E-05	1.18E-05	0	0	7.67E-05
Aminiphilaceae	0	1.32E-05	1.18E-05	3.82E-05	1.21E-05	1.28E-05
Caldilineaceae	5.08E-05	0	1.18E-05	0	2.41E-05	3.84E-05
Alteromonadaceae	0	2.64E-05	0	0	2.41E-05	7.67E-05
Carnobacteriaceae	0	0	2.36E-05	3.82E-05	0	0
Entomoplasmataceae	2.54E-05	7.93E-05	0	1.91E-05	0	6.4E-05
Methylophilaceae	2.54E-05	0	0	3.82E-05	2.41E-05	1.28E-05
Micrococcaceae	2.54E-05	2.64E-05	1.18E-05	3.82E-05	2.41E-05	1.28E-05
Rhodothermaceae	0	2.64E-05	1.18E-05	0	0	2.56E-05
Thermaceae	0	2.64E-05	1.18E-05	0	1.21E-05	1.28E-05
Erythrobacteraceae	2.54E-05	7.93E-05	0	0	1.21E-05	1.28E-05
Cellulomonadaceae	2.54E-05	0.000132	0	0	0	2.56E-05
Pseudanabaenaceae	0	7.93E-05	0	0	0	2.56E-05
Thermogemmatisporaceae	0	0	1.18E-05	0	2.41E-05	0
Carboxydocellaceae	0	0	0	1.91E-05	0	1.28E-05
Thermoactinomycetaceae	2.54E-05	2.64E-05	0	5.74E-05	0	1.28E-05
Rivulariaceae	0	0	1.18E-05	0	0	0.000537
Aurantimonadaceae	0	1.32E-05	0	0	0	2.56E-05
Dehalococcoidaceae	0	1.32E-05	1.18E-05	0	0	3.84E-05
Ignavibacteriaceae	2.54E-05	0.000132	0	0	0	1.28E-05
Rhabdochlamydiaceae	0	1.32E-05	0	0	0	0
Brachyspiraceae	0	5.29E-05	0	0	1.21E-05	2.56E-05
Brucellaceae	0	2.64E-05	1.18E-05	0	0	5.12E-05
Waddliaceae	0	1.32E-05	0	0	0	0
Thermicanaceae	0	0	1.18E-05	0	0	1.28E-05
Solirubrobacteraceae	2.54E-05	0	0	0	0	1.28E-05
Francisellaceae	0	0	0	0	0	2.56E-05
Chrysiogenaceae	0	0	0	0	0	0
Conexibacteraceae	2.54E-05	3.97E-05	1.18E-05	1.91E-05	0	0
Odoribacteraceae	0	0	0	0	0	0
Leptotrichiaceae	0	0	2.36E-05	0	0	0
Opitutaceae	0	0.00041	0	0	0	0

Family	39	38	37	36	35	34
Acidithiobacillaceae	0	1.32E-05	0	0	0	0
Nautiliaceae	0	0	0	0	0	0
Erysipelotrichaceae	0	0	1.18E-05	0	0	0
Gemellaceae	0	0	0	0	0	0
Myxococcaceae	0	0	0	0	0	0.000166
Haliangiaceae	5.08E-05	1.32E-05	0	0	2.41E-05	0
Pseudoalteromonadaceae	0	0	0	0	0	1.28E-05
Idiomarinaceae	2.54E-05	0	1.18E-05	0	0	0
Chroococcaceae	2.54E-05	0	0	0	0	1.28E-05
Methylacidiphilaceae	0	0	0	0	1.21E-05	0
Elusimicrobiaceae	0	0	0	0	0	0
Kineosporiaceae	0	3.97E-05	1.18E-05	1.91E-05	0	1.28E-05
Microcystaceae	0	0	1.18E-05	0	0	1.28E-05
Rikenellaceae	0	0	0	0	0	0
Verrucomicrobiaceae	0	1.32E-05	2.36E-05	0	0	0
Prevotellaceae	0	0	0	0	0	0
Halobacteroidaceae	0	0	0	0	0	0
Dietziaceae	2.54E-05	0	0	0	1.21E-05	1.28E-05
Listeriaceae	0	0	2.36E-05	0	0	7.67E-05
Flammeovirgaceae	0	0	0	0	3.62E-05	0
Isosphaeraceae	0	0	0	0	0	0
Kouleothrixaceae	5.08E-05	1.32E-05	0	0	0	0
Gemmatimonadaceae	0	0	0	0	0	1.28E-05
Tsukamurellaceae	0	1.32E-05	0	0	0	0
Dehalobacteriaceae	0	0	0	0	0	0
Thiohalorhabdaceae	0	0	0	0	0	0
Gordoniaceae	0	0	0	0	0	0
Leptospiraceae	0	0	0	0	0	2.56E-05
Dermabacteraceae	0	0	0	0	0	0
Intrasporangiaceae	0	1.32E-05	0	0	0	0
Saccharospirillaceae	2.54E-05	0	0	0	0	0
Koribacteraceae	0	0	0	0	0	0
Puniceicoccaceae	0	1.32E-05	0	0	0	0
Aeromonadaceae	0	1.32E-05	0	0	0	0
Promicromonosporaceae	0	0	0	0	0	0
Moritellaceae	0	0	0	0	0	0
Dermacoccaceae	0	0	0	0	0	0
Methanocorpusculaceae	0	0	0	0	0	0
Patulibacteraceae	0	0	0	0	0	0
Methanosarcinaceae	0	0	0	0	0	0
Sanguibacteraceae	0	0	0	0	0	0
Alcanivoracaceae	0	0	0	0	0	0
Archaeoglobaceae	0	0	0	0	0	0
Bogoriellaceae	0	0	0	0	0	0
Chthonomonadaceae	0	0	0	0	0	0
Nitrospinaceae	0	0	0	0	0	0
Catenulisporaceae	0	0	0	0	0	0

Nocardiopsaceae	0	0	0	0	0	0
Roseiflexaceae	0	0	0	0	0	0
Anaeroplasmataceae	0	0	0	0	0	0
Oscillochloridaceae	0	0	0	0	0	0
Chloroflexaceae	5.08E-05	0	0	0	0	0
Sporichthyaceae	2.54E-05	0	0	0	0	0
Armatimonadaceae	0	0	0	0	0	0
Fibrobacteraceae	0	0	0	0	0	0
Sulfolobaceae	0	0	0	0	0	0
Coprobacillaceae	0	0	0	0	0	0
Desulfomicrobiaceae	0	0	0	0	0	0
Cyanobacteriaceae	0	0	0	0	0	1.28E-05
Halobacteriaceae	0	0	0	0	0	0
Geodermatophilaceae	0	1.32E-05	0	0	0	0
Desulfurococcaceae	0	0	0	0	0	0
Methanobacteriaceae	0	0	0	0	0	0
Thermoproteaceae	0	0	0	0	0	0
Nitrospiraceae	0	0	0	0	0	0

	Sample Number					
Family	27	26	25	24	23	22
Clostridiaceae	0.754279	0.562211	0.787615	0.767954	0.92951	0.919137
Lactobacillaceae	0.23136	0.427451	0.198223	0.21284	0.00209	0.002757
Ruminococcaceae	0.000265	0.000337	0.000124	0.000325	0.016597	0.022612
Sporolactobacillaceae	0.000689	7.63E-05	4.51E-05	9.64E-05	0.012075	0.000152
Campylobacteraceae	0.000954	0.000862	0.000839	0.001012	0.017297	0.013178
Spirochaetaceae	0	4.49E-06	2.82E-05	0	1.46E-05	5.06E-05
Lachnospiraceae	8.83E-05	0.000103	0.000163	0.000181	0.000299	0.000177
Peptococcaceae	0.0003	0.000171	0.000163	0.000265	0.008412	0.011989
Veillonellaceae	0.000424	0.000198	0.000197	0.00041	0.000291	0.000253
Comamonadaceae	0.000141	0.000112	0.000304	0.000374	5.1E-05	0.000759
Oxalobacteraceae	0.000318	9.88E-05	0.000873	0.001832	7.28E-05	7.59E-05
Anaerolinaceae	1.77E-05	1.35E-05	1.69E-05	0	7.28E-06	2.53E-05
Rhodospirillaceae	0.000883	0.000557	0.000428	0.000578	0.000371	0.001366
Burkholderiaceae	0.000177	0.000108	0.000518	0.001325	7.28E-06	7.59E-05
Flavobacteriaceae	7.07E-05	9.88E-05	3.38E-05	9.64E-05	9.47E-05	0.000228
Flexibacteraceae	3.53E-05	4.49E-05	4.51E-05	7.23E-05	0.000117	0.000228
Sphingobacteriaceae	0.000124	0.000121	0.000113	0.00012	0.000204	0.000177
Solibacteraceae	0	0	0	0	0	0
Sphingomonadaceae	0.000389	0.000171	0.000265	0.000374	0.000269	0.008574
Phyllobacteriaceae	0.00023	4.94E-05	0.000496	0.001133	0.000117	0.000253
Desulfobacteraceae	0.000247	0.000157	0.000175	0.000133	0.00016	0.000228
Bradyrhizobiaceae	8.83E-05	5.84E-05	0.000417	0.000699	6.55E-05	0.000126

Family	27	26	25	24	23	22
Methylobacteriaceae	0.000159	6.29E-05	0.00013	0.000157	0.000102	0.00043
Xanthomonadaceae	5.3E-05	3.14E-05	0.000372	0.000627	4.37E-05	0
Desulfovibrionaceae	0.000159	0.000148	0.000197	0.000892	0.000153	0.000379
Rhodocyclaceae	7.07E-05	3.14E-05	1.69E-05	7.23E-05	1.46E-05	0
Chitinophagaceae	7.07E-05	5.84E-05	4.51E-05	6.02E-05	0.002243	0.003288
Caulobacteraceae	0.000318	0.00022	0.000321	0.000349	0.000138	0.000228
Paenibacillaceae	0.000336	0.000377	0.000332	0.000289	0.000328	5.06E-05
Thermotogaceae	8.83E-05	6.29E-05	7.89E-05	0.000108	0.000109	0.000278
Helicobacteraceae	0	8.98E-06	5.63E-06	2.41E-05	7.28E-06	0
Bacillaceae	0.000336	0.000422	0.0004	0.000313	0.000342	0.000101
Microbacteriaceae	0.000477	0.000189	0.000163	0.000217	0.000269	0.00086
Halanaerobiaceae	0.000883	0.000763	0.000546	0.000386	0.000437	0.000304
Planococcaceae	0.000212	0.00018	0.000225	0.000157	0.001755	0.000228
Thermoanaerobacteraceae	7.07E-05	4.49E-05	7.32E-05	2.41E-05	5.1E-05	7.59E-05
Propionibacteriaceae	7.07E-05	2.25E-05	0.000124	0.000241	5.83E-05	0.000177
Porphyromonadaceae	0	1.35E-05	1.13E-05	3.61E-05	0	0
Enterobacteriaceae	0.000389	0.000283	0.000434	0.000386	0.000371	0.000228
Geobacteraceae	7.07E-05	4.94E-05	0.000101	0.000181	0.000189	0.000177
Mycobacteriaceae	5.3E-05	8.08E-05	3.38E-05	4.82E-05	0.000102	0.000582
Rhodobacteraceae	0.000106	0.000557	0.000163	0.000181	0.000124	0.000329
Actinomycetaceae	0.0003	0.000207	0.000113	0.00012	0.00024	0.000734
Hyphomicrobiaceae	1.77E-05	8.08E-05	0.000124	0.000157	0.000124	0.000253
Acetobacteraceae	1.77E-05	1.35E-05	7.32E-05	0.000313	9.47E-05	0.000354
Caldicellulosiruptoraceae	0	3.14E-05	1.13E-05	1.2E-05	8.01E-05	7.59E-05
Piscirickettsiaceae	7.07E-05	1.8E-05	3.38E-05	1.2E-05	0.000503	0.000455
Xanthobacteraceae	0.000106	3.14E-05	9.58E-05	0.000265	0.000131	0.000278
Dethiosulfovibrionaceae	1.77E-05	0	3.38E-05	1.2E-05	7.28E-06	5.06E-05
Bdellovibrionaceae	0.000371	0.000242	0.000242	0.000193	0.000146	0.000253
Syntrophobacteraceae	0	1.35E-05	1.69E-05	2.41E-05	1.46E-05	0
Brevibacteriaceae	0.000159	8.08E-05	9.01E-05	0.000157	0.000124	0.000177
Phormidiaceae	3.53E-05	1.8E-05	6.2E-05	0.000181	7.28E-06	0
Exiguobacteraceae	0.000159	0.000153	0.001442	0.000157	6.55E-05	0
Syntrophaceae	5.3E-05	8.98E-06	2.25E-05	2.41E-05	4.37E-05	7.59E-05
Rhizobiaceae	5.3E-05	2.25E-05	0.000113	0.000422	0.00016	0.000582
Glycomycetaceae	0.000212	0.000108	9.01E-05	9.64E-05	0.000211	0.000228
Synergistaceae	0	2.69E-05	1.13E-05	0	0	0
Streptomycetaceae	0.000106	4.04E-05	6.76E-05	6.02E-05	8.74E-05	0.000253
Nocardioidaceae	5.3E-05	2.25E-05	7.89E-05	8.43E-05	8.74E-05	0.000152
Caldithrixaceae	7.07E-05	1.8E-05	1.13E-05	3.61E-05	2.18E-05	2.53E-05

Family	27	26	25	24	23	22
Thermodesulfovibrionaceae	0	0	3.94E-05	2.41E-05	2.18E-05	2.53E-05
Hydrogenophilaceae	0.000106	0	1.13E-05	1.2E-05	7.28E-06	0
Pseudomonadaceae	5.3E-05	1.35E-05	5.63E-05	8.43E-05	0.00016	0.001341
Heliobacteriaceae	3.53E-05	8.98E-05	0.000113	0.00012	6.55E-05	0.000202
Acidobacteriaceae	5.3E-05	3.14E-05	2.25E-05	4.82E-05	5.1E-05	2.53E-05
Sphaerochaetaceae	0	0	0	0	0	0
Desulfuromonadaceae	3.53E-05	6.29E-05	5.07E-05	4.82E-05	6.55E-05	0.000101
Pelobacteraceae	1.77E-05	2.69E-05	3.38E-05	2.41E-05	3.64E-05	2.53E-05
Litoricolaceae	1.77E-05	1.35E-05	5.63E-06	0	0.000211	0.000278
Mycoplasmataceae	0.000124	0.000193	0.000124	0.000157	4.37E-05	0
Thiotrichaceae	0.000106	1.8E-05	2.25E-05	1.2E-05	2.18E-05	0.000126
Deinococcaceae	7.07E-05	4.04E-05	5.07E-05	0	8.01E-05	0.000253
Pseudonocardiaceae	8.83E-05	4.04E-05	1.13E-05	2.41E-05	4.37E-05	7.59E-05
Bacteroidaceae	1.77E-05	0	0	1.2E-05	7.28E-06	0
Streptococcaceae	0.000124	8.98E-05	7.89E-05	9.64E-05	4.37E-05	5.06E-05
Hyphomonadaceae	1.77E-05	4.49E-05	7.32E-05	3.61E-05	3.64E-05	0
Desulfonatronumaceae	3.53E-05	1.35E-05	0	2.41E-05	0	0
Yaniellaceae	1.77E-05	0	5.63E-06	0	4.37E-05	5.06E-05
Desulfohalobiaceae	0	8.98E-06	5.63E-06	1.2E-05	7.28E-06	0
Syntrophomonadaceae	0	4.49E-06	3.38E-05	2.41E-05	8.01E-05	5.06E-05
Chromatiaceae	5.3E-05	4.04E-05	3.38E-05	3.61E-05	4.37E-05	5.06E-05
Symbiobacteriaceae	1.77E-05	4.49E-06	0	1.2E-05	7.28E-06	2.53E-05
Eubacteriaceae	1.77E-05	4.49E-06	5.63E-06	1.2E-05	7.28E-06	0
Thermodesulfobacteriaceae	8.83E-05	3.14E-05	7.32E-05	4.82E-05	6.55E-05	0.000101
Peptostreptococcaceae	0	0	0	0	0	5.06E-05
Sinobacteraceae	1.77E-05	1.8E-05	5.63E-06	4.82E-05	2.91E-05	7.59E-05
Alcaligenaceae	5.3E-05	8.98E-06	2.82E-05	2.41E-05	2.18E-05	0.000101
Actinosynnemataceae	7.07E-05	4.04E-05	3.38E-05	4.82E-05	7.28E-06	5.06E-05
Amoebophilaceae	8.83E-05	4.49E-06	2.25E-05	0	6.55E-05	0
Polyangiaceae	0	2.25E-05	2.25E-05	1.2E-05	7.28E-06	2.53E-05
Thermobaculaceae	0	8.98E-06	2.25E-05	2.41E-05	0	0
Deferribacteraceae	0	0	0	0	7.28E-06	2.53E-05
Chlorobiaceae	3.53E-05	4.49E-06	5.63E-06	1.2E-05	2.91E-05	0
Nannocystaceae	0.000106	1.35E-05	5.07E-05	4.82E-05	8.01E-05	0
Enterococcaceae	1.77E-05	6.29E-05	0.000101	3.61E-05	1.46E-05	2.53E-05
Rickettsiaceae	5.3E-05	2.69E-05	2.25E-05	2.41E-05	5.83E-05	0.000101
Moraxellaceae	3.53E-05	2.69E-05	2.82E-05	4.82E-05	3.64E-05	0.000101
Streptosporangiaceae	7.07E-05	1.35E-05	3.38E-05	3.61E-05	4.37E-05	5.06E-05
Coriobacteriaceae	3.53E-05	2.25E-05	1.13E-05	2.41E-05	2.18E-05	2.53E-05

Family	27	26	25	24	23	22
Micromonosporaceae	1.77E-05	2.25E-05	2.25E-05	3.61E-05	7.28E-06	2.53E-05
Anaerobrancaceae	0	4.49E-06	5.63E-06	1.2E-05	2.18E-05	2.53E-05
Cystobacteraceae	0	0	1.69E-05	4.82E-05	0	5.06E-05
Ectothiorhodospiraceae	1.77E-05	1.35E-05	2.25E-05	2.41E-05	0	7.59E-05
Caldisericaceae	3.53E-05	4.49E-06	0	0	0	0
Leuconostocaceae	7.07E-05	0.000117	8.45E-05	6.02E-05	6.55E-05	2.53E-05
Contubernalisaceae	3.53E-05	1.35E-05	1.69E-05	1.2E-05	0	2.53E-05
Neisseriaceae	1.77E-05	1.8E-05	5.63E-06	1.2E-05	1.46E-05	0.000101
Vibrionaceae	5.3E-05	3.59E-05	6.2E-05	4.82E-05	2.91E-05	2.53E-05
Actinopolysporaceae	1.77E-05	4.49E-06	5.63E-06	1.2E-05	0.000153	0.000101
Brocadiaceae	1.77E-05	4.49E-06	2.25E-05	0	0	0
Corynebacteriaceae	5.3E-05	8.98E-06	5.63E-06	1.2E-05	2.18E-05	0
Holophagaceae	1.77E-05	0	1.13E-05	0	0	0
Sulfobacillaceae	5.3E-05	0.000108	2.25E-05	8.43E-05	7.28E-06	2.53E-05
Kiloniellaceae	8.83E-05	1.35E-05	2.25E-05	1.2E-05	1.46E-05	0
Legionellaceae	1.77E-05	2.25E-05	3.38E-05	1.2E-05	7.28E-06	0
Methylocystaceae	0	0	0	6.02E-05	7.28E-06	0
Halomonadaceae	1.77E-05	4.49E-06	0	2.41E-05	2.91E-05	2.53E-05
Borreliaceae	0	0	5.63E-06	0	1.46E-05	0
Thermovenabulum	0	0	0	0	0	0
Bifidobacteriaceae	3.53E-05	3.14E-05	2.82E-05	3.61E-05	1.46E-05	0
Gallionellaceae	1.77E-05	4.49E-06	0	0	0	0
Fusobacteriaceae	3.53E-05	1.8E-05	2.82E-05	1.2E-05	2.18E-05	0.000177
Psychromonadaceae	3.53E-05	8.98E-06	1.13E-05	0	8.01E-05	5.06E-05
Pelagicoccaceae	0	4.49E-06	0	1.2E-05	5.1E-05	2.53E-05
Acholeplasmataceae	0	4.49E-06	0	0	1.46E-05	7.59E-05
Acidimicrobiaceae	1.77E-05	8.98E-06	0	0	2.18E-05	2.53E-05
Microviridae	1.77E-05	4.49E-06	0	3.61E-05	1.46E-05	0
Anaplasmataceae	0	2.25E-05	5.63E-06	1.2E-05	1.46E-05	2.53E-05
Desulfobulbaceae	0	8.98E-06	0	1.2E-05	7.28E-06	0
Thermomonosporaceae	0	0	0	3.61E-05	7.28E-06	0
Saprospiraceae	1.77E-05	4.49E-06	5.63E-06	1.2E-05	1.46E-05	7.59E-05
Aerococcaceae	1.77E-05	0	4.51E-05	3.61E-05	1.46E-05	0
Shewanellaceae	1.77E-05	8.98E-06	2.25E-05	2.41E-05	2.18E-05	7.59E-05
Nostocaceae	0	0	5.63E-06	0	0	0
Oceanospirillaceae	1.77E-05	0	2.82E-05	1.2E-05	2.18E-05	0.000152
Euzebyaceae	0	0	5.63E-06	0	0	0
Beijerinckiaceae	0	0	0	1.2E-05	0	0
Turicibacteraceae	0	0	0	0	0	0

Family	27	26	25	24	23	22
Coxiellaceae	3.53E-05	8.98E-06	0	1.2E-05	2.18E-05	2.53E-05
Nocardiaceae	3.53E-05	1.35E-05	5.63E-06	1.2E-05	0	0
Aminiphilaceae	0	1.35E-05	0	0	7.28E-06	0
Caldilineaceae	0	4.49E-06	0	2.41E-05	0	0
Alteromonadaceae	0	0	0	2.41E-05	1.46E-05	7.59E-05
Carnobacteriaceae	0	2.25E-05	2.25E-05	3.61E-05	0	0
Entomoplasmataceae	3.53E-05	8.98E-06	5.63E-06	2.41E-05	0	2.53E-05
Methylophilaceae	3.53E-05	3.14E-05	1.13E-05	2.41E-05	7.28E-06	5.06E-05
Micrococcaceae	0	4.49E-06	0	1.2E-05	7.28E-06	0
Rhodothermaceae	0	0	0	0	0	2.53E-05
Thermaceae	0	4.49E-06	1.69E-05	0	1.46E-05	0
Erythrobacteraceae	0	4.49E-06	1.13E-05	1.2E-05	1.46E-05	0.000304
Cellulomonadaceae	1.77E-05	4.49E-06	0	0	0	7.59E-05
Pseudanabaenaceae	0	0	1.13E-05	0	0	0
Thermogemmatisporaceae	0	0	5.63E-06	0	0	0
Carboxydocellaceae	0	1.35E-05	5.63E-06	1.2E-05	0	0
Thermoactinomycetaceae	1.77E-05	1.35E-05	0	1.2E-05	0	0
Rivulariaceae	0	0	0	0	0	0
Aurantimonadaceae	0	4.49E-06	5.63E-06	0.000181	0	0
Dehalococcoidaceae	0	0	0	0	3.64E-05	0
Ignavibacteriaceae	0	0	5.63E-06	0	7.28E-06	0
Rhabdochlamydiaceae	0	8.98E-06	5.63E-06	0	0	2.53E-05
Brachyspiraceae	0	4.49E-06	0	1.2E-05	0	5.06E-05
Brucellaceae	0	8.98E-06	0	4.82E-05	7.28E-06	5.06E-05
Waddliaceae	1.77E-05	0	0	0	0	0
Thermicanaceae	1.77E-05	0	0	0	7.28E-06	0
Solirubrobacteraceae	0	0	0	0	0	0
Francisellaceae	0	4.49E-06	0	1.2E-05	0	0
Chrysiogenaceae	0	0	0	2.41E-05	7.28E-06	2.53E-05
Conexibacteraceae	0	0	0	0	0	0
Odoribacteraceae	0	0	0	0	0	0
Leptotrichiaceae	0	0	0	0	0	0
Opitutaceae	0	0	0	0	0	0
Acidithiobacillaceae	0	0	0	0	0	0
Nautiliaceae	0	0	0	0	0	0
Erysipelotrichaceae	0	4.49E-06	0	0	0	0
Gemellaceae	0	0	5.63E-06	0	0	0
Myxococcaceae	0	0	1.13E-05	0	0	0
Haliangiaceae	0	0	0	0	0	0

Family	27	26	25	24	23	22
Pseudoalteromonadaceae	0	4.49E-06	0	0	2.18E-05	0
Idiomarinaceae	1.77E-05	4.49E-06	0	1.2E-05	7.28E-06	5.06E-05
Chroococcaceae	0	0	0	0	0	0
Methylacidiphilaceae	0	0	0	0	0	0
Elusimicrobiaceae	0	0	0	0	0	0
Kineosporiaceae	0	0	0	0	0	0
Microcystaceae	0	0	0	0	0	0
Rikenellaceae	0	0	0	0	0	0
Verrucomicrobiaceae	0	4.49E-06	5.63E-06	1.2E-05	2.18E-05	0
Prevotellaceae	0	0	1.13E-05	0	0	0
Halobacteroidaceae	0	8.98E-06	0	0	0	0
Dietziaceae	0	4.49E-06	0	0	7.28E-06	0
Listeriaceae	0	0	5.63E-06	0	0	0
Flammeovirgaceae	0	0	0	0	7.28E-06	0
Isosphaeraceae	0	0	0	0	0	0
Kouleothrixaceae	0	0	5.63E-06	0	0	0
Gemmatimonadaceae	0	0	0	0	0	0
Tsukamurellaceae	0	0	0	0	1.46E-05	2.53E-05
Dehalobacteriaceae	0	0	0	0	0	0
Thiohalorhabdaceae	0	0	0	0	0	0
Gordoniaceae	0	0	0	0	0	2.53E-05
Leptospiraceae	0	0	0	1.2E-05	0	0
Dermabacteraceae	0	0	2.82E-05	0	0	0
Intrasporangiaceae	0	0	0	0	0	2.53E-05
Saccharospirillaceae	0	0	0	0	7.28E-06	0
Koribacteraceae	0	0	0	0	0	0
Puniceicoccaceae	0	0	0	0	0	0
Aeromonadaceae	0	4.49E-06	0	0	0	0
Promicromonosporaceae	0	0	0	0	7.28E-06	0
Moritellaceae	0	0	0	0	0	2.53E-05
Dermacoccaceae	0	0	1.13E-05	0	0	0
Methanocorpusculaceae	0	0	0	0	0	0
Patulibacteraceae	0	0	0	0	0	0
Methanosarcinaceae	0	0	0	0	0	0
Sanguibacteraceae	0	0	0	0	0	0
Alcanivoracaceae	0	0	0	0	0	0
Archaeoglobaceae	0	0	0	0	0	0
Bogoriellaceae	0	0	0	0	0	0
Chthonomonadaceae	0	0	0	0	0	0

Family	27	26	25	24	23	22
Nitrospinaceae	0	0	0	0	0	0
Catenulisporaceae	0	0	0	0	0	0
Nocardiopsaceae	0	0	0	0	0	0
Roseiflexaceae	0	0	0	0	0	0
Anaeroplasmataceae	0	0	5.63E-06	0	0	0
Oscillochloridaceae	0	0	0	0	0	0
Chloroflexaceae	0	0	0	0	0	0
Sporichthyaceae	0	0	0	0	0	0
Armatimonadaceae	0	0	0	0	0	0
Fibrobacteraceae	0	0	0	0	0	0
Sulfolobaceae	0	0	0	0	0	0
Coprobacillaceae	0	0	0	0	0	0
Desulfomicrobiaceae	0	0	0	0	0	2.53E-05
Cyanobacteriaceae	0	0	0	0	0	0
Halobacteriaceae	0	0	0	0	0	0
Geodermatophilaceae	0	0	0	0	0	0
Desulfurococcaceae	0	0	0	0	0	0
Methanobacteriaceae	0	0	0	0	0	0
Thermoproteaceae	0	0	0	0	0	0
Nitrospiraceae	0	0	0	0	0	0

	Sample Number					
Family	21	20	19	18	17	16
Clostridiaceae	0.960931	0.57533	0.761086	0.779167	0.909903	0.864721
Lactobacillaceae	0.002758	0.006764	0.006594	0.014332	0.005632	0.001893
Ruminococcaceae	0.002188	0.31472	0.037664	0.070642	0.022077	0.110041
Sporolactobacillaceae	0.00027	0.029274	0.120408	0.033506	0.000489	8.23E-05
Campylobacteraceae	0.015947	0.010459	0.048986	0.073102	0.028348	0.001963
Spirochaetaceae	0.00013	0.000297	1.74E-05	2.29E-05	9.99E-06	1.76E-05
Lachnospiraceae	0.00049	0.003876	0.000523	0.001222	0.00029	0.001951
Peptococcaceae	0.001099	0.001285	0.000872	0.000634	0.017404	0.000505
Veillonellaceae	0.0003	0.000807	0.00075	0.000474	0.00028	0.00067
Comamonadaceae	0.00031	0.001901	0.000227	0.000176	9.99E-05	0.000494
Oxalobacteraceae	0.000779	0.004757	6.98E-05	9.17E-05	0.000419	0.00151
Anaerolinaceae	9.99E-06	0.000138	0	2.29E-05	6.99E-05	2.35E-05
Rhodospirillaceae	0.00052	0.001019	0.00068	0.000458	0.000679	0.000329
Burkholderiaceae	0.00051	0.003865	0.000122	0.000153	0.00026	0.001452
Flavobacteriaceae	0.00031	0.000711	0.000105	0.000115	0.00014	0.000106
Flexibacteraceae	4E-05	0.001104	0.000715	0.001872	0.000569	0.000317
Sphingobacteriaceae	0.0002	0.000563	0.00014	0.000474	0.000409	0.0003
Solibacteraceae	0	2.12E-05	0	7.64E-06	0	0
Sphingomonadaceae	0.00028	0.001179	0.000244	0.000328	0.000689	0.000364

Family	21	20	19	18	17	16
Phyllobacteriaceae	0.00041	0.001826	0.000105	0.000122	0.00031	0.000811
Desulfobacteraceae	0.00021	0.000467	0.000157	0.000581	0.0002	0.000288
Bradyrhizobiaceae	0.00051	0.001773	5.23E-05	0.000138	0.000399	0.000782
Methylobacteriaceae	0.00015	0.000255	0.000122	0.000168	0.00021	0.000123
Xanthomonadaceae	0.00021	0.000998	8.72E-05	0.000115	4.99E-05	0.000417
Desulfovibrionaceae	0.00023	0.000945	0.000279	0.000565	0.00019	0.00037
Rhodocyclaceae	0.00013	0.00035	5.23E-05	0.000176	8.99E-05	9.99E-05
Chitinophagaceae	0.00036	0.001083	0.002128	0.000206	0.00022	6.47E-05
Caulobacteraceae	0.00028	0.001147	0.000227	0.000176	0.00014	0.000552
Paenibacillaceae	0.00024	0.001359	0.003053	0.000932	0.00015	0.000141
Thermotogaceae	9.99E-05	0.001179	0.000297	0.000833	0.00025	0.000517
Helicobacteraceae	9.99E-06	5.31E-05	1.74E-05	2.29E-05	7.99E-05	5.88E-06
Bacillaceae	0.00019	0.001062	0.002041	0.001184	0.00019	0.000176
Microbacteriaceae	0.00037	0.000648	0.000523	0.000848	0.000369	0.000353
Halanaerobiaceae	0.00036	0.000478	0.00014	0.000481	0.000439	0.000359
Planococcaceae	9.99E-05	0.000563	0.000593	0.000413	0.00014	0.000159
Thermoanaerobacteraceae	0.00011	0.002294	0.000192	0.000611	0.00016	0.000182
Propionibacteriaceae	0.00041	0.001497	8.72E-05	8.4E-05	8.99E-05	0.000165
Porphyromonadaceae	9.99E-06	4.25E-05	0	2.29E-05	9.99E-06	5.88E-06
Enterobacteriaceae	0.00039	0.000616	0.000174	0.000565	0.000629	0.000588
Geobacteraceae	0.00016	0.000435	0.000401	0.000481	0.000339	0.000241
Mycobacteriaceae	0.00012	0.000276	0.00014	0.000199	8.99E-05	0.000129
Rhodobacteraceae	0.00016	0.000722	0.000157	0.000275	0.000739	0.000188
Actinomycetaceae	0.00025	0.000488	0.000227	0.000321	0.00024	0.000253
Hyphomicrobiaceae	0.00016	0.000382	0.000105	6.11E-05	0.00011	7.64E-05
Acetobacteraceae	0.00012	0.000329	3.49E-05	0.00013	5.99E-05	9.99E-05
Caldicellulosiruptoraceae	2E-05	0.0012	8.72E-05	0.000199	0.00015	0.000323
Piscirickettsiaceae	0.000679	0.000234	0.00082	0.000924	0.000639	5.29E-05
Xanthobacteraceae	8.99E-05	0.000393	6.98E-05	5.35E-05	0.00015	0.000118
Dethiosulfovibrionaceae	0	0.001019	3.49E-05	8.4E-05	2E-05	0.000699
Bdellovibrionaceae	0.00018	0.000106	0.000122	0.000313	0.0002	0.000153
Syntrophobacteraceae	6E-05	0.000202	0.00014	1.53E-05	3.99E-05	2.35E-05
Brevibacteriaceae	0.00012	0.000478	0.000122	0.000283	0.00015	0.000376
Phormidiaceae	0.00013	0.000542	1.74E-05	2.29E-05	0	0.000353
Exiguobacteraceae	5E-05	0.000106	0.000105	0.00013	4.99E-05	5.88E-05
Syntrophaceae	0.00011	0.000138	0.000122	0.000191	3.99E-05	4.11E-05
Rhizobiaceae	4E-05	0.000478	5.23E-05	6.88E-05	8.99E-05	0.000118
Glycomycetaceae	0.00021	0.000202	0.00014	0.000168	0.00014	0.000118
Synergistaceae	0	6.37E-05	1.74E-05	1.53E-05	9.99E-06	5.88E-06
Streptomycetaceae	0.00016	0.000531	0.000122	0.000115	0.00016	0.000153
Nocardioidaceae	0.00011	0.000265	0.000192	0.000222	8.99E-05	5.29E-05
Caldithrixaceae	0.00013	0.000191	6.98E-05	0.000359	9.99E-06	5.29E-05
Thermodesulfovibrionaceae	6E-05	0.00035	0	7.64E-05	2E-05	5.29E-05
Hydrogenophilaceae	3E-05	0.000149	1.74E-05	7.64E-06	2E-05	2.94E-05
Pseudomonadaceae	0.00011	0.000616	3.49E-05	6.11E-05	0.00024	8.82E-05
Heliobacteriaceae	0.00013	0.000276	6.98E-05	8.4E-05	0.00011	8.82E-05

Family	21	20	19	18	17	16
Acidobacteriaceae	6.99E-05	0.000297	1.74E-05	5.35E-05	3E-05	5.29E-05
Sphaerochaetaceae	0.00014	6.37E-05	0	0	9.99E-06	5.88E-06
Desulfuromonadaceae	7.99E-05	0.000287	0.000541	0.00042	6.99E-05	7.05E-05
Pelobacteraceae	6E-05	0.000117	0.000925	0.000275	6.99E-05	0.000123
Litoricolaceae	0.0003	0.000181	0.000331	0.000275	0.00025	5.88E-05
Mycoplasmataceae	9.99E-06	7.43E-05	6.98E-05	0.000351	7.99E-05	2.35E-05
Thiotrichaceae	7.99E-05	0.000382	6.98E-05	0.000244	8.99E-05	5.29E-05
Deinococcaceae	3E-05	0.000319	8.72E-05	0.000176	8.99E-05	0.000188
Pseudonocardiaceae	7.99E-05	0.000308	3.49E-05	9.17E-05	9.99E-06	4.7E-05
Bacteroidaceae	2E-05	3.19E-05	0	0	0	0
Streptococcaceae	2E-05	0.000127	0	3.82E-05	9.99E-06	9.4E-05
Hyphomonadaceae	3E-05	0.001019	3.49E-05	3.82E-05	9.99E-06	5.88E-05
Desulfonatronumaceae	3E-05	5.31E-05	0	3.06E-05	0	5.88E-06
Yaniellaceae	9.99E-06	0.000191	0.000576	0.00178	0.0003	4.11E-05
Desulfohalobiaceae	2E-05	6.37E-05	0	1.53E-05	0	1.76E-05
Syntrophomonadaceae	7.99E-05	0.000138	5.23E-05	0.000115	9.99E-06	4.7E-05
Chromatiaceae	6E-05	0.000234	6.98E-05	0.000214	0.00019	7.05E-05
Symbiobacteriaceae	3E-05	0.000212	0.00068	0.001398	0.000499	2.94E-05
Eubacteriaceae	0.00025	9.56E-05	0	6.11E-05	9.99E-06	1.76E-05
Thermodesulfobacteriaceae	4E-05	0.000499	5.23E-05	0.000107	0	0.000129
Peptostreptococcaceae	4E-05	5.31E-05	1.74E-05	7.64E-06	9.99E-06	5.88E-06
Sinobacteraceae	3E-05	0.000191	0.000105	0.000275	5.99E-05	0.000106
Alcaligenaceae	6.99E-05	6.37E-05	3.49E-05	1.53E-05	3E-05	5.88E-06
Actinosynnemataceae	5E-05	0.000127	0.000105	6.11E-05	9.99E-06	2.35E-05
Amoebophilaceae	5E-05	0.000149	0.00014	0.00013	3.99E-05	1.76E-05
Polyangiaceae	6.99E-05	0.00034	0	4.58E-05	9.99E-06	2.35E-05
Thermobaculaceae	4E-05	0.000159	0	0	0	5.88E-06
Deferribacteraceae	3E-05	9.56E-05	3.49E-05	7.64E-06	9.99E-06	1.18E-05
Chlorobiaceae	9.99E-06	0.000223	0.000331	0.000466	8.99E-05	7.64E-05
Nannocystaceae	9.99E-05	7.43E-05	8.72E-05	0.000107	9.99E-05	4.11E-05
Enterococcaceae	5E-05	0.00017	0.00014	7.64E-05	2E-05	2.35E-05
Rickettsiaceae	7.99E-05	0.000127	8.72E-05	5.35E-05	7.99E-05	5.29E-05
Moraxellaceae	9.99E-05	0.000223	8.72E-05	9.17E-05	7.99E-05	0.000112
Streptosporangiaceae	4E-05	9.56E-05	0	3.06E-05	3E-05	1.76E-05
Coriobacteriaceae	7.99E-05	0.000138	0	0	2E-05	2.94E-05
Micromonosporaceae	3E-05	0.000287	6.98E-05	0.000283	8.99E-05	1.18E-05
Anaerobrancaceae	3E-05	0.000234	0.000105	0.000229	3.99E-05	0.000112
Cystobacteraceae	3E-05	0.000276	3.49E-05	3.82E-05	0	2.35E-05
Ectothiorhodospiraceae	7.99E-05	0.000255	3.49E-05	3.06E-05	7.99E-05	8.23E-05
Caldisericaceae	9.99E-06	0	1.74E-05	0	9.99E-06	6.47E-05
Leuconostocaceae	4E-05	4.25E-05	6.98E-05	3.82E-05	9.99E-06	1.18E-05
Contubernalisaceae	4E-05	5.31E-05	0	0.000145	9.99E-06	5.88E-05
Neisseriaceae	9.99E-06	0.000106	0	1.53E-05	3E-05	1.76E-05
Vibrionaceae	6.99E-05	4.25E-05	1.74E-05	3.82E-05	0.00011	3.53E-05
Actinopolysporaceae	9.99E-06	5.31E-05	1.74E-05	7.64E-06	0.00014	5.88E-06
Brocadiaceae	4E-05	9.56E-05	0	2.29E-05	9.99E-06	1.18E-05

Family	21	20	19	18	17	16
Corynebacteriaceae	0	0.00017	0	1.53E-05	0	1.76E-05
Holophagaceae	0	4.25E-05	0	0	0	0
Sulfobacillaceae	9.99E-06	2.12E-05	0	7.64E-06	2E-05	1.76E-05
Kiloniellaceae	5E-05	0.000159	0	3.06E-05	3.99E-05	1.76E-05
Legionellaceae	7.99E-05	0.000106	5.23E-05	0.000107	3E-05	3.53E-05
Methylocystaceae	3E-05	7.43E-05	0	7.64E-06	3E-05	2.35E-05
Halomonadaceae	0.00016	9.56E-05	0.000157	0.000122	0.00013	2.35E-05
Borreliaceae	9.99E-05	8.49E-05	1.74E-05	3.82E-05	9.99E-06	1.76E-05
Thermovenabulum	9.99E-06	3.19E-05	0	0	0	5.88E-06
Bifidobacteriaceae	9.99E-06	0.000117	1.74E-05	1.53E-05	9.99E-06	4.11E-05
Gallionellaceae	0	4.25E-05	0	0	9.99E-06	0
Fusobacteriaceae	6E-05	0	6.98E-05	0.000138	0.00011	2.94E-05
Psychromonadaceae	0.00011	7.43E-05	6.98E-05	0.000107	4.99E-05	5.88E-06
Pelagicoccaceae	6.99E-05	6.37E-05	3.49E-05	3.06E-05	9.99E-06	1.76E-05
Acholeplasmataceae	0.00019	0.000191	3.49E-05	1.53E-05	3E-05	4.11E-05
Acidimicrobiaceae	3E-05	5.31E-05	0	4.58E-05	9.99E-06	5.88E-06
Microviridae	9.99E-06	0.000361	0	0	0	2.35E-05
Anaplasmataceae	3E-05	9.56E-05	3.49E-05	1.53E-05	3.99E-05	5.88E-06
Desulfobulbaceae	9.99E-06	5.31E-05	0	0.000313	0	5.88E-05
Thermomonosporaceae	4E-05	0.000106	0	2.29E-05	0	1.18E-05
Saprospiraceae	2E-05	4.25E-05	0	0	3.99E-05	0
Aerococcaceae	3E-05	8.49E-05	0	3.06E-05	9.99E-06	1.18E-05
Shewanellaceae	0.00011	8.49E-05	0	2.29E-05	9.99E-06	1.18E-05
Nostocaceae	0	6.37E-05	0	0	9.99E-06	0
Oceanospirillaceae	0.00014	0.000308	0	0	9.99E-06	1.76E-05
Euzebyaceae	0	3.19E-05	0	7.64E-06	0	5.88E-06
Beijerinckiaceae	9.99E-06	0.00017	0	0	0	2.94E-05
Turicibacteraceae	0	1.06E-05	0	0	0	0
Coxiellaceae	3E-05	3.19E-05	0	0	0	1.18E-05
Nocardiaceae	9.99E-06	6.37E-05	3.49E-05	3.82E-05	9.99E-06	2.94E-05
Aminiphilaceae	0	1.06E-05	1.74E-05	2.29E-05	0	0
Caldilineaceae	0	2.12E-05	1.74E-05	3.06E-05	0	0
Alteromonadaceae	0.00013	0.000106	0	3.06E-05	3.99E-05	1.76E-05
Carnobacteriaceae	0	1.06E-05	8.72E-05	1.53E-05	9.99E-06	0
Entomoplasmataceae	3E-05	7.43E-05	0	0	9.99E-06	2.35E-05
Methylophilaceae	0	4.25E-05	1.74E-05	7.64E-06	9.99E-06	5.88E-06
Micrococcaceae	9.99E-06	6.37E-05	1.74E-05	7.64E-06	9.99E-06	1.18E-05
Rhodothermaceae	0	2.12E-05	0	2.29E-05	9.99E-06	1.76E-05
Thermaceae	0	6.37E-05	0	7.64E-06	0	7.05E-05
Erythrobacteraceae	9.99E-06	0	1.74E-05	0	0	1.18E-05
Cellulomonadaceae	9.99E-06	1.06E-05	1.74E-05	7.64E-06	9.99E-06	1.18E-05
Pseudanabaenaceae	2E-05	4.25E-05	0	0	0	1.76E-05
Thermogemmatisporaceae	0	1.06E-05	3.49E-05	6.11E-05	3E-05	1.18E-05
Carboxydocellaceae	0	0	0	3.06E-05	0	0
Thermoactinomycetaceae	9.99E-06	0	1.74E-05	7.64E-05	9.99E-06	0
Rivulariaceae	9.99E-06	0.000106	0	7.64E-06	0	0

Family	21	20	19	18	17	16
Aurantimonadaceae	0	1.06E-05	0	0	0	1.76E-05
Dehalococcoidaceae	0	5.31E-05	0	0	0	5.88E-06
Ignavibacteriaceae	0	0	0	0	2E-05	0
Rhabdochlamydiaceae	0	1.06E-05	1.74E-05	9.17E-05	0	5.88E-06
Brachyspiraceae	3E-05	7.43E-05	0	0	0	5.88E-06
Brucellaceae	2E-05	1.06E-05	0	0	9.99E-06	1.76E-05
Waddliaceae	0	0	0	0	0	0
Thermicanaceae	9.99E-06	2.12E-05	0	7.64E-06	0	1.18E-05
Solirubrobacteraceae	0	0	0	7.64E-06	0	0
Francisellaceae	0	0	0	0	0	0
Chrysiogenaceae	2E-05	4.25E-05	0	2.29E-05	0	0
Conexibacteraceae	0	1.06E-05	0	0	0	0
Odoribacteraceae	0	0	0	0	0	0
Leptotrichiaceae	0	1.06E-05	0	7.64E-06	0	5.88E-06
Opitutaceae	0	0	0	0	0	5.88E-06
Acidithiobacillaceae	0	0	0	0	0	0
Nautiliaceae	0	0	0	0	0	0
Erysipelotrichaceae	2E-05	0	1.74E-05	0	0	0
Gemellaceae	0	8.49E-05	0	2.29E-05	0	5.88E-06
Myxococcaceae	0	0	0	0	0	0
Haliangiaceae	0	1.06E-05	0	1.53E-05	9.99E-06	5.88E-06
Pseudoalteromonadaceae	0	0	0	7.64E-06	9.99E-06	0
Idiomarinaceae	3E-05	0	0	1.53E-05	9.99E-06	0
Chroococcaceae	0	1.06E-05	0	3.06E-05	9.99E-06	5.88E-06
Methylacidiphilaceae	0	0	0	0	9.99E-06	5.88E-06
Elusimicrobiaceae	0	0	0	0	0	0
Kineosporiaceae	0	0	0	0	0	0
Microcystaceae	0	0	0	0	9.99E-06	0
Rikenellaceae	0	0	0	0	0	0
Verrucomicrobiaceae	0	3.19E-05	0	0	9.99E-06	0
Prevotellaceae	0	7.43E-05	0	0	0	0
Halobacteroidaceae	0	0	0	0	0	5.88E-06
Dietziaceae	0	1.06E-05	1.74E-05	0	0	5.88E-06
Listeriaceae	0	1.06E-05	0	0	0	0
Flammeovirgaceae	0	0	0	0	9.99E-06	5.88E-06
Isosphaeraceae	0	0	0	0	0	0
Kouleothrixaceae	0	1.06E-05	0	0	0	0
Gemmatimonadaceae	0	0	0	0	0	0
Tsukamurellaceae	0	0	0	0	0	5.88E-06
Dehalobacteriaceae	0	0	0	0	0	0
Thiohalorhabdaceae	0	0	0	7.64E-06	0	0
Gordoniaceae	9.99E-06	1.06E-05	0	0	0	0
Leptospiraceae	0	1.06E-05	0	0	0	0
Dermabacteraceae	0	1.06E-05	0	0	0	0
Intrasporangiaceae	0	0	0	0	0	0
Saccharospirillaceae	0	0	0	0	0	5.88E-06

Family	21	20	19	18	17	16
Koribacteraceae	0	0	0	0	0	0
Puniceicoccaceae	0	0	0	0	0	0
Aeromonadaceae	0	0	0	0	0	0
Promicromonosporaceae	0	0	0	0	0	0
Moritellaceae	0	0	0	7.64E-06	0	0
Dermacoccaceae	0	0	0	0	0	0
Methanocorpusculaceae	0	0	0	0	0	0
Patulibacteraceae	0	0	0	0	0	0
Methanosarcinaceae	0	0	0	0	9.99E-06	0
Sanguibacteraceae	9.99E-06	0	0	0	0	0
Alcanivoracaceae	0	0	0	0	0	0
Archaeoglobaceae	0	2.12E-05	0	0	0	0
Bogoriellaceae	0	0	0	0	0	0
Chthonomonadaceae	0	0	0	0	0	0
Nitrospinaceae	0	1.06E-05	0	0	0	0
Catenulisporaceae	0	0	0	0	0	0
Nocardiopsaceae	0	1.06E-05	0	0	0	0
Roseiflexaceae	0	0	0	0	0	0
Anaeroplasmataceae	0	0	0	0	0	0
Oscillochloridaceae	9.99E-06	0	0	0	0	0
Chloroflexaceae	0	0	0	0	0	0
Sporichthyaceae	9.99E-06	0	0	0	0	0
Armatimonadaceae	0	0	0	0	0	0
Fibrobacteraceae	0	0	0	0	0	0
Sulfolobaceae	0	0	0	0	0	0
Coprobacillaceae	0	0	0	0	0	0
Desulfomicrobiaceae	0	0	0	0	0	0
Cyanobacteriaceae	0	0	0	0	0	0
Halobacteriaceae	0	0	0	0	0	0
Geodermatophilaceae	0	0	0	0	0	0
Desulfurococcaceae	0	0	0	0	0	0
Methanobacteriaceae	0	0	0	0	0	0
Thermoproteaceae	0	0	0	0	0	0
Nitrospiraceae	0	0	0	0	0	0

	Sample Number					
Family	15	14	13	12	11	10
Clostridiaceae	0.909944	0.903511	0.926123	0.640385	0.959065	0.967121
Lactobacillaceae	0.080004	0.083616	0.060747	0.343876	0.001708	0.001305
Ruminococcaceae	0.000282	0.000725	0.000525	0.000504	0.000809	0.000708
Sporolactobacillaceae	6.18E-05	1.08E-05	2.39E-05	1.61E-05	2.67E-05	0
Campylobacteraceae	0.001306	0.001515	0.001158	0.001077	0.024417	0.016571
Spirochaetaceae	8.82E-06	6.49E-05	0.000346	2.14E-05	5.34E-05	0.000111
Lachnospiraceae	0.000115	0.000368	0.000251	0.000241	0.000578	0.000541
Peptococcaceae	9.71E-05	7.57E-05	0.000107	0.00023	0.000231	0.000207
Veillonellaceae	0.000265	0.000184	0.000334	0.000289	0.000489	0.000247
Comamonadaceae	4.41E-05	0.000335	0.000179	0.000161	0.000169	0.000613
Oxalobacteraceae	0.00015	0.000216	0.000227	0.000552	0.000196	0.000183
Anaerolinaceae	0	8.65E-05	3.58E-05	0	4.45E-05	2.39E-05
Rhodospirillaceae	0.000406	0.000368	0.000668	0.000675	0.000498	0.000398
Burkholderiaceae	0.000106	9.74E-05	0.000322	0.000434	0.000151	0.000215
Flavobacteriaceae	0.000106	0.000314	5.97E-05	6.43E-05	0.000302	0.000191
Flexibacteraceae	6.18E-05	7.57E-05	5.97E-05	7.5E-05	8.89E-05	7.96E-05
Sphingobacteriaceae	0.000212	0.0004	0.000155	0.000204	0.000285	0.000382
Solibacteraceae	0	0	0	0	0	0
Sphingomonadaceae	0.000353	0.000411	0.000298	0.000273	0.000267	0.000939
Phyllobacteriaceae	9.71E-05	0.000141	0.000191	0.0003	0.000151	0.000199
Desulfobacteraceae	0.000185	0.000325	0.000119	0.000182	0.000196	0.000151
Bradyrhizobiaceae	6.18E-05	2.16E-05	0.000107	0.000209	0.000151	0.000103
Methylobacteriaceae	6.18E-05	0.000108	0.000275	0.000134	0.000151	9.55E-05
Xanthomonadaceae	7.06E-05	3.25E-05	7.16E-05	0.000129	7.12E-05	0.000127
Desulfovibrionaceae	0.000194	0.000281	0.000191	0.000295	0.000187	0.000231
Rhodocyclaceae	2.65E-05	0.000108	2.39E-05	2.68E-05	2.67E-05	2.39E-05
Chitinophagaceae	6.18E-05	5.41E-05	4.77E-05	0.000107	8.01E-05	0.000159
Caulobacteraceae	7.94E-05	0.000162	9.55E-05	0.000397	0.000151	0.000151
Paenibacillaceae	0.000141	0.000238	0.000203	0.000429	0.000213	0.000151
Thermotogaceae	3.53E-05	0.000238	0.000119	0.000107	0.00016	0.000119
Helicobacteraceae	1.76E-05	3.25E-05	8.36E-05	1.07E-05	4.45E-05	6.37E-05
Bacillaceae	0.000159	0.000173	0.00031	0.000595	0.00024	0.000135
Microbacteriaceae	0.000265	0.000216	0.000322	0.000257	0.000569	0.00078
Halanaerobiaceae	0.000759	0.000454	0.000537	0.000981	0.000774	0.000366
Planococcaceae	7.06E-05	6.49E-05	0.000155	0.000322	0.000133	9.55E-05
Thermoanaerobacteraceae	5.29E-05	4.33E-05	8.36E-05	9.11E-05	0.000151	0.000143
Propionibacteriaceae	4.41E-05	2.16E-05	4.77E-05	5.36E-05	7.12E-05	5.57E-05
Porphyromonadaceae	0	0	0	1.07E-05	0	0.000151
Enterobacteriaceae	0.000362	0.000465	0.000573	0.000413	0.000347	0.000541
Geobacteraceae	0.000141	0.000151	9.55E-05	0.000134	0.000365	0.000159
Mycobacteriaceae	7.94E-05	9.74E-05	7.16E-05	3.75E-05	0.000133	0.000167
Rhodobacteraceae	0.000176	0.000487	0.000107	0.000166	0.000169	0.000119
Actinomycetaceae	6.18E-05	0.000151	8.36E-05	0.000188	0.000205	0.000199
Hyphomicrobiaceae	0.000168	5.41E-05	9.55E-05	0.000139	8.89E-05	0.000127
Acetobacteraceae	7.06E-05	3.25E-05	8.36E-05	7.5E-05	6.23E-05	7.96E-05
Caldicellulosiruptoraceae	0	1.08E-05	0.000119	1.07E-05	5.34E-05	3.98E-05
Piscirickettsiaceae	4.41E-05	4.33E-05	4.77E-05	8.57E-05	0.000827	0.000629

Family	15	14	13	12	11	10
Xanthobacteraceae	9.71E-05	7.57E-05	0.000143	0.000129	0.000151	6.37E-05
Dethiosulfovibrionaceae	4.41E-05	1.08E-05	1.19E-05	2.68E-05	0	3.18E-05
Bdellovibrionaceae	0.000265	0.000151	0.000251	0.000348	9.78E-05	0.000207
Syntrophobacteraceae	0	2.16E-05	4.77E-05	3.75E-05	0.000294	2.39E-05
Brevibacteriaceae	0.000132	0.000173	0.000191	0.00015	8.01E-05	0.000175
Phormidiaceae	1.76E-05	1.08E-05	5.97E-05	9.65E-05	2.67E-05	3.18E-05
Exiguobacteraceae	6.18E-05	5.41E-05	0.00074	0.000225	7.12E-05	4.78E-05
Syntrophaceae	1.76E-05	2.16E-05	2.39E-05	4.82E-05	5.34E-05	9.55E-05
Rhizobiaceae	7.94E-05	7.57E-05	4.77E-05	6.43E-05	8.01E-05	0.000111
Glycomycetaceae	0.00015	0.000216	0.000155	0.000118	0.000231	0.000279
Synergistaceae	0	7.57E-05	0	0	1.78E-05	7.96E-06
Streptomycetaceae	7.06E-05	3.25E-05	3.58E-05	0.000102	0.000125	0.000111
Nocardioidaceae	7.06E-05	7.57E-05	7.16E-05	4.82E-05	0.000133	0.000103
Caldithrixaceae	2.65E-05	4.33E-05	5.97E-05	2.14E-05	0.000107	4.78E-05
Thermodesulfovibrionaceae	0	3.25E-05	1.19E-05	1.07E-05	7.12E-05	3.18E-05
Hydrogenophilaceae	1.76E-05	0	2.39E-05	2.14E-05	8.89E-06	1.59E-05
Pseudomonadaceae	0	0	4.77E-05	3.75E-05	2.67E-05	0.000111
Heliobacteriaceae	8.82E-05	0.000151	0.000119	0.000118	8.89E-05	9.55E-05
Acidobacteriaceae	2.65E-05	5.41E-05	4.77E-05	2.14E-05	4.45E-05	7.96E-06
Sphaerochaetaceae	0	0	0	0	2.67E-05	0
Desulfuromonadaceae	8.82E-05	0	4.77E-05	4.29E-05	7.12E-05	2.39E-05
Pelobacteraceae	3.53E-05	4.33E-05	2.39E-05	2.68E-05	6.23E-05	2.39E-05
Litoricolaceae	2.65E-05	7.57E-05	1.19E-05	5.36E-06	0.00032	0.000151
Mycoplasmataceae	4.41E-05	7.57E-05	8.36E-05	0.000268	0	2.39E-05
Thiotrichaceae	8.82E-06	5.41E-05	3.58E-05	4.29E-05	8.01E-05	8.76E-05
Deinococcaceae	4.41E-05	9.74E-05	4.77E-05	2.68E-05	8.01E-05	3.98E-05
Pseudonocardiaceae	7.06E-05	8.65E-05	3.58E-05	5.89E-05	5.34E-05	8.76E-05
Bacteroidaceae	0	4.33E-05	0	0	0.000391	0.000493
Streptococcaceae	1.76E-05	3.25E-05	8.36E-05	0.000295	2.67E-05	3.18E-05
Hyphomonadaceae	7.06E-05	7.57E-05	0.000155	6.97E-05	2.67E-05	3.98E-05
Desulfonatronumaceae	3.53E-05	4.33E-05	0	1.07E-05	0	2.39E-05
Yaniellaceae	0	0	1.19E-05	0	2.67E-05	4.78E-05
Desulfohalobiaceae	0	0	1.19E-05	0	2.67E-05	0
Syntrophomonadaceae	0	0	8.36E-05	3.22E-05	8.01E-05	6.37E-05
Chromatiaceae	4.41E-05	1.08E-05	1.19E-05	1.61E-05	2.67E-05	3.18E-05
Symbiobacteriaceae	1.76E-05	1.08E-05	4.77E-05	4.29E-05	0	7.96E-06
Eubacteriaceae	0	1.08E-05	1.19E-05	0	0	6.37E-05
Thermodesulfobacteriaceae	1.76E-05	5.41E-05	5.97E-05	3.75E-05	4.45E-05	4.78E-05
Peptostreptococcaceae	0	0	0	0	3.56E-05	1.59E-05
Sinobacteraceae	1.76E-05	1.08E-05	0	1.61E-05	2.67E-05	1.59E-05
Alcaligenaceae	3.53E-05	5.41E-05	2.39E-05	3.22E-05	8.89E-06	2.39E-05
Actinosynnemataceae	7.94E-05	2.16E-05	2.39E-05	8.04E-05	5.34E-05	6.37E-05
Amoebophilaceae	0	2.16E-05	0	2.14E-05	0.000116	5.57E-05
Polyangiaceae	4.41E-05	2.16E-05	3.58E-05	3.22E-05	8.89E-06	1.59E-05
Thermobaculaceae	0	0	4.77E-05	1.07E-05	8.89E-06	0
Deferribacteraceae	0	1.08E-05	1.19E-05	5.36E-06	8.89E-06	7.96E-06
Chlorobiaceae	8.82E-06	0	2.39E-05	4.82E-05	1.78E-05	3.98E-05
Nannocystaceae	7.06E-05	4.33E-05	3.58E-05	3.75E-05	9.78E-05	1.59E-05

Family	15	14	13	12	11	10
Enterococcaceae	8.82E-06	4.33E-05	7.16E-05	0.000247	0	7.96E-06
Rickettsiaceae	6.18E-05	3.25E-05	2.39E-05	5.36E-05	2.67E-05	8.76E-05
Moraxellaceae	5.29E-05	4.33E-05	3.58E-05	6.43E-05	4.45E-05	1.59E-05
Streptosporangiaceae	4.41E-05	2.16E-05	3.58E-05	1.61E-05	3.56E-05	0.000135
Coriobacteriaceae	0	2.16E-05	0	3.75E-05	5.34E-05	3.98E-05
Micromonosporaceae	1.76E-05	3.25E-05	2.39E-05	2.68E-05	3.56E-05	6.37E-05
Anaerobrancaceae	0	0	2.39E-05	0	3.56E-05	1.59E-05
Cystobacteraceae	8.82E-06	0	2.39E-05	5.36E-06	8.89E-06	0
Ectothiorhodospiraceae	2.65E-05	2.16E-05	1.19E-05	1.07E-05	3.56E-05	7.96E-06
Caldisericaceae	0	2.16E-05	0	0	0	7.96E-06
Leuconostocaceae	4.41E-05	0.000119	5.97E-05	0.000102	8.89E-06	1.59E-05
Contubernalisaceae	8.82E-06	1.08E-05	7.16E-05	1.07E-05	8.89E-06	3.98E-05
Neisseriaceae	8.82E-06	1.08E-05	0	1.61E-05	8.89E-06	0
Vibrionaceae	3.53E-05	4.33E-05	3.58E-05	6.97E-05	2.67E-05	3.18E-05
Actinopolysporaceae	0	1.08E-05	0	1.07E-05	8.89E-06	7.96E-06
Brocadiaceae	0	1.08E-05	0	5.36E-06	0	2.39E-05
Corynebacteriaceae	4.41E-05	1.08E-05	0	1.07E-05	5.34E-05	8.76E-05
Holophagaceae	0	0	0	0	8.89E-06	7.96E-06
Sulfobacillaceae	1.76E-05	4.33E-05	2.39E-05	4.82E-05	2.67E-05	7.96E-06
Kiloniellaceae	5.29E-05	0	3.58E-05	2.14E-05	8.89E-06	3.98E-05
Legionellaceae	1.76E-05	5.41E-05	2.39E-05	2.68E-05	1.78E-05	5.57E-05
Methylocystaceae	8.82E-06	0	0	5.36E-06	0	1.59E-05
Halomonadaceae	8.82E-06	0	0	5.36E-06	1.78E-05	0
Borreliaceae	0	0	3.58E-05	2.14E-05	1.78E-05	7.96E-06
Thermovenabulum	0	0	0	0	8.89E-06	7.96E-06
Bifidobacteriaceae	2.65E-05	2.16E-05	0	1.07E-05	6.23E-05	3.18E-05
Gallionellaceae	8.82E-06	1.08E-05	0	1.61E-05	0	0
Fusobacteriaceae	0	7.57E-05	0	5.36E-06	5.34E-05	3.98E-05
Psychromonadaceae	3.53E-05	4.33E-05	0	0	0.000125	7.16E-05
Pelagicoccaceae	0	0	0	0	3.56E-05	2.39E-05
Acholeplasmataceae	1.76E-05	1.08E-05	0	5.36E-06	8.89E-06	1.59E-05
Acidimicrobiaceae	2.65E-05	0	0	1.61E-05	1.78E-05	1.59E-05
Microviridae	0	3.25E-05	2.39E-05	3.75E-05	0	7.96E-06
Anaplasmataceae	2.65E-05	3.25E-05	2.39E-05	2.14E-05	8.89E-06	2.39E-05
Desulfobulbaceae	0	0	0	0	8.89E-06	0
Thermomonosporaceae	0	0	0	1.61E-05	1.78E-05	3.98E-05
Saprospiraceae	8.82E-06	0	0	1.61E-05	2.67E-05	3.18E-05
Aerococcaceae	0	3.25E-05	1.19E-05	0.000166	0	1.59E-05
Shewanellaceae	1.76E-05	1.08E-05	0	2.14E-05	1.78E-05	7.96E-06
Nostocaceae	0	2.16E-05	0	0	1.78E-05	0
Oceanospirillaceae	8.82E-06	0	1.19E-05	1.07E-05	8.89E-06	0
Euzebyaceae	8.82E-06	0	0	5.36E-06	8.89E-06	0
Beijerinckiaceae	0	0	0	0	0	7.96E-06
Turicibacteraceae	0	0	0	0	0	1.59E-05
Coxiellaceae	0	2.16E-05	2.39E-05	1.07E-05	0	0
Nocardiaceae	0	1.08E-05	1.19E-05	1.07E-05	1.78E-05	2.39E-05
Aminiphilaceae	8.82E-06	1.08E-05	0	0	8.89E-06	1.59E-05
Caldilineaceae	8.82E-06	0	1.19E-05	5.36E-06	8.89E-06	7.96E-06

Family	15	14	13	12	11	10
Alteromonadaceae	0	0	1.19E-05	1.07E-05	0	1.59E-05
Carnobacteriaceae	8.82E-06	0	4.77E-05	8.57E-05	1.78E-05	0
Entomoplasmataceae	2.65E-05	2.16E-05	2.39E-05	1.61E-05	0	2.39E-05
Methylophilaceae	8.82E-06	0	0	3.22E-05	0	7.96E-06
Micrococcaceae	1.76E-05	0	0	1.07E-05	1.78E-05	7.96E-06
Rhodothermaceae	0	0	0	5.36E-06	8.89E-06	0
Thermaceae	8.82E-06	0	1.19E-05	0	1.78E-05	7.96E-06
Erythrobacteraceae	2.65E-05	0	0	1.07E-05	8.89E-06	1.59E-05
Cellulomonadaceae	1.76E-05	0	0	0	8.89E-06	0
Pseudanabaenaceae	0	0	2.39E-05	0	0	0
Thermogemmatisporaceae	0	0	0	0	0	1.59E-05
Carboxydocellaceae	8.82E-06	1.08E-05	0	1.61E-05	0	0
Thermoactinomycetaceae	0	0	1.19E-05	2.68E-05	0	0
Rivulariaceae	0	0	0	0	0	0
Aurantimonadaceae	0	0	1.19E-05	1.61E-05	8.89E-06	0
Dehalococcoidaceae	0	0	0	5.36E-06	1.78E-05	2.39E-05
Ignavibacteriaceae	0	0	0	0	8.89E-06	1.59E-05
Rhabdochlamydiaceae	0	0	1.19E-05	1.07E-05	1.78E-05	7.96E-06
Brachyspiraceae	8.82E-06	0	0	5.36E-06	2.67E-05	1.59E-05
Brucellaceae	0	0	0	0	0	7.96E-06
Waddliaceae	0	0	0	5.36E-06	0	1.59E-05
Thermicanaceae	0	0	0	0	8.89E-06	0
Solirubrobacteraceae	0	0	0	5.36E-06	0	2.39E-05
Francisellaceae	0	1.08E-05	0	0	8.89E-06	0
Chrysiogenaceae	0	0	0	5.36E-06	8.89E-06	0
Conexibacteraceae	0	0	0	0	8.89E-06	7.96E-06
Odoribacteraceae	0	0	0	0	0	0
Leptotrichiaceae	0	0	0	0	1.78E-05	0
Opitutaceae	0	0	0	0	0	0
Acidithiobacillaceae	0	0	0	0	0	0
Nautiliaceae	0	2.16E-05	0	0	0	0
Erysipelotrichaceae	8.82E-06	0	0	0	0	0
Gemellaceae	0	0	0	1.07E-05	0	7.96E-06
Myxococcaceae	0	0	0	0	0	0
Haliangiaceae	8.82E-06	0	0	5.36E-06	0	0
Pseudoalteromonadaceae	8.82E-06	0	0	0	0	0
Idiomarinaceae	0	0	0	0	0	7.96E-06
Chroococcaceae	0	0	0	0	0	0
Methylacidiphilaceae	0	0	0	0	0	1.59E-05
Elusimicrobiaceae	0	0	0	0	0	0
Kineosporiaceae	0	0	0	0	0	0
Microcystaceae	0	1.08E-05	0	0	0	7.96E-06
Rikenellaceae	0	0	0	0	0	0
Verrucomicrobiaceae	0	0	0	0	0	0
Prevotellaceae	0	0	0	0	0	0
Halobacteroidaceae	0	0	1.19E-05	5.36E-06	0	0
Dietziaceae	0	0	0	0	0	7.96E-06
Listeriaceae	0	0	0	0	0	0

Family	15	14	13	12	11	10
Flammeovirgaceae	0	0	0	0	0	1.59E-05
Isosphaeraceae	0	0	0	5.36E-06	0	0
Kouleothrixaceae	0	0	0	0	0	0
Gemmatimonadaceae	0	0	0	0	0	0
Tsukamurellaceae	0	0	0	0	0	0
Dehalobacteriaceae	0	0	0	0	0	0
Thiohalorhabdaceae	0	0	0	0	0	0
Gordoniaceae	0	0	0	0	0	7.96E-06
Leptospiraceae	0	0	0	0	0	0
Dermabacteraceae	0	0	0	0	0	0
Intrasporangiaceae	0	0	0	0	0	0
Saccharospirillaceae	0	0	0	0	0	0
Koribacteraceae	0	0	0	0	0	0
Puniceicoccaceae	0	1.08E-05	0	5.36E-06	0	0
Aeromonadaceae	0	0	0	0	0	0
Promicromonosporaceae	0	0	1.19E-05	0	0	0
Moritellaceae	0	0	0	0	0	0
Dermacoccaceae	0	0	1.19E-05	0	0	0
Methanocorpusculaceae	0	0	0	0	0	0
Patulibacteraceae	0	0	0	0	0	0
Methanosarcinaceae	0	0	0	0	0	0
Sanguibacteraceae	0	0	0	0	0	0
Alcanivoracaceae	0	0	0	0	0	0
Archaeoglobaceae	0	0	0	0	0	0
Bogoriellaceae	0	0	0	0	8.89E-06	0
Chthonomonadaceae	0	0	0	0	0	0
Nitrospinaceae	0	0	0	0	0	0
Catenulisporaceae	0	0	0	0	0	0
Nocardiopsaceae	0	0	0	0	0	0
Roseiflexaceae	0	0	0	0	0	0
Anaeroplasmataceae	0	0	0	0	0	0
Oscillochloridaceae	0	0	0	0	0	0
Chloroflexaceae	0	0	0	0	0	0
Sporichthyaceae	0	0	0	0	0	0
Armatimonadaceae	0	0	0	0	0	0
Fibrobacteraceae	0	0	0	0	0	0
Sulfolobaceae	0	0	0	0	0	0
Coprobacillaceae	0	0	0	0	0	0
Desulfomicrobiaceae	0	0	0	0	0	0
Cyanobacteriaceae	0	0	0	0	0	0
Halobacteriaceae	0	0	0	0	0	0
Geodermatophilaceae	0	0	0	0	0	0
Desulfurococcaceae	0	0	0	0	0	0
Methanobacteriaceae	0	1.08E-05	0	0	0	0
Thermoproteaceae	0	0	0	0	8.89E-06	0
Nitrospiraceae	0	0	0	0	0	0

	Sample Number					
Family	9	8	7	6	5	4
Clostridiaceae	0.967464	0.270761	0.774186	0.739404	0.953725	0.668189
Lactobacillaceae	0.001284	0.002626	0.004264	0.003422	0.001849	0.00339
Ruminococcaceae	0.000517	0.67136	0.129899	0.084813	0.001496	0.289283
Sporolactobacillaceae	2.21E-05	2.79E-05	0.000125	6.77E-05	0	0
Campylobacteraceae	0.014386	0.007795	0.04695	0.036902	0.025279	0.004211
Spirochaetaceae	5.9E-05	5.03E-05	0.001513	0.000764	0.000122	0.000107
Lachnospiraceae	0.000709	0.00756	0.010049	0.090071	0.000517	0.004903
Peptococcaceae	0.000295	0.001827	0.000895	0.000735	0.000313	0.001427
Veillonellaceae	0.00034	0.000481	0.010885	0.00894	0.000612	0.002469
Comamonadaceae	0.000354	0.000609	0.000142	0.000155	0.000286	0.000442
Oxalobacteraceae	0.000635	0.001257	0.0001	0.000164	0.000585	0.001577
Anaerolinaceae	4.43E-05	5.59E-05	0.000125	0.000106	6.8E-05	5E-05
Rhodospirillaceae	0.000502	0.000631	0.000385	0.000599	0.000965	0.000671
Burkholderiaceae	0.000531	0.001324	0.000134	0.000193	0.000422	0.001327
Flavobacteriaceae	0.000221	0.000307	0.000226	0.000261	0.000218	0.000264
Flexibacteraceae	0.000103	0.001285	0.00066	0.000493	5.44E-05	0.000963
Sphingobacteriaceae	0.000214	0.0009	0.000276	0.000367	0.000367	0.000471
Solibacteraceae	7.38E-06	0	0.000125	9.67E-05	5.44E-05	1.43E-05
Sphingomonadaceae	0.000229	0.000291	0.000159	0.000232	0.000476	0.00035
Phyllobacteriaceae	0.000221	0.000799	0.000117	9.67E-05	0.000394	0.000557
Desulfobacteraceae	0.000177	0.00019	0.00015	0.000348	0.00034	0.000378
Bradyrhizobiaceae	0.00028	0.000419	5.02E-05	0.000126	0.000598	0.0005
Methylobacteriaceae	8.12E-05	0.000129	0.000176	0.000271	0.000435	6.42E-05
Xanthomonadaceae	0.000266	0.000726	8.36E-05	0.000116	6.8E-05	0.000435
Desulfovibrionaceae	0.000332	0.000436	0.000209	0.00028	0.000177	0.000321
Rhodocyclaceae	6.64E-05	0.000319	5.02E-05	2.9E-05	0	0.000143
Chitinophagaceae	6.64E-05	0.000106	0.003043	0.000754	0.000462	0.000128
Caulobacteraceae	0.000332	0.001045	0.000159	0.000232	0.000109	0.000385
Paenibacillaceae	0.000221	0.000335	0.003879	0.00288	0.000231	0.000492
Thermotogaceae	0.000177	0.001928	0.000451	0.014643	0.000326	0.000871
Helicobacteraceae	5.17E-05	2.24E-05	8.36E-06	6.77E-05	4.08E-05	5E-05
Bacillaceae	0.000118	0.000503	0.000184	0.000232	0.000136	0.000557
Microbacteriaceae	0.000376	0.000402	0.000895	0.001073	0.000422	0.002655
Halanaerobiaceae	0.000354	0.00062	0.000326	0.000464	0.000313	0.00035
Planococcaceae	7.38E-05	0.000335	0.000134	0.000164	0.00019	0.000221
Thermoanaerobacteraceae	0.000103	0.004096	0.000201	0.000348	9.52E-05	0.000343
Propionibacteriaceae	8.86E-05	2.79E-05	5.02E-05	7.73E-05	8.16E-05	0.000228
Porphyromonadaceae	6.64E-05	1.12E-05	2.51E-05	9.67E-06	0	2.14E-05

Family	9	8	7	6	5	4
Enterobacteriaceae	0.00093	0.000564	0.000293	0.000203	0.000408	0.000357
Geobacteraceae	0.000177	0.000201	0.000159	0.000193	0.000544	0.000207
Mycobacteriaceae	0.000214	0.00019	9.2E-05	0.000145	5.44E-05	0.000121
Rhodobacteraceae	0.000155	0.000184	7.52E-05	0.000232	0.000435	0.000143
Actinomycetaceae	0.000295	0.000788	0.000493	0.000561	0.000354	0.000571
Hyphomicrobiaceae	0.000118	6.15E-05	0.000109	0.000155	0.000122	7.85E-05
Acetobacteraceae	0.00014	0.000173	9.2E-05	6.77E-05	9.52E-05	9.28E-05
Caldicellulosiruptoraceae	2.95E-05	0.002034	0.00015	9.67E-05	5.44E-05	0.000471
Piscirickettsiaceae	0.000487	7.26E-05	0.000953	0.000657	0.000925	3.57E-05
Xanthobacteraceae	0.000111	0.000196	0.000184	0.000184	0.000258	0.000186
Dethiosulfovibrionaceae	5.9E-05	0.001822	0.000125	4.83E-05	5.44E-05	0.001399
Bdellovibrionaceae	0.000273	0.000145	0.000109	0.000155	8.16E-05	0.000114
Syntrophobacteraceae	2.21E-05	2.79E-05	5.02E-05	6.77E-05	4.08E-05	1.43E-05
Brevibacteriaceae	0.000199	0.000738	0.0001	0.000232	9.52E-05	0.000221
Phormidiaceae	6.64E-05	0.000134	2.51E-05	6.77E-05	0	0.000207
Exiguobacteraceae	6.64E-05	0.000112	5.85E-05	4.83E-05	0	6.42E-05
Syntrophaceae	0.000103	0.000184	8.36E-05	0.000145	6.8E-05	5E-05
Rhizobiaceae	2.95E-05	6.71E-05	7.52E-05	0.000116	0.000204	0.000193
Glycomycetaceae	0.000185	0.000279	0.000109	0.000193	0.00015	0.0002
Synergistaceae	7.38E-06	2.24E-05	0	0.000203	1.36E-05	1.43E-05
Streptomycetaceae	0.00017	0.000402	0.000142	0.0003	0.000136	0.000457
Nocardioidaceae	0.000118	0.000196	0.000109	9.67E-05	0.000177	0.000164
Caldithrixaceae	0.000111	0.000156	7.52E-05	0.000174	8.16E-05	3.57E-05
Thermodesulfovibrionaceae	0.000162	0.000319	3.34E-05	3.87E-05	2.72E-05	3.57E-05
Hydrogenophilaceae	2.95E-05	4.47E-05	8.36E-06	1.93E-05	0	0.000136
Pseudomonadaceae	5.9E-05	4.47E-05	6.69E-05	5.8E-05	0.000272	0.000128
Heliobacteriaceae	8.12E-05	0.00043	0.000125	6.77E-05	0.000136	0.000171
Acidobacteriaceae	8.12E-05	0.000302	5.02E-05	0.000155	8.16E-05	5.71E-05
Sphaerochaetaceae	7.38E-05	1.68E-05	8.36E-06	9.67E-06	0	7.14E-06
Desulfuromonadaceae	0.000103	0.000145	0.000142	0.000193	9.52E-05	0.00015
Pelobacteraceae	3.69E-05	6.15E-05	9.2E-05	6.77E-05	0.000136	5E-05
Litoricolaceae	0.000192	0.000235	0.000268	0.000338	0.00053	0.000107
Mycoplasmataceae	2.95E-05	5.59E-05	0.000134	7.73E-05	2.72E-05	0.000193
Thiotrichaceae	5.17E-05	0.000682	0.00015	0.000174	5.44E-05	0.000164
Deinococcaceae	0.000103	0.000631	8.36E-06	9.67E-06	8.16E-05	0.000307
Pseudonocardiaceae	0.000103	0.00019	6.69E-05	0.000116	0.000109	9.99E-05
Bacteroidaceae	7.38E-06	1.12E-05	0	1.93E-05	0	7.14E-06
Streptococcaceae	2.95E-05	3.35E-05	1.67E-05	1.93E-05	1.36E-05	2.85E-05
Hyphomonadaceae	3.69E-05	3.91E-05	0.0001	7.73E-05	4.08E-05	7.85E-05

Family	9	8	7	6	5	4
Desulfonatronumaceae	2.21E-05	2.24E-05	4.18E-05	1.93E-05	0	5.71E-05
Yaniellaceae	4.43E-05	0.000156	0.000368	0.000184	1.36E-05	0.0003
Desulfohalobiaceae	1.48E-05	2.79E-05	5.02E-05	5.8E-05	0	0.000164
Syntrophomonadaceae	0.000103	0.00019	0.000242	0.0003	2.72E-05	8.56E-05
Chromatiaceae	4.43E-05	0.000134	5.85E-05	7.73E-05	2.72E-05	9.99E-05
Symbiobacteriaceae	4.43E-05	0.000218	0.000251	0.000164	2.72E-05	3.57E-05
Eubacteriaceae	9.6E-05	1.12E-05	8.36E-06	1.93E-05	0	2.14E-05
Thermodesulfobacteriaceae	6.64E-05	0.000391	0.000109	5.8E-05	4.08E-05	0.000178
Peptostreptococcaceae	0.000413	0.00076	2.51E-05	1.93E-05	1.36E-05	3.57E-05
Sinobacteraceae	3.69E-05	0.000324	0.000134	9.67E-05	1.36E-05	0.000114
Alcaligenaceae	7.38E-05	3.91E-05	0	1.93E-05	2.72E-05	7.14E-05
Actinosynnemataceae	0.000125	0.000123	8.36E-05	7.73E-05	8.16E-05	0.000128
Amoebophilaceae	4.43E-05	8.38E-05	0.000109	8.7E-05	4.08E-05	4.28E-05
Polyangiaceae	8.86E-05	7.82E-05	3.34E-05	4.83E-05	2.72E-05	2.85E-05
Thermobaculaceae	1.48E-05	3.35E-05	1.67E-05	1.93E-05	0	7.14E-06
Deferribacteraceae	7.38E-06	3.35E-05	0	0	0	0
Chlorobiaceae	7.38E-06	1.68E-05	0.000184	0.000271	2.72E-05	9.99E-05
Nannocystaceae	4.43E-05	3.91E-05	0.00015	9.67E-05	5.44E-05	7.14E-05
Enterococcaceae	2.95E-05	3.91E-05	1.67E-05	1.93E-05	0	2.14E-05
Rickettsiaceae	2.21E-05	5.03E-05	9.2E-05	5.8E-05	6.8E-05	0.000114
Moraxellaceae	2.21E-05	0.000145	4.18E-05	9.67E-05	9.52E-05	7.14E-05
Streptosporangiaceae	5.9E-05	0.00014	4.18E-05	2.9E-05	9.52E-05	5.71E-05
Coriobacteriaceae	2.95E-05	6.71E-05	5.02E-05	2.9E-05	5.44E-05	2.85E-05
Micromonosporaceae	1.48E-05	0.000106	8.36E-05	0.000116	1.36E-05	3.57E-05
Anaerobrancaceae	2.95E-05	0.000402	0.0001	0.000126	4.08E-05	0.000178
Cystobacteraceae	5.17E-05	5.59E-05	3.34E-05	4.83E-05	0	3.57E-05
Ectothiorhodospiraceae	1.48E-05	0.000246	8.36E-06	3.87E-05	1.36E-05	0.000121
Caldisericaceae	0	8.38E-05	0	0	0	5E-05
Leuconostocaceae	4.43E-05	3.91E-05	2.51E-05	9.67E-06	2.72E-05	5E-05
Contubernalisaceae	5.17E-05	6.15E-05	0.000109	0.000203	1.36E-05	7.14E-05
Neisseriaceae	2.95E-05	1.12E-05	0	1.93E-05	1.36E-05	2.14E-05
Vibrionaceae	8.86E-05	5.59E-06	4.18E-05	1.93E-05	5.44E-05	5E-05
Actinopolysporaceae	7.38E-05	8.94E-05	2.51E-05	1.93E-05	0	2.14E-05
Brocadiaceae	2.21E-05	4.47E-05	2.51E-05	1.93E-05	0	5.71E-05
Corynebacteriaceae	2.95E-05	6.71E-05	0	2.9E-05	2.72E-05	2.85E-05
Holophagaceae	2.95E-05	1.68E-05	0	0	0	4.28E-05
Sulfobacillaceae	2.21E-05	1.68E-05	1.67E-05	9.67E-06	0	0.000107
Kiloniellaceae	0.000103	1.68E-05	2.51E-05	1.93E-05	1.36E-05	2.85E-05
Legionellaceae	2.95E-05	7.82E-05	6.69E-05	4.83E-05	1.36E-05	2.85E-05

Family	9	8	7	6	5	4
Methylocystaceae	7.38E-06	4.47E-05	0	0	0	2.14E-05
Halomonadaceae	1.48E-05	0.000106	0.000134	7.73E-05	4.08E-05	2.14E-05
Borreliaceae	3.69E-05	5.03E-05	5.02E-05	9.67E-06	2.72E-05	2.14E-05
Thermovenabulum	7.38E-06	3.35E-05	0	9.67E-06	1.36E-05	0
Bifidobacteriaceae	5.17E-05	5.59E-05	3.34E-05	2.9E-05	4.08E-05	3.57E-05
Gallionellaceae	0	0	0	0	0	0
Fusobacteriaceae	3.69E-05	1.68E-05	5.02E-05	4.83E-05	0	2.85E-05
Psychromonadaceae	8.12E-05	7.26E-05	2.51E-05	0.000126	0.000109	4.28E-05
Pelagicoccaceae	3.69E-05	7.26E-05	2.51E-05	2.9E-05	4.08E-05	2.14E-05
Acholeplasmataceae	8.86E-05	2.24E-05	3.34E-05	0	5.44E-05	4.28E-05
Acidimicrobiaceae	2.21E-05	8.94E-05	8.36E-06	1.93E-05	1.36E-05	5.71E-05
Microviridae	1.48E-05	3.35E-05	1.67E-05	2.9E-05	0	7.14E-06
Anaplasmataceae	7.38E-06	5.59E-06	2.51E-05	1.93E-05	2.72E-05	2.14E-05
Desulfobulbaceae	1.48E-05	0	0	6.77E-05	0	3.57E-05
Thermomonosporaceae	4.43E-05	7.82E-05	2.51E-05	1.93E-05	0.00015	2.14E-05
Saprospiraceae	1.48E-05	1.68E-05	2.51E-05	0	6.8E-05	7.14E-06
Aerococcaceae	7.38E-06	2.24E-05	8.36E-06	1.93E-05	1.36E-05	3.57E-05
Shewanellaceae	3.69E-05	1.12E-05	0	9.67E-06	4.08E-05	7.14E-06
Nostocaceae	2.21E-05	1.12E-05	1.67E-05	3.87E-05	0	7.14E-06
Oceanospirillaceae	2.21E-05	1.12E-05	0	0	1.36E-05	1.43E-05
Euzebyaceae	2.21E-05	5.59E-06	8.36E-06	0	2.72E-05	0
Beijerinckiaceae	7.38E-06	0	0	0	1.36E-05	3.57E-05
Turicibacteraceae	0.000103	0.000274	0	0	0	0
Coxiellaceae	7.38E-06	1.12E-05	8.36E-06	0.000222	0	2.14E-05
Nocardiaceae	2.21E-05	5.03E-05	2.51E-05	9.67E-06	0	0.000107
Aminiphilaceae	2.95E-05	2.79E-05	0	9.67E-06	0	7.14E-06
Caldilineaceae	7.38E-06	2.79E-05	1.67E-05	9.67E-06	1.36E-05	7.14E-06
Alteromonadaceae	2.95E-05	2.79E-05	0	3.87E-05	1.36E-05	1.43E-05
Carnobacteriaceae	7.38E-06	0	5.02E-05	5.8E-05	0	1.43E-05
Entomoplasmataceae	1.48E-05	3.35E-05	2.51E-05	4.83E-05	0	3.57E-05
Methylophilaceae	0	1.12E-05	0	9.67E-06	0	7.14E-06
Micrococcaceae	7.38E-06	1.12E-05	3.34E-05	3.87E-05	5.44E-05	4.28E-05
Rhodothermaceae	7.38E-06	6.71E-05	1.67E-05	1.93E-05	0	5E-05
Thermaceae	1.48E-05	2.24E-05	8.36E-06	0	0	0
Erythrobacteraceae	2.21E-05	3.91E-05	8.36E-06	9.67E-06	0	7.14E-06
Cellulomonadaceae	2.21E-05	0.000112	8.36E-06	2.9E-05	5.44E-05	0
Pseudanabaenaceae	0	1.68E-05	0	0	0	2.85E-05
Thermogemmatisporaceae	1.48E-05	5.59E-06	8.36E-06	2.9E-05	0	7.14E-06
Carboxydocellaceae	1.48E-05	1.12E-05	0	9.67E-06	1.36E-05	1.43E-05

Family	9	8	7	6	5	4
Thermoactinomycetaceae	0	5.59E-06	3.34E-05	2.9E-05	0	0
Rivulariaceae	0	0	0	0	0	0
Aurantimonadaceae	0	1.12E-05	8.36E-06	0	0	1.43E-05
Dehalococcoidaceae	0	5.59E-06	0	0	0	4.28E-05
Ignavibacteriaceae	2.21E-05	0	0	0	0	1.43E-05
Rhabdochlamydiaceae	1.48E-05	5.59E-06	1.67E-05	0	0	2.14E-05
Brachyspiraceae	0	5.59E-06	8.36E-06	9.67E-06	0	7.14E-06
Brucellaceae	0	0	0	9.67E-06	0	0
Waddliaceae	7.38E-06	0	0	9.67E-06	0	0
Thermicanaceae	0	1.68E-05	1.67E-05	9.67E-06	0	2.85E-05
Solirubrobacteraceae	1.48E-05	1.12E-05	0	1.93E-05	0	1.43E-05
Francisellaceae	0	0	0	0	0	0
Chrysiogenaceae	7.38E-06	1.68E-05	8.36E-06	2.9E-05	1.36E-05	0
Conexibacteraceae	1.48E-05	2.79E-05	8.36E-06	0	5.44E-05	7.14E-06
Odoribacteraceae	0	0	0	0	0	0
Leptotrichiaceae	0	1.12E-05	8.36E-06	9.67E-06	0	7.14E-06
Opitutaceae	0	5.59E-06	0	0	0	0
Acidithiobacillaceae	7.38E-06	0	0	0	0	0
Nautiliaceae	0	0	0	0	0	7.14E-06
Erysipelotrichaceae	1.48E-05	1.12E-05	0	0	0	0
Gemellaceae	0	5.59E-06	8.36E-06	3.87E-05	0	0
Myxococcaceae	0	5.59E-06	0	0	0	0
Haliangiaceae	0	5.59E-06	0	0	0	0
Pseudoalteromonadaceae	0	0	0	9.67E-06	0	7.14E-06
Idiomarinaceae	0	5.59E-06	0	9.67E-06	1.36E-05	0
Chroococcaceae	1.48E-05	1.68E-05	0	9.67E-06	0	0
Methylacidiphilaceae	0	5.59E-06	0	0	0	1.43E-05
Elusimicrobiaceae	0	0	0	0	0	0
Kineosporiaceae	0	0	0	0	0	7.14E-06
Microcystaceae	0	5.59E-06	0	0	0	0
Rikenellaceae	0	0	0	0	0	0
Verrucomicrobiaceae	0	5.59E-06	0	9.67E-06	0	0
Prevotellaceae	0	0	0	0	0	0
Halobacteroidaceae	0	1.12E-05	3.34E-05	0	0	0
Dietziaceae	0	2.24E-05	0	9.67E-06	1.36E-05	0
Listeriaceae	0	0	0	0	1.36E-05	7.14E-06
Flammeovirgaceae	7.38E-06	0	8.36E-06	0	0	0
Isosphaeraceae	0	0	0	0	2.72E-05	0
Kouleothrixaceae	0	5.59E-06	0	0	0	0

Family	9	8	7	6	5	4
Gemmatimonadaceae	0	0	0	0	0	0
Tsukamurellaceae	7.38E-06	0	0	0	0	7.14E-06
Dehalobacteriaceae	0	0	0	0	0	0
Thiohalorhabdaceae	0	5.59E-06	0	0	0	0
Gordoniaceae	0	0	8.36E-06	0	1.36E-05	0
Leptospiraceae	0	1.12E-05	0	0	0	0
Dermabacteraceae	0	0	0	0	0	0
Intrasporangiaceae	0	0	0	9.67E-06	0	7.14E-06
Saccharospirillaceae	0	0	0	0	0	0
Koribacteraceae	0	0	0	0	0	0
Puniceicoccaceae	0	5.59E-06	0	0	0	0
Aeromonadaceae	7.38E-06	0	0	0	0	0
Promicromonosporaceae	0	0	0	9.67E-06	0	0
Moritellaceae	0	0	0	0	0	0
Dermacoccaceae	0	0	0	0	0	0
Methanocorpusculaceae	0	0	0	0	0	0
Patulibacteraceae	0	5.59E-06	0	0	0	0
Methanosarcinaceae	0	0	0	0	0	0
Sanguibacteraceae	0	0	0	0	0	0
Alcanivoracaceae	0	0	8.36E-06	0	0	0
Archaeoglobaceae	0	5.59E-06	0	0	0	0
Bogoriellaceae	7.38E-06	0	0	0	0	0
Chthonomonadaceae	7.38E-06	0	0	0	0	0
Nitrospinaceae	0	5.59E-06	0	0	0	0
Catenulisporaceae	0	5.59E-06	0	0	0	0
Nocardiopsaceae	0	0	0	0	0	0
Roseiflexaceae	0	0	0	0	0	0
Anaeroplasmataceae	0	0	0	0	0	0
Oscillochloridaceae	0	0	0	0	0	0
Chloroflexaceae	0	0	0	0	0	0
Sporichthyaceae	0	0	0	0	0	0
Armatimonadaceae	0	0	0	0	0	0
Fibrobacteraceae	0	0	0	0	0	0
Sulfolobaceae	0	0	0	0	0	0
Coprobacillaceae	0	0	0	0	0	0
Desulfomicrobiaceae	0	0	0	0	0	0
Cyanobacteriaceae	0	0	0	0	0	0
Halobacteriaceae	0	0	0	0	0	0
Geodermatophilaceae	0	0	0	0	0	0

Family	9	8	7	6	5	4
Desulfurococcaceae	0	0	0	0	0	7.14E-06
Methanobacteriaceae	0	0	0	0	0	0
Thermoproteaceae	0	0	0	0	0	0
Nitrospiraceae	0	0	0	0	0	0

	Sample Number		
Family	3	2	1
Clostridiaceae	0.761856	0.049541	0.403276
Lactobacillaceae	0.107224	0.005416	0.001346
Ruminococcaceae	0.000684	0.015168	0.013091
Sporolactobacillaceae	5.01E-05	1.77E-05	0
Campylobacteraceae	0.001618	0.012407	0.001544
Spirochaetaceae	1.67E-05	0.042833	0.182724
Lachnospiraceae	0.000606	0.004035	0.000623
Peptococcaceae	0.000573	0.003982	0.003287
Veillonellaceae	0.003164	0.003575	0.057097
Comamonadaceae	0.008665	0.100798	0.002054
Oxalobacteraceae	0.025388	0.001381	0.000255
Anaerolinaceae	3.34E-05	0.006354	0.080346
Rhodospirillaceae	0.001585	0.008301	0.040336
Burkholderiaceae	0.015016	0.006407	0.000142
Flavobacteriaceae	0.000428	0.076957	0.00034
Flexibacteraceae	0.000117	0.05301	0.007608
Sphingobacteriaceae	0.000273	0.061222	0.000482
Solibacteraceae	0	0.002177	0.060837
Sphingomonadaceae	0.000651	0.037894	0.002026
Phyllobacteriaceae	0.010411	0.002071	0.00792
Desulfobacteraceae	0.000256	0.054762	0.001091
Bradyrhizobiaceae	0.01382	0.001805	9.92E-05
Methylobacteriaceae	0.000384	0.000655	0.045635
Xanthomonadaceae	0.012191	0.009115	0.000269
Desulfovibrionaceae	0.00218	0.035877	0.002635
Rhodocyclaceae	0.0002	0.059948	0.00034
Chitinophagaceae	0.002013	0.012177	0.000496
Caulobacteraceae	0.002842	0.027647	0.000326
Paenibacillaceae	0.000395	0.001204	0.000368
Thermotogaceae	0.0003	0.001451	0.000482
Helicobacteraceae	1.67E-05	0.042302	0.007466
Bacillaceae	0.000957	0.00377	0.004987

Family	3	2	1
Microbacteriaceae	0.001001	0.00092	0.000283
Halanaerobiaceae	0.000645	0.000195	0.000383
Planococcaceae	0.000161	0.00023	0.000765
Thermoanaerobacteraceae	0.000751	0.002389	0.00418
Propionibacteriaceae	0.003832	0.000407	0.000227
Porphyromonadaceae	5.56E-06	0.033417	2.83E-05
Enterobacteriaceae	0.000595	0.000726	0.00034
Geobacteraceae	0.000473	0.015752	0.00051
Mycobacteriaceae	0.000206	0.022053	0.001077
Rhodobacteraceae	0.000356	0.005292	0.001771
Actinomycetaceae	0.000484	0.000496	0.000255
Hyphomicrobiaceae	0.000678	0.006885	0.001941
Acetobacteraceae	0.000957	0.002	0.007183
Caldicellulosiruptoraceae	3.89E-05	0.000779	0.002805
Piscirickettsiaceae	0.000267	0.000265	2.83E-05
Xanthobacteraceae	0.000951	0.002974	0.003287
Dethiosulfovibrionaceae	6.12E-05	0.000885	0.001629
Bdellovibrionaceae	0.000295	0.000265	0.000595
Syntrophobacteraceae	8.34E-05	0.003239	0.008642
Brevibacteriaceae	0.000167	0.000248	0.000283
Phormidiaceae	0.002797	0.000124	4.25E-05
Exiguobacteraceae	0.000133	1.77E-05	2.83E-05
Syntrophaceae	4.45E-05	0.008531	0.000326
Rhizobiaceae	0.000373	0.002708	0.000893
Glycomycetaceae	0.0003	0.000389	7.08E-05
Synergistaceae	0	0.012283	0.000312
Streptomycetaceae	0.000217	0.000885	0.000354
Nocardioidaceae	0.000145	0.002195	0.000581
Caldithrixaceae	7.23E-05	0.003929	0.001573
Thermodesulfovibrionaceae	6.67E-05	0.006106	0.000765
Hydrogenophilaceae	7.79E-05	0.008425	7.08E-05
Pseudomonadaceae	0.000745	0.00023	9.92E-05
Heliobacteriaceae	0.0001	0.000796	0.000354
Acidobacteriaceae	0.000161	0.002106	0.000907
Sphaerochaetaceae	1.67E-05	0.008567	0.00085
Desulfuromonadaceae	0.000117	0.002389	0.000113
Pelobacteraceae	3.89E-05	0.004443	0.000227
Litoricolaceae	6.12E-05	0.001009	9.92E-05
Mycoplasmataceae	0.000122	0.001416	0.000128

Family	3	2	1
Thiotrichaceae	0.000334	0.000726	0.000227
Deinococcaceae	6.67E-05	0.000761	0.000241
Pseudonocardiaceae	0.0002	0.00069	0.000907
Bacteroidaceae	5.56E-06	0.006956	2.83E-05
Streptococcaceae	0.000534	0.000177	0
Hyphomonadaceae	8.34E-05	0.001186	0.001941
Desulfonatronumaceae	5.01E-05	0.007186	0.000156
Yaniellaceae	1.67E-05	5.31E-05	0
Desulfohalobiaceae	2.78E-05	0.001876	0.004505
Syntrophomonadaceae	0.00025	0.001788	0.001275
Chromatiaceae	0.000122	0.003115	0.000312
Symbiobacteriaceae	2.22E-05	0.000177	0.000113
Eubacteriaceae	6.12E-05	0.005699	0
Thermodesulfobacteriaceae	5.56E-05	0.00069	7.08E-05
Peptostreptococcaceae	0	0.00285	1.42E-05
Sinobacteraceae	7.79E-05	0.001097	0.000128
Alcaligenaceae	0.00015	0.002779	0.000425
Actinosynnemataceae	8.34E-05	0.000496	0.000383
Amoebophilaceae	6.67E-05	0.002389	0.000269
Polyangiaceae	0.000567	0.000354	0.000128
Thermobaculaceae	2.78E-05	0.00046	0.003329
Deferribacteraceae	1.11E-05	0.00069	0.003514
Chlorobiaceae	6.67E-05	0.000496	9.92E-05
Nannocystaceae	0.000133	0.000389	0.000156
Enterococcaceae	0.000156	5.31E-05	0.000128
Rickettsiaceae	0.000139	0.000566	0.000411
Moraxellaceae	0.000172	0.00023	8.5E-05
Streptosporangiaceae	6.67E-05	0.00069	9.92E-05
Coriobacteriaceae	7.79E-05	0.001451	0.000227
Micromonosporaceae	7.23E-05	0.000177	0.000198
Anaerobrancaceae	5.56E-06	0.000212	0.000213
Cystobacteraceae	0.000317	0.000743	0.000255
Ectothiorhodospiraceae	0.00015	0.000743	5.67E-05
Caldisericaceae	0	0.000938	0.000142
Leuconostocaceae	2.22E-05	0.000283	7.08E-05
Contubernalisaceae	2.22E-05	0.000106	0.000779
Neisseriaceae	0.00015	0.001823	5.67E-05
Vibrionaceae	4.45E-05	0.000265	2.83E-05
Actinopolysporaceae	1.67E-05	0.000673	1.42E-05

Family	3	2	1
Brocadiaceae	8.34E-05	0.001274	0.000113
Corynebacteriaceae	0.000295	7.08E-05	5.67E-05
Holophagaceae	5.56E-06	0.002974	0
Sulfobacillaceae	5.56E-05	0.000336	7.08E-05
Kiloniellaceae	0.0001	0.000319	0.000156
Legionellaceae	6.67E-05	0.000425	0
Methylocystaceae	0.000178	0.001628	1.42E-05
Halomonadaceae	5.56E-05	0.000124	5.67E-05
Borreliaceae	3.89E-05	0.001168	0.000298
Thermovenabulum	3.34E-05	0.002283	0.000255
Bifidobacteriaceae	0.000167	0.000283	0.000113
Gallionellaceae	5.56E-06	0.002814	2.83E-05
Fusobacteriaceae	2.78E-05	0.000584	0
Psychromonadaceae	0	7.08E-05	1.42E-05
Pelagicoccaceae	1.67E-05	0.000283	0.000496
Acholeplasmataceae	3.89E-05	0.000283	0.000113
Acidimicrobiaceae	2.78E-05	0.000195	0.000156
Microviridae	0.000111	8.85E-05	0
Anaplasmataceae	2.22E-05	0.00092	9.92E-05
Desulfobulbaceae	1.67E-05	0.000903	4.25E-05
Thermomonosporaceae	2.22E-05	0.000248	9.92E-05
Saprospiraceae	2.22E-05	0.000619	4.25E-05
Aerococcaceae	0.0001	0	4.25E-05
Shewanellaceae	6.67E-05	0.00046	1.42E-05
Nostocaceae	2.22E-05	0.001381	4.25E-05
Oceanospirillaceae	6.12E-05	3.54E-05	1.42E-05
Euzebyaceae	3.34E-05	0.001257	9.92E-05
Beijerinckiaceae	0.0003	0.000442	4.25E-05
Turicibacteraceae	0	0.000832	0
Coxiellaceae	7.23E-05	0.000496	0.000156
Nocardiaceae	5.01E-05	0.000159	2.83E-05
Aminiphilaceae	2.22E-05	0.001115	5.67E-05
Caldilineaceae	0	0.000814	0.000113
Alteromonadaceae	1.11E-05	0.000212	4.25E-05
Carnobacteriaceae	6.67E-05	8.85E-05	2.83E-05
Entomoplasmataceae	3.34E-05	3.54E-05	2.83E-05
Methylophilaceae	5.01E-05	0.000142	0
Micrococcaceae	2.78E-05	0.000106	7.08E-05
Rhodothermaceae	5.56E-06	0.000159	0.000383

Family	3	2	1
Thermaceae	1.67E-05	0.00046	2.83E-05
Erythrobacteraceae	1.11E-05	0.000248	0
Cellulomonadaceae	1.67E-05	1.77E-05	1.42E-05
Pseudanabaenaceae	0.0002	3.54E-05	0
Thermogemmatisporaceae	1.67E-05	0.000425	2.83E-05
Carboxydocellaceae	2.22E-05	8.85E-05	0.000283
Thermoactinomycetaceae	2.22E-05	0	0.000142
Rivulariaceae	0	7.08E-05	0
Aurantimonadaceae	8.34E-05	5.31E-05	5.67E-05
Dehalococcoidaceae	2.22E-05	0.000124	0
Ignavibacteriaceae	0	0.000442	0
Rhabdochlamydiaceae	5.56E-06	7.08E-05	0
Brachyspiraceae	1.11E-05	3.54E-05	9.92E-05
Brucellaceae	7.79E-05	3.54E-05	0
Waddliaceae	0	0.000673	0
Thermicanaceae	0	0.000212	5.67E-05
Solirubrobacteraceae	0	7.08E-05	0.000326
Francisellaceae	0	0.000619	4.25E-05
Chrysiogenaceae	1.67E-05	8.85E-05	7.08E-05
Conexibacteraceae	0	7.08E-05	4.25E-05
Odoribacteraceae	0	0.000673	0
Leptotrichiaceae	5.56E-06	0.000389	1.42E-05
Opitutaceae	0	7.08E-05	0
Acidithiobacillaceae	0	0.000496	0
Nautiliaceae	0	0.000425	1.42E-05
Erysipelotrichaceae	0	0.000283	1.42E-05
Gemellaceae	5.56E-06	1.77E-05	0
Myxococcaceae	0	5.31E-05	8.5E-05
Haliangiaceae	5.56E-06	0	7.08E-05
Pseudoalteromonadaceae	5.56E-06	0.000124	1.42E-05
Idiomarinaceae	5.56E-06	1.77E-05	1.42E-05
Chroococcaceae	1.11E-05	0	0
Methylacidiphilaceae	0	0.000124	4.25E-05
Elusimicrobiaceae	0	0.000372	0
Kineosporiaceae	5.56E-06	0.000124	0
Microcystaceae	5.56E-06	0.000159	2.83E-05
Rikenellaceae	0	0.000354	0
Verrucomicrobiaceae	1.11E-05	1.77E-05	0
Prevotellaceae	0	0.000177	0

Family	3	2	1
Halobacteroidaceae	5.56E-06	1.77E-05	0
Dietziaceae	1.11E-05	0	0
Listeriaceae	1.67E-05	0	1.42E-05
Flammeovirgaceae	1.11E-05	1.77E-05	1.42E-05
Isosphaeraceae	0	0.000159	2.83E-05
Kouleothrixaceae	0	0	0
Gemmatimonadaceae	0	0.000159	0
Tsukamurellaceae	5.56E-06	3.54E-05	0
Dehalobacteriaceae	0	0.000159	1.42E-05
Thiohalorhabdaceae	5.56E-06	7.08E-05	1.42E-05
Gordoniaceae	0	3.54E-05	0
Leptospiraceae	0	3.54E-05	0
Dermabacteraceae	0	0	0
Intrasporangiaceae	0	1.77E-05	2.83E-05
Saccharospirillaceae	1.11E-05	0	0
Koribacteraceae	0	0	4.25E-05
Puniceicoccaceae	0	1.77E-05	0
Aeromonadaceae	5.56E-06	0	0
Promicromonosporaceae	0	0	1.42E-05
Moritellaceae	1.11E-05	0	0
Dermacoccaceae	0	0	0
Methanocorpusculaceae	0	0	1.42E-05
Patulibacteraceae	0	0	1.42E-05
Methanosarcinaceae	0	1.77E-05	0
Sanguibacteraceae	0	3.54E-05	0
Alcanivoracaceae	0	1.77E-05	0
Archaeoglobaceae	0	0	0
Bogoriellaceae	0	0	0
Chthonomonadaceae	0	0	1.42E-05
Nitrospinaceae	0	0	0
Catenulisporaceae	0	1.77E-05	0
Nocardiopsaceae	0	0	0
Roseiflexaceae	0	0	0
Anaeroplasmataceae	0	0	0
Oscillochloridaceae	0	0	0
Chloroflexaceae	0	0	0
Sporichthyaceae	0	0	0
Armatimonadaceae	0	0	2.83E-05
Fibrobacteraceae	0	1.77E-05	0

Family	3	2	1
Sulfolobaceae	0	1.77E-05	0
Coprobacillaceae	0	1.77E-05	0
Desulfomicrobiaceae	0	0	0
Cyanobacteriaceae	0	0	0
Halobacteriaceae	0	0	0
Geodermatophilaceae	0	0	0
Desulfurococcaceae	0	0	0
Methanobacteriaceae	0	0	0
Thermoproteaceae	0	0	0
Nitrospiraceae	0	0	0

Aerotolerance Study (Chapter 4)

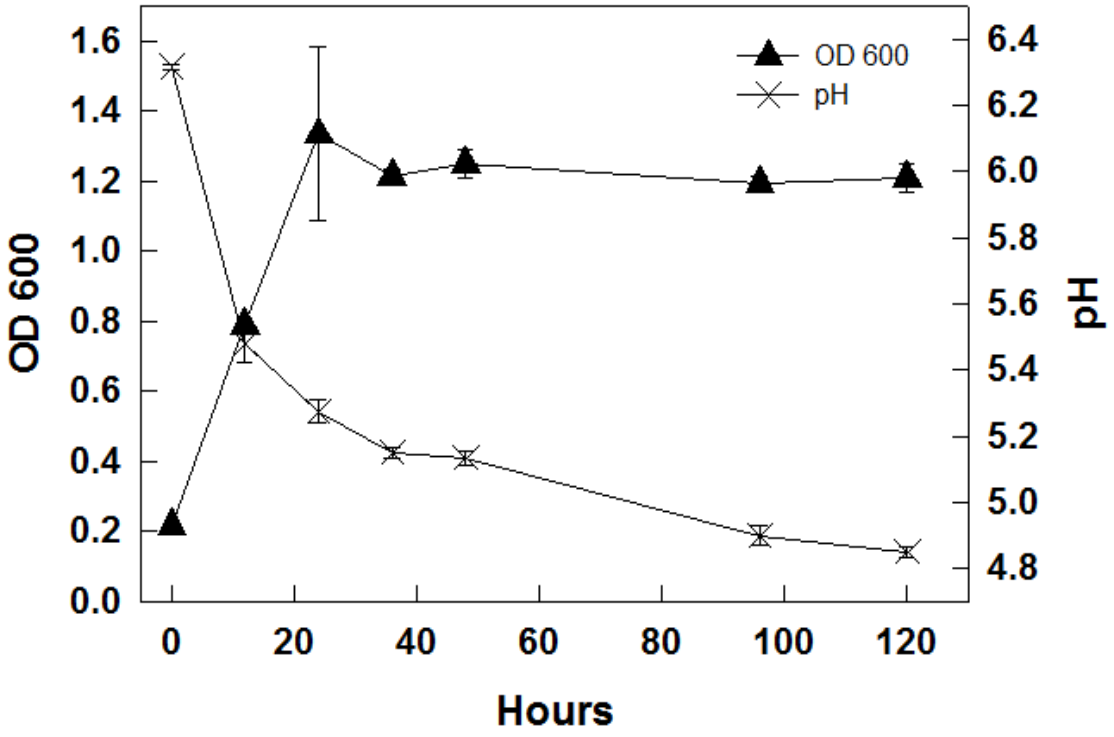

Figure A-3: *Clostridium* C10 growth 100mL in 250mL Erlenmeyer flask and 30 g/L glucose; 80 rpm agitation; foam topper. Error bars represent standard deviation between replicates.

Electrode Study (Chapter 5)

	Butanol g/L	Biomass g/L	α	dx/dt	α * dx/dt	g/Lh Butanol	β	q Butanol
3% Glucose Control	4.03	0.94	7.30	0.02	0.11	0.07	0.07	0.18
3% Glucose Electrode	6.88	1.19	5.76	0.09	0.51	0.13	0.11	0.62
6% Glucose Control	3.88	0.85	7.52	0.02	0.16	0.08	0.09	0.25
6% Glucose Electrode	6.37	1.31	4.87	0.06	0.29	0.12	0.09	0.38
3% Xylose Control	2.19	0.72	3.04	0.02	0.05	0.02	0.03	0.08
3% Xylose Electrode	4.98	1.41	3.52	0.07	0.24	0.15	0.10	0.35
6% Xylose Control	3.06	0.73	4.22	0.02	0.07	0.05	0.07	0.14
6% Xylose Electrode	5.78	1.49	3.88	0.08	0.32	0.20	0.13	0.46

Table A-7: Specific butanol production calculations for electrode study using the Luedeking-Piret equation for mixed growth associated products.

OD600	3% Xylose Control	SD	Electrode	6% Xylose Control	SD	Electrode	3% Glucose Control	SD	Electrode	6% Glucose Control	SD	Electrode
0	0.17	0.00	0.17	0.15	0.00	0.21	0.17	0.01	0.17	0.18	0.01	0.19
12	0.29	0.03	0.32	0.64	0.02	0.58	0.56	0.02	0.73	0.60	0.02	0.85
24	0.69	0.10	1.01	1.26	0.05	2.82	1.11	0.01	2.40	1.13	0.08	2.92
36	1.50	0.04	3.14	1.61	0.04	4.46	1.24	0.05	3.58	1.34	0.04	3.30
48	1.58	0.04	3.60	1.68	0.04	4.61	1.30	0.02	3.76	1.48	0.13	3.31
72	1.62	0.03	4.02	1.82	0.01	4.49	1.71	0.06	3.65	1.92	0.08	3.20
96	1.92	0.01	3.99	2.06	0.08	4.39	1.86	0.16	3.74	2.15	0.07	3.41
120	2.04	0.01	4.00	2.37	0.04	4.21	2.11	0.01	3.70	2.05	0.07	3.39

pH	3% Xylose Control	SD	Electrode	6% Xylose Control	SD	Electrode	3% Glucose Control	SD	Electrode	6% Glucose Control	SD	Electrode
0	5.80	0.01	5.81	5.73	0.01	5.71	5.85	0.01	5.86	6.08	0.01	6.06
12	5.54	0.06	5.50	5.43	0.04	5.16	5.47	0.01	5.59	5.36	0.03	5.36
24	5.60	0.02	5.30	5.78	0.04	5.23	5.76	0.05	5.94	5.84	0.04	6.02
36	5.61	0.04	5.70	5.77	0.02	5.45	5.77	0.01	5.64	5.95	0.03	5.95
48	5.58	0.03	5.56	5.68	0.01	5.16	5.75	0.09	5.43	6.02	0.01	6.00
72	5.50	0.02	5.40	5.49	0.04	5.20	5.52	0.01	5.32	5.36	0.06	5.88
96	5.47	0.01	5.34	5.21	0.01	5.20	5.31	0.05	5.20	5.41	0.01	5.49
120	5.45	0.03	5.33	5.26	0.04	5.18	5.42	0.06	5.20	5.41	0.01	5.50

Table A-8: Strain C10 optical density and pH measurements for electrode study.

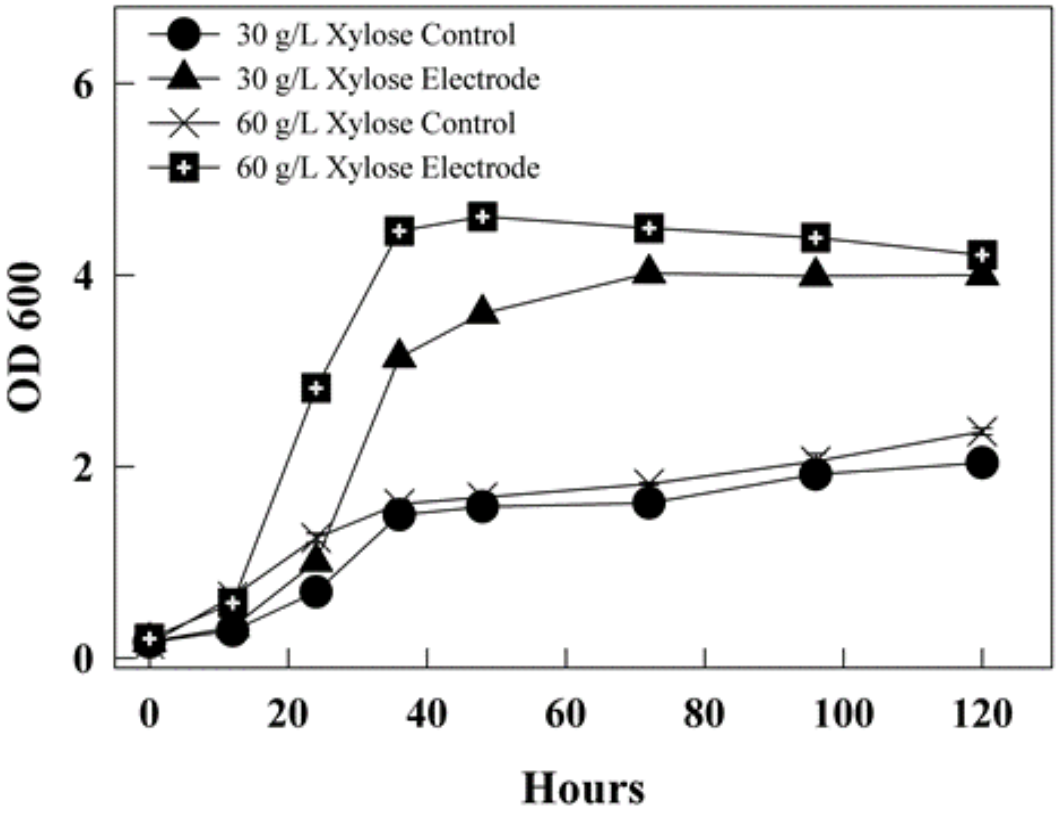

Figure A-4: Optical density readings for xylose-fed fermentations in the presence and absence of the electrode system. Error bars represent standard deviation between samples

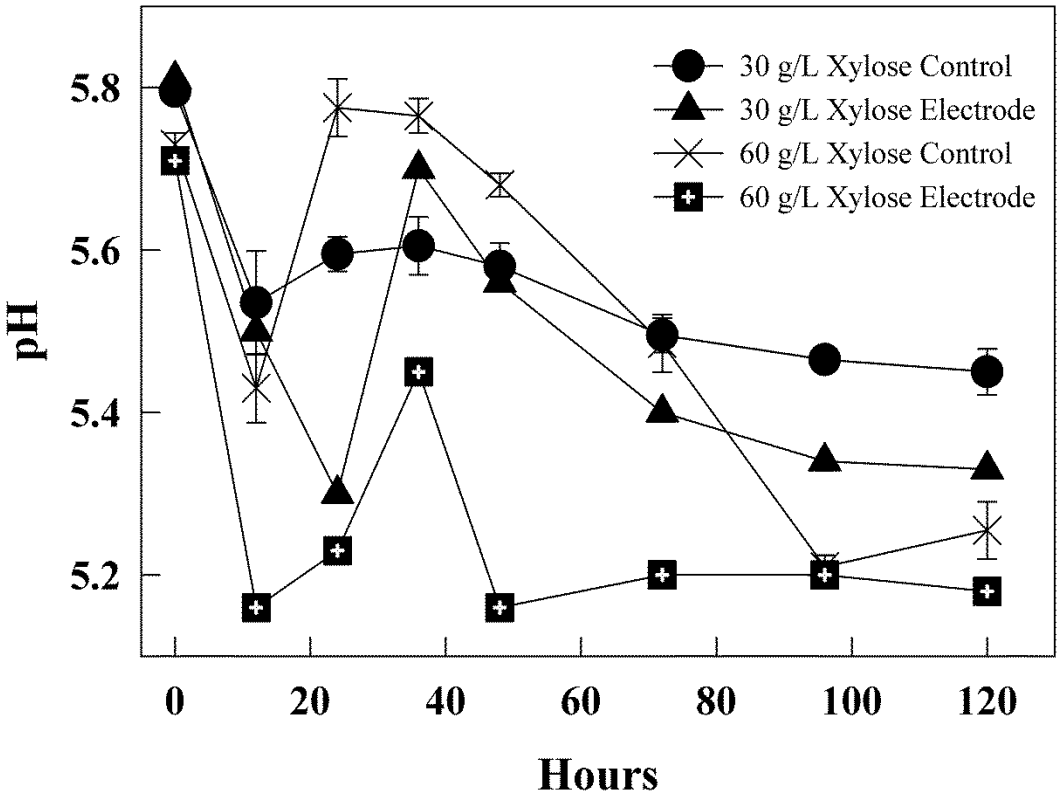

Figure A-5: pH readings for xylose-fed fermentations in the presence and absence of the electrode system. Error bars represent standard deviation between samples.

Strain Characterizations

Full 16S Consensus Sequences

CELL 1 (DC-1)

1217 bp

5’ – CGGATATTTTCTTTGTGTGGCGGACTGCAGCTCTGCAGGTGCGTGTAGCAATAC
ATCACCGCCGGACGGGTGCGTAACACGTGGGAATGTACCTAGTGGTTCGGAACAACG
CTTGGAAACGAGTGCTAATACCGGATGTGCCCGAGAGGGGAAAGATTCATCGCCACT
AGATCAGCCCGCGCAGGATTAGCTAGTTGGTAGGGTAATGGCCTACCAAGGCTCCGA
TCCTTAGCTGTTCTGAGAGGAAGATCAGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTTGGGAATCTTGGACAATGGGCGAAAGCCTGATCCAGCC
ATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTACCCGGGAAGATA
ATGACGGTACCGGGAGAATAAGCTCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATA
CGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGGCCTT
TTAAGTCAGGGGTGAAAGCCCAGAGCTCAACTCTGGAATTGCCTTTGAAACTATTGG
GCTTGAGTGCGGGAGAGGTGAGTGGAATTCCCAGTGTAGAGGTGAAATTCTTAGATA
TTGGGAAGAACACCGGTGGCGAAGGCGGCTCACTGGCCCGTTTCTGACGCTCATGCA
CGATAGCGTGGGGATCAAACAGGATGATATACCCTGGTAGTCCACGCTGTAAACTAT
GGACGCTAGCCGTTGGGCAGCTTGCTGTTCAGTGGCGCATCTAACGCATTAAGCGTC
CCGCCTGGGGAGTACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGAGCCCGC
ACGAGCAGTGGAGAATGTGGCTCAGGTCTATGCAACCGCGCAGAAACTGACCAGGG
TTTGACATTCTGTGCTCGGCTAATGACAGATAAGATTTTCGCGCCACGGGACACAAA
CGACAGGCTGCTGGCATGGGATGTCGTCAGCTCGCTGTCTTGACCTGTAGCGTCATGT
ATCCTGTCAACGGAGTCAGCAAGAGCTCTACTTTTCACGTTAGGCCACTTACTATACA
CTCGAGCAGGTCTAGAGTTGAACTCACGGCTTCAGCCAATGATGCAGGATTGGGCAT
GCATCGCTGCAGTTCCGTCGATGGACTGAACGGTGGTCTAGATACCAGTGCTGAACC
ATGAGCTTGTTTCGTTCACTCAG – 3’

Cellulose 9 (C9)

1352 BP

5’-GTGAGTCGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTGAGT
AACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAATA
CCGCATAAGATTGTAGTGCCGCATGGCATAGCAATTAAAGGAGTAATCCGCTATGAG
ATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCA
ACGCCGCGTGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTCTTCAGGGACGATA
ATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCRGWTTWACTGNGGYGTAAANNKGGAGNCGTNAG
KTGGATATTNNTAAGTGGGATGTGAAATACTSGGNGCTTNAACMTGGGTGYKRCATT
CCAAACTGGATATCTAGAGYGCAGGAGAGGAAAGTAGAANTTCYTRGTGTAGCGGT
GAAATGCGTASAGATTAGGAAGAATACCAGTGGCGAAGRCGACTTTSTGGACYGTAA
CWKACAMTGAGGCTYGAAAGCGTGGGGAGCAAACAGGATTAKATACCYTGGTAGT
CCACGCCGTAAWCGATGAATACTAKGTGTAGGGGTTGTCATGACCTCTGTGCCGCCG
CTAACRCATTAMGTATTNCCTCCTGGGGAGTACGGTCGCARGATTAAAACTCAAAGS
NAATKGACGGGNGGCCCGCACAMGCAKCGGAGCATGTGGTTTAANTTMGAAGNNC
AMCGTNNSARGAACCTTACYTAGNACTTGACATCTSCTGNAATTACNMCTTAATNCG
GGGAAGCCCTTCGGGGCAGGAAGACAGTTGGTGCATGGTTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTCCCATT
TAGTTGAGCACTCTAGCGAGACTCCCCGGGTTAACCGGGAGGAAGGTTGGGATGACG
TTAAATCTTCATGCCCCTTATGTCTACGGCTACACACGTGTTACAATGGTTGCTACAG
AGAGATGTTAAACCGCGAGGTGGAGCCAAACTTTAAAACCAGTCTCAGTTCGGATTG
TAGGCTGAAACTCGCCTACATGAAGCTGGAGTTTCTAGTAATCGCGAATCAGAATGT
CGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGA - 3’

Cellulose 10 (C10)

1360 bp

5’- GTCGGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTGAGTAAC
ACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAATACCG
CATAAGATTGTAGTGCCGCATGGCATAGCAATTAAAGGAGTAATCCGCTATGAGATG
GACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGT
AGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCT
ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACG
CCGCGTGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTCTTCAGGGACGATAATG
ACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGTGGATATTT
AAGTGGGATGTGAAATACTCGGGCTTAACCTGGGTGCTGCATTCCAAACTGGATATC
TAGAGTGCAGGAGAGGAAAGTAGAATTCYTAGTGTAGCGGTGAAATGCGTAGAGAT
TAGGAAGAATACCAGTGGCGAAGGCGACTTTCTGGACTGTAACTGACACTGAGGCTC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGCCGCCGCTAACGCATTAAGTAT
TCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCNAAAGGAATTGACGGGGGCCC
GCACAAGCAGCGGAGCATGTGGNTTTAATTCGAAGCAACGCGAAGAACCTTACCTA
GACTTGACATCTCCTGAATTACCCTTAATCGGGGAAGCCCTTCGGGGCAGGAAGACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCACTCTAGCGAGACTGCC
CGGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTA
GGGCTACACACGTGCTACAATGGCTGGTACAGAGAGATGCTAAACCGCGAGGTGGA
GCCAAACTTTAAAACCAGTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAA
GCTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCT
TGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTTCGTGAGCTAA
CG – 3’

Cellulose 3 (C3)

1381 BP

5’ – TGCAGTCGAGCGAGGAATTGCTTCGGTAATTTCCTAGCGGCGGACGGGTGAGTA
ACACGTGGGCAACCTGCCTGATAGAGGGGGATAGCCTCCCGAAAGGGAGATTAATA
CCGCATAAAGTCAAATGAAGGCATCTTCAAATGACCAAAGGAGTAATCCGCTATCAG
ATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGACT
CCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCA
ACGCCGCGTGAGTGATGAAGGCCTTCGGGTTGTAAAGCTCTGTCTTCAGGGACGATA
ATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGCGGAT
ACTTAAGTGGGATGTGAAATACCTGGGCTTAACTTGGGTGCTGCATTCCAAASTGKG
TGTCTAGAGTGTKGGAGAGSMAAGTGGARTTCCTAGTGTAGCGGTGAAAKSNCGTAG
AGATTAGGAWGAACMCYASTGGYGRAGGCSACTKTCTGGRCAATARCTGACSCTGA
GGCTCGAAASCGTGGGGAGCAMACAGGATTAGATAYCMTGGTMGTCCAYGCYGTAA
AMRATGGSTAYTAGSTGTAGGGGGTATCGACTCCYCYTGTGCCGCCGTTAACACAAT
AAGTACYCCGCYNTGGGGAGTACGGTCGCWAGATTNAAMACTYWNAAGGAATTGA
CGGGGGCYCGCACACGCAGCGGAGCATGTGGTTTAATTTGAAGCAACGCGAAGAAC
CTTACCTCGACTTGACATCTCCTGACTTACTCCTAATCGAGGAAGTTCTCCCTTCGGG
GAGGACAGGAAGACAGGTGGTGCATGGTTGTCGTCAGCTTGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCAC
TCTAGCAAGACTGCCGTGGTTAACGCGGAGGAAGGTGGGGATGACGTCAAATCATC
ATGCCCCTTATGTCTAGGGCTACACACGTGCTACAATGGCGAGTACAAAGAGACGCA
AAGCCGCGAGGTGGAGCAAAACTTATAAAACTCGTCTCAGTTCGGATTGCAGGCTGA
AACTCGCCTGCATGAAGCTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCC
GAAGTCCGTAGCCTAACCTTTAGGA – 3’

Xylan 5 (X5)

1367 BP

5’ – GCAGTCGAGCGATGAGTTCCTTCGGGAACGGATTAGCGGCGGACGGGTGAGTA
ACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAATAC
CGCATAAGATTGTAGTGCCGCATGGCATAGCAATTAAAGGAGTAATCCGCTATGAGA
TGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAA
CGCCGCGTGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTCTTCAGGGACGATAA
TGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGTGGATAT
TTAAGTGGGATGTGAAATACTCGGGCTTAACCTGGGTGCTGCATTCCAAACTGGATA
TCTAGAGTGCAGGAGAGGAAAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAGAG
ATTAGGAAGAATACCAGTGGCGAAGGCGACTTTCTGGACTGTAACTGACACTGAGGC
TCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGAATACTAKGTGTAGGGGTTGTCATGACCTCTGTGCCGCCGCTAACGCATTAAGT
ATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCC
CGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTAG
ACTTGACATCTCCTGAATTACCCTTAATCGGGGAAGCCCTTCGGGGCAGGAAGACAG
GTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCACTCTAGCGAGACTGCCC
GGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTAG
GGCTACACACGTGCTACAATGGCTGGTACAGAGAGATGCTAAACCGTGAGGTGGAG
CCAAACTTTAAAACCAGTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAG
CTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTTCGTGAGCTAAC
GCGCAAGCG – 3’

Xylose 1 (X1)

1359 BP

5’ – GCAAGTCGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTGAG
TAACACGTGGGTAACCTGCCTCATAGAGGGGAATAGCCTTTCGAAAGGAAGATTAAT
ACCGCATAAGATTGTAGTGCCGCATGGCATAGCAATTAAAGGAGTAATCCGCTATGA
GATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGAT
GCGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGC
AACGCCGCGTGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTCTTCAGGGACGAT
AATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAA
TACGTAGGTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGTGGAT
ATTTAAGTGGGATGTGAAATACTCGGGCTTAACCTGGGTGCTGCATTCCAAACTGGA
TATCTAGAGTGCAGGAGAGGAAAGTAGAATTCCTAGTGTAGCGGTGAAATGCGTAG
AGATTAGGAAGAATACCAGTGGCGAAGGCGACTTTCTGGACTGTAACTGACACTGAG
GCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGAATACTAGGTGTAGGGGTTGTCATGACCTCTGTGCCGCCGCTAACGCATTAA
GTATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCT
AGACTTGACATCTCCTGAATTACCCTTAATCGGGGAAGCCCTTCGGGGCAGGAAGAC
AGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCTTATTGTTAGTTGCTACCATTTAGTTGAGCACTCTAGCGAGACTGC
CCGGGTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCT
AGGGCTACACACGTGCTACAATGGCTGGTACAGAGAGATGCTAAACCGCGAGGTGG
AGCCAAACTTTAAAACCAGTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGA
AGCTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCC
TTGTACACACCGCCCGTCACACCATGAGAGTTGGCAATACCCAAAGTTCGTGAGCTA
A – 3’

Xylose 2 (X2)

1314 BP

5’ – TCGAGCGATGAAGCTCCTTCGGGAGTGGATTAGCGGCGGACGGGTGAGTAACA
CGTGGGTAACCTGCCTCATAGAGGGAATAGCCTTTCGAAAGGAAGATTAATACCGCA
TAAGATTGTAGTGCCGCATGGCATAGCAATTAAAGGAGTAATCCGCTATGAGATGGA
CCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAG
CCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACG
GGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCG
CGTGAGTGATGACGGTCTTCGGATTGTAAAGCTCTGTCTTCAGGGACGATAATGACG
GTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTGTCCGGATTTACTGGGCGTAAAGGGAGCGTAGGTGGATATTTAAG
TGGGATGTGAAATACTCGGGCTTAACCTGGGTGCTGCATTCCAAACTGGATATCTAG
AGTGCAGGAGAGGNAAAGTAGAATTCYTAGTGTAGCGGTGAAATGCGTAGAGATTA
GGAAGAATACCAGTGGCGAAGGCGACTTTSTGGACYGTAAYTGACACTGAGGCTCG
AWAGCGTGGGGAGCAAACAGGATTAGATACCMTGGTAGTCCACGCCGTAWACGAT
GAATACTAKGTGTAGGGGTTGTCATGACCTCTGTGCCGCCGCTAACGCATTAAGTAT
TCCKCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCAGCGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACTTAGAC
TTGACATCTCCTCAATTACCCTTAATCGGGGAAGCCCTTCGGGGCAGGAAGACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTTATTGTTAGTTGCTCCCATTTAGTTGAGCACTCTAGCGAGACTGCCCGG
GTTAACCGGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTAGGG
CTACACACGTGTTACAATGGTTGCTACAGAGAGATGTTAAACCGCGAGGTGGAGCCA
AACTTTAAAACCAGTCTCAGTTCGGATTGTAGGCTGAAACTCGCCTACATGAAGCTG
GAGTTTCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGT – 3’

KRH-YZ

1305 BP

5’-
GGGTGTGTGTGCCTCAGCGCGTGCCGTCGCGGGGGGGACACTTCGAAAAAACTGCTC
TCTACCGTATAATCTCTGGGTGAAAAAGGGGGAGCCCGAGGCGTCTCGCGCTCTTGG
CGGCGCATATGTCATATTGTGTTTGGGGGGGTAAAAGGGCTCACCGCCCCAACTATC
TGGCTGGGGTGAGAGAAGAACACCCCCCACTGGGGGTGAGACACGGGCCCCACACT
CCTAGGGGGGGCCACTGGGGAAAATTGGAGACAGGGGGCGCAAGCCAGATCCCAAT
GCCCCGCGCGCGAAAAAAAGCTTTTGGTTGTAAACTGTTTTTGTGTAGAGAACAAAG
GTCCTGGGGTTTATACCGGGGTGATGAGACTACCCCGAAAAAATAAGCACGGTATCA
CTGCGTGCCCACCCCCGCTAATATATAKGGTNNGCAMGCGTNAATCNNAATTACTGG
GCGTAWAGMGTGCGCASGCGGTKTTGTNRKACASGCGTGAAATMYCCGSGCTCWMC
NCTGNNNNATWGYGCTYGTGACWGYRMGRCTSKAGTGYGNCASAGRGGRKATRGW
AYTCCGYGTGTAGYNGWGAWRTGCRTAKATATGCNNNRGAACACNCKATSGCNARS
GCWATCCYSTGSGYSTGCWSTGACKCNTCATGCANGAANNCGTGKRGAGCAMACAS
GAKTAKAKACMCTGKKAGTMCWCGCCCTAAACGATGTCAACTGGTTGTTGGGTCTC
TTCTGACTCAGTAACGAAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGCCGC
AAGGTTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGT
TTAATTCGATGCAACGCGAAAAACCTTACCCACCTTTGACATGTACGGAATTTGCCA
GAGATGGCTTAGTGCTCGAAAGAGAACCGTAACACAGGTGCTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGCCATTAG
TTGCTACGAAAGGGCACTCTAATGGGACTGCCGGTGACAAACCGGAGGAAGGTGGG
GATGACGTCAAGTCCTCATGGCCCTTATAGGTGGGGCTACACACGTCATACAATGGC
CGGTACAAAGGGTAGCCAACCCGCGAGGGGGAGCCAATCCCACAAAGCCGGTCGTA
GTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGG
ATCAGCATGTCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCA
TGGGA – 3’

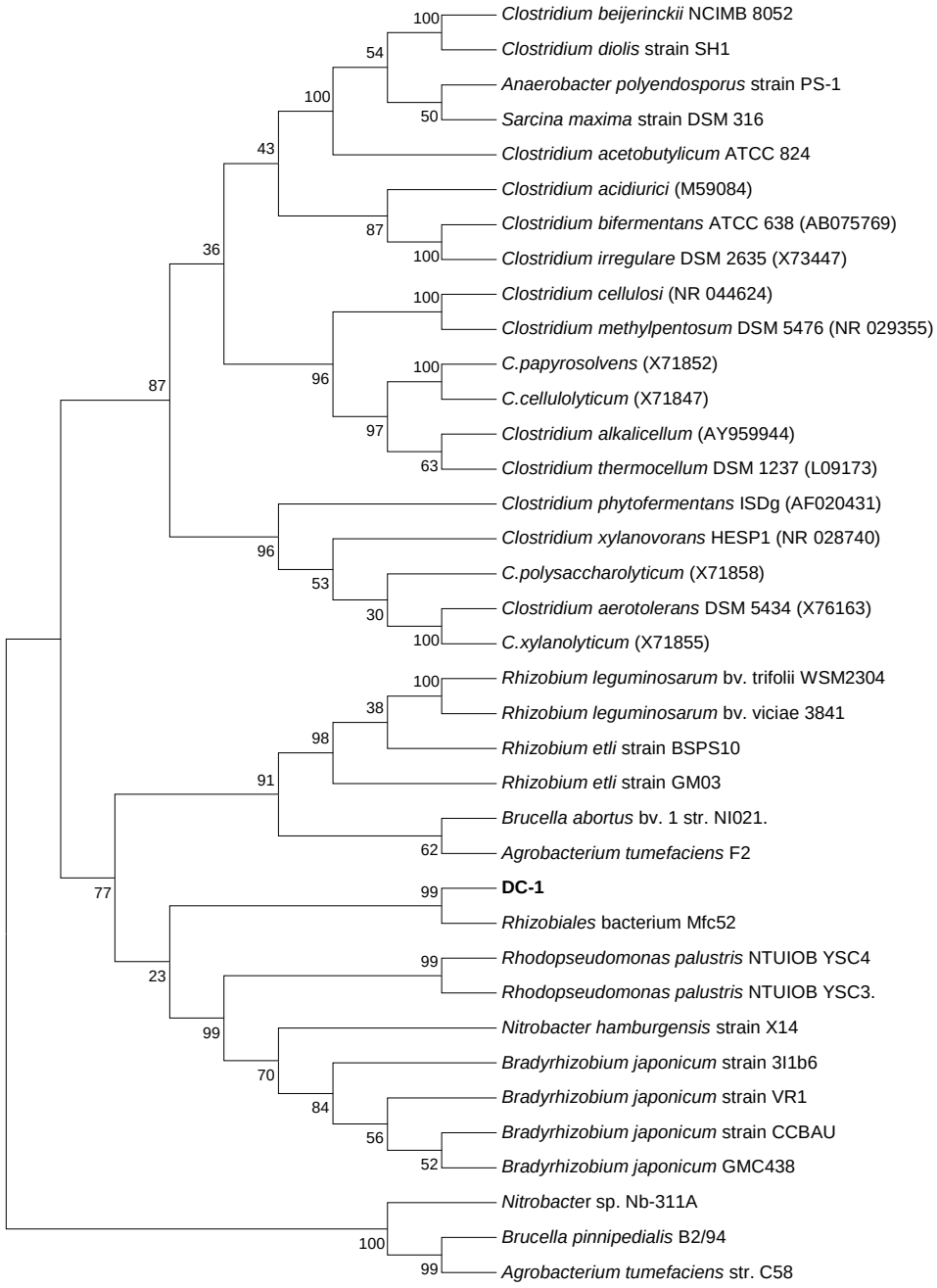

Figure A-6: Strain DC-1 molecular phylogenetic analysis by maximum likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model

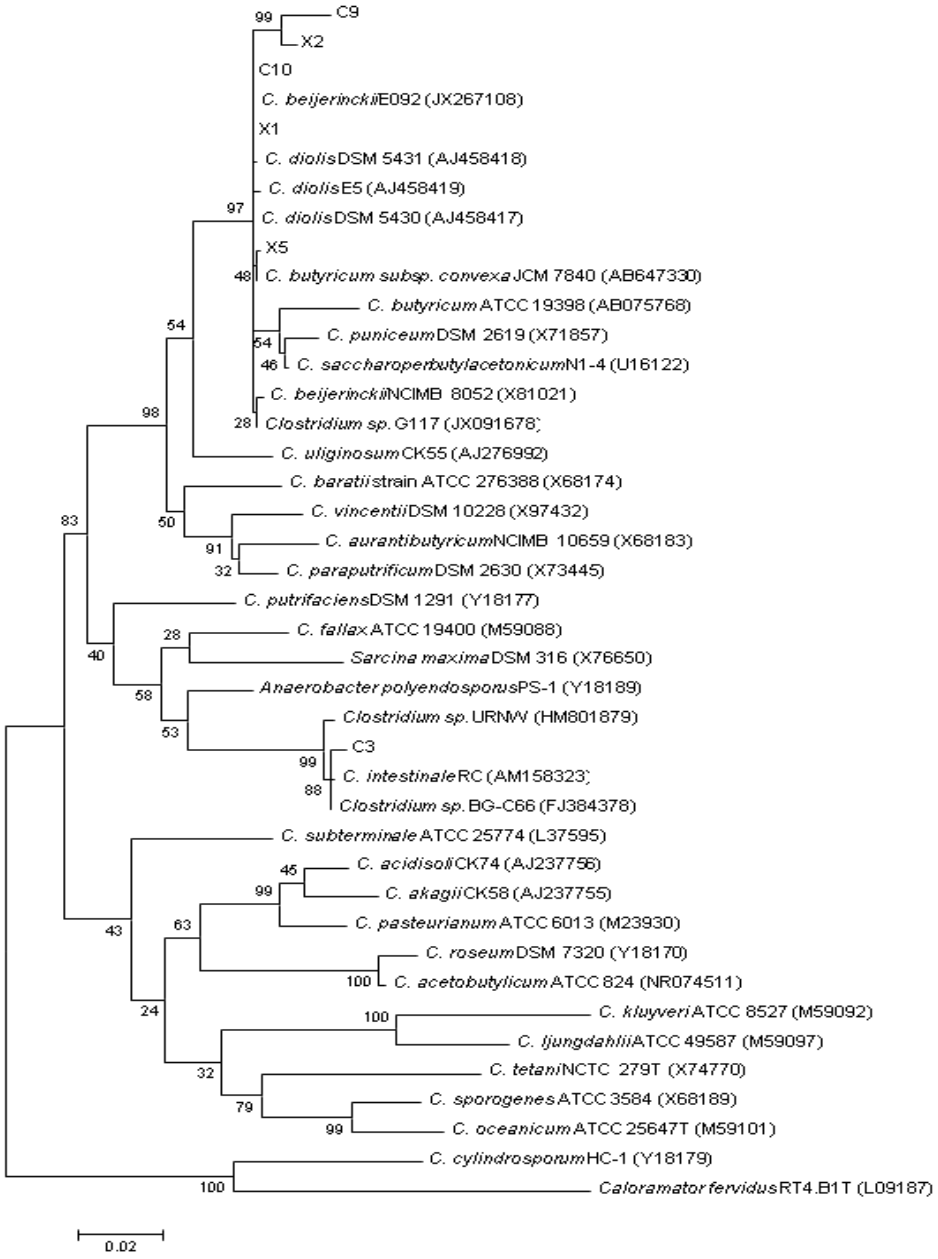

Figure A-7: Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [1]. The tree with the highest log likelihood (-9095.2448) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

	1	2	3	4	5
C. beijerinckii E092	94%	99%	100%	99%	99%
C. beijerinckii NCIMB 8052^T	94%	99%	100%	99%	99%
Clostridium sp. G117	94%	99%	99%	98%	99%
C. diolis DSM 5430^T	94%	99%	100%	99%	99%
C. diolis SH1^T	94%	99%	99%	98%	99%
C. saccharoperbuytl. N1-4^T	94%	99%	99%	98%	99%
C. roseum strain 653^T	94%	99%	99%	98%	99%
C. butyricum subsp. convexa	94%	99%	99%	98%	99%

Table A-9: 16S rDNA sequence similarities. 1, C9 (1352 bp); 2, C10 (1360 bp); 3, X1 (1359 bp); 4, X2 (1314 bp); 5, X5 (1367 bp)

	C3 (1381 bp)
Clostridium sp. BG-C66	96%
C. intestinale RC^T	96%
Clostridium sp. P301	96%
Clostridium sp. Kas301-1	96%
C. fallax VA24831^T	91%
A. polyendosporous PS-1^T	92%

Table A-10: 16S rDNA sequence similarities for strain C3

	***C. progen*. DC1 (FAME %)**
C8:0 FAME	1.21
C10:0 FAME	0.15
C12:0 FAME	0.78
C14:0 FAME	4.13
C15:1 FAME	1.62
C16:0 FAME	41.11
C16:1 FAME	1.49
C17:1T FAME	7.10
C18:0 FAME	2.64
C18:1 FAME	0.59
C18:1-11C FAME	0.59
C18:1-12C FAME	1.48
C19:0 FAME	36.18
C18:2 FAME	0.16
C24:1 FAME	0.77

Table A-11: Fatty acid methyl ester composition for strain DC-1. Data were normalized on a percent basis.

	X1	X2	X5	C9	C10
C6:0 FAME	0	0	0	0.17	0
C8:0 FAME	1.18	1.48	1.52	0.73	0.91
C12:0 FAME	0.81	0.69	0.51	0.68	0.57
C14:0 FAME	4.30	3.45	2.09	3.89	3.26
C15:1 FAME	1.58	1.32	0.71	1.34	1.41
C16:0 FAME	41.55	34.36	34.29	38.68	33.99
C16:1 FAME	1.61	1.26	1.47	1.39	1.20
C17:1T FAME	7.06	5.81	6.25	6.57	6.07
C18:0 FAME	2.07	2.13	2.54	1.88	1.14
C18:1 FAME	0.59	0.66	0.56	0.55	0.40
C18:1-11C FAME	0.50	0.53	0.77	0.51	0.50
C18:1-12C FAME	1.39	1.24	0.88	1.20	1.14
C19:0 FAME	36.39	45.92	46.00	41.59	48.97
C18:2 FAME	0.51	0.23	1.42	0.18	0.43
C24:1 FAME	0.45	0.90	0.99	0.64	0.00

Table A-12: Fatty acid methyl ester composition for isolated *Clostridium* strains. Data were normalized on a percent basis.

	C3
C8:0 FAME	1.31
C12:0 FAME	1.22
C14:0 FAME	5.26
C15:1 FAME	0.34
C16:0 FAME	5.40
C16:1 FAME	3.70
C18:0 FAME	0.47
C18:1 FAME	1.99
C18:1-11C FAME	4.02
C19:0 FAME	76.29

Table A-13: Fatty acid methyl ester composition for strain C3.

	C. progen. **DC-1**	***R. electr. MFC-52***	***C. beijer.***
G+C mol%	41.59%	64.7%	26-28%
Gram	+, rod w/ terminal inclusion body	-, Stalked rod	+, rod
Salinity	3%	1%	<6.5%
pH Range	2-8.5	5-7.5	
Optimal pH	3-7	6.7	4.6-5.4
Temperature Range	18-42	20-40	25-45
Optimal Temperature	37	30	37
Fermentation Products	Acetone, Butanol, Ethanol, butyrate, acetate, lactate, fumarate, succinate, Hydrogen	Lactate, Acetate, Fumarate, Hydrogen	Acetone, Butanol, Ethanol, Propanol, butyrate, acetate, lactate, succinate, formate, Hydrogen
Nitrate Reduction	past Nitrite	To Nitrite	
Motility	Motile	Motile	Motile
Esculin	+	N/A	+
Starch	+	+	+/-
Arabinose	-	+	+/-
Cellobiose	+	+	+
Citrate	-	N/A	N/A
Crystalline Cellulose	+	-	-
Dextrin	+	N/A	+
Dulcitol	-	N/A	+
Glycerol	-	N/A	W
Glucose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Ribose	-	+	+/-
Sorbitol	+		+/-
Trehalose	+	+	+/-
Xylose	+	+	+

Table A-14: Strain DC-1 characteristics. +, growth/positive reaction; -, no growth/reaction; +/-, occurs in some strains; W, weak growth/reaction; T, terminal; C, central; N/A, not available; NT, not tested

Initial pH	Growth Rate (hr^{-1})	R^2	Lag (hrs)	Doubling Time (hrs)	OD600 Max
9	No Growth				
8.5	0.0906	0.9488	25.061	7.650631132	1.723
8	0.1563	0.9698	17.4943	4.434722844	1.646
7	0.0892	0.9724	<0.01	7.770708302	1.641
6	0.1007	0.9916	<0.01	6.883288784	1.74
5	0.0852	0.994	<0.01	8.135530288	1.757
4	0.1748	0.983	7.0901	3.965372886	1.777
3	0.1153	0.9911	<0.01	6.011684133	1.893
2	0.1091	0.9829	<0.01	6.353319712	1.386

Table A-15: pH tolerance for strain DC-1. This study was performed in tri-buffered TYG media. The modified Gompertz equation was used to fit optical density data in order to determine growth rate.

Characteristics	1	2	3	4	5	6	7	8	9
Isolation Source	Marsh	Marsh	Wet-wood	Marsh	Marsh	N/A	Straw	Soil, rumen	N/A
G+C mol%	38.03%	42.58%	41.59%	34.88%	39.61%	26-28%	N/A	27-28%	31%
Endospore	T	T	C	T	T				oval
Gram Stain	+, rod	+; rod	+; rod	+; rod	+; rod	+, rod	+, rod and filament	+; rod	+; rod
Salinity (as NaCl)	3%	3%	2%	2%	3%	<6.5%			
pH Range	2-8.5	3-8	2-9	2-8	2-8.5		5.5-8.5		
Optimal pH	3-7	7	7	3-6	7	4.6-5.4	7		5.6-6.7
Temperature Range	18-42	18-42	18-45	18-42	18-42	25-45	N/A		N/A
Products from PYG	A, B, E, H2	A, B, E, H2	A, B, E, H2	A, B, E, H2	A, B, E, H2	A, B, E, propanol, butyrate, acetate, lactate, H2	butyrate, acetate, 1,3-propanediol	butyrate, acetate, formate, lactate, B, E, H2	A, B, E, acetate, butyrate, H2
Nitrate Reduction	to Nitrite	to Nitrite	to Nitrite	Past Nitrite	past Nitrite	N/A	N/A		N/A
Motility	+	+	+	+	+	+	+	+/-	+
Esculin Hydrolysis	+	+	+	+	+	+	+		+
Starch Hydrolysis	+	+	+	+	+	+/-	-		N/A
Lecithinase	-	-	-	-	-	-			N/A
Lipase	-	-	-	-	-	-			N/A
Acetate	-	W	-	W	-	N/A	N/A		
Arabinose	W	W	-	W	W	+/-	W		+
Cellobiose	+	+	+	+	+	+	+		+
Citrate	W	-	W	W	W	N/A	N/A		N/A
Crystalline Cellulose	+	+	NT	NT	NT	-	-		N/A
Dextrin	+	+	+	+	+	+			+
Dulcitol	-	-	+	+	-	+			+
Glycerol	-	-	+	+	-	W			-
Glucose	+	+	+	+	+	+	+		+
Lactate	+	+	-	-	-	N/A			N/A
Lactose	-	W	+	+	-	+			+/-
Maltose	+	+	+	+	+	+			+
Ribose	-	+	+	+	-	+/-	W		-
Sorbitol	-	+	-	+	+	+/-	W		+
Trehalose	+	+	+	+	-	+/-	+		+
Xylan	NT	NT	+	NT	NT	+			N/A
Xylose	+	+	+	+	+	+	+		N/A

Table A-16: Characteristics of *Clostridium* isolates. 1, C9; 2, C10; 3, X5; 4, X1; 5, X2; 6, *C. beijerinckii*; 7, *C. diolis*; 8, *C. butyricum*; 9, *C. saccharoperbutlyacetonicum*; A, acetone; B, butanol; E, ethanol; +, growth/positive reaction; -, no growth/reaction; +/-, occurs in some strains; W, weak growth/reaction; T, terminal; C, central; N/A, not available; NT, not tested

Characteristics	1	2	3	4	5
Isolation Source	Woodland marsh	Feces, soil, penile lesions, pond mud	Meadow-gley soil	Spoiled meat, urine specimens	Soil, feces, marine sediment, wounds
G+C mol%	45.55%	26-28%	29%	22-25%	26%
Endospore					
Gram Stain	+; rod	+, rod	oval/spherical	+; rod	+; rod
pH Range	3-6	N/A	5.5-8.5	5.7-8.5	
Optimal pH	6	N/A	6.5-7.5	6.2-7.4	
Temperature Range (ºC)	NT	N/A	15-45	5-30	25-45
Optimal Temp. (ºC)	37	37	25-35	15-22	
Products from PYG	acetate, butyrate, H2	acetate, butyrate, lactate, formate, succinate	acetate, lactate, butyrate, ethanol, butanol, H2	acetate, formate, lactate, succinate	acetate, butyrate, lactate, pyruvate, succinate, H2
Nitrate Reduction	-	-	-	-	+/-
Motility	-	+	-	-	+/-
Esculin Hydrolysis	-	+	-	-	+
Starch Hydrolysis	-	-	+	-	-
Lecithinase	-		-	-	-
Lipase	-		-	-	-
Acetate	+	N/A	N/A	N/A	N/A
Arabinose	+	-	-	-	-
Cellobiose	+	+	+	-	W
Citrate	-	N/A	N/A	N/A	N/A
Crystalline Cellulose	+	-	N/A	N/A	N/A
Dextrin	W	N/A	N/A	N/A	N/A
Dulcitol	-	-	N/A	N/A	N/A
Glycerol	+	-	N/A	N/A	N/A
Glucose	W	+	+	+	+
Lactate	W	N/A	+	N/A	N/A
Lactose	W	+	-	-	W
Maltose	W	-	+	-	+
Ribose	+	N/A	N/A	-	W
Sorbitol	+	+	+	-	-
Trehalose	W	+	+	-	-
Xylan	NT	-	N/A	N/A	N/A
Xylose	W	-	+	-	-

Table A-17: Characteristics of strain C3. 1, strain C3; 2, *C. intestinale*; 3, *A. polyendosporus*; 4, *C. putrifaciens*; 5, *C. fallax*; +, growth/positive reaction; -, no growth/reaction; W, weak growth/reaction; +/-, occurs in some strains; N/A, not available; NT, not tested; T, terminal

C. beijerinckii NCIMB 8052 Reference (C.b.8052)	Hybrid Pair	Tm at which 50% is fluorescence lost	ΔTm (Tm homologous - Tm Hybrid)	%RBR
	C.b. 8052 + X 5	75.82	0.45	88.0565
	C.b. 8052 + X 1	74.14	2.13	79.5723
	C.b. 8052 + X 2	76.49	-0.22	91.44
	C.b. 8052 + C9	76.16	0.11	89.7735
	C.b. 8052 + C10	75.15	1.12	84.6729

Table A-18: DNA-DNA hybridization data for isolates

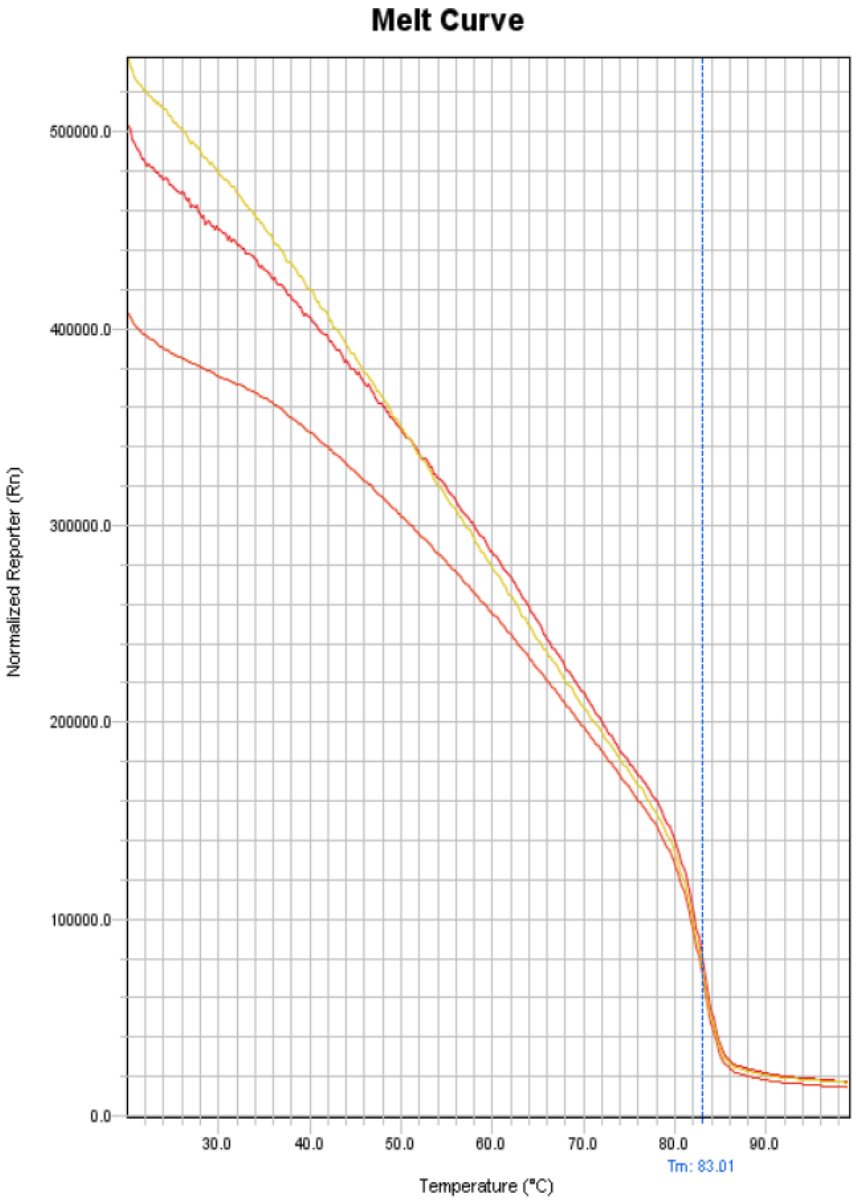

Figure A-8: Melt curve for *C. beijerinckii* 8052 and strain C10 hybrid

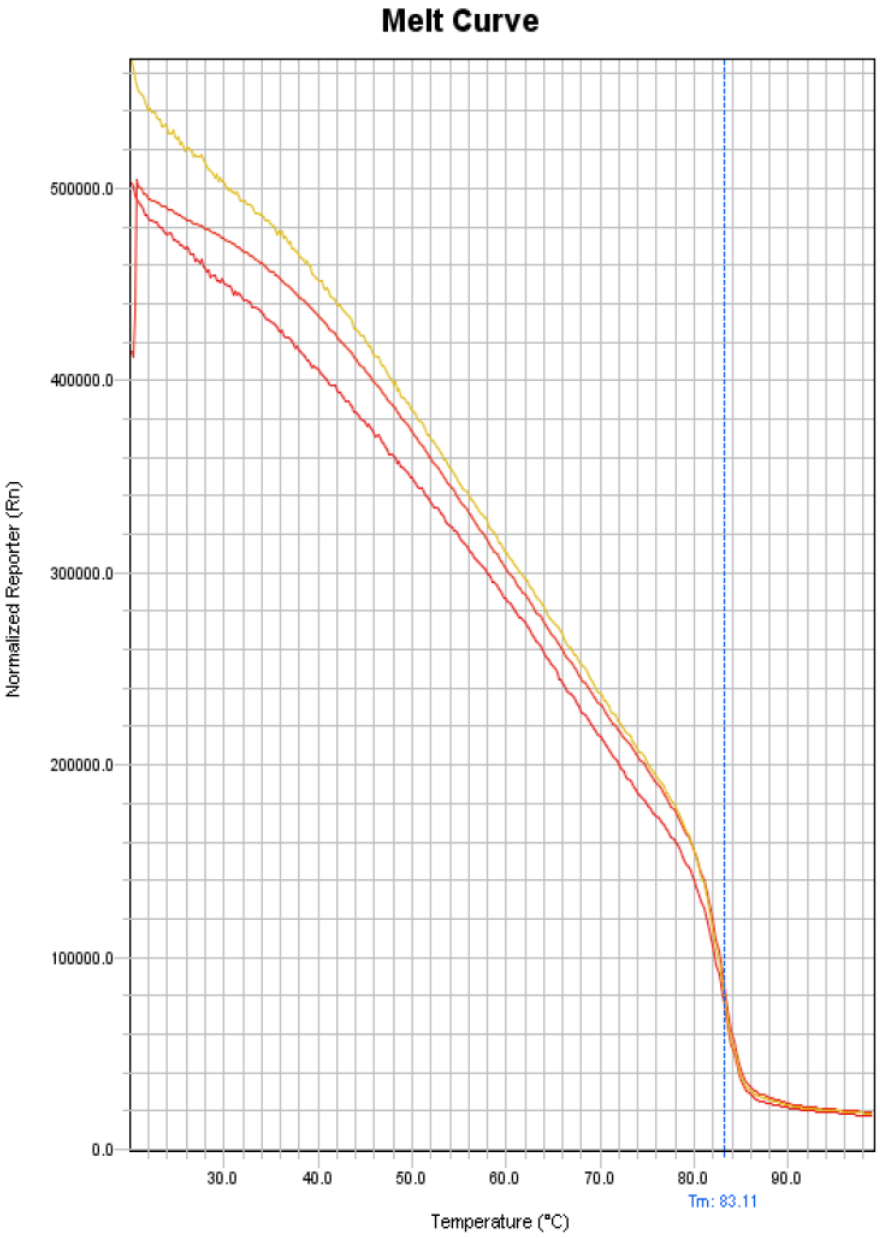

Figure A-9: Melt curve for *C. beijerinckii* 8052 and strain X2 hybrid

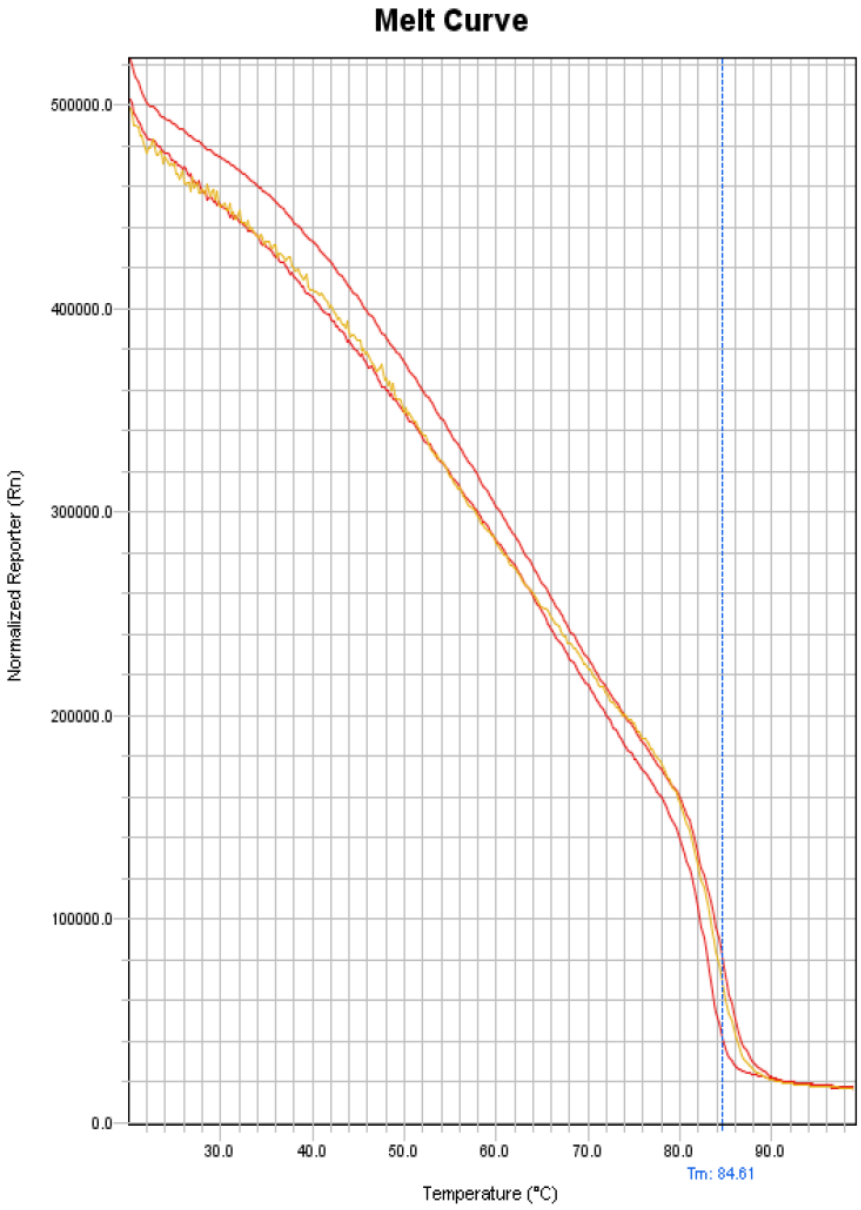

Figure A-10: Melt curve for *C. beijerinckii* 8052 and strain X5 hybrid

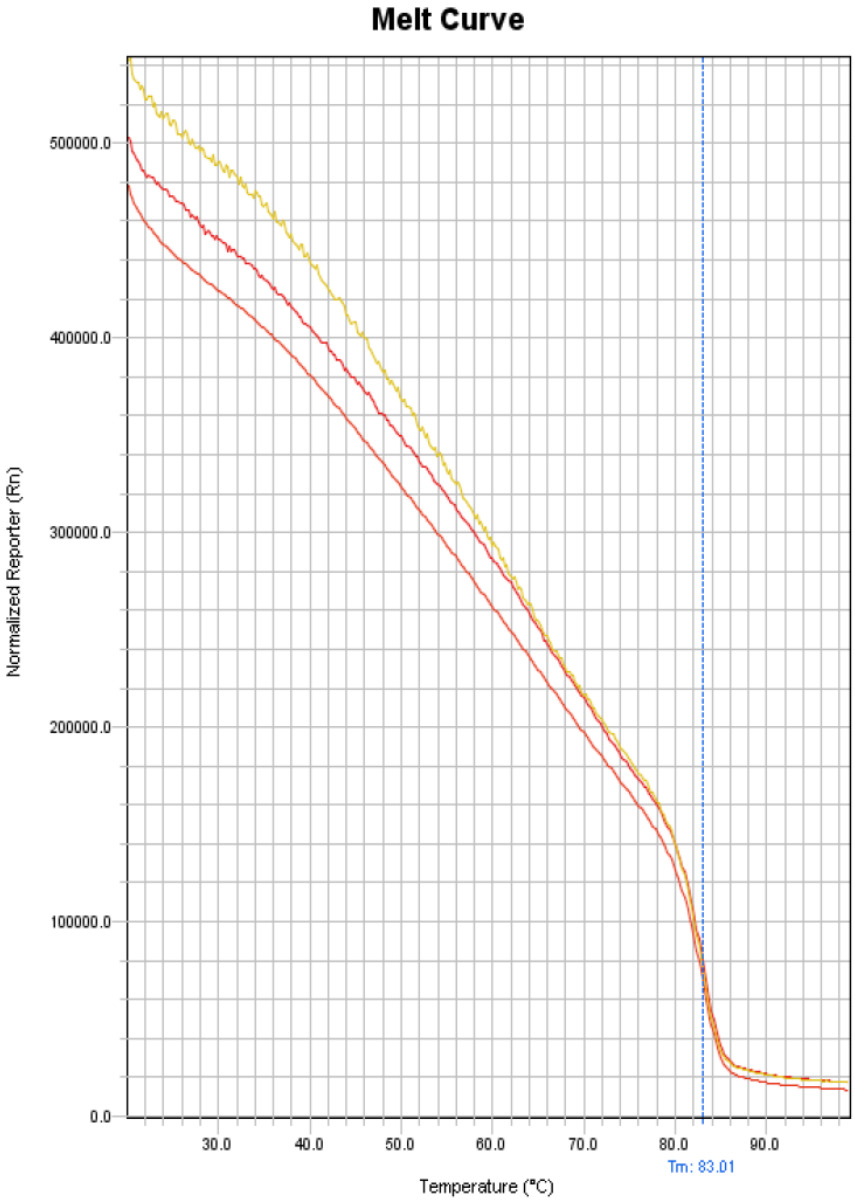

Figure A-11: Melt curve for *C. beijerinckii* 8052 and strain C9 hybrid

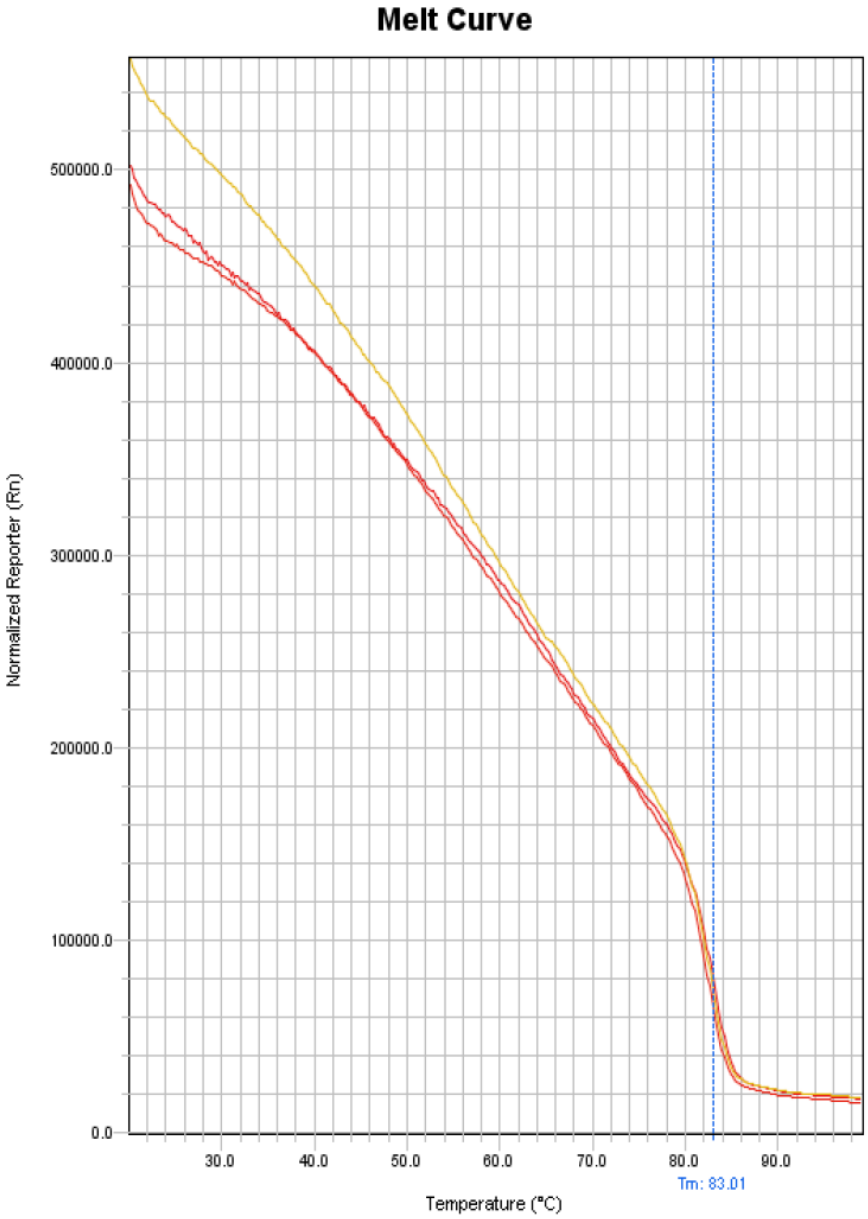

Figure A-12: Melt curve for *C. beijerinckii* 8052 and strain X1 hybrid

Correlation Curves and Method QA

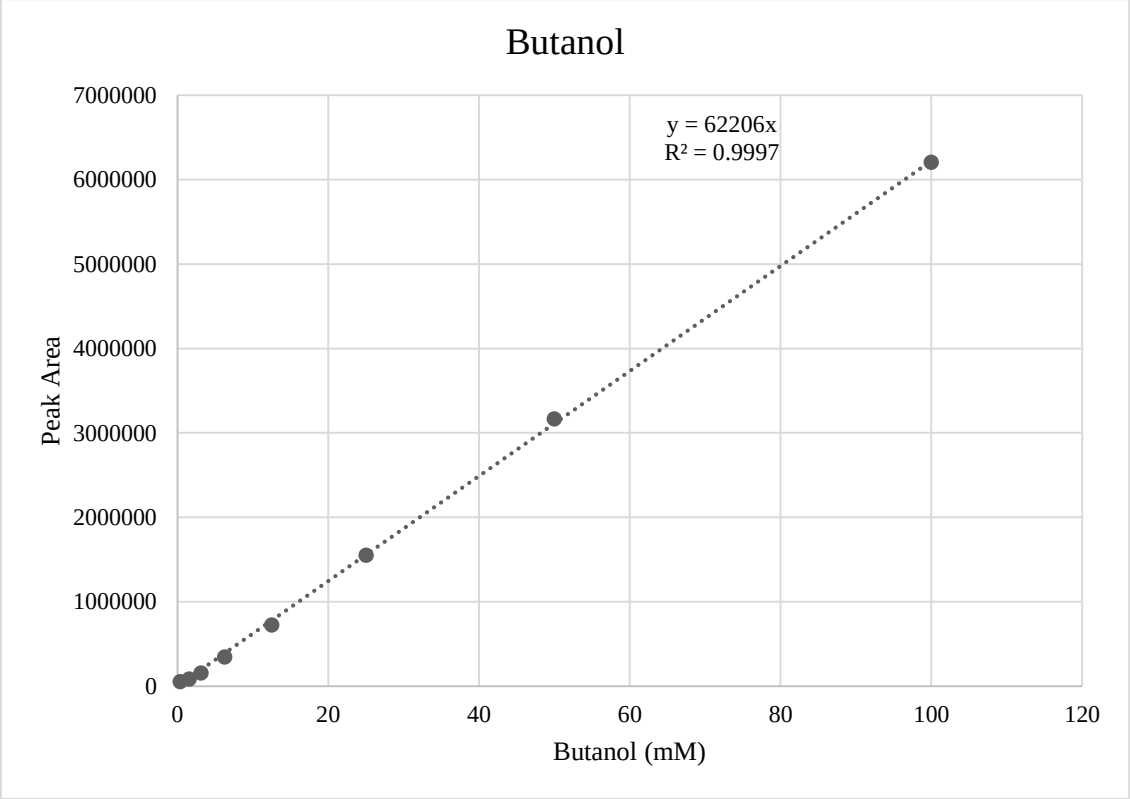

Figure A-13: Butanol standard curve for GC-FID analysis. The temperature program is as follows: 40ºC dwell for 2 minutes, followed by a temperature ramp of 50ºC/min until the oven reached 220ºC. This was held for 1 minute until restarting the temperature program. The injector and detector temperatures were set to 200ºC and 300ºC, respectively. Helium was used as the carrier gas at a linear velocity of 80.3 cm/sec.

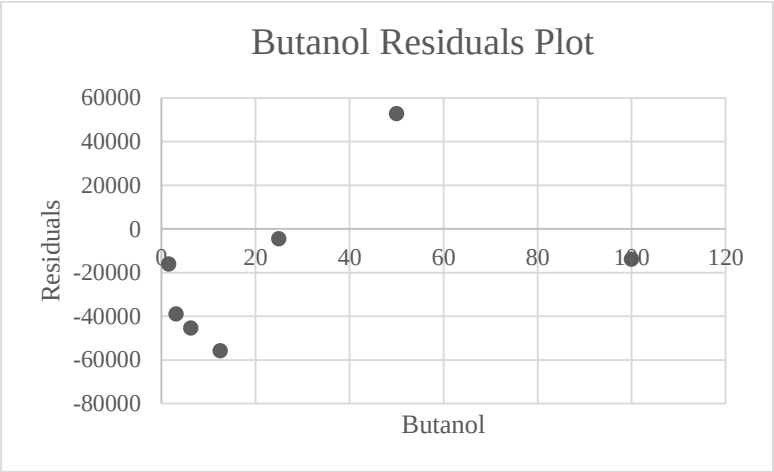

Figure A-14: Butanol standard curve residual plot

Regression Statistics	
Multiple R	0.999903576
R Square	0.999807162
Adjusted R Square	0.833140495
Standard Error	40723.78713
Observations	7

Table A-19: Linear regression statistics for butanol standard curve

Residual Output

Observation	*Predicted 50883.7*	*Residuals*	*Standard Residuals*
1	97040.76624	-16091.96624	-0.426809767
2	194392.5606	-38968.86058	-1.033577255
3	388785.1212	-45381.62116	-1.203663919
4	777570.2423	-55789.74232	-1.479720163
5	1555140.485	-4566.584633	-0.121120247
6	3110280.969	52774.33073	1.399741924
7	6220561.939	-13966.63853	-0.370439364

Table A-20: Residual outputs for butanol standard curve

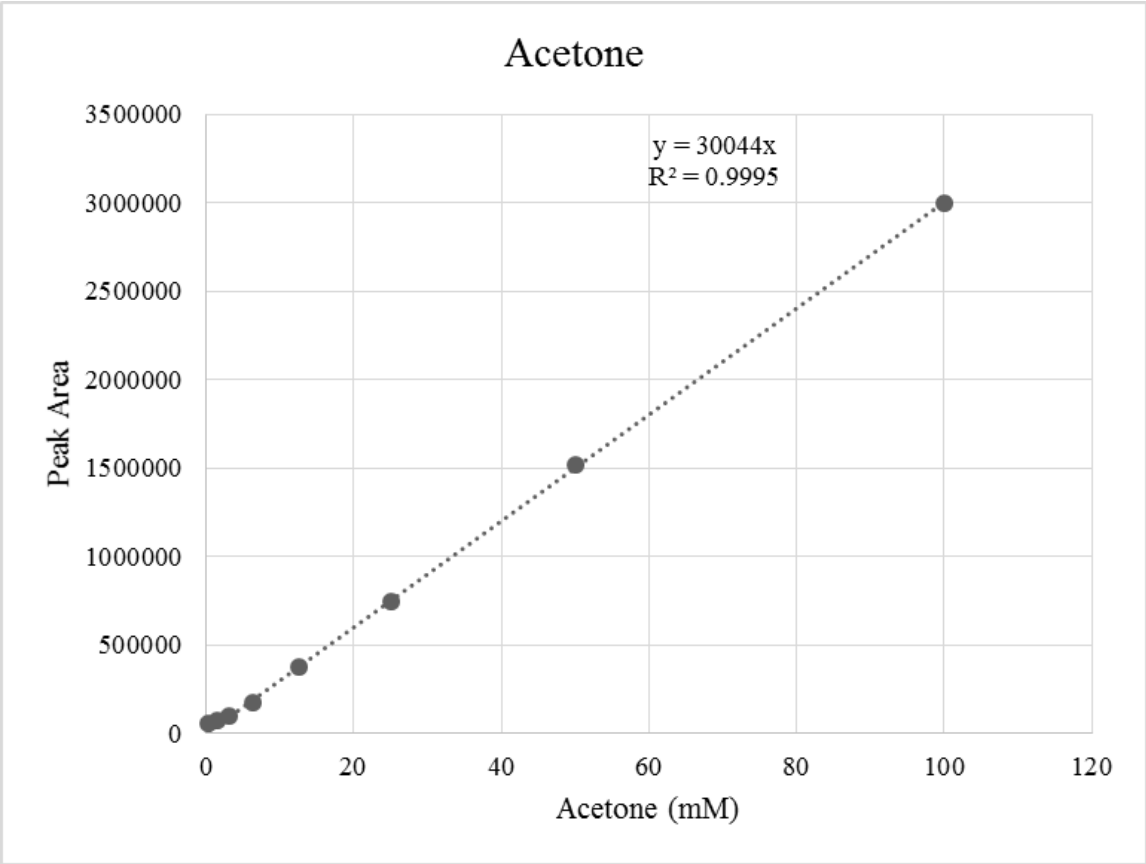

Figure A-15: Acetone standard curve for GC-FID analysis. The temperature program is as follows: 40 °C dwell for 2 min, followed by a temperature ramp of 50 °C/min until the oven reached 220 °C. This was held for 1 min until restarting the temperature program. The injector and detector temperatures were set to 200 °C and 300 °C, respectively. Helium was used as the carrier gas at a linear velocity of 80.3 cm/s.

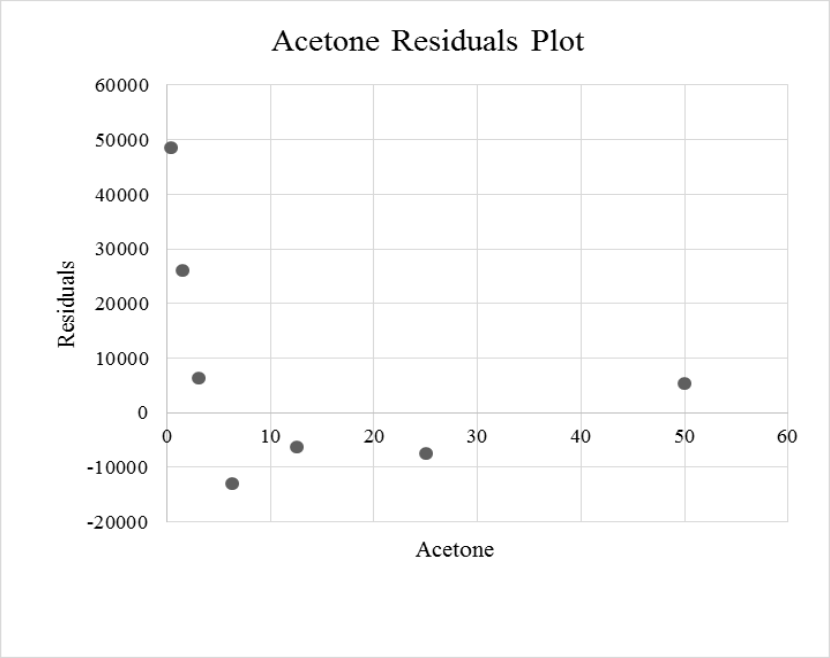

Figure A-16: Acetone standard curve residual plot

Regression Statistics	
Multiple R	0.999448666
R Square	0.998897637
Adjusted R Square	0.83223097
Standard Error	23658.88559
Observations	7

Table A-21: Linear regression statistics for acetone standard curve

Observation	*Predicted*	*Residuals*	*Standard Residuals*
1	10878.68016	48458.61984	2.212331237
2	47140.94736	26093.35264	1.191266679
3	94432.98749	6291.112508	0.287214633
4	188865.975	-12912.77498	-0.589520204
5	377731.95	-6284.249967	-0.28690133
6	755463.8999	-7419.299933	-0.338720935
7	1510927.8	5338.600134	0.243728606

Table A-22: Residual outputs for acetone standard curve

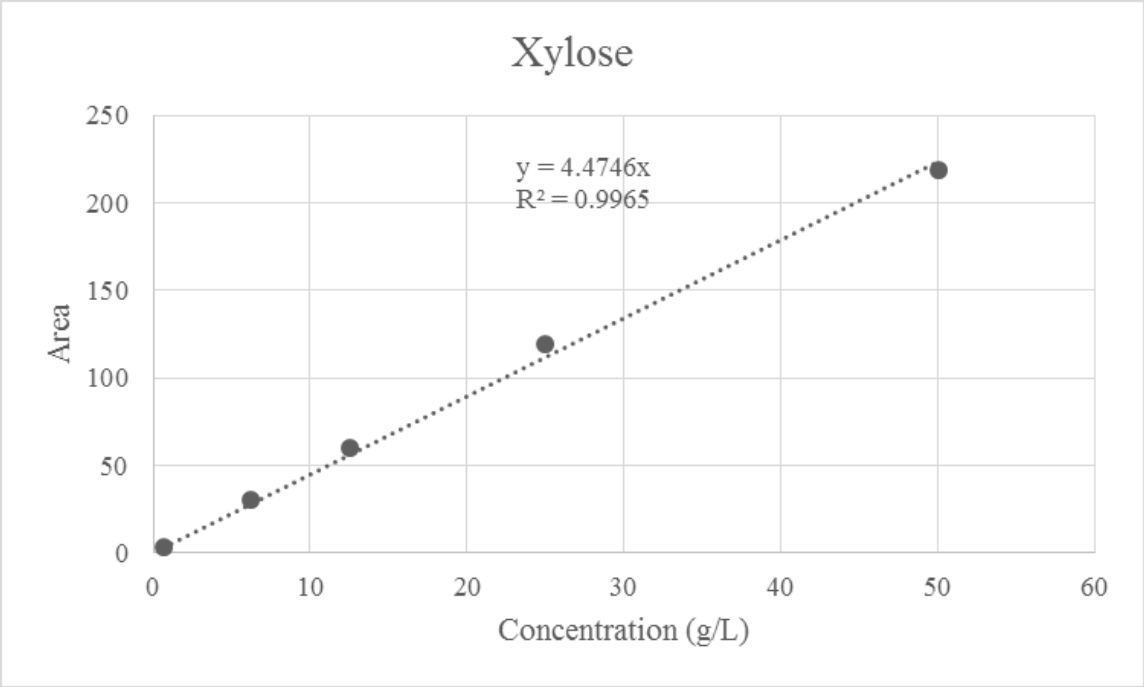

Figure A-17: Xylose standard curve for HPLC-RI analysis. Xylose was separated using a Biorad HP-aminex column, using sonicated 5 mM H_2SO_4 as the mobile phase. Samples were injected in volumes of 25 µL. The mobile phase was set at a constant flow of 0.6 mL/minute, and the column oven was set to 60 ºC. Xylose was analyzed using a refractive index detector.

Regression Statistics	
Multiple R	0.998819206
R Square	0.997639807
Adjusted R Square	0.996853076
Standard Error	4.81142269
Observations	5

Table A-23: Linear regression statistics for xylose standard curve

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	6.517940824	-3.508940824	-0.842115964
2	31.08185632	-0.997156324	-0.23930904
3	58.37509577	1.453704233	0.348876656
4	112.9615747	6.494625345	1.558654865
5	222.1345324	-3.44223243	-0.826106517

Table A-24: Residual outputs for xylose standard curve

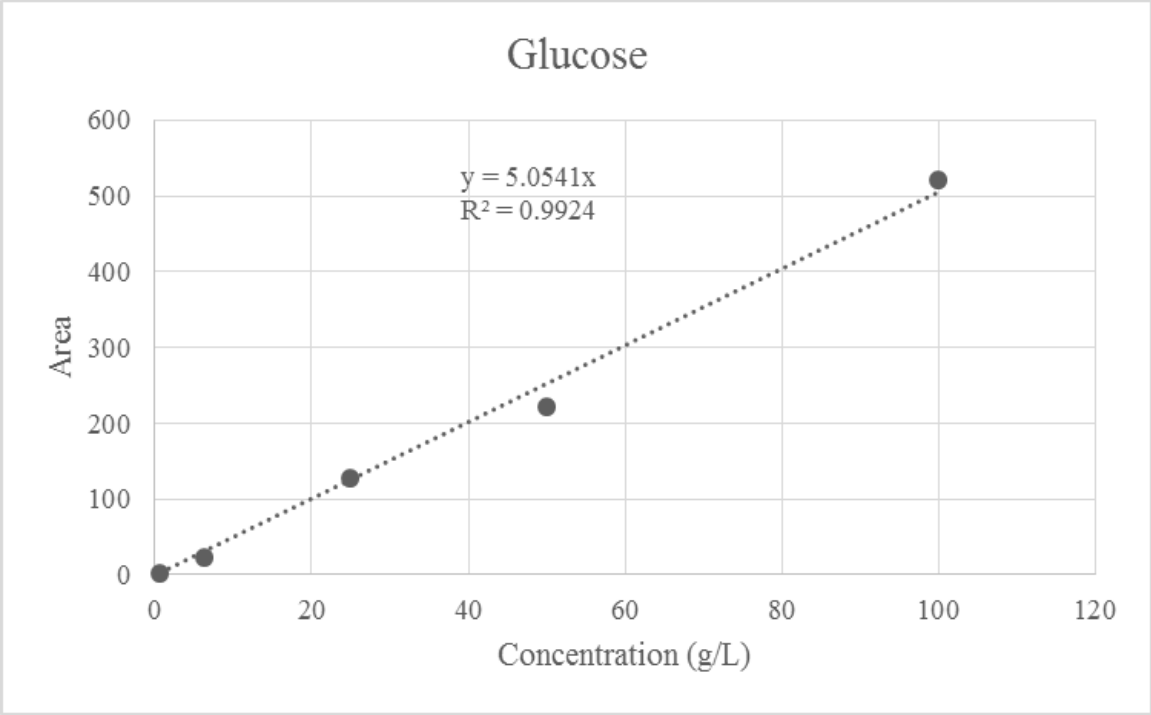

Figure A-18: Glucose standard curve for HPLC-RI analysis. Glucose was separated using a Biorad HP-aminex column, using sonicated 5 mM H_2SO_4 as the mobile phase. Samples were injected in volumes of 25 µL. The mobile phase was set at a constant flow of 0.6 mL/min, and the column oven was set to 60 ºC. Glucose was analyzed using a refractive index detector.

Regression Statistics	
Multiple R	0.996879652
R Square	0.993769042
Adjusted R Square	0.991692055
Standard Error	19.1995941
Observations	5

Table A-25: Linear regression statistics for glucose standard curve

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	-6.565427543	8.871328	0.533538711
2	22.62640092	0.426899	0.025674532
3	119.9324958	6.834304	0.411028208
4	249.6739556	-28.6837	-1.725090249
5	509.1568752	12.55112	0.754848797

Table A-26: Residual outputs for glucose standard curve

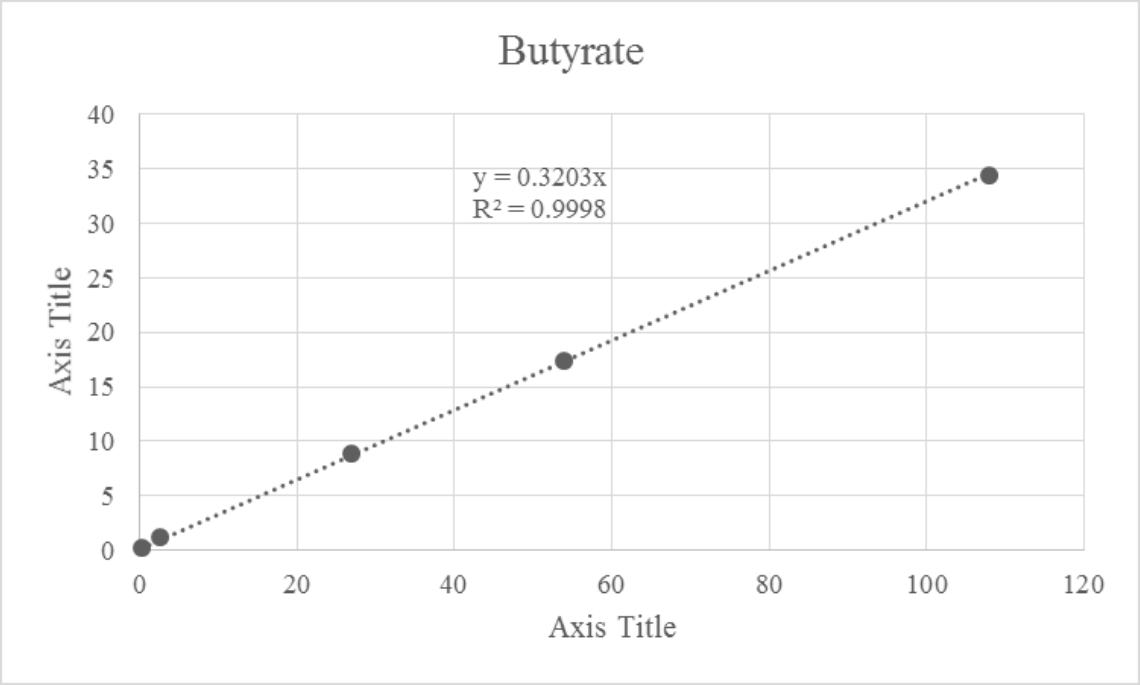

Figure A-19: Butyrate standard curve for HPLC-UV analysis. Butyrate was separated using a Biorad HP-aminex column, using sonicated 5 mM H_2SO_4 as the mobile phase. Samples were injected in volumes of 25 µL. The mobile phase was set at a constant flow of 0.6 mL/min, and the column oven was set to 60 ºC. Butyrate was analyzed with a variable wavelength detector set at 210 nm.

Regression Statistics	
Multiple R	0.999969032
R Square	0.999938065
Adjusted R Square	0.99991742
Standard Error	0.405021097
Observations	5

Table A-27: Linear regression statistics for butyrate standard curve

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	-0.277459336	0.547459336	1.560786817
2	3.026088969	-0.326088969	-0.929667888
3	27.20988999	-0.209889989	-0.598388786
4	54.15058635	-0.150586351	-0.429316254
5	107.860894	0.139105973	0.396586111

Table A-28: Residual outputs for butyrate standard curve

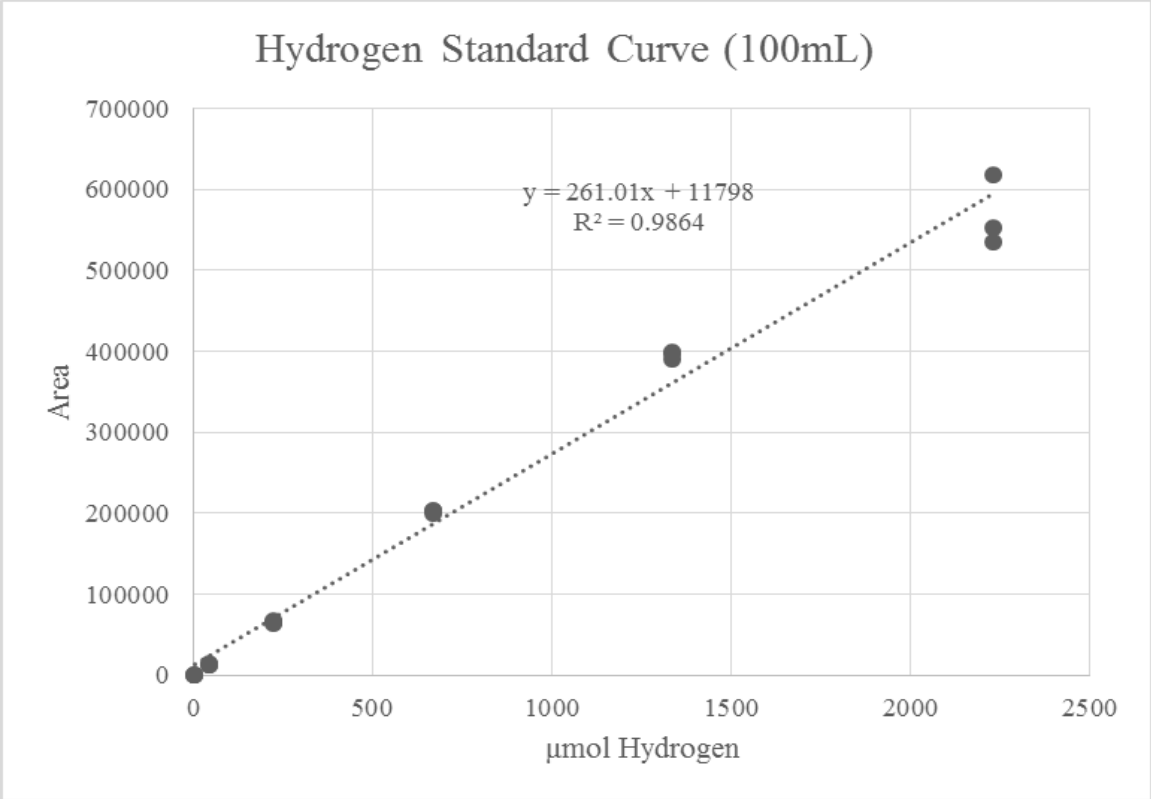

Figure A-20: Hydrogen standard curve (160 mL bottles). Hydrogen headspace was analyzed using a Shimadzu GC-8A equipped with an internal TCD and a 100/120 Carbosieve SII column (10' length x 1/8" outer diameter). Nitrogen was the carrier gas at a constant pressure of 400 kPa . The TCD voltage was set at 60V; the column temperature was set at 50 °C, and the injector and detector temperatures were both set at 150 °C. A VICI gas-tight syringe was used to deliver a 0.5 mL injection volume for each sample analyzed. Total hydrogen present in both liquid and gas phases were quantified using the dimensionless Henry's constant for hydrogen at 25 °C (0.01907).

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	12972.59296	-12407.89296	-0.489498307
2	12972.59296	-12098.39296	-0.477288359
3	12972.59296	-12070.09296	-0.476171908
4	23439.08998	-9881.989975	-0.389850024
5	23439.08998	-9645.789975	-0.380531802
6	23439.08998	-9529.089975	-0.375927922
7	70003.25625	-4878.856246	-0.192473604
8	70003.25625	-5089.456246	-0.200781891
9	70003.25625	-2192.656246	-0.086501514
10	186413.6719	12727.32808	0.502100201
11	186413.6719	17832.12808	0.703487412
12	186413.6719	17919.52808	0.706935391
13	361029.2954	36523.90456	1.440888434
14	361029.2954	38552.20456	1.520906002
15	361029.2954	29893.60456	1.179319396
16	593850.1268	-41198.32679	-1.625297001
17	593850.1268	-58063.72679	-2.290646451
18	593850.1268	23607.57321	0.931331948

Table A-29: Residual outputs for hydrogen standard curve (160mL bottles)

Regression Statistics	
Multiple R	0.993172263
R Square	0.986391143
Adjusted R Square	0.98554059

Table A-30: Linear regression statistics for hydrogen standard curve (160mL bottles)

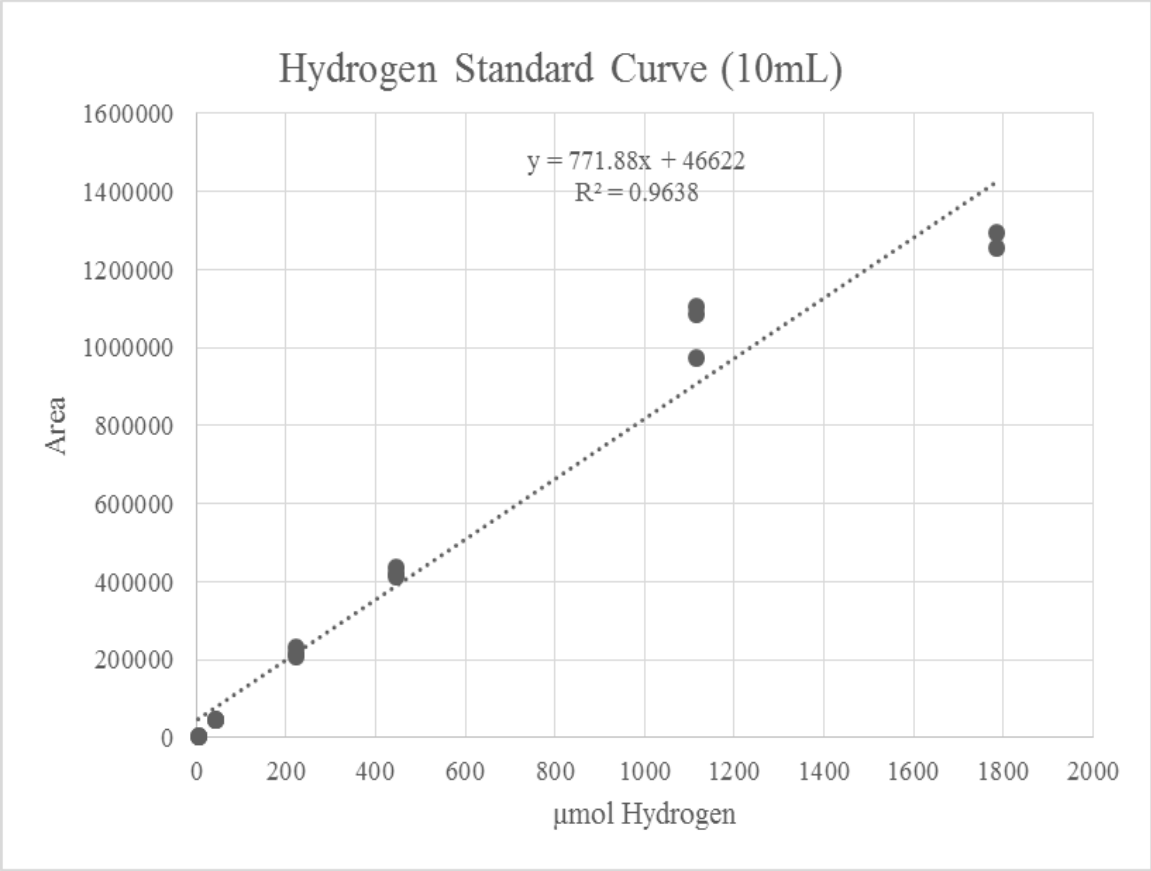

Figure A-21: Hydrogen standard curve (10 mL tubes). Hydrogen headspace was analyzed using a Shimadzu GC-8A equipped with an internal TCD and a 100/120 Carbosieve SII column (10' length x 1/8" outer diameter). Nitrogen was the carrier gas at a constant pressure of 400 kPa . The TCD voltage was set at 60V; the column temperature was set at 50 ºC, and the injector and detector temperatures were both set at 150 ºC. A VICI gas-tight syringe was used to deliver a 0.5 mL injection volume for each sample analyzed. Total hydrogen present in both liquid and gas phases were quantified using the dimensionless Henry's constant for hydrogen at 25 ºC (0.01907).

Observation	*Predicted Y*	*Residuals*	*Standard Residuals*
1	50095.33264	-45286.13264	-0.493996657
2	50095.33264	-44971.63264	-0.490565983
3	50095.33264	-45506.93264	-0.496405219
4	81047.7235	-34260.9235	-0.373729897
5	81047.7235	-34919.6235	-0.380915222
6	81047.7235	-36010.8235	-0.392818406
7	218751.1282	11859.97178	0.129372637
8	218751.1282	-4297.628219	-0.046880002
9	218751.1282	-11092.82822	-0.121004372
10	390880.3841	21199.91588	0.231255949
11	390880.3841	32024.31588	0.349332214
12	390880.3841	46632.41588	0.508682376
13	907268.1518	179383.0482	1.956771773
14	907268.1518	196813.8482	2.146912914
15	907268.1518	66951.14817	0.730326072
16	1423655.92	-168546.8195	-1.838566477
17	1423655.92	-129971.3195	-1.417771702

Table A-31: Residual outputs for hydrogen standard curve (10 mL tubes)

Regression Statistics	
Multiple R	0.981739106
R Square	0.963811671
Adjusted R Square	0.961399116

Table A-32: Linear regression statistics for hydrogen standard curve (10 mL tubes)

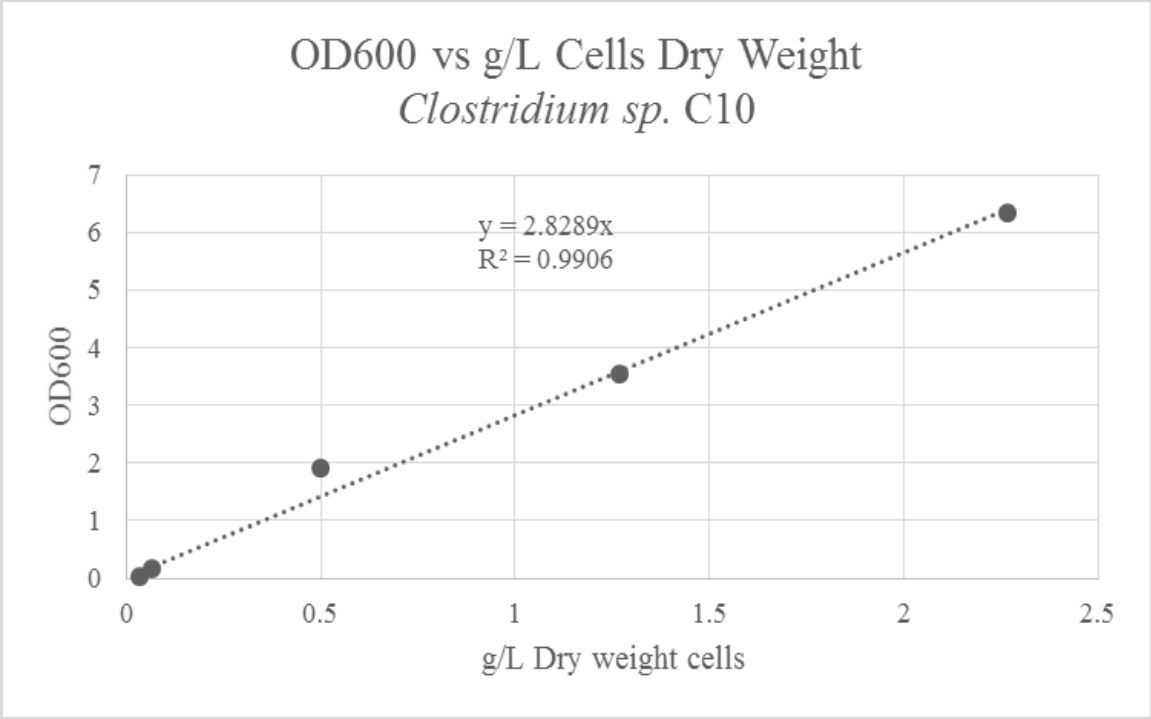

Figure A-22: Optical density vs. cell dry weight correlation curve for *Clostridium sp.* C10

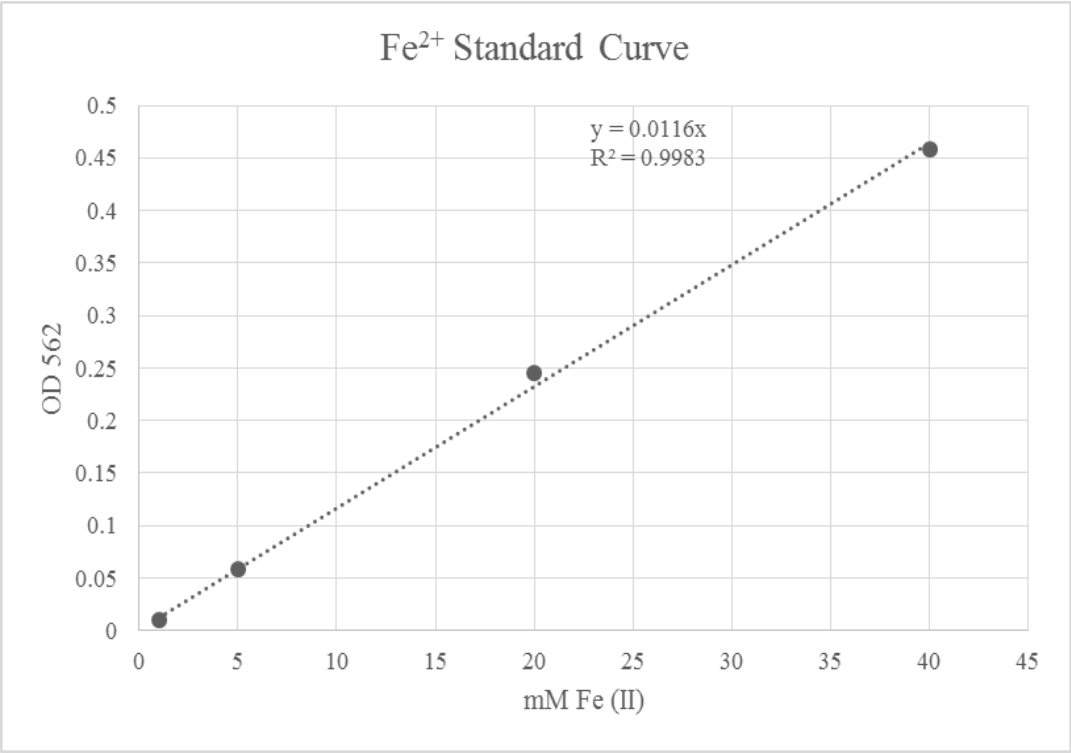

Figure A-23: Ferrous iron standard curve as determined by the spectrophotometric ferrozine assay. Ferrous sulfate standards were prepared anaerobically and added (0.1 mL) to 4.9 mL 0.5 N HCl. The iron-HCl digestate was added (0.1 mL) to 4.9 mL of room temperature ferrozine solution and analyzed immediately after mixing.