BUTYRIC ACID PRODUCTION FROM RENEWABLE RESOURCES

By

Adam Marschall Jaros

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Food Science – Doctor of Philosophy

2014

ABSTRACT

BUTYRIC ACID PRODUCTION FROM RENEWABLE RESOURCES

By

Adam Marschall Jaros

Butyric acid is globally marketed with uses ranging from flavor additive to cattle feed enhancer to chemical feedstock for construction materials. Naturally found in milks, butter and numerous cheeses, butyric acid imparts a buttery flavor to food products. The manufacture and sale of butyric acid as a food flavoring for human consumption is subject to market demand for all-natural products. The "all-natural" labeling requirements preclude the use of synthetic or petro-chemical sourced compounds as a component of the product. In order for producers to cater to the demand for all-natural products alternative methods for generating butyric acid must be developed and evaluated.

The fermentation production of butyric acid is an all-natural method utilizing the anaerobic bacteria *Clostridium tyrobutyricum* from hydrolyzed lignocellulosic biomass, a renewable resource. The overall hypothesis of this dissertation is that real-world lignocellulosic hydrolysates are feasible fermentation substrates for generating butyric acid and challenges from using lignocellulosics, such as acetate inhibition, can be overcome.

Hydrolysis of hemicellulose, a component of lignocellulose, through hot water extraction provides a low-cost source of xylose for the fermentation production of butyric acid but also yields acetate, a microbial inhibitor. This dissertation provides a characterization of the effects of acetate on the fermentation production of butyrate (dissociated form of butyric acid at fermentation pH) and reveals that the lowered productivity is due to an extended lag-phase in bacterial growth. However, once the acetate induced lag-phase is overcome, acetate (26.3 g/L)

challenged batch fermentations of *C. tyrobutyricum* demonstrate higher butyrate yields (12.6%) owing to an acetate re-uptake metabolic mechanism.

Selective adaptation of *C. tyrobutyricum* cultures to the presence of 26.3 g/L acetate developed tolerant cultures with high butyrate productivity rates compared with non-adapted *C. tyrobutyricum* cultures under comparable batch conditions. Enzyme activity for the metabolic enzyme acetate kinase (responsible for acetate production) was evaluated and reduced activity was observed equally in both acetate tolerant and control *C. tyrobutyricum* in the presence of acetate.

Lignocellulosic hydrolysate material having undergone pretreatments of either ammonia-fiber expansion (AFEX), alkaline hydrogen peroxide (AHP) or extracted ammonia-fiber expansion (E-AFEX) was obtained for fermentation trials on *C. tyrobutyricum* and all were adequate as fermentation substrates. A 1-liter batch fermentation of AFEX hydrolysate was conducted and similar sugar consumption and butyrate production was observed compared with a control batch. The *C. tyrobutyricum* culture fermenting AFEX hydrolysate experienced half the specific growth rate during log-phase growth than the control batch. Taken together, these results indicate that renewable lignocellulosic biomass is a feasible fermentation substrate for the fermentation production of butyric acid.

ACKNOWLEDGEMENTS

Foremost, I would like to thank my Ph.D advisor Professor Kris A Berglund for accepting me into his lab and providing me with the opportunity to undertake graduate research in both the Department of Food Science and Human Nutrition at Michigan State University and the Division of Sustainable Process Engineering at Luleå Technical University. Completing these programs has been a lot of hard work but Dr. Berglund has been an inspiration of ideas and research concepts the entire time. I thank him for his confidence in my work and motivation to follow through on my research.

I would also like to thank my dissertation guidance committee members Dr. Strasburg, Dr. Linz and Dr. Hodge. All three have been there for me over the past several years to provide guidance and constructively challenge me.

I appreciate the time I have spent with my lab-mates from the Berglund lab including Dr. Tim Petrik and John Jeffery. Conversations with the two of them were always inspiring and led to some interesting experimentation, especially in beverage distillation. Thanks also to fellow laboratory colleagues Jacob Rochte for always covering for me at the distillery and Stephen Peabody for tirelessly assisting with lab work.

I thank my parents, Squire Jaros and Dr. Christel Marschall who have always supported me to follow my dreams and instilled the wisdom in me to pursue my education. I love you both and appreciate the opportunities you have granted me in life.

I am grateful for my supportive friends, especially John Baer III, Nick and Lauren Noel, Christopher Singleton, Erica Clark, Dr. Lisa Harlow, Dr. Brenna Flannery and Annie Ledbury are always there for me when I need support and I am thankful for it.

TABLE OF CONTENTS

LIST OF TABLES……ix

LIST OF FIGURES……...x

INTRODUCTION……...1
Overall Hypothesis…….....1
Chapter Summaries……..……..……3
REFERENCES ……..……..10

CHAPTER 1: LITERATURE REVIEW……...12
Motivation……..……..12
Biorefinery……..…… 14
Lignocellulosic Chemistry and Pretreatments……..…...17
Microbial Inhibition……..……...20
Clostridial Fermentation……..……23
Production of Butyric Acid by *Clostridia tyrobutyricum* Fermentation……..……24
REFERENCES…….……..…..29

CHAPTER 2: THE EFFECT OF ACETATE ON THE FERMENTATION PRODUCTION OF BUTYRATE……...36
Abstract……36
Introduction……..37
Experimental: Bacterial Strain, Media and Growth……39
Fermentations……...40
Analytical Methods……..41
Cell Biomass Determination……41
Results and Discussion……41
Acetate Re-Utilization……48
Conclusion……...51
Acknowledgements……..52
APPENDIX……..……53
REFERENCES……60

CHAPTER 3: ACETATE ADAPTATION OF CLOSTRIDIA TYROBUTYRICUMFOR IMPROVED FERMENTATION PRODUCTION OFBUTYRATE….....63
Abstract……63
Introduction…….....64
Methods……68
Microorganism and Adaptation……...68
Fermentations……...69
Analytical Methods…….……70
Dry Cellular Weight Determination……..……...……71
Specific Growth Rate (μnet)...……71

Acetate Kinase Assay……..71
Results……..72
Fermentation Kinetics……..72
Acetate Kinase Activity……...79
Discussion……81
Conclusion……...85
Acknowledgements……..86
APPENDIX……..……87
REFERENCES…….…..…100

CHAPTER 4: BUTYRATE FERMENTATION PRODUCTION FROM PLANT BIOMASS HYDROLYSATE.....…..103
Introduction……103
Materials and Methods……...108
AFEX-CS……...108
E-AFEX-CS……...109
AHP-CS…….....109
Specific Growth Rate (μ_{net})……...110
Fermentations……110
Analytical Methods……111
Results and Discussion……..111
Conclusions……122
APPENDIX……124
REFERENCES……..127

CHAPTER 5: ADAPTATION TO SUBSTRATE INHIBITION FROM HIGH INITIAL GLUCOSE CONCENTRATION……..……..…130
Introduction……130
Methods……..134
Adaptation Selection……..134
Fermentations……135
Analytical Methods……135
Dry Cellular Weight Determination……...136
Specific Growth Rate (μ_{net})……...136
Results and Discussion……..136
Conclusions……139
APPENDIX……140
REFERENCES……..144

CHAPTER 6: THE APPLICATION OF INDUCED WHOLE-CELL *PICHIA PASTORIS* IN A NATURAL BIOCONVERSION OF BUTANOL TO BUTYRALDEHYDE…….....146
Abstract……..147
Introduction……147
Background……148
The Whole-Cell Bioconversion System……...……..149
Materials and Methods……..……151
Preparation of Induced *Pichia pastoris* Cells……..……..……151

Analytical Methods……153
AOX Assay……153
Catalase Assay……...…154
Results and Discussion……154
Conclusion……163
REFERENCES ...……..165

CHAPTER 7: CONCLUSIONS……..….168

LIST OF TABLES

Table 1. Effect of acetate inhibition on butyrate, acetate and biomass production in *C. tyrobutyricum* cultures……47

Table 2. Raw data used to generate Figures 4 and 5 in Chapter 2.....54

Table 3. Raw data used to generate Figure 6 in Chapter 2. OD, optical density. DCW, dry cellular weight...57

Table 4. Fermentation kinetics of *C. tyrobutyricum* cultures run in batch with or without selection for acetate tolerance and with or without acetate inhibition 75

Table 5. The effect of acetate inhibition on butyric acid yield in batch fermentations of *C. tyrobutyricum* with an initial 60 g/L glucose or xylose and run until completion.....79

Table 6. The impact of the presence of acetate on enzymatic Acetate Kinase activity in *C. tyrobutyricum* fermentations……80

Table 7. Raw data used to generate Figure 7a-b in Chapter 3…88

Table 8. Raw data used to generate Figures 7c in Chapter 3. OD, optical density. DCW, dry cellular weight…91

Table 9. Raw data used to generate Figure 8a-b in Chapter 3……94

Table 10. Raw data used to generate Figures 8c in Chapter 3……97

Table 11. Raw data used to generate Figures 13, 14 and 15a-c in Chapter 4. Table 11 includes OD and DCW data mentioned but not shown in the text. OD, optical density. DCW, dry cellular weight. ……125

Table 12. Raw data used to generate Figures 16a-c in Chapter 5……141

Table 13. Summarization of data on Butyraldehyde production from 4 separate bioconversions……162

LIST OF FIGURES

Figure 1. *n*-butanol, *n*-butyraldehyde and butyric acid……...12

Figure 2. Schematic of the metabolic production of butyric acid in *Clostridia.* Adapted from Zhang *et al* (2009) (12).....25

Figure 3. Schematic of xylose metabolism and production of butyric acid in *Clostridia*. Adapted from Zhu and Yang (2004) (51).....26

Figure 4. Effect of acetate inhibition on xylose consumption in *C. tyrobutyricum* batch fermentation system. Fermentations performed as 1-liter batches in 2.5-liter working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$. Ten-mL samples were withdrawn from the fermentation and analyzed by HPLC...……43

Figure 5. Effect of acetate inhibition on butyrate production in *C. tyrobutyricum* batch fermentation system. Fermentations performed as 1-liter batches in 2.5-liter working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$. Ten-mL samples were withdrawn from the fermentation and analyzed by HPLC……...……44

Figure 6. Effect of acetate inhibition on cell biomass production in *C. tyrobutyricum* batch fermentation system. Fermentations performed as 1-liter batches in 2.5-liter working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$. Ten-mL samples were withdrawn from the fermentation and analyzed for OD by spectrophotometer at a wavelength of 600nm…….……45

Figure 7-a,b,c. Effect of acetate inhibition on xylose consumption, butyrate production and biomass generation in *C. tyrobutyricum* fermentations using non-adapted or acetate-adapted cultures. Fermentations performed as 1 L batches in 2.5 L working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$ and except for the control 26.3 g/L acetate……....73

Figure 8-a,b,c. Effect of acetate inhibition on glucose consumption, butyrate production and biomass generation in *C. tyrobutyricum* fermentations using non-adapted or acetate adapted cultures. Fermentations performed as 1 L batches in 2.5 L working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at

6.0 with 5M NaOH. The media contained 60 g/L glucose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$ and except for the control 26.3 g/L acetate……..77

Figure 9. Effect of acetate inhibition on relative acetic acid fermentation kinetics of *C. tyrobutyricum* xylose batches. Fermentations performed as described in Fig. 7……..…81

Figure 10a-b. *Clostridia tyrobutyricum* fermentation trials of pH adjusted AFEX-CS hydrolysate. **a)** Treatment 2 is AFEX-CS hydrolysate pH adjusted to 7.5 with 5M NaOH. **b)** Treatment 3 is AFEX-CS hydrolysate pH adjusted to 7.5 with 5M NaOH and further diluted 2x with ddH_2O. Batch fermentations were performed in sterile nitrogen sparged serum bottles at 50ml volume, 5 ml *C. tyrobutyricum* inoculums, 36°C and 50 rpm shaking…….113

Figure 11. *Clostridia tyrobutyricum* fermentation trial of pH adjusted E-AFEX-CS Hydrolysate. E-AFEX-CS hydrolysate batch fermentation was performed with 50 ml working volume in a nitrogen sparged serum bottle kept at 36°C with 100 rpm shaking and inoculated with 5ml of a *C. tyrobutyricum* culture $OD_{600nm} > 2$..……..115

Figure 12a-b. Dextrose consumption and butyric acid production for *Clostridia tyrobutyricum* fermentation of Alkaline Hydrogen Peroxide pretreated corn-stover. Treatment 2 is pure hydrolysate and Treatment 5 is hydrolysate diluted 2x (ddH_2O). Hydrolysate was passed through a sterile filter (0.22 µm) prior to pH adjustment. Both treatments were initially pH adjusted to 7.5 with 5M NaOH. Fermentations were performed in sterile nitrogen sparged serum bottles at 50 ml volume, 5 ml *C. tyrobutyricum* inoculum, 36°C and 50 rpm shaking……..….117

Figure 13. Fermentation kinetics for *Clostria tyrobutyricum* fermentation of AFEX pre-treated corn stover hydrolysate. Batch was performed at 1L working volume under anaerobic conditions with 250 rpm agitation, 36 °C temperature control and pH 6.0 maintained with 5M NaOH. Component analysis was performed by HPLC on an Aminex 87H column……...118

Figure 14. Fermentation kinetics for *Clostria tyrobutyricum* fermentation of diauxic control media containing 56.151 g/L dextrose, 40.16 g/L xylose and 6 g/L yeast extract. Batch was performed at 1L working volume under anaerobic conditions with 250 rpm agitation, 36 °C temperature control and pH 6.0 maintained with 5M NaOH. Component analysis was performed by HPLC on an Aminex 87H column.…….119

Figure 15a-c. Kinetics for *Clostria tyrobutyricum* fermentation of AFEX pre-treated corn stover hydrolysate and a comparable control diauxic-fermentation (containing 6 g/L yeast extract). Each batch was performed at 1L working volume under anaerobic conditions with 250 rpm agitation, 36 °C temperature control and pH 6.0 maintained with 5M NaOH. Component analysis was performed by HPLC on an Aminex 87H column…….120

Figure 16a-c. *Clostridia tyrobutyricum* fermentation of control media (150 g/L glucose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$) at a 1 liter volume with pH controlled at 6.0 with 5M NaOH, 250 rpm agitation and a controlled temperature of 36°C. The selectively adapted batch was inoculated with 50ml of a 150 g/L glucose adapted culture of *C. tyrobutyricum* and

the control batch inoculated with 50ml of a standard culture. **a.** Dry Cellular Weight (DCW) **b.**Butyric acid production. **c.** glucose consumption……..…….**.....**137

Figure 17. Butanol to Butyraldehyde Bioconversion using a whole-cell system. Induced *P. pastoris* 1 liter bioconversion took place at pH 7.25 to 7.4 using a 0.1 M potassium phosphate buffer and a 10 L/ min 75:25 air:O_2 sparging stream. The definition of a unit of AOX activity is that one unit will oxidize 1.0 µmole of methanol to formaldehyde per minute at pH 7.5 at 25°C. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation……155

Figure 18. Butanol to Butyraldehyde Bioconversion using a continuous cell lysing system. Induced *P. pastoris* 1L bioconversion took place in pH 6.5 potassium phosphate buffer using a 10 L/min 50:50 air:O_2 sparging system. Cell-extraction and catalase assay described in the methods section. The definition of a unit of catalase activity is the decomposition of 1.0 µmole of H_2O_2 to oxygen and water per minute at pH 7.0 at 25°C with a substrate composition of 50 mM H_2O_2……...…..157

Figure 19. Effect of 0.5M Tris-Borate buffer binding butyraldehyde product during bioconversion. The 0.5 M Tris-Borate buffer maintained the pH at 8.4 during induced *P. pastoris* 1L bioconversion using a 10 L min^{-1} 50:50 air:O_2 sparging system. The definition of a unit of AOX activity is that one unit will oxidize 1.0 µmole of methanol to formaldehyde per minute at pH 7.5 at 25°C..……..….159

Figure 20. Butanol to Buytraldehyde bioconversion pressurized to 8psi with pure O_2. Induced *P.pastoris* 1L bioconversion. This batch has no sparging occurring and is run with a 2M Tris-Borate buffer keeping the pH at 8.3. Agitation at 900 rpm. The definition of a unit of AOX activity is that one unit will oxidize 1.0 µmole of methanol to formaldehyde per minute at pH 7.5 at 25°C…….…….**....**160

INTRODUCTION

Overall Hypothesis

Green chemical fermentation production offers a sustainable alternative to petro-chemical synthesis yet substantial research into this field is required for the development of economically feasible processes. Fermentable xylose from a hot water extracted hemicellulose pulp and paper waste stream is an economical and sustainable carbon source but challenges subsequent fermentation production with the microbial inhibitor acetate. The objective of this dissertation is to characterize previously unexamined levels of acetate inhibition on bacterial fermentation and develop tolerant bacterial cultures to increase butyrate yields. The guiding hypothesis of this research is that butyrate fermentation yields can be increased through the presence of the acetate inhibitor allowing for the use of agricultural waste streams as substrate.

The hypothesis is examined in this dissertation through three specific aims beginning with a thorough characterization of the impact of acetate on fermentation. Through selective adaptation of the fermenting organism *Clostridium tyrobutyricum*, the inhibition of butyrate production by acetate present in the media can be overcome and lead to higher yields. The mechanism of acetate microbial inhibition is understood to be pH independent and will be examined here in the specific case of the inhibition on the enzymes involved with the *C. tyrobutyricum* metabolic pathway.

The feasibility of farm raised plant biomass residues as a substrate for *C. tyrobutyricum* fermentation production of butyrate will be demonstrated. *C. tyrobutyricum* cultures will be

adapted to overcome further technical aspects of plant biomass substrates such as high initial sugar content resulting in inhibitive high osmotic pressure.

Chapter Summaries

Chapter 2 examines the effects of acetate on the fermentation production of butyrate by the anaerobic bacteria *Clostridium tyrobutyricum*. *C. tyrobutyricum* consumes both five and six carbon monomeric sugars and unlike other members of the genus, does not generate solvent metabolic by-products, thus increasing the carbon efficiency of butyrate production (1, 2). Butyrate is the main metabolic product of *C. tyrobutyricum* fermentation with acetate and lactate being lesser produced co-products (2). Developing a natural process for butyrate production is important, as many of the applications associated with either butyrate or butyric acid derivatives are related to the food and flavor industry with strong consumer sentiment against petro-chemical synthesized materials.

An economic and renewable source of fermentable xylose comes from the hot water extraction of the hemicellulose waste stream of the pulp and paper industry. Depending on processing, hardwood derived xylose is found in the hydrolysate in concentrations of up to 70 g/L (3). A concern is that the auto-hydrolysis of this low-cost substrate generates high levels of acetate in the range of 40 g/L, inhibitory to subsequent microbial fermentations (3). The work in Chapter 2 focuses on the impact of high levels of acetate on *C. tyrobutyricum* fermentation in order to develop a process for using xylose extracted from hemicellulose as a fermentative substrate without requiring the removal of acetate from the feedstock.

The studies performed challenged *C. tyrobutyricum* batch fermentations with acetate in order to characterize the effects of acetate inhibition on lag and log phase culture growth, butyrate and acetate production rates as well as final yields. These effects were demonstrated on 1-liter batch fermentations of minimal media with inoculums of *C. tyrobutyricum* conditioned to either glucose or xylose consumption. The inoculum culture had to be conditioned to one

specific carbon source for fermentation as *C. tyrobutyricum* exhibits diauxic growth (4). The presence of a more utilizable carbon source, in this case glucose, represses the metabolic activities associated with the consumption of the lesser utilizable carbon, xylose. As the purpose of this study was to examine the effects of acetate inhibition and metabolic switching would have generated inhibition, each culture was exposed to only one sole carbon source for the duration of the fermentations.

1-liter batch fermentations were performed with 950 mL minimal media (6 g/L yeast extract, 5 ppm $FeSO_4$ 7 H_2O, and 200 mL xylose or glucose at 300 g/L autoclaved separately and added later) and variable amounts of acetate equivalents in the form of sodium acetate (0, 4.4, 8.8, 17.6 or 26.3 g/L). Log phase 50 mL inoculumn *C. tyrobutyricum* cultures conditioned to either xylose or glucose were used once the optical density of the inoculum reached an absorbance of 2.0 at 600 nm. Batches were run at 36°C, 250 rpm agitation with pH adjusted to 6.0 with automatic addition of 5 M NaOH. Anaerobiosis was achieved by sparging the vessel with nitrogen gas. Samples were withdrawn aseptically and analyzed by HPLC for sugar and acid quantities as well as optical density for dry cellular weight determination.

The most obvious effect of the initial acetate inhibition was an extended lag phase of the culture, preventing sugar consumption and butyrate production. Xylose consuming batches demonstrated significant lag phase extensions when challenged with 17.6 and 26.3 g/L acetate (Table 1). The 17.6 g/L acetate challenged culture required 45 hours to adapt to the inhibitory level of acetate and begin log phase growth while the 26.3 g/L challenged culture required 118 hours. These extended lag phase periods is also reflected in the delay of sugar consumption and butyrate production in these batches. Of note is the significant drop in the overall production rate of butyrate in the 26.3 g/L acetate challenged culture (Table 1). The control xylose consuming

culture generated butyrate at 0.23 g/L/h and the 26.3 g/L challenged culture only generated 0.14 g/L/h.

Glucose consuming cultures challenged with lower concentrations of acetate (i.e., 4.4 and 8.8 g/L) were less effected by the inhibition and demonstrated no lag phase in growth. Glucose batches with higher levels of initial acetate (17.6 and 26.3 g/L) were similar to xylose batches in exhibiting extended lag phases as well as lowered productivity (0.12 g/L/h butyrate with 26.3 g/L initial acetate).

The results of this work generate interest for further studies of adapting or selecting *C. tyrobutyricum* cultures to acetate tolerance. Despite extended lag phases in growth and lowered butyrate production rates, *C. tyrobutyricum* eventually overcame even the highest levels of acetate inhibition and fully utilized all available sugar. Once the cultures had adapted to the challenge they functioned similar to the controls in sugar consumption and then performed exceptionally in terms of butyrate production given the activation of the acetic acid re-uptake mechanism. Acetic acid re-uptake is a metabolic mechanism that converts acetate from the media into butyrate. These factors make *C. tyrobutyricum* an interesting candidate for further adaptation studies as is the focus of Chapter 3**.**

A characterization of the impact of high concentrations of initial acetate on *C. tyrobutyricum* fermentation for butyrate production was carried out in Chapter 2. Chapter 3 continues upon the conclusions reached in Chapter 2 by examining the effects of challenging acetate adapted cultures with high concentrations of acetate. The overall purpose is similar, in that by determining the impacts of acetate on butyrate fermentation, these studies evaluate the

use of xylose extracted from hemicellulose as a fermentative substrate without requiring the removal of acetate from the feedstock.

Currently, hemicellulose streams from the pulp and paper industry are burned for energy; such streams could be hydrolyzed to generate a low-cost source of xylose feedstock for microbial fermentation production. Hot water extraction techniques are an inexpensive method for hydrolyzing xylose monomers from the xylan chains, but such techniques not only yield the fermentable sugar but levels of acetate inhibitory for microbial growth (3). This is due to the auto-hydrolysis of the xylan backbone which occurs during hot-water-extraction increasing the techniques effectiveness as a hydrolysis method but releasing the inhibitor. Hardwood xylan contains up to 7 acetyl groups for every 10 xylose units, facilitating the release of xylose by auto-hydrolysis as well as acetic acid (5).

Results presented in Chapter 2 suggest that while initial acetate concentrations of 17.6 and 26.3 g/L caused extended lag phase growth in *C. tyrobutyricum* cultures and lowered overall butyrate productivity rates, these challenging levels of acetate were eventually overcome by the culture and the fermentations ran to completeness. Due to the acetic acid re-uptake mechanism of *C. tyrobutyricum*, acetate in the media can be recycled into the metabolic pathway for butyrate production by converting the free acetate to acetyl-CoA within the cell and then feeding the butyryl-CoA to the butyrate end-product branch (2, 6). The uptake phenomenon was observed in the previous studies resulting in higher final yields of butyrate in cultures challenged with acetate than controls (Chapter 2).

Previous research has selectively adapted *Clostridial* strains for increased tolerance to butanol and ethanol enhancing the strains fermentative productivity (7, 8). Challenging

organisms with such toxic products complicates the adaptive selection process while adapting a culture for organic acid tolerance requires only simple selection also known as evolutionary engineering. As *C. tyrobutyricum* has no solvent producing metabolic pathways, it is an excellent strain for our work producing butyrate as there is no carbon lost to solvents when fermenting with this strain. *C. tyrobutyricum* has been selectively adapted for organic acid tolerance utilizing an immobilized fibrous-bed bio-reactor leading to increases in butyrate yields (9). The immobilized bed bio-reactor method requires a substantial (72 hours) lag phase of growth similar to the high acetate challenged batch fermentation. Overcoming the extended lag phase in growth is simple for batch fermentations as the inoculation culture can be pre-adapted to acetate tolerance, shortening the lag phase significantly.

The metabolic pathways of butyrate, acetate and lactate production in *Clostridia* have been fully elucidated allowing for an examination of enzymatic activities to understand factors influencing butyric acid production. Glucose enters the Embden-Meyerhof-Parnas pathway generating pyruvate while xylose is catabolised to pyruvate in the Hexose Monophosphate pathway (10, 11). Pyruvate is co-oxidized with cellular CoA to acetyl-CoA, the branch point node of the acetate and butyrate end-product pathways (11, 12). Acetate kinase is the final enzyme in the metabolic pathway responsible for acetate production. The activity of this enzyme was examined in acetate challenged versus control batch fermentations. The data indicate that acetate kinase is inhibited by the presence of acetate whether or not the culture has been adapted for acetate tolerance.

This study demonstrated that an evolutionarily engineered culture of *C. tyrobutyricum* adapted for acetate tolerance was able to drastically reduce the lag growth phase caused by an initial concentration of 26.3 g/L acetate during batch fermentation. The use of an adapted culture

increased the overall productivity of butyrate production compared to control cultures challenged with acetate. In xylose batch fermentations, external acetate is ultimately consumed and converted to butyrate, further increasing the yield of the fermentation.

In chapter 4, farm-raised corn stover (agricultural waste) undergoing several types of common lignocellulosic pretreatment methods were utilized as a substrate for *C. tyrobutyricum* fermentation. The pretreatment methods employed for processing the corn stover were ammonia-fiber expansion (AFEX), alkaline hydrogen peroxide (AHP) treatment and extracted ammonia-fiber expansion (E-AFEX).

The lignin fraction of lignocellulosic material can block enzymatic saccharification through physically blocking the cellulose and hemicellulose enzyme targets as well as binding structural carbon in ester linkages. Pre-treatment methods chemically and physically degrade ligin and lignin-carbohydrate complexes (LCCs) prior to enzymatic hydrolysis thus increasing sugar availability for fermentation. AFEX is the exposure of plant biomass to ammonia during heating and pressurization with subsequent de-pressurization in order to expand lignocellulosic fibers. AHP is the loading of plant biomass with H_2O_2 and sodium hydroxide (NaOH) to break down covalent lignin bonds with subsequent acidification for enzymatic hydrolysis. E-AFEX ™ is similar to the AFEX process with an extraction step involving the lignin removal from the plant biomass prior to ammonia fiber expansion.

Batch fermentations using the above mentioned hydrolysates were conducted with *C. tyrobutyricum* with analysis of sugar consumption and butyrate production along with monitoring of specific growth rates on the different media. Ultimately, AFEX, AHP and E-AFEX ™ hydrolysates were all observed to be usable substrates for *C. tyrobutyricum* based on

the criteria that the bacteria demonstrated growth on all three with the concomitant generation of butyrate. Given these results, pH-controlled batch fermentation was performed with AFEX-CS material and a comparative synthetic fermentation. The pH-controlled synthetic fermentation resulted in higher final butyrate yields and specific growth rates than the AFEX fermentation yet overall the batches were remarkably similar.

Chapter 5 examines the effects of high osmotic pressure from lignocellulosic hydrolysate on *C. tyrobutyricum* fermentation. Lignocellulosic hydrolysates often yield free sugar concentrations greater than 100 g/L, inhibitory to bacterial growth. Chapter 5 presents the comparative study of adapting a *C. tyrobutyricum* culture to growth on a media containing 150 g/L glucose. While the adapted culture begins log phase growth several hours sooner than a control culture, the two cultures demonstrated equivalent specific growth rates and butyrate yields.

REFERENCES

REFERENCES

1. Wiegel, J., Tanner, R., and Rainey, F. A. (2006) An Introduction to the Family Clostridiaceae. In *The Prokaryotes* (Dworkin, M., ed) Vol. 4 pp. 654-678, Springer, Singapore

2. Zhang, C., Yang, H., Yang, F., and Ma, Y. (2009) Current Progress on Butyric Acid Production by Fermentation. *Current Microbiology* **59**, 656-663

3. Helmerius, J., Walter, J. V. v., Rova, U., Berglund, K. A., and Hodge, D. B. (2010) Impact of hemicellulose pre-extraction for bioconversion on birch Kraft pulp properties. *Bioresource Technology* **101**, 5996-6005

4. Shuler, M. L., and Kargi, F. (2002) How Cells Grow. In *Bioprocess Engineering Basic Concepts* (Amundson, N. R., ed) pp. 155-200, Prentice Hall PTR, Upper Saddle River, New Jersey

5. Teleman, A., Tenkanen, M., Jacobs, A., and Dahlman, O. (2002) Characterization of O-acetyl-(4-O-methylglucurono)xylan isolated from birch and beech. *Carbohydrate Research* **337**, 373-377

6. Michel-Savin, D., Marchal, R., and Vandecasteele, J. P. (1990) Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. *Applied Microbiology and Biotechnology* **32**, 387-392

7. Herrero, A. A., and Gomez, R. F. (1980) Development of Ethanol Tolerance in Clostridium thermocellum: Effect of Growth Temperature. *Applied and Environmental Microbiology* **40**, 571-577

8. Lin, Y.-L., and Blaschek, H. P. (1983) Butanol Production by a Butanol-Tolerant Strain of Clostridium acetobutylicum in Extruded Corn Broth. *Applied and Environmental Microbiology* **45**, 966-973

9. Zhu, Y., and Yang, S.-T. (2003) Adaptation of Clostridium tyrobutyricum for Enhanced Tolerance to Butyric Acid in a Fibrous-Bed Bioreactor. *Biotechnology Progress* **19**, 365-372

10. Liu, X., Zhu, Y., and Yang, S.-T. (2006) Butyric acid and hydrogen production by Clostridium tyrobutyricum ATCC 25755 and mutants. *Enzyme and Microbial Technology* **38**, 521-528

11. Madigan, M. T., Martinko, J. M., Dunlap, P. V., and Clark, D. P. (2009) Metabolic Diversity: Catabolism of Organic Compounds. In *Biology of Microorganisms* (Brock, T. D., ed) pp. 612-651, Pearson Education, San Francisco, CA

12. Zhu, Y., and Yang, S.-T. (2004) Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum. *Journal of Biotechnology* **110**, 143-157

CHAPTER 1: LITERATURE REVIEW

Motivation

Each year, the global chemical industry observes increased market demand for 4-carbon oxychemicals including butyric acid and *n*-butyraldehyde (Fig 1). The increased application of 4-carbon oxychemicals is not only limited to direct use but often for utilization as chemical feedstocks to generate other commercially important compounds (1-3). For example, the period between 1988 and 1991 saw the commercial production of 24,000 tons of butyric and isobutyric acid in the U.S. alone, with most of the material applied to the production of the plasticizer cellulose acetate butyrate (3, 4).

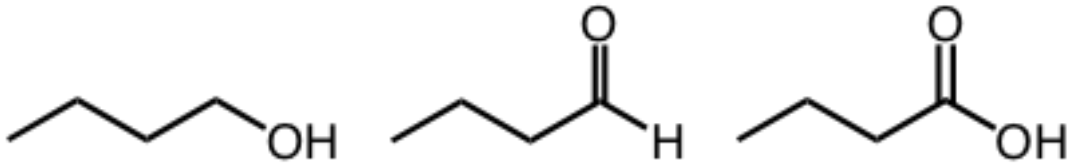


Figure 1. *n*-butanol, *n*-butyraldehyde and butyric acid.

The mainstream commercial production of butyric acid occurs by the air-oxidation of butyraldehyde, synthetically produced from the oxo reaction of propylene, carbon monoxide and hydrogen (1, 3). The oxo reaction, also known as hydroformylation, is the addition of a hydrogen atom and carbon monoxide to the double bond of an alkene with heat and pressure and the aid of a metal catalyst (1, 5). For decades, the industrial process of the oxo reaction has been the predominant method for the production of both *n*-butyraldehyde and the isomer isobutyraldehyde (1). Oxidation of *n*- butyraldehyde is the common method for generating butyric acid (1).

As industrial synthesis of these chemicals increases world-wide, several issues have begun to develop. The foremost is that while the production volume is increasing the originating building block molecule, propylene, is generated from non-renewable sources. The bulk of global propylene feedstock results from the steam-cracking of hydrocarbons in the refining of gas oil by the petro-chemical industry (6). Other sources of propylene exist such as that extracted from natural gas refining and during coke production as coal is carbonized, both sources as non-renewable as petroleum (6). Given that commercial synthesis of butyric acid and butyraldehyde and compounds derived from these building blocks is dependent on the oxo reaction of propylene, the production of these chemicals is based on a non-renewable source. Increasing demand for butyric acid and butyraldehyde for developing new products requires that alternative, renewable processes for producing these chemicals be developed.

An issue that butyric acid distributors face is the strong "all-natural" labeling market for food and personal care products, which has generated an incentive to produce flavor and cosmetic ingredients without utilizing petro-chemical based sources or genetically modified organisms. Butyric acid is a natural fermentation product in butter and some cheeses and thus imparts a buttery quality in terms of food use. Butyric acid is approved by the Food and Drug Administration (US) for use as a synthetic flavoring and is specifically listed on the Code of Federal Regulations of substances generally recognized as safe (GRAS) (7). GRAS substances are allowed to be sold to consumers in the US under the Federal Food, Drug and Cosmetics Act (7). Similarly, butyric acid is registered in the European commission database (FLAVIS) of flavor additives usable in or on foodstuffs in the EU (8). The applications of butyric acid in the dairy, food flavoring and pharmaceutical industries are widespread (9). Butyric acid enhances the butter-like notes of food flavors while esters generated from butyric acid include aromatic fruit

fragrances useful for flavoring (9, 10). Due to the useful qualities of butyric acid, the development of natural production from bacterial fermentation has been studied for decades and is still a major concern (9, 11, 12). Concurrently, butyraldehyde is a useful flavor compound as it directly imparts buttery or cheesy characteristics to a product and can be processed into 2-ethyl butyraldehyde found in chocolate and cocoa (13, 14). As such, a market exists for butyric acid and butyraldehyde produced non-synthetically or "all-naturally" requiring that alternative processes be developed to generate these chemicals from non-petro-chemical means.

Biorefinery

The generation of heat and electricity by burning biomass is a common industrial practice and there are also several processes for the manufacture of bio-based chemical building blocks from plant biomass (i.e, starch, cellulose, hemicellulose) (15). Biorefineries are similar to petroleum refineries in that they are able generate a diverse spectrum of products and energy but unlike petroleum refineries, are able to produce these products from biomass rather than petrochemical sources (15). Of the multiple conversion platforms that exist in biorefineries, thermochemical processing transforms biomass through gasification and the subsequent conditioning of break-down products to generate fuels and chemicals (16). Alternatively, biomass can be converted into fuels and chemicals by hydrolysing fermentatable sugars from the material for use as a fermentation substrate.

The research examined herein is based upon conversion of plant material to fermentable sugar through chemical and enzymatic hydrolysis for bio-processing. Depending on the process and the biomass source, different fermentable sugars are released. Starch and cellulose hydrolyse into sucrose, glucose and fructose. Hemicellulose (xylan) is a heteroglycan consisting of a variety of sugars including xylose, mannose, glucose, galactose, arabinose and rhamnose (17).

The type of sugar available determines the fermentation method and resulting chemical product. Fermentation of sucrose, glucose and fructose by *Saccharomyces cerevisiae* is a common industrial means of producing bioethanol (18). Biodiesel, bioethanol and biobutanol are examples of fuels currently being produced industrially in biorefineries both through thermochemical and biochemical means (15). Currently, yeast fermentation of food-grade sugar and enzymatic treated starch is the most common industrial method producing bioethanol (15). While some bioethanol is sold as beverage spirits, most is produced for fuel use reducing reliance on oil imports and fluctuations in the oil market (19, 20). An important feature of bacterial fermentations is that they utilize various sugars including xylose and other pentose sugars which non-modified *S. cerevisiae* is incapable of consuming (18). Thus, bacterial fermentation platforms can generate a wide array of final products ranging from organic acids to long chain alcohols diversifying the final product profile of a bio-process. Historically, chemical and fuel production utilizing *Clostridial* bacterial fermentation has been a major industry at times only second to yeast based ethanol in terms of production capacity (21).

The international forestry industry produced 403 million of tons of pulp and paper in 2011 with long term trends demonstrating production volume increases around 1% annually (22). Industry models predict solid annual increases in global demand for pulp and paper products at least until 2030 (23). With a large amount of wood material being processed, the traditional wood processing mill is in a unique position to branch out into biorefinery production, converting lignocellulosic biomass into fuels and chemicals. On the domestic side, abundant agricultural waste and forest resources provide a sustainable source of lignocellulosic biomass for biorefinery use (24). Lignocellulosic material is the most abundant renewable resource on the planet and is considered a waste stream from both the agricultural and pulp and paper industries

(25). The conversion of this type of biomass to a fermentable substrate of monomeric sugars requires hydrolysis of the tough plant structural carbohydrates (cellulose, hemicellulose and lignin). Typically the cellulose fraction of wood material is utilized in the production of pulp and paper and the leftover hemicelluloses and lignin are burned for energy (26). The alternative to burning the hemicellulose fraction is to convert this fraction into fermentable monomeric sugars either enzymatically or chemically (27). As with chemical production, the cost of fermentation substrate is a critical factor for practical application (28). Converting biorefinery by-products into value added products may further reduce associated process costs (19). Due to the abundance and sustainable sources of lignocellulose, it has the potential to become a cost effective source of fermentation substrate.

The further motivation of butyric acid production from sustainable sources encompasses issues of national dependence on foreign petro-chemical sources. In 2012, the United States Defense Logistics Agency procured and distributed over 22 million barrels of Jet Propulsion Fuel Formulation 8 (JP-8) to US war-fighters with a total value of over $3 billion USD (29). With the U.S. refining industry supplying only 50% of the Department of Defense jet fuel demand, much of the liquid fuels used for national defense originate from non-U.S. sources (30). JP-8, like most kerosene-type fuels, consists primarily of aliphatic alkanes (i.e., decane, dodecane, hexadecane) purified by the fractional distillation of light sweet crude oil (31). Non-petrochemical methods for producing alkanes exist though, namely the Clemmensen reduction of ketones. Heptanone and other ketones can be generated by the ketonization of butyric acid, thus the building blocks of jet fuels can be generated from renewable substrates (32).

Ketonization is considered a green process for chemical production as no polluting by-products are generated in the process (32). 4-heptanone production through ketonization requires

no solvent extraction step and the ketone itself is a useful solvent (32, 33). Ketonization of two butyric acid molecules by manganese and cerium oxide catalysts generates one 4-heptanone molecule, a flavor additive considered fit for human consumption to impart fruit flavors as well as a chemical feedstock for diesel additives. Reports from the World Health Organization and Food and Agricultural Organization (UN) have concluded that 4-heptanone presents no safety concern with regard to use as a flavoring agent (34). The sustainable generation of butyic acid through microbial fermentation and further sustainable chemical processing through ketonization and reduction provides an alternative means of generating solvents, flavors and jet fuels necessary for national security.

Lignocellulosic Chemistry and Pretreatments

Depending on the source of biomass, the dry weight percentage of fermentable carbohydrate ranges from 56 to 72% (35). The main constituent of biomass is cellulose, ranging from 35-48% by dry weight depending on the source (i.e, softwood, hardwood, grass, farm or agricultural waste) (35). Cellulose is a polysaccharide of glucose monomers linked in a chain by β-1, 4 glycosidic linkages. Unlike starch or glycogen which are polysaccharides of glucose in α-1,4 glycosidic linkages, β-1,4 glycosidic bonds are not easily hydrolyzed thus making it significantly more difficult to convert cellulose to fermentable sugars as is done commercially with starch (36). Within plant cells, cellulose forms a tight crystalline structure as microfibrils embedded in the lignin-hemicellulose matrix. While this complex provides the plant cell with a tough rigid structure, it imbues biomass with recalcitrance to hydrolysis.

After cellulose, the second major component of lignocellulosic biomass is hemicellulose, a more complex polysaccharide consisting of a heterogeneous mix of hexose (glucose, galactose, mannose) and pentose (xylose, arabinose) monosaccharides residues (35, 37). Of the

polysaccharides constituting hemicellulose, the main component is xylan, an acetylated xylose heteropolysaccharide consisting of a backbone of β-1,4-linked β-D-xylopyranosyl residues (38). Depending on the source, xylan displays varied branching and backbone compositions, such a structure makes xylan more flexible than cellulose (38). The xylan backbone from grass sources has α-1,3-linked L-arabinofuranosyl residue substitutions and is thus an arabinoxylan (38); however, xylan from wood sources is quite different. Hardwood xylan such as from birch and beech contains a xylan backbone with a 4-O-methylglucuronic acid substituent for every 15 xylose residues and 0.4 to 0.7 degrees of acetylation for every xylose (38, 39). The hardwood xylan acetylation ratio of acetyl:xylose units of 0.7:1, permits the release of xylose in hot water by autohydrolysis (39). Softwood xylan on the other hand has little to no acetylation, limiting the solubility of hemicellulose derived from softwood sources as increased acetylation and side chains leads to increased solubility in water (38, 39).

Lignin-carbohydrate ester/ether linkages within plant cell walls provide a tough structural complex inhibiting enzymatic degradation of hemicellulose and cellulose (37). Biomass from high lignin content plants such as forage and turf grass are especially resistant to saccharification (40). While lignocellulosic research has engaged in genetically altering grasses and agricultural crops to reduce lignin synthesis and increase forage digestibility, these alterations have undesirable morphological effects (40). Structural changes from reduced lignin synthesis that negatively impact the value of the plant include weakened upright growth habit and diminished seed yield (40). Treatments and processes designed to degrade these recalcitrant properties exist and are examined in this work as they do not require genetic and subsequent negative morphological changes of lignocellulosic biomass sources. Multiple biomass pretreatment methods are currently being evaluated for both efficient and cost effective conversion of

cellulose and hemicellulose into saccharifiable residues. Pretreatment methods including the ones described below destroy or degrade lignin-carbohydrate linkages opening up hemicellulose and cellulose to saccharification enzymes, allowing for greater efficiency and activity of enzymatic hydrolysis (41).

Alkaline hydrogen peroxide pretreatment involves adding H_2O_2 (~4 % v/v) to milled biomass and then increasing the pH of the slurry to over 11.5 for a delignification reaction to occur, exposing cellulose and hemicellulose to saccharification enzymes (18, 42, 43). The mechanism behind this method for degrading lignin is that at such a high pH, H_2O_2 dissociates into H^+ and HOO^-. H_2O_2 decomposition products OH and O_2^-·then oxidize lignin, releasing lignin from the lignin-carbohydrate complex and hydrating the cellulose polymer (42). Other pretreatments include dilute sulfuric acid steam treatment and ammonia fiber expansion to increase the efficiency of biomass hydrolysis also by degrading the lignin-hemicellulose ester/ether linkages (44).

Integration of pretreatment and fermentation processes into current industrial operations is imperative for the economic viability of the conversion of sustainable biomass to useful fuels and chemicals. Pretreatment accounts for 30% of the cost associated with processing biomass (35). Auto-hydrolysis or hot water extraction is an alternative and milder process design compared with harsh chemical pretreatment (37, 45). The mechanism behind hot water extraction/autohydrolysis works as the hydronium ions from protonated H_2O depolymerize the hemicellulose chain, releasing bound acetic acid and further depolymerizing the xylan (45). Hot water extraction of wood chips and other biomass solubilizes hemicelluloses and thus allows for the fractionation of hemi-cellulose (xylan) from plant material, leaving a purer fraction of cellulose for industrial processes such as pulp and paper production (37). As hemicellulose is a

heterogeneous polysaccharide, it is more readily hydrolysable into component monosaccharides than cellulose (35). Due to this nature of xylan, autohydrolysis, the simple treatment of lignocellulosic material or a hemicellulose fraction with heat and water, is an appropriate method for hydrolyzing the polysaccharide to shorter xylose chains allowing for enzymatic hydrolysis to xylose monomers (37).

Microbial Inhibition

A drawback to the use of lignocellulosic-derived hydrolysates in the production of fermentation media is that many microbial inhibitors exist naturally in biomass and are formed as well in the chemical pretreatment and degradation process. One of the main sources of inhibitors comes from the degradation products of lignin, a biopolymer found in plant cell walls constituting 15-27% of the dry weight of lignocellulosic biomass (35). Lignin is a heterogeneous polymer of methoxylated monolignols (coniferyl alcohol, ρ-coumaryl alcohol and sinapyl alcohol) which pretreatment degrades into constituent monomers. Pretreatment degradation products of plant cell walls includes syringic acid, syringaldehyde, ρ-coumaric acid, ferulic acid, sinapic acid as well as organic acids such as lactate, formate and acetate, all microbial inhibitors to varying degrees (44). Pretreatment and hydrolysis of biomass can also lead to the release or generation of furfural and hydroxymethyl furfural (HMF) along with toxic phenolics (25).

Different plant sources contain varying amounts of lignin or nascent anti-microbials. Corn fiber from wet-milling operations may contain less than 8% lignin and thus generate far less monolignols from pretreatment than other sources of biomass (46). Alkaline hydrogen peroxide (AHP) pretreated and enzymatically hydrolysed wheat straw contains no detectable level of the inhibitors furfural or hydroxymethyl furfural (18). AHP wheat straw does contain

inhibitory levels of salts, enough to necessitate an electrodialytic removal of salt from the hydrolysate before fermentation (47).

Organic acids, especially acetic acid are common microbial inhibitors released through the hydrolysis of hemicellulose due to the heavy acetylation of the xylan polysaccharide (39, 48, 49). While concentrations of acetic acid of up to 40 g/L may be released through the complete hydrolysis of hemicellulose to obtain fermentable xylose, concentrations as low as 9.0 g/L acetic acid cause 50% growth inhibition in bacteria (27, 48). The toxicity of organic acids is pH-and hydrophobicity-dependent leading to the current theory as to the mechanism of inhibition given that the undissociated form of the acid is able to diffuse through the cellular plasma membrane and into the cell where the higher internal pH allows for dissociation (48). The uncoupling of the acid into the anion and proton forms lowers the pH within the cytosol collapsing the pH gradient and thus inhibiting the cellular transport functions dependent upon the pH gradient across the plasma membrane (48). With a decrease of pH gradient dependent cellular transport function, the cell loses the ability to import essential nutrients and cofactors needed to sustain it and produce energy. Not only does an acetic-acid-inhibited cell starve for nutrients, but as the internal proton concentration increases the cell expends ATP energy to drive out the excess protons with the plasma membrane ATPase pump (49). The expenditure of ATP to rid the cytosol of protons and the inability to internalize nutrients inhibits cell growth causing for lowered productivity for bacterial fermentations exposed to high levels of acetic acid. Acetic acid inhibition is a significant factor in utilizing hemicellulose derived xylose due to the cost associated with physical treatments for the removal of the acid from the hydrolysate.

Fortunately, acetic acid is a natural metabolic product of many bacteria which commonly exhibit mechanisms of tolerance to the inhibitory effects of the acid as well as consume it as a

carbon source (12, 48). Native systems for acetic acid tolerance must exist as acetic acid is less inhibitory to cellular growth than is expected from the relationship of organic acid hydrophobicity to toxicity (48). In addition to inherent tolerance, multiple tolerance enhancing strategies exist including the use of alternative fermentation strategies, genetic engineering of the organism and strain adaptation by evolutionary selection (49-51). The simplest method for reducing inhibition is to dilute the hydrolysate feed stream in a fermentation so that exposure to the inhibitor is below that of the cells inherent capacity for detoxification (49). This is often impractical from an economic point of view as diluting the fermentation feed stream to lower inhibition also dilutes the carbon source and subsequently the volumetric production rate. This lower rate results in significant increased capital costs due the increased fermentation volume. Direct genetic modification is also impractical in many industrial settings as alterations in the natural metabolic pathway of an organism often require external maintenance to prevent reversion. Genetic modification often weakens the organisms ability to compete against non-modified contaminants as a common outcome is mutants with lowered growth rates (12).

The directed selection of a strain to acetic acid tolerance through adaptation, also known as evolutionary engineering, requires none of the external maintenance of genetic modification while generating a strain with increased tolerance to the inhibitor (50, 51). The approach to evolutionary engineering is to increase exposure of a culture of a microorganism to an inhibitor through multiple passages given the theory that each surviving culture passage is more tolerant to the inhibitor than the previous one (49, 52). This method is able to imbue a strain with a desired adaptation while maintaining the endogenous enzyme systems necessary for metabolism and healthy cellular growth.

Clostridial Fermentation

Species of the anaerobic bacteria *Clostridia* ferment glucose, xylose, fructose, maltose and ribose to useful chemical products but cannot utilize dimeric, oligomeric or polymeric sugars (23). In order to efficiently utilize the carbon in lignocellulosic material the full hydrolysis of lignocellulosic feedstock to monomeric sugars is required prior to fermentation. As discussed earlier, hydrolysis of lignocellulosic material generates a wide variety of monosaccharides thus the capability of *Clostridia* to consume a diverse array of sugars makes it a candidate for fermenting hydrolysates. *Clostridium tyrobutyricum* is a species which has been studied in particular for the fermentation production of butyric acid (10, 12).

Studies regarding the bacterial production of organic acids often describe the acid in either the protonated form, (i.e., acetic acid and butyric acid) or dissociated form (butyrate and acetate) (53-55). The form of either acid is dependent upon the pH of the fermentation media with the pKa of butyric acid at 4.82 and pKa of acetic acid at 4.76 (56).

Anaerobic, butyrate-producing bacteria such as *Clostridia* metabolize glucose to pyruvate through the Embden-Meyerhof-Parnas (EMP) pathway and concomitantly generate acetate, butyrate, H_2 and CO_2 as major metabolic end-products (12). Bacteria within the genus *Clostridium* are considered butyric acid bacteria but many of the *Clostridium sensu stricto* species produce mixtures of acids and alcohols during fermentation (57). The acid-producing (acidogenic) and solvent-producing (solventogenic) stages of *Clostridial* fermentation are dependent upon the growth stage of the fermentation (58). During both batch and fed-batch fermentations of solventogenic *Clostridia*, the exponential growth stage generates the majority of the acid products while during the stationary growth phase solvents are produced (58, 59). Butanol is a well-known industrial product of *Clostridial* fermentation in the solventogenic ABE

(Acetone-Butanol-Ethanol) fermentation. While ABE utilizes the same initial metabolic pathway as acidogenic producers, the acids are used as electron acceptors and that moiety is reduced to the pH neutral alcohol (57). However, between species the *Clostridia* have diverged in their evolution of metabolic pathways with several completely unable to produce solvent (acetone, butanol and ethanol) of any kind. Species such as *C. tyrobutyricum* are useful in this regard as the carbon efficiency of butyric acid production is increased with the lack of concomitant solventogenic metabolism. On the other hand, butanol is an important chemical commodity which may be produced by other species such as *C. beijerinckii* or *C. acetobutylicum* given the appropriate pH control (25, 52).

Production of Butyric Acid by *Clostridia tyrobutyricum* Fermentation

As seen in Figure 2, *C. tyrobutyricum* is able to generate 4 adenosine triphosphates (ATPs) from the fermentation production of 2 acetate molecules from a single glucose molecule while only 3 ATP are received by fermenting glucose to a butyrate (12). These differing energy outputs effect the acid production during cellular growth stages of batch fermentation. As more ATP is generated from acetate production, this is the main product made during logarithmic growth phase due to the higher energetic costs of cellular division and biomass generation (60). Once the *C. tyrobutyricum* culture reaches stationary phase growth, the high energy demands are no longer required and the entire culture switches over to mainly butyric acid production which generates less of a pH drop.

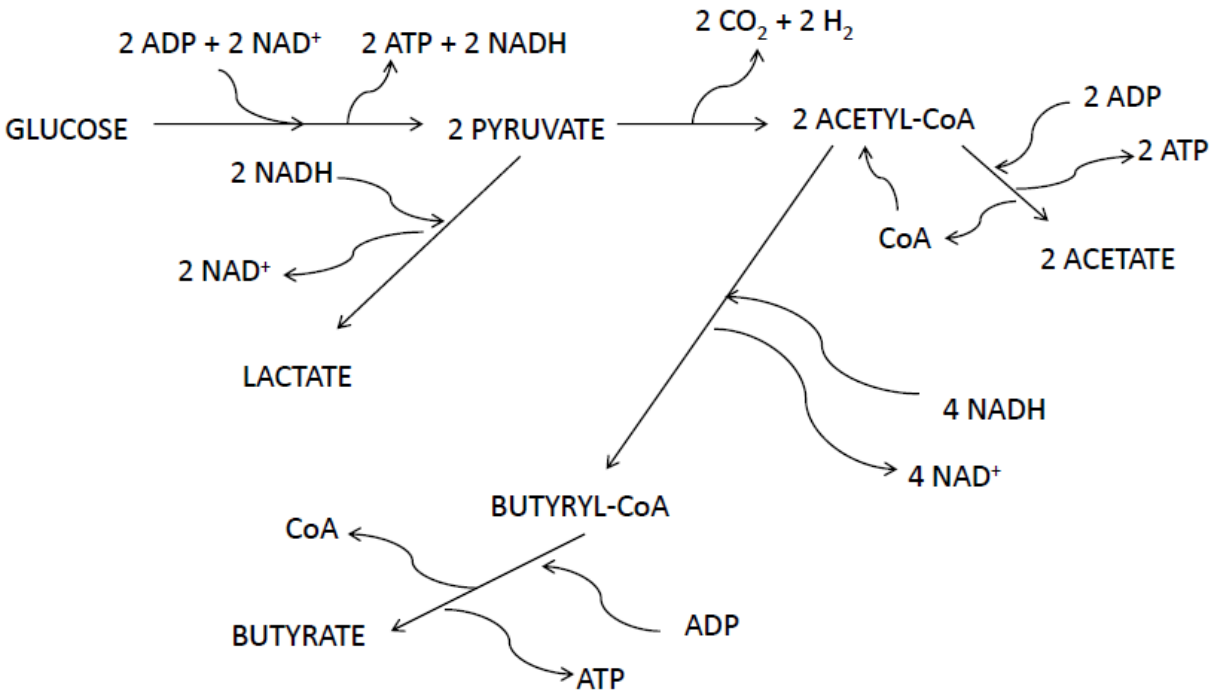


Figure 2. Schematic of the metabolic production of butyric acid in *Clostridia*. Adapted from Zhang *et al* (2009) (12).

Figure 3 demonstrates the outcome of xylose metabolism with production of either acid generating fewer ATP than with glucose consumption (51). Transport of xylose across the cell membrane and into the cytoplasm consumes energy and is the responsibility of an alternative transport system than the glucose transport system, thus the net gain of ATP from xylose consumption is reduced (54). Due to this, the presence of glucose represses the mechanism of xylose metabolism in *Clostridia* which exhibits diauxic growth, consuming glucose prior to the consumption of other carbon sources with an extended lag period as the proper metabolic transport machinery is activated (61). One advantage of utilizing a hemicellulose-derived substrate stream is that the monomeric sugar is all xylose, rather than a mixture of sugar monomers common to other types of hydrolysate. This feature reduces fermentation time by avoiding the lag period caused by diauxic growth.

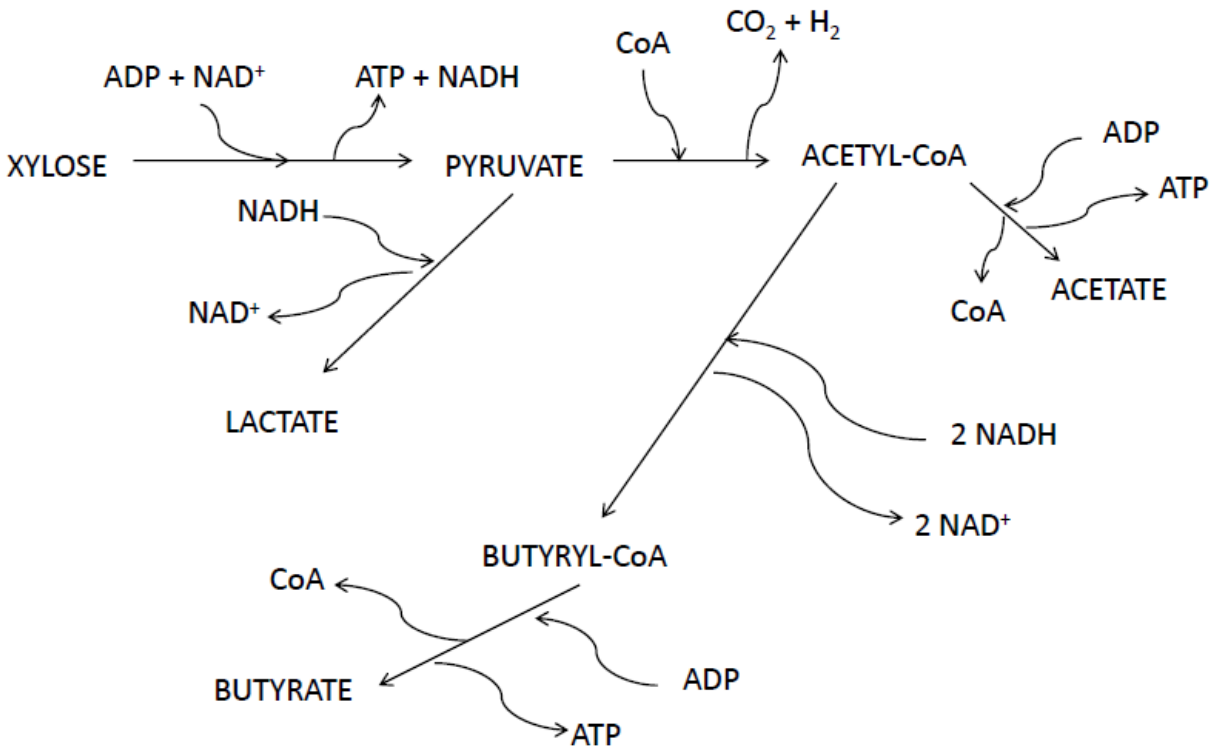


Figure 3. Schematic of xylose metabolism and production of butyric acid in *Clostridia*. Adapted from Zhu and Yang (2004) (51).

Although acetate is a known inhibitor of microbial fermentations, many studies have found the presence of externally added acetate to be beneficial in terms of final product yields for bacterial fermentations, specifically in fermentations utilizing *Clostridia* (58, 59, 62-64). External pH is one major factor regulating the metabolic pathways of fermenting organisms, especially for *Clostridia* (51). In *C. tyrobutyricum*, pH changes in the external media induce metabolic shifts in terms of acid end-products. pH 5.0 leads to predominantly hetero-fermentive production of lactate and acetate while at pH 6.0 the same fermentation generates primarily butyrate (51). It has been reported that pH-induced metabolic shift is ultimately the result of pH effects on the expression levels of acid-forming enzymes in the *Clostridial* metabolic pathway rather than the effect of pH on acid-forming enzyme activities (51). An external pH of 5.3

generates a higher level of expression of phosphotransacetylase (PTA), one of the enzymes responsible for the acetate formation metabolic pathway, than at an external pH of 6.3 (51). Butyrate-forming enzymes such as phosphotransbutyrylase (PTB) have higher expression at pH 6.3 than at 5.3(51). The correlation between pH and metabolism is an important aspect to examine for optimizing the production of specific acids from *Clostridial* fermentation.

Another advantage of using a high acetate substrate such as hemicellulose derived xylose for butyrate production is that *C. tyrobutyricum* and several other *Clostridia* can assimilate acetate from the media to generate even higher levels of butyrate (12, 65). The mechanism of acetate re-utilization involves the free acetate in the media being internalized and exchanging into the acetyl-CoA pool to result in butyrate production. The culture mainly generates acetate as a product during logarithmic phase growth, yet the energetic cost of cellular maintenance given a high external acetate environment provides a reason behind the evolution of an acetate re-utilization mechanism (60). Hot-water extraction and other methods of deriving xylose from hemicellulose cause for the release of up to 40 g/L free acetate in the hydrolysate increasing butyrate production carbon efficiency due to the re-utilization mechanism (27).

Clostridial fermentation for butanol generates acetate during logarithmic growth phase and the secondary solvent fermentation is enhanced by the presence of these weak organic acids (both acetate and butyrate) generated during the primary fermentation stage (58). Similar to butyrate production, despite acetate being a microbial inhibitor the presence of externally added acetate to the media enhances and stabilizes solvent production (58, 59, 64). The acetate uptake mechanism assimilates the external acetate into solvent products including acetone, butanol and ethanol (59). In *C. acetobutylicum* fermentations, the external additions of 4 to 5 g/L acetate and/or butyrate demonstrated 4- to 6-fold increases in solvent production in a 5 g/L/day glucose

fed-batch fermentation system (58). External additions of 4 to 5 g/L acetate and/or butyrate with the same 5 g/L/day glucose fed batch *C. acetobutylicum* fermentations also increased the solvent producing: sugar fermented ratio 3-fold (58). A similar fed-batch fermentation utilizing xylose as the sugar source also increased solvent production by a factor of 6-fold with the external addition of 4 g/L acetate and 4 g/L butyrate as well as increasing xylose consumption and the solvent produced: sugar fermented ratio by 3.3 (58). External acetate in *Clostridial* fermentations while inhibiting microbial growth, increases product formation and efficiency.

REFERENCES

REFERENCES

1. Billig, E. (2001) Butyraldehydes. In *Kirk-Othmer Encyclopedia of Chemical Technology* Vol. 4 pp. 459-470, John Wiley & Sons, Inc

2. Billig, E. (2001) Butyl Alcohols. In *Kirk-Othmer Encycopedia of Chemical Technology* Vol. 4 pp. 393-402, John Wiley & Sons, Inc.

3. Sauer, E. T. (2000) Carboxylic Acids, Economic Aspects. In *Kirk-Othmer Encyclopedia of Chemical Technology* pp. 1-7, John Wiley & Sons, Inc

4. International, S. (1991) Chemical Economics Handbook. Menlo Park, California

5. Zachry, J. B. (1965) The Oxo Reaction. *Annals of the New York Academy of Sciences* **125**, 154-160

6. Calamur, N., and Carrera, M. (2001) Propylene. In *Kirk-Othmer Encyclopedia of Chemical Technology* Vol. 20 pp. 1-25, John Wiley & Sons, Inc.

7. Administration, F. a. D. (2013) Title 21 Food and Drugs; Part 182 SUBSTANCES GENERALLY RECOGNIZED AS SAFE; Subpart A General Provisions. (Services, H. a. H., ed) Vol. 3, Code of Federal Regulations, Silver Spring, MD

8. Communities, C. o. t. E. (2000) Commission Regulation (EC) No. 1565/2000. (Food, T. S. C. o., ed) Vol. 1565/2000 pp. L 180/188-L180/116, Official Journal of the European Communities, Official Journal of the European Communities

9. Sturdik, J. Z. E. (2000) Advances in biotechnological production of butyric acid. *Journal of Industrial Microbiology and Biotechnology* **24**, 153-160

10. Yang, Z. W. S.-T. (2003) Extractive Fermentation for Butyric Acid Production From Glucose by *Clostridium tyrobutyricum*. *BIOTECHNOLOGY AND BIOENGINEERING* **82**, 93-102

11. Sturdik, D. V. M. T. J. Z. E. (1995) Short Note: Effect of growth supplements and whey pretreatment on butyric acid production by *Clostridium butyricum*. *World Journal of Microbiology and Biotechnology* **11**, 363

12. Zhang, C., Yang, H., Yang, F., and Ma, Y. (2009) Current Progress on Butyric Acid Production by Fermentation. *Current Microbiology* **59**, 656-663

13. Yolles, S. (1974) Controlled Release Flavor Compositions. Vol. 3,857,964, David E. Brook, United States

14. Ronzio, R. (2003) The Encyclopedia of Nutrition and Good Health. p. 737, Facts On File, Inc., New York, NY

15. Briens, C., Piskorz, J., and Berruti, F. (2008) Biomass Valorization for Fuel and Chemicals Production - A Review. *International Journal of Chemical Reactor Engineering* **6**, 49

16. Jong, E. d., and Ree, R. v. (2009) Biorefineries: adding value to the sustainable utilisation of biomass. In *Task 42* (42, I. E. A. B. T., ed) pp. 1-15, Implementing Agreement on Bioenergy, Online

17. Shimizu, K. (1991) Chemistry of Hemicelluloses. In *Wood and Cellulosic Chemistry* (David N S Hon, N. S., ed) pp. 177-214, Marcel Dekker, Inc., New York

18. Saha, B. C., and Cotta, M. A. (2006) Ethanol Production from Alkaline Peroxide Pretreated Enzymatically Saccharified Wheat Straw. *Biotechnology Progress* **22**, 449-453

19. Anoop Singh, D. P., Nicholas E Korres, Abdul-Sattar Nizami, Shiv Prasad, Jerry D Murphy (2010) Key issues in life cycle assessment of ethanol production from lignocellulosic biomass: Challenges and perspectives. *Bioresource Technology* **101**, 5003-5012

20. Gisella M. Zanin, C. C. S., Elba P.S. Bon, Raquel C.L. Giordano, Flavio F. de Moraes, Silvio R. Andrietta, Carlos Coelho de Carvalho Neto, Isaias C. Macedo, Djalma Lahr Fo, Luiz P. Ramos, Jose D. Fontana (2000) Brazilian Bioethanol Program. *Applied Biochemistry and Biotechnology* **84-86**, 1147-1161

21. Durre, P. (1998) New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. *Applied Microbiology and Biotechnology* **49**, 639-648

22. United Nations. (2011) 2011 Global Forest Products Facts and Figures. (Organization, F. a. A., ed) p. 17, United Nations

23. Hytonen, E., Szabo, L., Soria, A., Forsstrom, J., Keranen, J. T. (2009) A world model of the pulp and paper industry: Demand, energy consumption and emission scenarios to 2030. *Environmental Science and Policy* **12**, 257-269

24. Werpy, T., and Petersen, G. (2004) Top Value Added Chemicals from Biomass Vol. 1: Results of Screening for Potential Candidates from Sugars and Synthesis Gas p. 76, United States Department of Energy, Springfield, VA

25. Ezeji, T., Qureshi, N., and Blaschek, H. P. (2007) Butanol Production From Agricultural Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation. *Biotechnology and Bioengineering* **97**, 1460-1469

26. Heiningen, A. v. (2006) Converting a kraft pulp mill into an integrated forest biorefinery. *Pulp & Paper Canada* **107**, 38-43

27. Helmerius, J., Walter, J. V. v., Rova, U., Berglund, K. A., and Hodge, D. B. (2010) Impact of hemicellulose pre-extraction for bioconversion on birch Kraft pulp properties. *Bioresource Technology* **101**, 5996-6005

28. Qureshi, N., and Blaschek, H. P. (2000) Economics of Butanol Fermentation Using Hyper-Butanol Producing Clostridium beijerinckii BA101. *Trans IChemE Part C* **78**, 139-144

29. Agency, U. S. D. L. (2012) Fact Book Fiscal Year 2012. (Defense, D. o., ed) p. 84, Defense Logistics Agency, Fort Belvoir, VA

30. Andrews, A. (2009) Department of Defense Fuel Spending, Supply, Acquisition, and Policy. (Defense, D. o., ed)

31. Lenhert, D. B. (2004) The Oxidation of JP-8 and its Surrogates in the Low and Intermediate Temperature Regime. In *Mechanical Engineering and Mechanics* Vol. Doctor of Philosophy p. 246, Drexel, Philadelphia PA

32. Ambareesh D. Murkute, J. E. J., Dennis J. Miller (2011) Supported mesoporous solid base catalysts for condensation of carboxylic acid. *Journal of Catalysis* **278**, 189-199

33. Venkata K.S. Pappu, V. K., Arati Santhanakrishnan, Carl T. Lira, Dennis J. Miller (2013) Butyric acid esterification kinetics over Amberlyst solid acid catalysts: The effect of alcohol carbon chain length. *Bioresource Technology* **130**, 793-797

34. (JECFA), J. F. a. A. O. U. W. H. O. E. C. o. F. A. (1999) SAFETY EVALUATION OF CERTAIN FOOD ADDITIVES. (Safety, I. P. o. C., ed), World Health Organization, Geneva

35. Blaschek, H. P., and Ezeji, T. C. (2007) Science of Alternative Feedstocks. In *Corn-Based Ethanol in Illinois and the U.S.: A Report from the Department of Agricultural and Consumer Economics* pp. 112-128, University of Illinois, Urbana-Champaign, Illinois

36. Shuler, M. L., and Kargi, F. (2002) Cell Construction. In *Bioprocess Engineering Basic Concepts* (Amundson, N. R., ed) pp. 25-46, Prentice Hall PTR, Upper Saddle River, New Jersey

37. Helmerius, J. (2010) Integration of a Hemicelluloses Extraction Step into a Forest Biorefinery for Production of Green Chemicals. In *Chemical Engineering and Geosciences* Vol. Licentiate p. 34, Lulea University of Technology, Lulea Sweden

38. Bronnenmeier, K., and Staudenbauer, W. L. (1993) Molecular Biology and Genetics of Substrate Utilization in Clostridia. In *The Clostridia and Biotechnology* (Woods, D. R., ed) pp. 261-309, Butterworth-Heinemann, Boston

39. Teleman, A., Tenkanen, M., Jacobs, A., and Dahlman, O. (2002) Characterization of O-acetyl-(4-O-methylglucurono)xylan isolated from birch and beech. *Carbohydrate Research* **337**, 373-377

40. James C Baldwin, J. E. D., Ruth C Martin, Gary M Banowetz (2007) Differentially expressed genes associated with post-harvest processing in *Lolium temulentum L.* . *Plant Science* **173**, 73-83

41. Chundawat, S. P. S. (2009) Ultrastructural and physicochemical modifications within ammonia treated lignocellulosic cell walls and their influence on enzymatic digestibility. In *Chemical Engineering* Vol. Doctor of Philosophy p. 438, Michigan State University, Ann Arbor, MI

42. Gould, J. M. (1985) Studies on the Mechanism of Alkaline Peroxide Delignification of Agricultural Residues. *BIOTECHNOLOGY AND BIOENGINEERING* **27**, 225-231

43. Banerjee, G., Car, S., Liu, T., Williams, D. L., Meza, S. L., Walton, J. D., and Hodge, D. B. (2012) Scale-Up and Integration of Alkaline Hydrogen Peroxide Pretreatment, Enzymatic Hydrolysis, and Ethanolic Fermentation. *Biotechnology and Bioengineering* **109**, 922-931

44. Chundawat, S. P., Vismeh, R., Sharma, L. N., Humpula, J. F., Sousa, L. d. C., Chambliss, K. C., Jones, D. A., Balan, V., and Dale, B. E. (2010) Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. *Bioresource Technology* **101**, 8429-8438

45. Garrote, G., Dominguez, H., and Parajo, J. C. (1999) Mild autohydrolysis: an environmentally friendly technology for xylooligosaccharide production from wood. *Journal of Chemical Technology and Biotechnology* **74**, 1101-1109

46. Saha, B. C., Dien, B. S., and Bothast, R. J. (1998) Fuel Ethanol Production from Corn Fiber Current Status and Technical Prospects. *Applied Biochemistry and Biotechnology* **70-72**, 115-125

47. Qureshi, N., Saha, B. C., Hector, R. E., and Cotta, M. A. (2008) Removal of fermentation inhibitors from alkaline peroxide pretreated and enzymatically hydrolyszed wheat straw: Production of butanol from hydrolysate using *Clostridium beijerinckii* in batch reactors. *Biomass and Bioenergy* **32**, 1353-1358

48. Zaldivar, J., and Ingram, L. O. (1999) Effect of organic acids on the growth and fermentation of ethanologenic *Escherichia coli* LY01. *Biotechnology and Bioengineering* **66**, 203-210

49. Almeida, J. R., Modig, T., Petersson, A., Hahn-Hagerdal, B., Liden, G., and Gorwa-Grauslund, M. F. (2007) Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *Journal of Chemical Technology and Biotechnology* **82**, 340-349

50. Kuyper, M., Toirkens, M. J., Diderich, J. A., Winkler, A. A., Dijken, J. P. v., and Pronk, J. T. (2005) Evolutionary engineering of mixed-sugar utilization by a xylose-fermenting *Saccharomyces cerevisiae* strain. *Federation of European Microbiological Societies Yeast Research* **5** 925-934

51. Zhu, Y., and Yang, S.-T. (2004) Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum. *Journal of Biotechnology* **110**, 143-157

52. Lin, Y.-L., and Blaschek, H. P. (1983) Butanol Production by a Butanol-Tolerant Strain of Clostridium acetobutylicum in Extruded Corn Broth. *Applied and Environmental Microbiology* **45**, 966-973

53. Liu, X., Zhu, Y., and Yang, S.-T. (2006) Butyric acid and hydrogen production by Clostridium tyrobutyricum ATCC 25755 and mutants. *Enzyme and Microbial Technology* **38**, 521-528

54. Ling Jiang, J. W., Shizhong Liang, Xiaoning Wang, Peilin Cen, Zhinan Xu (2010) Production of Butyric Acid from Glucose and Xylose with Immobilized Cells of *Clostridium tyrobutyricum* in a Fibrous-bed Bioreactor. *Applied Biochemistry and Biotechnology* **160**, 350-359

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

56. JR Martin, H. P., J Ballongue, R Gay (1983) Effects of Acetic and Butyric Acids on Solvents Production by Clostridium Acetobutylicum. *Biotechnology Letters* **5**, 89-94

57. Wiegel, J., Tanner, R., and Rainey, F. A. (2006) An Introduction to the Family Clostridiaceae. In *The Prokaryotes* (Dworkin, M., ed) Vol. 4 pp. 654-678, Springer, Singapore

58. Olivier Fond, G. M.-A., H. Petitdemange, J.M. Engasser (1985) The role of acids on the production of acetone and butanol by *Clostridium acetobutylicum*. *Applied Microbiology and Biotechnology* **22**, 195-200

59. Chen, C.-K., and 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**, 499-505

60. Michel-Savin, D., Marchal, R., and Vandecasteele, J. P. (1990) Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. *Applied Microbiology and Biotechnology* **32**, 387-392

61. Wilfrid J. Mitchell, K. A. A., Mohsen Yazdanian (1995) Factors affecting utilization of carbohydrates by clostridia. *FEMS Microbiology Reviews* **17**, 317-329

62. He, Q., Lokken, P. M., Chen, S., and Zhou, J. (2009) Characterization of the impact of acetate and lactate on ethanolic fermentation by *Thermoanaerobacter ethanolicus*. *Bioresource Technology* **100**, 5955-5965

63. Zhu, Y., and Yang, S.-T. (2003) Adaptation of Clostridium tyrobutyricum for Enhanced Tolerance to Butyric Acid in a Fibrous-Bed Bioreactor. *Biotechnology Progress* **19**, 365-372

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

65. Chun-Hui Zhang, Y.-J. M., Fang-Xiao Yang, Wei Liu, Yue-Dong Zhang (2009) Optimization of medium composition for butyric acid production by *Clostridium thermobutyricum* using response surface methodology. *Bioresource Technology* **100**, 4284-4288

CHAPTER 2: THE EFFECT OF ACETATE ON THE FERMENTATION PRODUCTION OF BUTYRATE

Data in this chapter has been published in:

Jaros, A., Rova, U., Berglund, K.A. (2012) EFFECT OF ACETATE ON FERMENTATION PRODUCTION OF BUTYRATE. *Cellulose Chemistry and Technology* 46, 341-347

Abstract

A carbon source for the fermentation production of butyrate is xylose extracted from ligno-cellulosic material by hot water extraction. Although this auto-hydrolysis of hemicellulose can provide a low-cost source of xylose, the process generates a high level of acetic acid that might inhibit subsequent fermentations. This study focuses on the effects of acetate on the production of butyrate from xylose by batch fermentations with a selected strain of *Clostridium tyrobutyricum*.

At initial acetate concentrations of 17.6 g/L and 26.3 g/L in the media, *C. tyrobutyricum* cultures exhibited a lag phase (45 and 118 hours, respectively) in terms of sugar consumption, butyrate production and cell biomass growth, lowering the overall production rate. Butyrate fermentations performed with high concentrations of acetate in the media demonstrated a re-uptake of acetate into the butyrate production pathway and after the lag phase, all cultures adapted to the inhibitory acetate which increased the final butyrate yields 12.6% (32.6 g/L compared to 28.5 g/L).

Key words: *Clostridium tyrobutyricum*, butyrate, xylose fermentation, hemicellulose utilization, acetate inhibition

Introduction

Economically feasible fermentation processes involving low cost renewable substrates have potential applications in manufacturing butyrate as a food additive or as a bulk organic commodity for further refinement into biodegradable plastics (1). In terms of a food product, butyrate is listed by the Food and Drug Administration (US) as a flavoring substance generally recognized as safe (GRAS). As a flavor additive, butyrate has a wide array of uses from enhancing the sensorial quality of cattle feed, to acting as a chemical feedstock in the production of flavor esters.

Butyrate is mainly produced industrially from petrochemical sources, but can also be made by fermentation of sugar or other starch sources by anaerobic bacteria. Most of these bacteria are obligate anaerobes, meaning they require oxygen-free conditions for them to perform their metabolism, typical of the conditions in the gastrointestinal tracts of mammals from which many of these bacteria are isolated. Microorganisms from the genus *Clostridium* are the most studied in terms of butyrate fermentation and within this group, the Gram-positive anaerobic spore-former *Clostridium tyrobutyricum* can produce butyrate utilizing either glucose or xylose as a carbon source (1, 2). Using xylose as a carbon source, yields up to 0.4 g/g have been obtained with immobilized *C. tyrobutyricum* in a fibrous-bed reactor (3).

Hemicellulose is a valuable biomass resource and hemicellulose extraction could be integrated into current industrial pulp and paper processes yielding hemicellulosic sugars for fermentation while retaining the cellulose fraction to be used in the subsequent pulp production (4). One of the main issues concerning an integration is that near-neutral pre-extraction of hemicellulose from hardwood releases not only xylose, but also acetic acid, a microbial growth inhibitor influencing energy generation and intracellular pH homeostasis. Depending on process constraints, a hydrolysate of hardwood extracted xylose, conceivable for fermentation, typically contains up to 70 g/L xylose and 40 g/L acetic acid (4). A representative process design for such an extraction involves isothermally heating wood chips to 160°C and allowing the wood to extract for 2 hours.

Butyric acid production by *C. tyrobutyricum* is an attractive potential industrial approach for lingo-cellulosic based fermentations, but in order for hemicellulose derived xylose to be competitive as a low cost substrate, the inhibiting acetic acid must either be removed from the hydrolysate or the fermentation strain adapted to tolerate such high levels. Previous research has shown *Clostridia* to be tolerant to high levels of initial acetate and butyrate and that the presence of these organic acids results in increased product yields not only for butyrate fermentation but butanol fermentation as well (2, 5). Addition of 8.9 g/L sodium acetate (6.518 g/L acetic acid equivalents) to a *Clostridium beijerinckii* BA101 fermentation increased the final butanol produced by 14% (5). *Clostridium thermobutyricum* fermentations challenged with 30 to 360 mM acetate (1.8 to 21.6 g/L) exhibited a 2-to-4 fold increase in butyric acid formation (6). Furthermore, reinforcement of a cassava-based medium with 30 mM ammonium acetate (1.8 g/L acetic acid equivalent) increased *Clostridium acetobutylicum* strain EA 2018 butyric acid

fermentation by 2.6 fold (7). As *C. tyrobutyricum* is from the same genus as these bacteria and share analogous metabolic pathways one can expect an increase in butyric acid yield when challenging *C. tyrobutyricum* with acetic acid.

As *Clostridia* are prone to tolerance adaptation regarding toxic fermentation products, *C. tyrobutyricum* is a candidate for selective enrichment towards acetate tolerance. The present work focuses on the impact of high levels of acetate/acetic acid on *C. tyrobutyricum* fermentation growth kinetics and product yields in order to use xylose extracted from hemicellulose as a fermentative substrate without requiring the removal of the acetate from the feedstock.

Experimental: Bacterial Strain, Media and Growth

A lyophilized stock culture of *C. tyrobutyricum* (ATCC 25755) was re-hydrated under sterile anaerobic conditions in Reinforced Clostridial Medium (RCM; Difco) broth. Stock cultures were maintained at -70°C in CRYOBANK™ vials. To prepare cultures for glucose fermentations, RCM which contains 5 g/L glucose, was used while a medium was prepared for the xylose batches with the following composition per liter: 10 g peptone, 10 g beef extract, 3 g yeast extract, 5 g sodium chloride, 0.5 g L-cysteine, 3 g sodium acetate anhydrous, 0.5 g agar and 900 mL distilled water. The medium was sterilized at 121 °C for 20 min; thereafter 10 mL of a separately sterilized xylose solution (50 g/L) were aseptically added to bring the xylose concentration to 5 g/L. The inoculum for each batch fermentation in these studies was prepared by anaerobically inoculating 50 mL Screw Cap Corning tubes containing 35 mL sterile glucose or xylose based RCM with 5 mL of the stock culture. The inoculated tubes were cultivated under

anaerobic conditions at 36 °C, 80 rpm, until log phase, approximately when the optical density at 600 nm had reached a value of 2. The reason behind two types of reinforced inoculate media is to avoid any substrate based growth lag. *C. tyrobutyricum* inocula were pre-conditioned to the correct sugar substrate in the inoculation media prior the batch fermentation.

Fermentations

One-liter batch fermentations were conducted in New Brunswick Bioflo 310 reactors, 2.5 L working volume at 36°C. For each batch 950 mL minimal media of the following composition was used; 6 g/L yeast extract, 5 ppm $FeSO_4 \cdot 7\ H_2O$, and 200 mL xylose or glucose at 300 g/L sterilized separately. To examine the effect of acetate on butyrate production by *C. tyrobutyricum* various amounts of sodium acetate (4.4, 8.8, 17.6 or 26.3 g/L) were added to the media before sterilization. Fermentations without acetate are referred to as controls. Anaerobiosis was reached by sparging the vessel with nitrogen prior to inoculation. The batches were inoculated with 50 mL log phase *C. tyrobutyricum* cultures. Agitation was kept at 250 rpm and the nitrogen sparging was maintained until logarithmic growth was observed.

In order to maintain the *C. tyrobutyricum* cultures in the acidogenic phase, pH 6.0 was sustained by automatic addition of 5 M NaOH throughout the fermentation. Samples (10 mL) were withdrawn aseptically at regular intervals for analytical measurements. The batch fermentations were conducted until all sugar had been consumed.

Analytical Methods

Organic acids and residual sugar were analyzed by HPLC (LC-20AT dual pump and 10A RI detector, Shimadzu) equipped with an ion exchange column (Aminex HPX-87H, 9 um, 7.8mm x 300mm, Bio-Rad) and a cation-H guard column (Micro-guard, 30mm x 4.6 mm) using 50 mM sulfuric acid as the mobile phase. The flow rate of the mobile phase was maintained at 1 mL/min during analysis with 20 μL of sample injected into the system with an auto-injector (SIL-20AHT, Shimadzu) with the column and guard maintained at 65°C in a column oven (CT0-20A, Shimadzu). To remove cellular debris samples were centrifuged at 10 000 rpm for 5 min in a micro-centrifuge prior analyses. Data for each sample was acquired and analyzed with Shimadzu EZ Start 7.4 SP1 chromatography software using standards with known concentrations of glucose, xylose, butyrate, acetate and lactate.

Cell Biomass Determination

Cell growth was monitored during fermentation by measuring the optical density at 600 nm. The biomass from 40 mL cell suspension, removed in triplicate, was dried in a 80°C dryer for 48 hours and and the dry cell weight (DCW, g/L) determined. The optical densities were then converted to dry cell weight using the following equation: $DCW = 0.38(OD_{600})$.

Results and Discussion

Although *Clostridia* prefer glucose as a sugar source, other metabolic pathways enable utilization of alternate sugar sources such as xylose. Once glucose is either completely consumed

or lacking in the fermentation media, these alternate pathways are activated (8). Repression of xylose metabolism by the presence of glucose is a common trait of these strains and causes a significant lag phase in substrate uptake during diauxic fermentations between glucose depletion and the xylose being consumed. Previous studies conclude this xylose consumption lag is caused by the requirement of the new metabolic system to be activated by the *Clostridia* in addition to an alternate xylose transport system (9). *C. tyrobutyricum* activation of the xylose metabolism systems requires 48 hours to overcome (10).

By avoiding the growth lag of *C. tyrobutyricum* caused by altered metabolic pathways for the carbon source, a direct study of the growth lag generated by inhibitory acetate was made using xylose as sugar source. *C. tyrobutyricum* cultures inoculated into media with 8.8 g/L initial acetate or less began log phase growth and thus sugar consumption at roughly the same time (Fig. 4). Cultures grown in 17.6 g/L acetate required 45 hours to adapt to the acetate environment before the consumption of xylose started. The lag phase for the 26.3 g/L initial acetate batch was even longer, stretching over 118 hours as the culture adapted to the acid. For all of the trials performed in this study, once the lag phase had passed, the xylose consumption occurred at nearly similar rates (Fig. 4). Other than the inhibitory acetate, the conditions were ideal for the *C. tyrobutyricum* fermentation as the control, 4.4 g/L and 8.8 g/L acetate initial concentration batches all entered log phase within the first 24 hours. The extended lag phase for the 26.3 g/L initial acetate concentration is one of the main drawbacks of utilizing a xylose feed derived from hardwood hemicellulose, as the acetate level, similar to those found in the hydrolyzate, is so inhibitory to cellular growth that the extended lag phase lowers productivity. Although it took over 100 hours to enter the logarithmic growth phase, the 26.3 g/L initial acetate concentration

batch did eventually reach the same biomass levels as the control and lower initial acetate concentrations batches as well as surpassed them in final butyrate yield (Fig. 5 and Fig. 6). When xylose was used as carbon source the 26.3 g/L initial acetate concentration batch resulted in 32.6 g/L butyric acid compared to the control batches (28.5 g/L butyric acid), a 12.6% increase. Given previous reports of the tolerance adaptability of *C. tyrobutyricum*, it appears that given a long exposure time even at 26.3 g/L acetate the culture adapts and then carries out a butyric acid fermentation fully utilizing the xylose present. The glucose fermentations with additional acetate did not show any increase in yield and were also less sensitive according to the response in lag time.

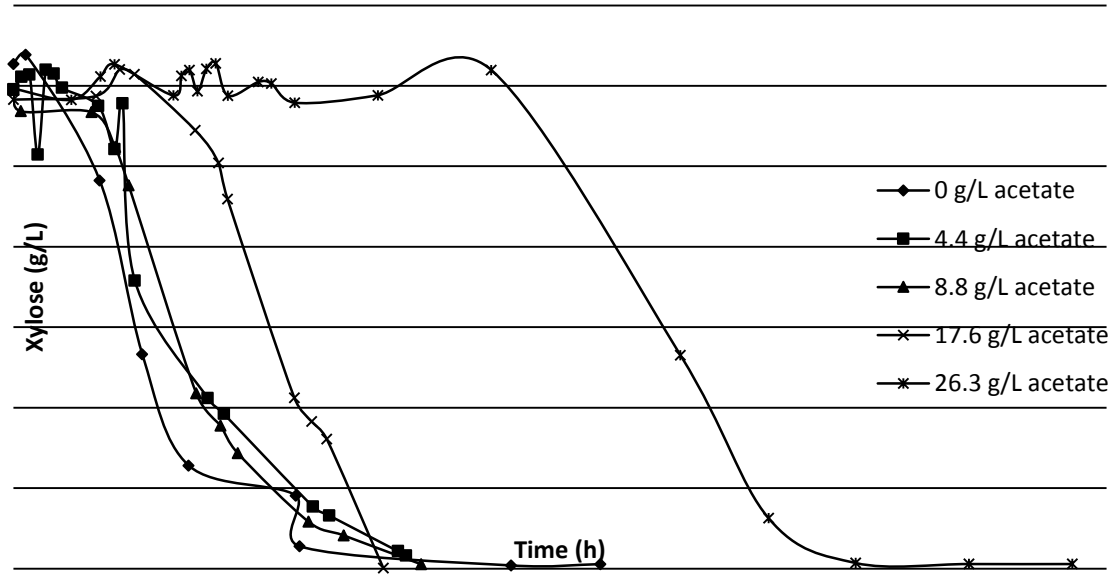


Figure 4. Effect of acetate inhibition on xylose consumption in *C. tyrobutyricum* batch fermentation system. Fermentations performed as 1-liter batches in 2.5-liter working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm,

and pH at 6.0 with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$. Ten-mL samples were withdrawn from the fermentation and analyzed by HPLC.

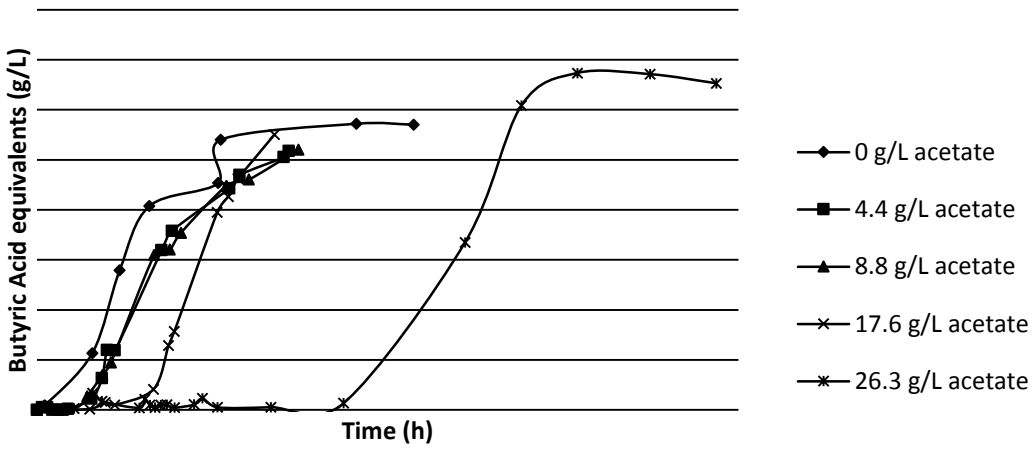


Figure 5. Effect of acetate inhibition on butyrate production in *C. tyrobutyricum* batch fermentation system. Fermentations performed as 1-liter batches in 2.5-liter working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$. Ten-mL samples were withdrawn from the fermentation and analyzed by HPLC.

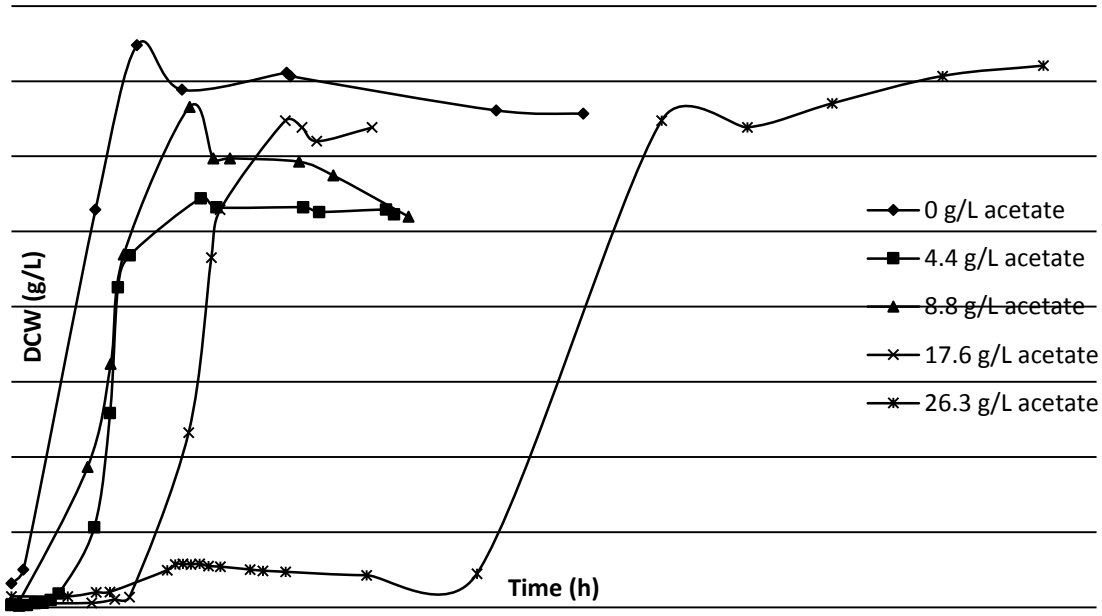


Figure 6. Effect of acetate inhibition on cell biomass production in *C. tyrobutyricum* batch fermentation system. Fermentations performed as 1-liter batches in 2.5-liter working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$. Ten-mL samples were withdrawn from the fermentation and analyzed for OD by spectrophotometer at a wavelength of 600nm.

Slower cell-mass generation and sugar uptake result in reduced overall productivity as fermentations with higher initial acetate concentrations exhibited periods of limited xylose consumption (Fig. 4 and Fig.6). Control batches on glucose and xylose generated butyrate at a rate of 0.24 and 0.23 g/L/h respectively, while the presence of 26.3 g/L acetate in the media resulted in a productivity decrease to 0.12 and 0.14 g/L/h (Table 1). The controls or fermentations with lower initial acetate concentration (i.e., 8.8 g/L or less) required 100 hours for

consumption of nearly all sugar, and most of the butyrate was produced within this time (Fig. 4 and Fig.5). However, the fermentation with initial 26.3 g/L acetate took over 100 hours to begin consuming xylose or producing butyric acid at detectable levels and more than 200 hours were required before all xylose was converted. As stated above, inoculation cultures were pre-conditioned with the appropriate sugar source prior to inoculation so adjustment to a new sugar source was not the cause of the lag.

Unfortunately, the lengthy lag phase presents an issue for the large-scale feed-stock potential of hemicellulose derived xylose as such long periods of cellular non-growth leaves fermentation tanks open to acetate tolerant contaminating micro-organisms. The issue of lowered equipment turnover due to the low 0.14 L/h overall production rate increases the economic burden on plant design. Also, operating tanks must be held at under anaerobic conditions until the culture enters log phase and starts generating CO_2 requiring a much longer period of nitrogen sparging due to inhibitory acetate.

Table 1. Effect of acetate inhibition on butyrate, acetate and biomass production in *C. tyrobutyricum* cultures.

Sugar	Acetate (g/L)	Lag time[2] (h)	Sugar cons[3] (g/L/h)	Butyrate Yield[4] (mol/mol)	Final concentration (g/L)			Overall produc.[6] (g/L/h)
					Butyrate	Acetate	Bio-mass[5]	
Glu[1]	0	0	1.09	0.72	23.6	5.3	3.3	0.24
Glu	4.4	0	0.97	0.80	23.0	11.3	2.4	0.32
Glu	8.8	0	0.77	0.80	22.0	11.0	3.1	0.30
Glu	17.6	26	1.68	0.85	24.4	21.4	3.2	0.34
Glu	26.3	118	0.72	0.76	22.3	27.7	3.0	0.12
Xyl[1]	0	0	1.12	0.62	28.5	2.0	3.3	0.23
Xyl	4.4	24	1.71	0.56	24.8	8.6	2.6	0.33
Xyl	8.8	20	1.07	0.60	26.0	11.6	2.6	0.26
Xyl	17.6	45	1.10	0.73	32.3	14.8	3.2	0.28
Xyl	26.3	118	0.86	0.90	32.6	22.0	3.6	0.14

[1] Glucose and xylose respectively

[2] Calculated as time until sugar consumption started

[3] Calculated for the linear sugar consumption phase

[4] Yield was calculated as mol butyrate per mol glucose or xylose consumed during fermentation

[5] Calculated as DCW g/L

[6] Overall productivity calculated from the start of the fermentation until the sugar source were completed

Acetate Re-Utilization

Of note is the increased final butyrate concentration at high acetate concentrations with xylose as compared to glucose as a feed-stock, 32.6 g/L and 22.3 g/L butyric acid, respectively (Table 1). In fact, all of the xylose feedstock batches performed better in terms of butyric acid production than the comparable glucose batches (Table 1). Glucose is preferred over xylose as a carbon source for *C. tyrobutyricum*, yet our findings demonstrate that there are no adverse effects on acid product selectivity or final butyric acid concentration with a xylose feed-stock batch fermentation. In contrast, *C. tyrobutyricum* immobilized onto a fibrous-bed bioreactor exhibit higher productivity on glucose feeds compared to xylose as the lower energy efficiency of metabolizing xylose lowered butyric acid yields (3). To the best of our knowledge, this is the first report challenging *C. tyrobutyricum* with such high concentrations of initial acetic acid (17.6 and 26.3 g/L) in batch fermentations. Research has been performed on similar *C. tyrobutyricum* fermentations in a glucose fed-batch style with the dilution rate of D= 0.1 h^{-1} and an acetate input of 10 g/L leading to utilization of acetate and conversion into butyric acid (11). As discussed below, providing *C. tyrobutyricum* with a high initial acetic acid equivalent not only challenges the fermentation, but has been reported to increase butyric acid yields as re-utilization of acetate for conversion to the 4-carbon product might be energy favorable for the microorganism (12, 13).

In the fermentations with the xylose feed, there is a correlation between the initial acetate concentration and the butyrate production with the highest butyrate yield of 0.9 mol butyrate/mol xylose occurring with the highest initial acetate concentration, 26.3 g/L (Table 1 and Fig. 5).

This result indicates that the acetate end-product pathway in the organism is being inhibited by the acetate and less of the carbon is entering that metabolic pathway. This result is a beneficial aspect of using xylose derived from a hemicellulose substrate streams since the high concentration of acetate found in these streams will push the fermentation towards butyrate and away from generating more acetate.

The experiments with 17.6 and 26.3 g/L initial acetate concentrations and xylose as the carbon source show reduced levels of acetate at the end of the fermentation (14.8 and 22.0 g/L respectively), suggesting re-utilization of acetate. The utilization of acetate is also evident in the 26.3 g/L acetate batches of both feed-stocks which have lower sugar consumption rates than the batches with lower acetate concentrations as acetate is being used as an additional carbon source. The control batches demonstrated sugar consumption rates of 1.09 and 1.12 g/L/h for glucose and xylose feed-stocks respectively while the 26.3 g/L acetate batch rates were 0.72 and 0.86 g/L/h (Table 1). Re-utilization of acetate is a known phenomenon for *Clostridium* strains being used for acetone-butanol-ethanol (ABE) fermentations where free acetate present in the media exchanges with the acetyl-CoA pool obtained from glycolysis and results in higher butyrate production (14). *C. tyrobutyricum* utilizes the Embden-Meyerhof-Parnas metabolic pathway after glycolysis to generate ATP with butyrate, acetate and lactate as end products (6). The main metabolic pathway generates butyrate but the presence of acetate or lactate in the media induces even higher use of this pathway and the production of butyrate (15). The hypothetical evolutionary function of this mechanism is in uncoupling the cellular membrane pH gradient from undissociated acids in the environment (11). The butyrate pathway generates less ATP than the one producing acetate but this energy sacrifice is made by the cell to maintain a functional

pH gradient (11). An important factor determining acid product selectivity is that during log phase growth the cell requires more ATP than during stationary phase, leading to the necessity of acetate production during log phase but mainly butyrate production during stationary phase (3). Nevertheless, the acetate uptake pathway provides another positive aspect of xylose substrate stream derived from hemicellulose as some of the inhibitory acetate is converted into the butyrate product.

While the xylose-fed 17.6 and 26.3 g/L initial acetic acid concentration batches demonstrated acetate uptake, this phenomenon was not seen in glucose-fed batches at the same initial acetic acid concentrations (Table 1). Glucose-fed 17.6 and 26.3 g/L initial acetic acid batches generated a net gain of acetic acid, 3.8 g/L and 1.4 g/L, respectively. An interpretation of these results stems from the lower energy gains of xylose metabolism compared to that of glucose. Cells consuming xylose rather than glucose thus have less free energy to devote to maintaining the pH gradient across the cell wall and in turn must activate the acetate uptake mechanism to partially nullify the external acetic acid. It is possible that our glucose metabolizing batches with high initial acetic acid had enough energy throughout the fermentation to carry-out maintenance of the pH gradient and thus continued generating acetic acid. Future work would test this theory by exposing glucose metabolizing cultures to even higher initial acetic acid concentrations than those presented in this study and noting acetate uptake.

Coinciding to the acetate uptake results, acetic acid had less of an effect on extending lag phase in growth on glucose-fed cultures than on *C. tyrobutyricum* consuming xylose (Table 1). Only at the 26.3 g/L initial acetic acid concentration did the glucose batch match the xylose batch in sustaining a 118-hr long lag phase. It required 17.6 g/L acetic acid before the glucose

consuming *C. tyrobutyricum* even demonstrated a lag phase while some lag phase was noticed in xylose fed cultures with only 4.4 g/L acetic acid present. Such a marked difference in response to acetic acid inhibition likely emanates from the lowered energetic value of metabolizing xylose. Xylose consumption leads to less free energy for cellular maintenance than glucose. In the case of lag phase, the lower free energy from xylose causes an extension of the lag as the cells take longer to adapt to the high acetate environment. Further studies regarding the issue of lag phase both with glucose and xylose consuming *C. tyrobutyricum* will be conducted in order to determine if an adaptation technique can overcome the lag phase all together.

Conclusion

Xylose derived from hardwood by near-neutral pre-extraction results in a feedstock stream that contains up to 40 g/L acetic acid (4). The work presented here evaluates the challenges to design a butyric acid fermentation based on a xylose stream with similar inhibitory levels of acetic acid. Xylose is an excellent alternative to glucose as a feedstock for batch fermentation as a higher final butyric acid concentration was obtained compared to the glucose batches.

High initial acetic acid concentrations proved inhibitory to the *C. tyrobutyricum* and generated an extended growth-lag phase before the culture could begin fermentation. Despite lowered production rates caused by the lag, the acetic acid challenged cultures all eventually adapted to the acid environment and fully consumed the xylose substrate. A benefit of the presence of acetate in the media was the acetate re-utilization mechanism which induced higher final butyric acid concentrations. The re-utilization of acetate from the media back into the

acidogenic pathway increased product yield up to 45%. Increased product yield is of economic importance in an acetic acid containing feedstock as *C. tyrobutyricum* will ferment carbon from the acid as well as the xylose, increasing the butyric acid yields in batch production.

The adaptive ability of *C. tyrobutyricum* makes hemicellulose-derived xylose a feasible and attractive substrate for fermentations, as acetate present in the hydrolyzate can be used as a carbon source, thus saving the requirements of detoxification. Future work will focus on maintaining pre-adapted cultures to overcome the production inhibiting lag phase. In addition, examining the xylose metabolism at a molecular level will reveal insight into metabolic pathways affected by the acetic acid which might illuminate a potential solution to overcome the inhibition. Experimentally blocking the acetic acid uptake mechanism during fermentation in the presence of acetate would demonstrate proof of the theory of acetic acid uptake in *C. tyrobutyricum*. A further exploration of fermentation kinetics on acetic acid tolerant pre-adapted strains will provide a better understanding of hemicellulose derived xylose as a production feedstock.

Acknowledgements

This work was supported by the Swedish Energy Agency (STEM), Swedish Governmental Agency for Innovation Systems (VINNOVA), the United States Defense Logistics Agency, and Bio4Energy (funded by the Swedish National Research Council (VR) and STEM).

APPENDIX

Table 2. Raw data used to generate Figures 4 and 5 in Chapter 2.

			g/L			
Added Acetate (g/L)	Hours	Sample	Xylose	Lactic Acid	Acetic Acid	Butyric Acid
0	0.0	T0 (+)	62.70	0	0.257	0
0	3.1	T1	63.87	0	0.236	0.451
0	21.3	T2	48.25	0.308	1.402	5.673
0	31.8	T3	26.64	0	1.259	13.969
0	43.3	T4	12.82	0	1.455	20.38
0	69.8	T5	9.06	0	1.226	22.695
0	70.7	T6	2.77	0.505	0.87	27.01
0	122.9	T7	0.39	0	1.266	28.617
0	144.9	T8	0.55	0	1.991	28.524
4.4	0.0	T0(-)		0.64		0.20
4.4	0.0	T0(+)	59.56	0.17	4.68	0
4.4	2.0	T1	61.11	0.26	4.7	0.26
4.4	4.0	T2	61.40	0.13	4.59	0.26
4.4	6.0	T3	51.45	0.29	4.24	0
4.4	8.0	T4	62.00	0.16	4.76	0
4.4	10.0	T5	61.53	0.17	4.85	0
4.4	12.0	T6	59.75	0.15	4.69	0.10
4.4	21.0	T7	57.48	0	4.76	1.13
4.4	25.0	T8	52.13	0.2	4.90	3.17
4.4	27.0	T9	57.81	0.38	6.10	5.98
4.4	30.0	T10	35.78	0.73	5.15	5.96
4.4	48.0	T11	21.21	0.94	5.65	15.96
4.4	52.0	T12	19.23	0.78	5.74	17.90
4.4	74.0	T13	7.72	0.97	6.03	22.14
4.4	78.0	T14	6.61	1.02	6.23	23.48
4.4	95.0	T15	2.17	1.00	6.08	25.27
4.4	97.0	T16	1.63	1.10	6.24	25.90

Table 2 (cont'd)

			g/L			
Added Acetate (g/L)	Hours	Sample	Xylose	Lactic Acid	Acetic Acid	Butyric Acid
8.8	0.0	T0(+)	59.49	0.44	9.26	0.00
8.8	1.8	T1	56.84	0.26	8.81	
8.8	19.3	T2	56.73		9.23	1.35
8.8	25.2	T3	52.39	0.96	9.93	3.47
8.8	28.5	T4	47.62	0.61	9.39	4.75
8.8	45.2	T5	21.78	1.24	10.41	15.48
8.8	51.2	T6	17.77	1.40	10.30	16.02
8.8	55.4	T7	14.36	1.38	10.77	17.71
8.8	72.9	T8	5.833	1.28	10.66	22.40
8.8	81.6	T9	4.14	1.36	10.68	23.04
8.8	100.7	T10	0.57	1.30	11.63	26.03
17.6		T0(-)	60.69	0.111	17.554	0
17.6	0.0	T0(+)	58.29	0.124	16.776	0
17.6	20.4	T1	58.74	0	16.909	0.087
17.6	26.2	T2	62.01	0	17.492	0.74
17.6	29.9	T3	61.45	0	16.923	0.491
17.6	44.9	T4	54.49	0.297	17.077	2.067
17.6	50.7	T5	50.42	0.672	17.354	6.444
17.6	52.9	T6	45.91	0.358	16.561	7.841
17.6	69.4	T7	21.23	0.495	14.855	19.738
17.6	73.7	T8	18.30	1.059	15.324	21.328
17.6	77.4	T9	16.12	0.684	14.927	23.091
17.6	91.4	T10	0.08	0.586	13.996	27.505

Table 2 (cont'd)

			g/L			
Added Acetate (g/L)	Hours	Sample	Xylose	Lactic Acid	Acetic Acid	Butyric Acid
26.3		T0(-)		0.612		
26.3	0.0	T0(+)	59.63	0.165	24.78	0
26.3	14.3	T1	58.25	0.162	25.131	0.134
26.3	21.5	T2	61.16	0	25.136	1.662
26.3	25.0	T3	62.66	0	25.976	0.867
26.3	39.5	T4	58.79	0.822	25.44	0.203
26.3	41.5	T5	61.24	0.93	25.357	1.068
26.3	43.5	T6	61.95	1.266	25.654	0.494
26.3	45.5	T7	59.31	1.081	25.342	0.224
26.3	47.7	T8	62.1	1.30	25.659	0.49
26.3	50.0	T9	62.78	1.175	25.467	0.506
26.3	53.0	T10	58.75	1.079	25.361	0.234
26.3	60.5	T11	60.47	1.26	25.676	0.538
26.3	63.7	T12	60.26	1.041	25.35	1.154
26.3	69.5	T13	57.89	1.054	24.951	0.262
26.3	90.0	T14	58.82	1.097	25.189	0.276
26.3	118.0	T15	61.96	0.988	25.663	0.673
26.3	164.7	T16	26.5	0.857	22.912	16.736
26.3	186.5	T17	6.27	0.707	21.862	30.436
26.3	208.0	T18	0.71	0.806	20.567	33.678
26.3	236.0	T19	0.58	0.563	20.578	33.56
26.3	261.5	T20	0.581	0.856	22.088	32.65

Table 3. Raw data used to generate Figure 6 in Chapter 2. OD, optical density. DCW, dry cellular weight.

			600 nm		g/L
Added Acetate (g/L)	Hours	Sample	OD 6x dil	OD	DCW
0	0.0	T0 (+)	0.07	0.42	0.160
0	3.1	T1	0.11	0.66	0.251
0	21.3	T2	1.16	6.96	2.645
0	31.8	T3	1.64	9.84	3.739
0	43.3	T4	1.51	9.06	3.443
0	69.8	T5	1.56	9.36	3.557
0	70.7	T6	1.55	9.3	3.534
0	122.9	T7	1.45	8.7	3.306
0	144.9	T8	1.44	8.64	3.283
4.4	0.0	T0(-)		0.04	0.015
4.4	0.0	T0(+)		0.04	0.015
4.4	2.0	T1		0.03	0.011
4.4	4.0	T2		0.04	0.015
4.4	6.0	T3		0.07	0.027
4.4	8.0	T4		0.08	0.030
4.4	10.0	T5		0.13	0.049
4.4	12.0	T6		0.24	0.091
4.4	21.0	T7		1.40	0.532
4.4	25.0	T8		3.40	1.292
4.4	27.0	T9		5.60	2.128
4.4	30.0	T10		6.16	2.341
4.4	48.0	T11		7.16	2.721
4.4	52.0	T12		7.00	2.660
4.4	74.0	T13		7.00	2.660
4.4	78.0	T14		6.92	2.630
4.4	95.0	T15		6.96	2.645
4.4	97.0	T16		6.88	2.614

Table 3 (cont'd)

			600 nm		g/L
Added Acetate (g/L)	Hours	Sample	OD 6x dil	OD	DCW
8.8	0.0	T0(+)		0.07	0.027
8.8	1.8	T1		0.09	0.034
8.8	19.3	T2		2.46	0.935
8.8	25.2	T3		4.26	1.619
8.8	28.5	T4		6.18	2.348
8.8	45.2	T5		8.76	3.329
8.8	51.2	T6		7.86	2.987
8.8	55.4	T7		7.86	2.987
8.8	72.9	T8		7.80	2.964
8.8	81.6	T9		7.56	2.873
8.8	100.7	T10		6.84	2.599
17.6		T0(-)			0.000
17.6	0.0	T0(+)		0.07	0.027
17.6	20.4	T1		0.08	0.030
17.6	26.2	T2		0.14	0.053
17.6	29.9	T3		0.18	0.068
17.6	44.9	T4		3.06	1.163
17.6	50.7	T5		6.12	2.326
17.6	52.9	T6		6.96	2.645
17.6	69.4	T7		8.52	3.238
17.6	73.7	T8		8.40	3.192
17.6	77.4	T9		8.16	3.101
17.6	91.4	T10		8.40	3.192

Table 3 (cont'd)

			600 nm		g/L
Added Acetate (g/L)	Hours	Sample	OD 6x dil	OD	DCW
26.3		T0(-)		0.03	0.011
26.3	0.0	T0(+)		0.19	0.072
26.3	14.3	T1		0.19	0.072
26.3	21.5	T2		0.26	0.099
26.3	25.0	T3		0.27	0.103
26.3	39.5	T4		0.65	0.247
26.3	41.5	T5		0.75	0.285
26.3	43.5	T6		0.76	0.289
26.3	45.5	T7		0.75	0.285
26.3	47.7	T8		0.76	0.289
26.3	50.0	T9		0.72	0.274
26.3	53.0	T10		0.71	0.270
26.3	60.5	T11		0.66	0.251
26.3	63.7	T12		0.64	0.243
26.3	69.5	T13		0.62	0.236
26.3	90.0	T14		0.56	0.213
26.3	118.0	T15		0.59	0.224
26.3	164.7	T16	1.42	8.52	3.238
26.3	186.5	T17	1.4	8.40	3.192
26.3	208.0	T18	1.47	8.82	3.352
26.3	236.0	T19	1.55	9.30	3.534
26.3	261.5	T20	1.58	9.48	3.602

REFERENCES

REFERENCES

1. Francesco Canganella, J. W. (1993) The Potential of Thermophilic Clostridia in Biotechnology. In *The Clostridia and Biotechnology* (Woods, D. R., ed) pp. 393-429, Butterworth-Heinemann, Bosten

2. Zhu, Y., and Yang, S.-T. (2003) Adaptation of Clostridium tyrobutyricum for Enhanced Tolerance to Butyric Acid in a Fibrous-Bed Bioreactor. *Biotechnology Progress* **19**, 365-372

3. Ling Jiang, J. W., Shizhong Liang, Xiaoning Wang, Peilin Cen, Zhinan Xu (2010) Production of Butyric Acid from Glucose and Xylose with Immobilized Cells of *Clostridium tyrobutyricum* in a Fibrous-bed Bioreactor. *Applied Biochemistry and Biotechnology* **160**, 350-359

4. Helmerius, J., Walter, J. V. v., Rova, U., Berglund, K. A., and Hodge, D. B. (2010) Impact of hemicellulose pre-extraction for bioconversion on birch Kraft pulp properties. *Bioresource Technology* **101**, 5996-6005

5. Ezeji, T., Qureshi, N., and Blaschek, H. P. (2007) Butanol Production From Agricultural Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation. *Biotechnology and Bioengineering* **97**, 1460-1469

6. Canganella, F., Kuk, S.-U., Morgan, H., and Wiegel, J. (2002) Clostridium thermobutyricum: growth studies and stimulation of butyrate formation by acetate supplementation. *Microbiological Research* **157**, 149-156

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

8. Wilfrid J. Mitchell, K. A. A., Mohsen Yazdanian (1995) Factors affecting utilization of carbohydrates by clostridia. *FEMS Microbiology Reviews* **17**, 317-329

9. Joseph Aduse-Opoku, W. J. M. (1988) Diauxic growth of *Clostridium thermosaccharolyticum* on glucose and xylose. *FEMS Microbiology Letters* **50**, 45-49

10. Brumm, P. J. (1988) Fermentation of Single and Mixed Substrates by the Parent and an Acid-Tolerant, Mutant Strain of *Clostridium Thermoaceticum*. *BIOTECHNOLOGY AND BIOENGINEERING* **32**, 444-450

11. D. Michel-Savin, R. M., J.P. Vandecasteele (1990) Butyrate production in continuous culture of *Clostridium tyrobutyricum*: effect of end-product inhibition. *Applied Microbiology and Biotechnology* **33**, 127-131

12. Zhang, C., Yang, H., Yang, F., and Ma, Y. (2009) Current Progress on Butyric Acid Production by Fermentation. *Current Microbiology* **59**, 656-663

13. Michel-Savin, D., Marchal, R., and Vandecasteele, J. P. (1990) Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. *Applied Microbiology and Biotechnology* **32**, 387-392

14. Chen, C.-K., and 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**, 499-505

15. Jo, J. H., Lee, D. S., and Park, J. M. (2008) The effects of pH on carbon material and energy balances in hydrogen-producing *Clostridium tyrobutyricum* JM1. *Bioresource Technology* **99**, 8485-8491

CHAPTER 3: ACETATE ADAPTATION OF *CLOSTRIDIA TYROBUTYRICUM* FOR IMPROVED FERMENTATION PRODUCTION OF BUTYRATE

Data in this chapter has been published in:

Jaros, A.M., Rova, U., Berglund, K.A. (2013) Acetate adaptation of *Clostridia tyrobutyricum* for improved fermentation production of butyrate. *SpringerPlus* 2, 1-8

Abstract

Clostridium tyrobutyricum ATCC 25755 is an acidogenic bacterium capable of utilizing xylose for the fermentation production of butyrate. Hot water extraction of hardwood lingocellulose is an efficient method of producing xylose where autohydrolysis of xylan is catalysed by acetate originating from acetyl groups present in hemicellulose. The presence of acetic acid in the hydrolysate might have a severe impact on the subsequent fermentations. In this study the fermentation kinetics of *C. tyrobutyricum* cultures after being classically adapted for growth at 26.3 g/L acetate equivalents were studied. Analysis of xylose batch fermentations found that even in the presence of high levels of acetate, acetate-adapted strains had similar fermentation kinetics as the parental strain cultivated without acetate. The parental strain exposed to acetate at inhibitory conditions demonstrated a pronounced lag phase (over 100 hours) in growth and butyrate production as compared to the adapted strain (25 hour lag) or non-inhibited controls (0 lag).

Additional insight into the metabolic pathway of xylose consumption was gained by determining the specific activity of the acetate kinase (AK) enzyme in adapted versus control batches. AK activity was reduced by 63% in the presence of inhibitory levels of acetate, whether or not the culture had been adapted. This result indicates acetate adaptation is not based on an alteration of AK activity.

Key words: *Clostridium tyrobutyricum*, butyrate, xylose fermentation, hemicellulose utilization, acetate inhibition

Introduction

Butyric acid is approved by the Food and Drug Administration (US) as a flavor enhancer and several flavor esters used in the food industry are derived from butyric acid. There is a well established market for all-natural foods, where the components are not synthetically derived from petro-chemicals as well as a strong consumer bias against using genetically modified organisms (GMOs) in food production. Due to this, butyric acid fermented from biomass by wild type anaerobic bacteria can be developed as a saleable commodity.

Un-utilized hemicellulose streams from the pulp and paper industry can potentially, after hydrolysis, provide a low-cost source of xylose feedstock for organic acid fermentation. Hardwood xylan is extensively acetylated, i.e. up to seven acetyl groups per ten xylose units which facilitate xylose release by autohydrolysis (1). The resulting hemicellulose hydrolysate contains levels of acetate of up to 40 g/L acetic acid, inhibitory to microbial growth (2). When used in fermentation media, the inhibitory acetate generates a long lag period before log phase growth and butyric acid production (3). Previous work has shown that addition of 17.6 g/L and

26.3 g/L acetate in the media generates a lag phase of 45 and 118 hours, respectively, while un-inhibited controls begin fermentation and subsequently production almost immediately upon inoculation (3).

Multiple *Clostridial* strains have been classically selected for increased tolerance to both butanol and ethanol which successfully lead to higher solvent yields and higher overall productivity (4, 5). Due to the toxicity of these compounds, each step of the selection requires a short unchallenged incubation period, an exigency removed when challenging the organism with acetate.

For organic acid production, non-solventogenic *Clostridia* such a *C. tyrobutyricum* are used in fermentation processes where none of the typical toxic by-products such as butanol and ethanol are produced. *C. tyrobutyricum* cultures have been selectively adapted to tolerate the presence of inhibitory organic acids in order to increase acid product yields (6). Despite their success, these selections have been performed on immobilized *C. tyrobutyricum* cultures in fibrous-bed bioreactors requiring a 3-day cell growth period followed by a 36 to 48-hour cell immobilization period in order for a continuous feed fermentation to begin (6). Such a process allows for the eventual in-line adaptation of a *C. tyrobutyricum* culture to inhibitory acid products while simple adaptation techniques produce a tolerant culture ready to inoculate immediately into batch fermentation.

Through our work we have detected that *C. tyrobutyricum* demonstrates diauxic growth, the phenomena of a metabolic shift occurring in the middle of the growth cycle when the two carbon sources glucose and xylose are present (data not shown). The presence of a more

utilizable carbon source, in this case, glucose, prevents activation of the metabolic machinery required for the cells to consume the secondary substrate, xylose. Fortunately, *C. tyrobutyricum* readily consumes xylose if the culture has been pre-conditioned to xylose metabolism and no other sugar sources are available.

Anaerobic, butyrate-producing bacteria such as *Clostridia* metabolize glucose to pyruvate through the Embden-Meyerhof-Parnas (EMP) pathway and concomitantly generate acetate, butyrate, H_2 and CO_2 as major metabolic end-products (7). Xylose is specifically catabolised in the Hexose Monophosphate Pathway to pyruvate which is enzymatically co-oxidized with cellular coenzyme-A to acetyl coenzyme A (8, 9). Acetyl-CoA is the branch-point node of the acetate and butyrate end-product pathways where the enzymes phosphotransacetylase (PTA) and acetate kinase (AK) are responsible for the metabolism of acetyl-CoA to acetate if the branch-point does not follow the butyrate pathway (8). In attempts to force the carbon flux from the acetate to butyrate metabolic branch in *C. tyrobutyricum*, mutants have been developed using targeted integrational plasmid mutagenesis with inactivated *pta* and *ack* genes coding for PTA and AK respectively (10, 11). Fermentations with the mutants yielded more butyric acid compared to wild type *C. tyrobutyricum*, but both mutant strains demonstrated significantly slower growth kinetics than wild type and in both cases resulted in higher final acetic acid concentrations with increased acid tolerance (10, 11). These results exhibit a common issue of genetic engineering in that GMO's are typically less robust and demonstrate slower growth than wild type organisms and the complexity of most metabolic pathways allows for the re-routing of inactivated processes due to homeostasis. The presence of 17.6 g/L to 26.3 g/L initial acetate in the media has the similar effect of lowering acetate production in xylose-fermenting wild type

cultures (3). This simple means of directing carbon flux towards butyric acid production is an added benefit of working with high acetate media and is especially important in light of evidence that such levels of acetate are present in potential xylose feedstock streams (2, 3).

C. tyrobutyricum batch fermentations under high acetate-challenged conditions perform better with xylose as a carbon source than glucose. Fermentations with 26.3 g/L initial acetate generated 32.6 g/L butyric acid on xylose, while the comparable batch with glucose feed produced 22.3 g/L (3). Similar results were observed with all initial acetate concentrations (0, 4.4, 8.8, 17.6 g/L). However, batch fermentations utilizing high acetate (26.3 g/L initial acetate) xylose synthetic media resulted in an extended lag phase of 118 hours, lowering productivity (3). The extended lag phase generated by acetate is economically detrimental for batch fermentation of butyrate as it leads to a long period of reactor inactivity and potential exposure to microbial contamination. On the other hand, after lag phase the 26.3 g/L initial acetate-challenged batch obtained a similar biomass concentration as the lower acetate and control batches and surpassed them in final butyrate yield (3). The focus of this work is to adapt a strain of *C. tyrobutyricum* to increased acetate tolerance, thus decreasing the extended lag phase while maintaining the acetate re-utilization metabolic mechanism to deliver increased yields of butyric acid. As hardwood-derived hemicellulose hydrolysate feedstock gives rise to high levels of both xylose and acetate, a xylose-consuming strain capable of overcoming the acetate-induced lag and yet re-utilizing acetate to generate even more butyric acid would be of commercial value.

Methods

Microorganism and Adaptation

A lyophilized stock culture of *C. tyrobutyricum* (ATCC 25755) was re-hydrated under sterile anaerobic conditions in Reinforced Clostridial Media (RCM; Difco). Once the culture entered log phase, when the optical density (OD) at 600 nm was approximately 2.0, transfers were made to glycerol stock vials (CRYOBANK^{TM}) and the culture was maintained at -70°C. *C. tyrobutyricum* was classically adapted to 26.3 g/L inhibitory acetate equivalents by serially passaging log phase cultures into serum bottles with RCM containing subsequently higher concentrations of sodium acetate (starting at 0 g/L then, 6 g/L, 12 g/L, 24 g/L and 36 g/L sequentially) at each passage. As the molar mass of sodium acetate is 82.03 g/mol, these concentrations correspond with 0 g/L, 4.4 g/L, 8.8 g/L, 17.6 g/L, and 26.3 g/L acetic acid equivalents respectively.

The adaptation was performed on two sets of *C. tyrobutyricum* cultures, each culture solely conditioned to consuming either xylose or glucose so that the actual batch fermentations could be performed without a lag phase due to an altered sugar source. The glucose-conditioned culture was maintained with RCM from Difco with the appropriate additions of acetate equivalents in the form of sodium acetate. The xylose-conditioned culture bottles also received the appropriate amount of acetate equivalent from a media consisting of: 10 g peptone (Fisher), 10 g beef extract (Teknova), 3 g yeast extract (Bacto), 5 g sodium chloride (J.T. Baker), 0.5g L-cysteine (Sigma-Aldrich), 3g sodium acetate anhydrous (J.T. Baker), 0.5 g agar (Bacto) and 900 mL distilled water. For the xylose feed, 5 g of xylose (Acros) in 10 mL distilled water, separately

autoclaved at 121 °C for 20 min was added to the culture media. Prior to autoclaving all serum bottles were sparged with nitrogen to maintain an anaerobic atmosphere. Each serum bottle contained a total volume of 100 mL RCM (initial pH 6.5) with 5 mL from the previous stage used to inoculate the next higher acetate stage. During adaptation, serum bottles were incubated at 36°C in an incubator-shaker (New Brunswick Scientific Innova 40) with shaking at 80 rpm.

The cultures required 24 hours to adapt and reach log phase growth before passaging to the next level of selection with the exception of the last transfer of the 17.6 g/L acetate adapted cultures to the final 26.3 g/L. Glucose conditioned cultures required 48 hours to reach log phase when challenged with 26.3 g/L acetic acid and xylose conditioned required 96 hours of incubation to reach log phase.

C. tyrobutyricum inoculum for each batch fermentation were pre-conditioned to the correct sugar substrate in the inoculation media prior the batch fermentation by anaerobically inoculating 50 mL Screw Cap Corning tubes containing 35 mL sterile glucose or xylose based RCM with 5 mL of the stock culture. The inoculated tubes were cultivated under anaerobic conditions at 36°C, 80 rpm, until log phase, approximately when OD_{600} had reached a value of 2.

Fermentations

One-liter batch fermentations were conducted in New Brunswick Bioflo 310 2.5 L working volume reactors under anaerobic conditions at 36°C. For each batch, 950 mL media of the following composition was used; 6 g/L yeast extract, 5 ppm $FeSO_4$ 7 H_2O, and 200 mL

xylose or glucose at 300 g/L sterilized separately. Anaerobiosis was reached by sparging the vessel with nitrogen prior to inoculation. The batches were inoculated with 50mL log phase *C. tyrobutyricum* cultures. The nitrogen sparging was maintained until logarithmic growth in the vessel was observed.

Agitation was kept at 250 rpm and in order to maintain the *C. tyrobutyricum* cultures in acidogenic production, pH 6.0 was sustained with 5 M NaOH throughout the fermentation. Sodium acetate (0 – 36 g/L) was added to the initial media prior inoculation for studies assessing acetate inhibition. Fermentations without acetate are referred to as controls. Samples (10 mL) were withdrawn at regular intervals for analytical measurements. Data presented in the tables and figures of this study are the results of single batch fermentations while an analysis involving duplicate and triplicate fermentations is given in the discussion where stated.

Analytical methods

Organic acids and residual sugar were analyzed by HPLC (LC-20AT dual pump and 10A RI detector, Shimadzu) equipped with an ion exchange column (Aminex HPX-87H, 9 um, 7.8mm x 300mm, Bio-Rad) and a cation-H guard column (Micro-guard, 30mm x 4.6 mm) using 50 mM sulfuric acid as a mobile phase. The flow rate of the mobile phase was maintained at 1 mL/min during analysis with 20 μL of sample injected into the system with an auto-injector (SIL-20AHT, Shimadzu) with the column and guard maintained at 65°C in a column oven (CT0-20A, Shimadzu). Prior to analyses, samples were centrifuged at 10 000 rpm for 5 min in a micro-centrifuge (Microfuge 18, Beckman Coulter). Data for each sample was acquired with Shimadzu EZ Start 7.4 SP1 chromatography software using standards for glucose, xylose, butyrate, acetate and lactate.

Dry Cellular Weight Determination

Cell growth was monitored during fermentation by measuring the optical density at 600 nm. The biomass from 40 mL cell suspension, removed in triplicate, was dried in an 80°C dryer for 48 hours and the dry cell weight (DCW, g/L) determined. The optical densities were then converted to dry cell weight using the following equation: $DCW = 0.38(OD_{600})$. This optical density to dry cellular weight conversion formula was determined for the specific organism and media used in this study.

Specific Growth Rate (μ_{net})

DCW was used to determine the specific growth rate as described by Shuler *et al* (12). The DCW data points from the logarithmic growth phase were plotted on a semi-log graph to locate the period during that phase in which the culture experienced the fastest growth. These points were then used in the following equation: $\mu_{net}\ (1/h) = (\ln(DCW_X/DCW_0))/(Time_X - Time_0)$, where DCW was measured in g/L and time in hours. DCW_X is the last point during the fasted logarithmic growth period and DCW_0 is the first point. $Time_X$ and $Time_0$ are described similarly.

Acetate Kinase Assay

Bacterial cells from xylose-conditioned batches at log phase growth were chilled on ice and centrifuged at room temperature at 5,000 rpm for 5 min and washed in 25 mM Tris-HCl, pH 7.4 in order to remove acetate from the medium. After a second centrifugation the cell pellet was resuspended in 25 mM Tris-HCl, pH 7.4 and sonicated three intervals at 30 khz for 60 seconds,

while on ice, to lyse the cell wall. The supernatant was used for acetate activity studies using a method (13) where the conversion of acetate to acyl phosphates by acetate kinase is coupled to the formation of a ferric-hydroxamate complex detectable by UV-Vis at 540 nm. In summary, the enzyme activity was measured at 29ºC using UV/VIS spectroscopy where the absorbance of a 4 mL reaction mixture at 540 nm and the ferric-hydroxamate complex molar extinction coefficient of 0.169 mM^{-1} cm^{-1} was used to calculate the enzyme activity (6, 10). Acetate kinase activity was standardized to the total protein content of each sample, determined separately by Bradford (Bio-rad protein assay) using bovine serum albumin. One unit of acetate kinase is defined as the amount of enzyme producing 1 µmol of hydroxamic acid per minute at 29ºC and the specific activity calculated as units of activity/mg cellular protein. The results reported here are averages of enzyme assays run in triplicate.

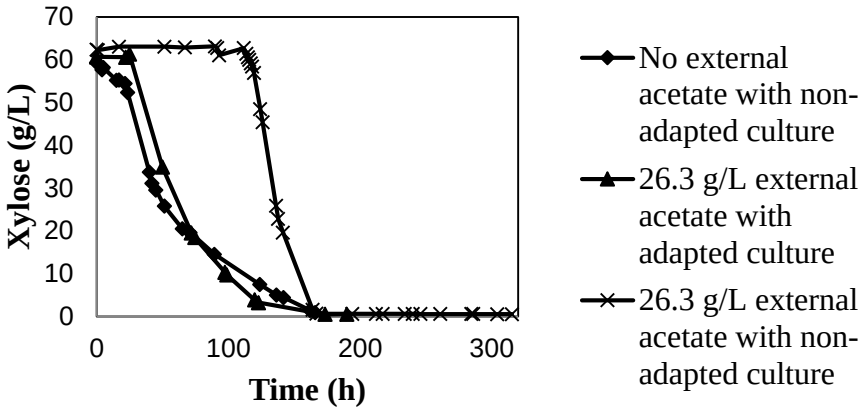
Results

Fermentation Kinetics

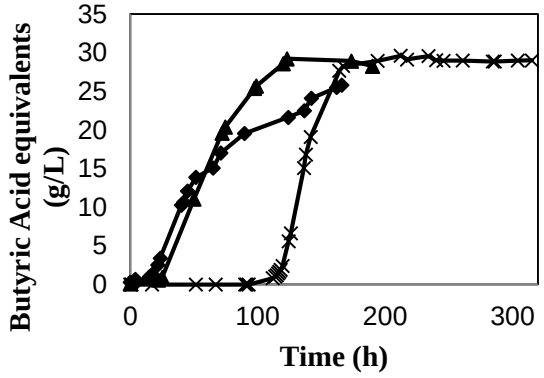
The non-adapted (control) *C. tyrobutyricum* culture inoculated into xylose-minimal media begins sugar consumption almost immediately with butyric acid production beginning 15 hours later (Fig. 7a and 7b). The same culture inoculated into xylose-minimal media containing 26.3 g/L acetate equivalents required over 100 hours to acclimate to the acetate despite both fermentations operating under the same conditions. The extended period of minimal metabolism and productivity is due to the acetate causing a delay in log phase cellular growth (Fig. 7c). Once the *C. tyrobutyricum* culture had adapted to the 26.3 g/L acetate media the culture performed like

the control, resulting in complete xylose utilisation and production of over 25 g/L of butyric acid and similar levels of cell mass.

a



b



c

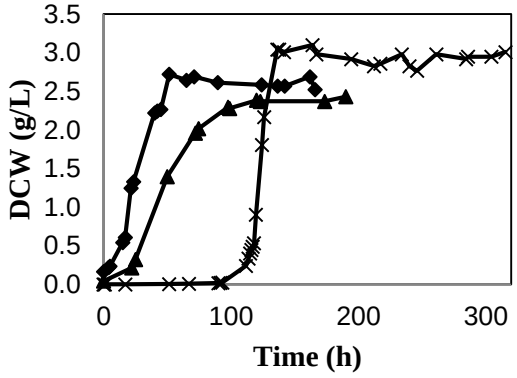


Figure 7-a, b, c. Effect of acetate inhibition on xylose consumption, butyrate production and biomass generation in *C. tyrobutyricum* fermentations using non-adapted or acetate-adapted cultures. Fermentations performed as 1 L batches in 2.5 L working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0

with 5M NaOH. The media contained 60 g/L xylose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$ and except for the control 26.3 g/L acetate

The acetate-adapted culture maintained tolerance to the 26.3 g/L acetate in the media and after a 22 hour lag in xylose consumption following inoculation, subsequently began producing butyric acid (Fig. 7a and 7b). The acetate-tolerant culture running under acetate inhibition conditions performed similarly to the control fermentation in that the xylose was fully utilized in 175 hours from inoculation and produced 28 g/L butyric acid compared to the controls production of 25.8 g/L butyric acid. Despite the increased product yield, the net specific growth rate (μ_{net}) of the acetate-tolerant culture was reduced by 28.7% compared to the control. The specific growth rate of the control fermentation was 0.093 1/h while the acetate-selected culture showed a log phase growth of 0.067 1/h (Table 2). This observation is not surprising as a similar yield increase corresponding with a growth rate reduction was seen in genetically modified *C. tyrobutyricum* where the *pta* gene had been deleted (10, 11).

The effectiveness of selective adaptation to generate an acetate tolerant *C. tyrobutyricum* culture is even more evident in glucose-consuming fermentations. The adapted inoculum under 26.3 g/L acetate conditions experienced no lag in growth and tracked almost exactly with the uninhibited control in terms of glucose consumption and butyric acid production (Fig. 8a and 8b). Unlike the xylose batches, the glucose-consuming cultures (control, non-adapted-inhibited and adapted-inhibited) generated similar levels of butyric acid between batches (25.61, 26.22 and 25.86 g/L respectively) (Table 2). Analogous to the xylose batches, the acetate- inhibited non-adapted culture experienced approximately 94 hours of lag phase before beginning to consume

glucose, produce butyric acid or generate DCW biomass (Fig. 8a-c, Table 2). Acetate adaptation allows the culture to overcome inhibition caused by 26.3 g/L acetate and the 94 hours of lag phase. A net production of acetate occurred in the glucose-consuming acetate adapted batch demonstrating the higher cellular energy made available from glucose consumption as compared to that of xylose. The xylose-consuming acetate-adapted batch activated the *Clostridial* acetate re-utilization pathway resulting in an overall consumption of acetate rather than production. This activation was likely necessitated by the lower amount of energy from xylose metabolism.

Table 4. Fermentation kinetics of *C. tyrobutyricum* cultures run in batch with or without selection for acetate tolerance and with or without acetate inhibition.

Sugar	Acetate (g/L)	*C. tyrobutyricum* [2]	Lag time[3] (h)	Complete utilization of carbon (h)	Sugar cons[4] (g/L/h)	Butyrate Yield[5] (mol/mol)
Glc[1]	0	non-adapted	0	77	1.07	0.85
Glc	26.3	non-adapted	94	171	1.09	0.89
Glc	26.3	adapted	0	75	1.21	0.87
Xyl[1]	0	non-adapted	0	166	0.56	0.74
Xyl	26.3	non-adapted	102	167	1.22	0.79
Xyl	26.3	adapted	25	174	0.6	0.81

Table 4 (cont'd)

Sugar	Acetate (g/L)	*C. tyrobutyricum* [2]	Final concentration (g/L)			Specific Growth Rate (μ_{net})[7] (1/h)	Overall produc.[8] (g/L/h)
			Butyrate	Acetate	Bio-mass[6]		
Glc[1]	0	non-adapted	25.61	8.38	3.4	0.306	0.28
Glc	26.3	non-adapted	26.22	27.85	3.59	0.274	0.15
Glc	26.3	adapted	25.86	32.03	2.77	0.206	0.32
Xyl[1]	0	non-adapted	25.8	4.24	2.72	0.093	0.16
Xyl	26.3	non-adapted	29	27.76	3.04	0.121	0.12
Xyl	26.3	adapted	28.92	24.46	2.3	0.067	0.17

[1] Glucose and xylose respectively

[2] Whether or not the inoculum had been selectively adapted to 26.3 g/L

[3] Calculated as time until sugar consumption started

[4] Calculated for the linear sugar consumption phase

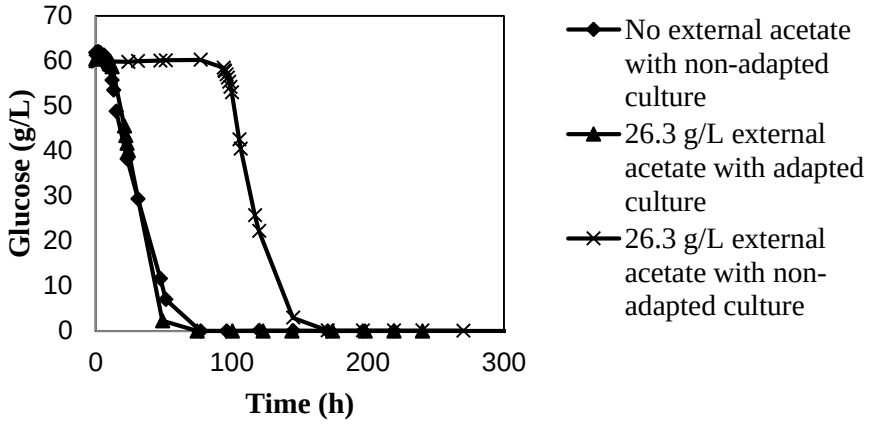
[5] Yield was calculated as mol butyrate per mol glucose or xylose consumed during fermentation

[6] Calculated as DCW g/L

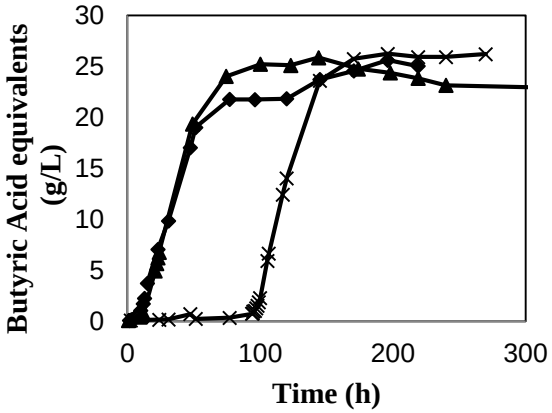
[7] As determined by the formula μnet (h^{-1}) = $(\ln(DCW_X/DCW_0))/(Time_X-Time_0)$

[8] Overall productivity calculated from the start of the fermentation until the sugar source were completed

a



b



c

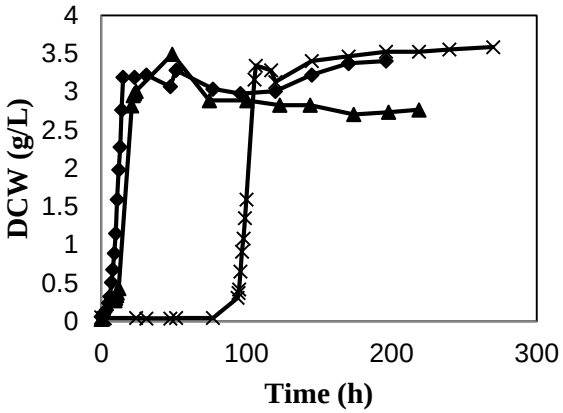


Figure 8-a, b, c. Effect of acetate inhibition on glucose consumption, butyrate production and biomass generation in *C. tyrobutyricum* fermentations using non-adapted or acetate-adapted cultures. Fermentations performed as 1 L batches in 2.5 L working volume vessels under anaerobic conditions. Temperature was maintained at 36°C, agitation at 250 rpm, and pH at 6.0 with 5M NaOH. The media contained 60 g/L glucose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$ and except for the control 26.3 g/L acetate

Similar to the xylose batches, the acetate-tolerant culture consuming glucose also exhibited a 32.7% reduction in specific growth rate compared with the glucose control culture (Table 2). The glucose control batch demonstrated a 0.306 1/h specific growth rate and the adapted culture dropped to 0.206 1/h during acetate inhibition (26.3 g/L). The non-adapted culture under acetate inhibition (26.3 g/L) dropped to 0.274 1/h, only a 10.5% reduction compared to the glucose control batch.

The specific growth rates of glucose consuming batches were two to three times higher than those of the xylose consuming *C. tyrobutyricum* batches (Table 2). Lowered specific growth rates are a consequence of xylose consumption due to the lowered energetic value of xylose metabolism over glucose. With less free energy from sugar consumption, the xylose consuming batches have less energy to perform cellular maintenance and growth thus, in general have lower specific growth rates than glucose consuming batches.

The xylose-consuming acetate-inhibited batches exhibited higher final yields of butyric acid than the control culture (Table 3). Both the acetate tolerant and non-adapted cultures yielded 0.48 g/g butyric acid from the initial 60 g/L xylose compared to the control cultures 0.43 g/g. Glucose-consuming cultures demonstrated no significant change in butyric acid yield between the 3 batches (Table 3).

The selection pressure during cultivation in 26.3 g/L acetate medium with xylose or glucose resulted in a strain with improved butyrate production while exposed to high acetate concentrations during fermentation. However, this phenotype was only preserved to some extent for the glucose-fermenting acetate-adapted strain. When this adapted strain, stored at -70°C, was

used directly to inoculate a 26.3 g/L acetate challenged media, the lag phase was increased to 42 hours (results not shown), compared to 94 hours for the non-adapted strain. In contrast, there was a complete reversion of the acetate-adapted strain during xylose fermentation using an inoculum from cryogenic storage (-70°C). Further characterization of strain stability and the molecular mechanisms resulting in increased tolerance for acetate is needed to identify target enzyme pathways or individual genes important for the desired phenotype. The induced tolerance of *C. tyrobutyricum* enables one to use adaptation as a tool to identify alteration of the organism's own enzyme systems that can be targeted for further permanent genetic modification.

Table 5. The effect of acetate inhibition on butyric acid yield in batch fermentations of *C. tyrobutyricum* with an initial 60 g/L glucose or xylose and run until completion.

	Butyric acid yield (g/g)		
Carbon source	No external acetate with non-adapted culture	26.3 g/L external acetate with non-adapted culture	26.3 g/L external acetate with adapted culture
Glucose	0.43	0.44	0.43
Xylose	0.43	0.48	0.48

Acetate Kinase Activity

The metabolic selectivity in *C. tyrobutyricum* is influenced by growth stage, with exponentially growing cultures producing both butyric and acetic acids, while slower stationary growth rates tend towards butyric acid (14). As such, during log phase growth of each batch, culture samples were removed and analyzed for acetate kinase activity. Acetate kinase (AK) is the last enzyme on the metabolic arm converting acetyl-CoA to acetate, thus AK activity under particular fermentation conditions is related to acetate production (11). Table 4 presents the

specific activity in relation to cellular protein. The presence of inhibitory acetate (26.3 g/L) in the media reduced the AK activity to 3.15 U/mg in both the adapted and non-adapted cultures as the control culture exhibited 8.42 U/mg (Table 4). In both cases of acetate inhibition, whether the culture was acetate tolerant or not, the acetate kinase activity was reduced leading to the inhibition of metabolic acetate production (Fig. 9, Table 2).

Table 6. The impact of the presence of acetate on enzymatic Acetate Kinase activity in *C. tyrobutyricum* fermentations.

	No external acetate with non-adapted culture	26.3 g/L external acetate with non-adapted culture	26.3 g/L external acetate with adapted culture
Acetate Kinase activity (Units/mg cellular protein)	8.42	3.15	3.15

Results reported here are averages of enzymes assays run in triplicate as described in the methods.

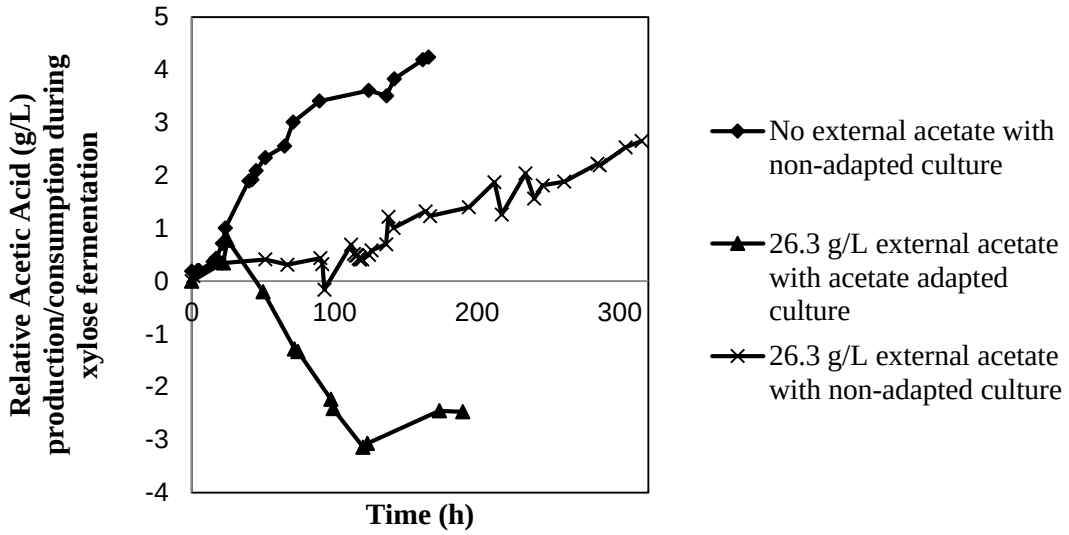


Figure 9. Effect of acetate inhibition on relative acetic acid fermentation kinetics of *C. tyrobutyricum* xylose batches. Fermentations performed as described in Fig. 7

The AK specific activity results correlate strongly to the production data in Fig. 9, where the control culture with the highest AK activity also generated the most acetic acid equivalents, 4.24 g/L. The non-adapted batch with 26.3 g/L initial acetic acid equivalents and the lowered AK activity generated only an additional 2.65 g/L acetic acid by the time the xylose had been completely utilized (Fig. 9). The selected batch run under the same initial acetic acid conditions performed with even higher carbon flux away from the acetate branch as acetate re-uptake mechanisms allowed the culture to consume 2.47 g/L of the initial acetate from the media (Fig. 9).

Discussion

Acetate-tolerant *C. tyrobutyricum* cultures consuming xylose overcame the acetate-induced lag growth phase four times faster than the comparable non-selected cultures under the

same acetate inhibition conditions (26.3 g/L) (Fig. 7a-c, Table 2). The selected culture also maintained lowered utilization of the acetate metabolic pathway under challenged conditions (Fig. 9 and Table 4). The acetate-producing metabolic pathway yields more ATP than the butyrate pathway, so an inhibition of acetate kinase (AK) or phosphotransacetylase (PTA) leads to increased carbon flux towards phosphotransbutyrylase (PTB) and butyrate kinase (BK) as the butyrate pathway must compensate for the energy loss (8, 14). Rather than lower energy consumption and less biomass generated, the acetate-inhibited *C. tyrobutyricum* cultures generated a similar amount of biomass as the control by increasing butyrate production to overcome the energy inefficiency (Fig. 7c, 8c). Similar to our results, *C. tyrobutyricum* fermentations with genetic inactivation of *pta* also had higher butyric yields and inactivated (or in our case, inhibited) acetate producers still developed similar levels of biomass as controls (Fig. 7c, 8c) (10).

Both acetate kinase and phosphotransacetylase are more sensitive to product inhibition by butyrate than the enzymes responsible for the butyrate pathway, butyrate kinase and phosphotransbutyrylase (6). This natural inhibition is beneficial from an industrial standpoint as shortly after the culture enters the exponential growth phase *C. tyrobutyricum* stops co-producing both acid products and singularly forms butyrate (14). The metabolic selectivity towards butyrate is further increased with the presence of acetate in the media as the acetate pre-adapted culture produced negligible quantities of acetic acid even during the beginning log phase stage (Fig. 9).

Other than AK inhibition, another innate mechanism pushing the carbon flux of the *Clostridial* metabolism towards butyrate and away from acetate is the re-uptake of acetate from the media back into the usable acetyl-CoA pool by the CoA transferase enzyme (14). This re-

utilization mechanism of acetate provides no energy benefits to the cell but allows for the control of environmental acetate and utilizes protons in the acetate-to-butyrate conversion process (14). Acetate re-uptake can be exploited under the conditions pertaining to a microbial inhibiting level of acetate present in the feed stream since the supposed contaminant in this case can potentially be used as a carbon source (2, 3). Some of the re-assimilated acetyl-CoA enters the butyrate pathway and thus this mechanism contributes to carbon efficiency (15). Acetate re-uptake occurred in the xylose consuming pre-adapted fermentation, not only is the final butyric concentration (28.92 g/L) higher than the control (25.8 g/L) but the initial acetate concentration decreases during the course of the study (Fig. 7b and 9). Unfortunately, CoA transferase is also implicated in a redundant pathway leading to acetate generation directly from acetyl-CoA, so information concerning this enzyme's specific activity may not provide useful information concerning the acetate re-uptake mechanism (11).

The selective adaptation of acetate-tolerant glucose-consuming cultures completely eliminated the acetate-induced lag phase in growth under inhibitory conditions (Fig. 8a-c, Table 2). The higher energetic value of glucose consumption over that of xylose appears to allow acetate-selected cultures consuming glucose to begin fermentation immediately even under 26.3 g/L acetate inhibition (Fig. 8a). This is remarkable given that the non-selected glucose consuming batch still required a 94 hour lag-phase to overcome acetate inhibition, similar to the 102 hours seen in the xylose consuming non-selected culture under the same conditions (Table 2). The selective adaptation of *C. tyrobutyricum* for acetate tolerance is more effective for glucose consuming cultures than xylose consumers.

The energetic differences between xylose and glucose consumption appear to also affect the final butyric acid yields for 26.3 g/L acetate inhibited batches (data not shown). Duplicate fermentations of 60 g/L xylose produced an average of 27.16 g/L butyric acid with a standard deviation of (+/- 1.93) while duplicate fermentations of 60 g/L dextrose average 24.34 g/L butyric acid (+/- 0.99), a non-significant difference. Challenging the fermentations with 26.3 g/L acetic acid exacerbates the difference between carbon sources and leads to a significant increase in butyric acid yield for xylose consuming batches (data not shown). Given 26.3 g/L acetic acid inhibition, triplicate non-adapted batches consuming 60 g/L xylose generated an average of 30.45 g/L butyric acid (+/- 2.80) with duplicate batches of challenged glucose consumers producing only 25.20 g/L butyric acid (+/- 1.44).

The overall higher specific growth rates of glucose batches compared to the xylose batches is another result of the higher energetic value of glucose metabolism (Table 2). Due to this, the specific growth rates of the glucose batches are all two-to-three times faster than the corresponding xylose batches. As would be expected, acetate inhibition slows the specific growth rates in glucose batches but surprisingly, the non-adapted acetate inhibited xylose batch had a faster specific growth rate (0.121 1/h) than the control 0.093 1/h (Table 2). This can be explained by the long 102 hours of lag-phase that the non-adapted xylose batch had to adapt to the high level of acetate.

The overall butyric acid productivity of the non-adapted acetate-inhibited xylose batch was only 0.12 g/L/h despite the faster specific growth rate. For industrial practices, the 102-hour lag-phase of the non-adapted xylose batch to start consumption is far too long a period of inactivity. The week of non-growth as the non-selected culture undergoes lag-phase would tie up

fermentation capacity and potentially allow for contamination of the batch with other acetate tolerant microbes. The acetate-adapted *C. tyrobutyricum* culture required only a 25 hour lag-phase until xylose consumption began, greatly reducing the time involved in complete batch fermentation.

The final yield of the selected acetate- challenged culture is 0.48 g/g (butyric acid/xylose), 0.05 g/g higher than control (0.43 g/g) (Table 3). This indicates the power of a simple selection method to adapt a culture which increases yield without the use of genetic modification. As one of the markets for bacterially fermented butyrate is as an all-natural food enhancer, a production process not utilizing genetically modified organisms might be a requirement.

Conclusion

A simple selective adaptation for acetate tolerance generated a *C. tyrobutyricum* culture capable of reducing the acetate-induced lag-phase by 75% for a xylose-consuming fermentation and completely negated lag-phase in a glucose batch. Specific growth rates for acetate-inhibited (26.3 g/L) batches of adapted cultures were reduced compared to non-inhibited control batches but despite this, the adapted cultures demonstrated greater overall butyric acid production than controls for either carbon source. Enzymatic data collected on acetate kinase demonstrated reduced activity in cultures fermenting xylose in the presence of acetate whether or not the culture had been selected for acetate tolerance. Acetate adapted glucose consuming cultures preserved in cryogenic (-70°C) storage maintain partial acetate tolerance. As selective adaption

is a simpler technique to perform than genetic modification, the work here presents the potential for industrially producing all-natural butyric acid for consumer use.

Acknowledgments

The authors gratefully acknowledge the support by the Swedish Energy Agency, Swedish Governmental Agency for Innovation Systems (VINNOVA), the United States Defense Logistics Agency, and Bio4Energy, a strategic research environment appointed by the Swedish government.

APPENDIX

Table 7. Raw data used to generate Figures 7a-b in Chapter 3.

				g/L			
Acetate adapted culture	External Acetate (g/L)	Hours	Sample	Xylose	Lactic Acid	Acetic Acid	Butyric Acid
No	0		T0(-)	65.36	0.35	0	0
No	0	0	T0(+)	59.30	0.10	0.19	0.30
No	0	4	T1	57.63	0	0.20	0.70
No	0	5	T2	58.13	0.13	0.20	0.37
No	0	15	T3	55.17	0	0.37	1.02
No	0	17	T4	55.26	0.15	0.43	1.31
No	0	21.5	T5	54.43	0.11	0.72	2.53
No	0	23.5	T6	52.36	0.20	1.01	3.42
No	0	40	T7	33.75	0.54	1.90	10.28
No	0	42	T8	31.08	0.55	1.92	10.67
No	0	45	T9	29.55	0.57	2.09	12.12
No	0	51.5	T10	25.77	0.35	2.34	13.87
No	0	65	T11	20.52	0.33	2.56	15.11
No	0	71	T12	19.62	0.37	3.01	17.03
No	0	89.5	T13	14.54	0.38	3.41	19.56
No	0	124	T14	7.45	0.36	3.61	21.64
No	0	136.5	T15	5.03	0.61	3.51	22.50
No	0	142	T16	4.43	0.39	3.83	24.11
No	0	162	T17	1.46	0.41	4.19	25.51
No	0	166	T18	0.94	0.44	4.24	25.80

Table 7 (cont'd)

				g/L			
Acetate adapted culture	External Acetate (g/L)	Hours	Sample	Xylose	Lactic Acid	Acetic Acid	Butyric Acid
Yes	26.3		T0(-)	63.73	0.54	26.96	0.27
Yes	26.3	0	T0(+)	60.65	0.14	26.91	0.12
Yes	26.3	22	T1	60.56	0.28	27.26	0.59
Yes	26.3	25	T2	61.28	0.28	27.69	0.92
Yes	26.3	50	T3	34.89	0.14	26.71	11.08
Yes	26.3	72	T4	19.51	0.17	25.63	19.62
Yes	26.3	74.5	T5	18.47	0.16	25.58	20.38
Yes	26.3	97.5	T6	10.30	0.16	24.68	25.39
Yes	26.3	99	T7	9.69	0.17	24.50	25.69
Yes	26.3	120	T8	3.88	0.16	23.77	28.58
Yes	26.3	123	T9	3.26	0.15	23.84	29.22
Yes	26.3	173.5	T10	0.53	0.15	24.46	28.92
Yes	26.3	190	T11	0.57	0.14	24.44	28.27
No	26.3		T0(-)	68.606	1.072	28.719	
No	26.3	0	T0(+)	62.333	0.339	25.109	0
No	26.3	1	T1	62.207	0.357	25.208	0
No	26.3	17	T2	63.024	0.334	25.442	0
No	26.3	52	T3	63.044	0.326	25.521	0
No	26.3	67	T4	62.849	0.314	25.423	0
No	26.3	90	T5	63.124	0.303	25.545	0
No	26.3	92	T6	62.74	0.311	25.435	0
No	26.3	93	T7	61.029	0.334	24.95	0
No	26.3	112	T8	62.699		25.801	0.839
No	26.3	114	T9	61.379		25.627	1.051
No	26.3	115	T10	60.556		25.595	1.235
No	26.3	116	T11	59.92		25.598	1.455
No	26.3	117	T12	59.117		25.544	1.686

Table 7 (cont'd)

				g/L			
Acetate adapted culture	External Acetate (g/L)	Hours	Sample	Xylose	Lactic Acid	Acetic Acid	Butyric Acid
No	26.3	118	T13	58.376		25.518	1.91
No	26.3	120	T14	56.873		25.521	2.401
No	26.3	124	T15	48.421	0.416	25.611	5.591
No	26.3	126	T16	45.354	0.473	25.692	6.644
No	26.3	136	T17	25.847	0.703	25.809	15.074
No	26.3	138	T18	22.78	1.101	26.328	16.825
No	26.3	142	T19	19.595	1.006	26.119	19.125
No	26.3	164	T20	1.599	0.99	26.433	27.671
No	26.3	167	T21	0.651	0.982	26.342	28.296
No	26.3	194	T22	0.623	0.586	26.51	28.944
No	26.3	212	T23	0.617	0.532	26.982	29.603
No	26.3	217	T24	0.625	0.543	26.374	29.132
No	26.3	234	T25	0.609	0.532	27.152	29.573
No	26.3	240	T26	0.609	0.534	26.672	29.024
No	26.3	246	T27	0.597	0.523	26.924	28.979
No	26.3	261	T28	0.59	0.522	26.992	28.996
No	26.3	285	T29	0.586	0.526	27.333	28.863
No	26.3	286	T30	0.578	0.521	27.301	28.862
No	26.3	304	T31	0.569	0.512	27.643	28.986
No	26.3	315	T32	0.557	0.515	27.763	29.003

Table 8. Raw data used to generate Figure 7c in Chapter 3. OD, optical density. DCW, dry cellular weight.

Acetate adapted culture	External Acetate (g/L)	Hours	Sample	OD 600 nm	DCW (g/L)
No	0		T0(-)	0	0.000
No	0	0	T0(+)	0.44	0.167
No	0	4	T1	0.58	0.220
No	0	5	T2	0.63	0.239
No	0	15	T3	1.43	0.543
No	0	17	T4	1.61	0.612
No	0	21.5	T5	3.28	1.246
No	0	23.5	T6	3.50	1.330
No	0	40	T7	5.85	2.223
No	0	42	T8	5.91	2.246
No	0	45	T9	5.97	2.269
No	0	51.5	T10	7.16	2.721
No	0	65	T11	6.96	2.645
No	0	71	T12	7.08	2.690
No	0	89.5	T13	6.88	2.614
No	0	124	T14	6.80	2.584
No	0	136.5	T15	6.76	2.569
No	0	142	T16	6.76	2.569
No	0	162	T17	7.08	2.690
No	0	166	T18	6.64	2.523

Table 8 (cont'd)

Acetate adapted culture	External Acetate (g/L)	Hours	Sample	OD 600 nm	DCW (g/L)
Yes	26.3		T0(-)	0.03	0.011
Yes	26.3	0	T0(+)	0.12	0.046
Yes	26.3	22	T1	0.57	0.217
Yes	26.3	25	T2	0.85	0.323
Yes	26.3	50	T3	3.68	1.398
Yes	26.3	72	T4	5.16	1.961
Yes	26.3	74.5	T5	5.31	2.018
Yes	26.3	97.5	T6	6.04	2.295
Yes	26.3	99	T7	6.0	2.280
Yes	26.3	120	T8	6.28	2.386
Yes	26.3	123	T9	6.24	2.371
Yes	26.3	173.5	T10	6.24	2.371
Yes	26.3	190	T11	6.40	2.432
No	26.3		T0(-)		
No	26.3	0	T0(+)	0.01	0.0
No	26.3	1	T1	0.01	0.0
No	26.3	17	T2	0.01	0.0
No	26.3	52	T3	0.02	0.0
No	26.3	67	T4	0.03	0.0
No	26.3	90	T5	0.05	0.0
No	26.3	92	T6	0.06	0.0
No	26.3	93	T7	0.07	0.0
No	26.3	112	T8	0.64	0.2
No	26.3	114	T9	0.89	0.3
No	26.3	115	T10	1.06	0.4
No	26.3	116	T11	1.18	0.4
No	26.3	117	T12	1.29	0.5
No	26.3	118	T13	1.41	0.5
No	26.3	120	T14	2.38	0.9
No	26.3	124	T15	4.76	1.8

Table 8 (cont'd)

Acetate adapted culture	External Acetate (g/L)	Hours	Sample	OD 600 nm	DCW (g/L)
No	26.3	126	T16	5.7	2.2
No	26.3	136	T17	8.0	3.0
No	26.3	138	T18	8.0	3.0
No	26.3	142	T19	7.92	3.0
No	26.3	164	T20	8.16	3.1
No	26.3	167	T21	7.84	3.0
No	26.3	194	T22	7.68	2.9
No	26.3	212	T23	7.44	2.8
No	26.3	217	T24	7.52	2.9
No	26.3	234	T25	7.84	3.0
No	26.3	240	T26	7.44	2.8
No	26.3	246	T27	7.28	2.8
No	26.3	261	T28	7.84	3.0
No	26.3	285	T29	7.68	2.9
No	26.3	286	T30	7.76	2.9
No	26.3	304	T31	7.76	2.9
No	26.3	315	T32	7.92	3.0

Table 9. Raw data used to generate Figures 8a-b in Chapter 3.

				g/L			
Acetate adapted culture	External Acetate (g/L)	Hours	Sample	Glucose	Lactic Acid	Acetic Acid	Butyric Acid
No	0	0	T0(+)	61.856		0.138	0
No	0	1.5	T1	62.047		0.144	0.094
No	0	2.5	T2	61.888			0.128
No	0	3.5	T3	61.776		0.125	0.177
No	0	5	T4				
No	0	6	T5	61.268		0.345	0.36
No	0	7	T6	60.795		0.37	0.437
No	0	8	T7	60.339		0.484	0.609
No	0	9	T8	59.531		0.584	0.799
No	0	10	T9	58.491	0.096	0.717	1.056
No	0	11	T10				
No	0	12	T11	55.729	0.152	1.028	1.72
No	0	13	T12	53.499	0.155	1.258	2.268
No	0	14	T13				
No	0	15	T14	48.816	0.234	1.771	3.739
No	0	23	T15	38.256	0.355	2.46	7.063
No	0	31	T16	29.345	0.408	3.052	9.849
No	0	47.5	T17	11.636	0.201	4.387	17.044
No	0	51.5	T18	6.995	0.467	4.792	19.029
No	0	77	T19	0	0.172	6.501	21.779
No	0	96	T20	0	0.145	6.665	21.753
No	0	120	T21	0.102	0.129	6.788	21.84
No	0	145	T22	0.077	0.123	7.397	23.726
No	0	170.5	T23	0	0.117	7.651	24.561
No	0	196.25	T24	0	0.544	8.103	25.611
No	0	219	T25	0	0.54	8.382	25.044

Table 9 (cont'd)

				g/L			
Acetate adapted culture	External Acetate (g/L)	Hours	Sample	Glucose	Lactic Acid	Acetic Acid	Butyric Acid
Yes	26.3		T0(-)	63.875	0.543	26.03	
Yes	26.3	0	T0(+)	60.444		24.97	0
Yes	26.3	1	T1	60.46		25.02	0.087
Yes	26.3	9.5	T2	59.776		25.35	0.451
Yes	26.3	10	T3	59.365		25.19	0.515
Yes	26.3	10.5	T4	59.485		25.48	0.603
Yes	26.3	11	T5	59.299		25.44	0.643
Yes	26.3	12	T6	58.733		25.39	0.778
Yes	26.3	21	T7	45.54	0.278	25.93	4.964
Yes	26.3	22	T8	43.403		26.04	5.694
Yes	26.3	23	T9	41.75		26.14	6.259
Yes	26.3	24	T10	40.114	0.175	26.02	6.789
Yes	26.3	49	T11	2.261	0.125	25.8	19.35
Yes	26.3	74.5	T12	0		31.12	24.037
Yes	26.3	100.25	T13	0		31.47	25.232
Yes	26.3	123	T14	0		31.55	25.112
Yes	26.3	144	T15	0		32.03	25.862
Yes	26.3	174	T16	0		32.12	24.779
Yes	26.3	198	T17	0		32.3	24.383
Yes	26.3	219	T18	0		32.4	23.853
Yes	26.3	240	T19	0		32.52	23.147
Yes	26.3	357.5	T20	0		32.66	22.807

Table 9 (cont'd)

				g/L			
Acetate adapted culture	External Acetate (g/L)	Hours	Sample	Glucose	Lactic Acid	Acetic Acid	Butyric Acid
No	26.3	0	T0(+)	59.956	0.11	23.85	0
No	26.3	1.5	T1	59.836		23.73	
No	26.3	2.5	T2	59.969		23.8	0.093
No	26.3	24	T3	59.796		23.71	0.163
No	26.3	31	T4	59.98		23.9	0.197
No	26.3	47.5	T5	60.10		23.92	0.715
No	26.3	51.5	T6	60.141		23.85	0.246
No	26.3	77	T7	60.264		24.01	0.347
No	26.3	94	T8	58.484		23.96	0.741
No	26.3	94.5	T9	57.954		24.03	0.798
No	26.3	95	T10	57.699		24.15	0.98
No	26.3	96	T11	56.999		24.06	1.042
No	26.3	97	T12	56.324		24.18	1.295
No	26.3	98	T13	55.344		24.2	1.539
No	26.3	99	T14	54.263		24.26	1.891
No	26.3	100	T15	52.994		24.33	2.277
No	26.3	105.5	T16	42.545	0.286	24.64	5.940
No	26.3	106.5	T17	40.502	0.306	24.63	6.650
No	26.3	117	T18	25.699	0.172	24.53	12.44
No	26.3	120	T19	22.234	0.363	24.75	14.032
No	26.3	145	T20	2.960	0.150	26.44	23.608
No	26.3	170.5	T21	0.097	0.135	27.32	25.751
No	26.3	196.25	T22	0.096	0.125	28.00	26.254
No	26.3	219	T23	0.105	0.316	27.86	25.93
No	26.3	240	T24	0.111	0.311	27.85	25.927
No	26.3	270	T25	0.076	0.123	27.85	26.22

Table 10. Raw data used to generate Figure 8c in Chapter 3.

Acetate adapted culture	External Acetate (g/L)	Hours	Sample	OD 600 nm	DCW (g/L)
No	0		T0(-)	0.02	0.01
No	0	0	T0(+)	0.17	0.06
No	0	1.5	T1	0.23	0.09
No	0	2.5	T2	0.32	0.12
No	0	3.5	T3	0.40	0.15
No	0	5	T4	0.64	0.24
No	0	6	T5	0.86	0.33
No	0	7	T6	1.34	0.51
No	0	8	T7	1.78	0.68
No	0	9	T8	2.34	0.89
No	0	10	T9	3.03	1.15
No	0	11	T10	4.20	1.60
No	0	12	T11	5.22	1.98
No	0	13	T12	6.00	2.28
No	0	14	T13	7.28	2.77
No	0	15	T14	8.40	3.19
No	0	23	T15	8.40	3.19
No	0	31	T16	8.48	3.22
No	0	47.5	T17	8.08	3.07
No	0	51.5	T18	8.64	3.28
No	0	77	T19	8.00	3.04
No	0	96	T20	7.84	2.98
No	0	120	T21	7.92	3.01
No	0	145	T22	8.48	3.22
No	0	170.5	T23	8.88	3.37
No	0	196.25	T24	8.96	3.40

Table 10 (cont'd)

Acetate adapted culture	External Acetate (g/L)	Hours	Sample	OD 600 nm	DCW (g/L)
Yes	26.3		T0(-)	0	0.00
Yes	26.3	0	T0(+)	0.08	0.03
Yes	26.3	1	T1	0.09	0.03
Yes	26.3	9.5	T2	0.72	0.27
Yes	26.3	10	T3	0.8	0.30
Yes	26.3	10.5	T4	0.89	0.34
Yes	26.3	11	T5	0.98	0.37
Yes	26.3	12	T6	1.13	0.43
Yes	26.3	21	T7	7.42	2.82
Yes	26.3	22	T8	7.77	2.95
Yes	26.3	23	T9	7.84	2.98
Yes	26.3	24	T10	7.92	3.01
Yes	26.3	49	T11	9.2	3.50
Yes	26.3	74.5	T12	7.6	2.89
Yes	26.3	100.25	T13	7.6	2.89
Yes	26.3	123	T14	7.44	2.83
Yes	26.3	144	T15	7.44	2.83
Yes	26.3	174	T16	7.12	2.71
Yes	26.3	198	T17	7.2	2.74
Yes	26.3	219	T18	7.28	2.77

Table 10 (cont'd)

Acetate adapted culture	External Acetate (g/L)	Hours	Sample	OD 600 nm	DCW (g/L)
No	26.3		T0(-)	0	0.00
No	26.3	0	T0(+)	0.14	0.05
No	26.3	1.5	T1	0.12	0.05
No	26.3	2.5	T2	0.11	0.04
No	26.3	24	T3	0.12	0.05
No	26.3	31	T4	0.10	0.04
No	26.3	47.5	T5	0.10	0.04
No	26.3	51.5	T6	0.12	0.05
No	26.3	77	T7	0.12	0.05
No	26.3	94	T8	0.81	0.31
No	26.3	94.5	T9	0.98	0.37
No	26.3	95	T10	1.10	0.42
No	26.3	96	T11	1.72	0.65
No	26.3	97	T12	2.4	0.91
No	26.3	98	T13	2.85	1.08
No	26.3	99	T14	3.56	1.35
No	26.3	100	T15	4.2	1.60
No	26.3	105.5	T16	8.32	3.16
No	26.3	106.5	T17	8.8	3.34
No	26.3	117	T18	8.64	3.28
No	26.3	120	T19	8.24	3.13
No	26.3	145	T20	8.96	3.40
No	26.3	170.5	T21	9.12	3.47
No	26.3	196.25	T22	9.28	3.53
No	26.3	219	T23	9.28	3.53
No	26.3	240	T24	9.36	3.56
No	26.3	270	T25	9.44	3.59

REFERENCES

REFERENCES

1. Teleman, A., Tenkanen, M., Jacobs, A., and Dahlman, O. (2002) Characterization of O-acetyl-(4-O-methylglucurono)xylan isolated from birch and beech. *Carbohydrate Research* **337**, 373-377

2. Helmerius, J., Walter, J. V. v., Rova, U., Berglund, K. A., and Hodge, D. B. (2010) Impact of hemicellulose pre-extraction for bioconversion on birch Kraft pulp properties. *Bioresource Technology* **101**, 5996-6005

3. Jaros, A. M., Rova, U., and Berglund, K. A. (2012) The Effect of Acetate on the Fermentation Production of Butyrate. *Cellulose Chemistry and Technology* **46**, 341-347

4. Lin, Y.-L., and Blaschek, H. P. (1983) Butanol Production by a Butanol-Tolerant Strain of Clostridium acetobutylicum in Extruded Corn Broth. *Applied and Environmental Microbiology* **45**, 966-973

5. Herrero, A. A., and Gomez, R. F. (1980) Development of Ethanol Tolerance in Clostridium thermocellum: Effect of Growth Temperature. *Applied and Environmental Microbiology* **40**, 571-577

6. Zhu, Y., and Yang, S.-T. (2003) Adaptation of Clostridium tyrobutyricum for Enhanced Tolerance to Butyric Acid in a Fibrous-Bed Bioreactor. *Biotechnology Progress* **19**, 365-372

7. Zhang, C., Yang, H., Yang, F., and Ma, Y. (2009) Current Progress on Butyric Acid Production by Fermentation. *Current Microbiology* **59**, 656-663

8. Zhu, Y., and Yang, S.-T. (2004) Effect of pH on metabolic pathway shift in fermentation of xylose by Clostridium tyrobutyricum. *Journal of Biotechnology* **110**, 143-157

9. Madigan, M. T., Martinko, J. M., Dunlap, P. V., and Clark, D. P. (2009) Metabolic Diversity: Catabolism of Organic Compounds. In *Biology of Microorganisms* (Brock, T. D., ed) pp. 612-651, Pearson Education, San Francisco, CA

10. Zhu, Y., Liu, X., and Yang, S.-T. (2005) Construction and Characterization of pta Gene-Deleted Mutant of Clostridium tyrobutyricum for Enhanced Butyric Acid Fermentation. *Biotechnology and Bioengineering* **90**, 154-166

11. Liu, X., Zhu, Y., and Yang, S.-T. (2006) Butyric acid and hydrogen production by Clostridium tyrobutyricum ATCC 25755 and mutants. *Enzyme and Microbial Technology* **38**, 521-528

12. Shuler, M. L., and Kargi, F. (2002) How Cells Grow. In *Bioprocess Engineering Basic Concepts* (Amundson, N. R., ed) pp. 155-200, Prentice Hall PTR, Upper Saddle River, New Jersey

13. Rose, I. A. (1955) Acetate Kinase of Bacteria (Acetokinase). *Methods in Enzymology* **1**, 591-595

14. Michel-Savin, D., Marchal, R., and Vandecasteele, J. P. (1990) Control of the selectivity of butyric acid production and improvement of fermentation performance with Clostridium tyrobutyricum. *Applied Microbiology and Biotechnology* **32**, 387-392

15. Canganella, F., Kuk, S.-U., Morgan, H., and Wiegel, J. (2002) Clostridium thermobutyricum: growth studies and stimulation of butyrate formation by acetate supplementation. *Microbiological Research* **157**, 149-156

CHAPTER 4: BUTYRATE PRODUCTION FROM FERMENTATION OF PLANT BIOMASS HYDROLYSATE

Introduction

The global shift towards the utilization of renewable substrates in the manufacture of man-made products has developed an upsurge in process designs boycotting the use of petro-chemical synthesis. Consumer preference towards natural products has generated increased demand for the discovery of non-synthetic methods to produce commonly consumed chemicals. Integrating the production of value added bio-based products to bio-refinery fuel and power output increases the profitability of related products and operational efficiency of the plant (1). Specifically, maximizing the use of feedstock components increases operational efficiency while the development of byproducts from waste streams enhances refinery profitability, thus making investment in biorefineries more economically attractive (1). Decades of research developing cellulosic biofuel processes has indicated that biomass can be incorporated into bio-refinery plant design and processes to generate bio-based fuels and chemicals (2).

Wood biomass from forests and municipal solid wastes are potential sources of lignocellulosic biomass, along with crops and agricultural residues (3). Lignocellulosic material is the most abundant resource in the world (4). The majority of fermentable carbon in plant biomass is locked in the form of cellulose and hemi-cellulose. In nature, brown rot fungi (*Gloeophyllum trabeum, Postia placenta* and *Merulius lacrymans* are examples) utilize oxidative Fenton reactions and available transition metals (Fe^{2+} and theoretically Ti^{3+}) to control the formation of reduced oxygen species ($O_2^{\bullet-}$/$HOO^{\bullet}$, HOO^-/H_2O_2, $HO^{\bullet}$) (5). The hydroxyl radicals

attack the carbohydrates of lignocellulosic material degrading the cellulose and hemicelluloses for the microbe's consumption and growth.

On the industrial scale, the necessary conversion of plant structural carbohydrates to fermentable sugars occurs much faster than in nature through enzymatic hydrolysis. The microbial fermentation production of fuels and chemicals requires this saccharification step in order for such fermentations to take place efficiently (6). Lignin inhibits saccharification enzymes from actively degrading cellulose and hemicelluose by acting as a physical and chemical barrier preventing the enzymes from binding to the proper substrate (7). This inhibition makes the removal of lignin an important element of industrial biomass processing (8).

Lignin is the component of plants binding hemicellulose within the plant cell wall to stabilize cell size and intercellular connections to conjugate nearby cells providing rigidity and strength to the plant. The presence of lignin varies between types of plants with the polymer comprising less than 20% of the mass of grasses and up to 30% of the mass of wood (9). Lignin is formed by the plant through enzymatic dehydrogenation of phenylpropanes, namely, originating units of coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol (9). Often these originating units are derivatized during or prior to polymerization, i.e. guaiacyl and syringyl derivatives of coniferyl alcohol are found in the composition of hardwood lignin (9). Lignification is the polymerization of the phenylpropanes through the mechanism of dehydrogenation and radical coupling (9, 10). Radical coupling leads to the formation of non-patterned carbon-carbon bonds and some ether linkages generating a nebulous polymer but with a tight structure difficult to either chemically or enzymatically degrade (10). These properties protect plants physical integrity and from microbial infection as the tight structure of lignin prevent attacking microbes from gaining a foothold or cleaving fermentable carbon. Fully

formed lignin polymers contain a wide variety of covalent linkages between lignin monomers including phenyl-alkyl ether linkages resistant to acidic and alkali hydrolysis as well as ester linkages resistant to acidic hydrolysis (10). The ether linkages between lignin monomers are the most readily hydrolyzable and other carbon-carbon bonds are more resistant to chemical degradation.

Lignin hydrolysis and fractionation is an important element in the processing of plant biomass for use as a cellulosic substrate and not only for the increased efficiency of enzymatic saccharification. Without the removal of lignin from the material, lignin degradation products remain present after the saccharification of cellulose and hemicellulose and inhibit subsequent fermentations (11). The class of lignin-based inhibitors present in pretreated media is dependent upon pretreatment method. Acid treatments generate carboxylic acid-based lignin derivatives and furfurals while ammonia-based pretreatments leave phenolic amide residues in the media (11). Several production models exist involving a consequent detoxification step of fermentation media generated from pretreated lignocellulosic material.

Rather than detoxify pretreated material, removal of the lignin fraction prior to or during pretreatment limits the formation of toxic lignin derivatives from the process. The removal of the lignin fraction from biomass material is not only required to limit toxic derivative formation but also for the enhanced enzymatic saccharification of cellulose and hemi-cellulose. One common type of fractionation employed to separate lignin generates “Ethanol process lignin” by solubilizing the cellulose and hemicelluloses fractions of lignocellulosic material for fermentation and leaving a residual insoluble lignin residue (12). Despite the name ethanol process lignin, fermentable sugars derived from this process could similarly be used in other bacterial fermentations including the fermentation production of butyric acid. Alternatively, there

are ammonia-based aqueous treatments solubilizing lignin from cellulose and hemicellulose removing saccharification inhibiting lignin and down-stream toxic derivatives allowing for the later recovery and refining of the lignin fraction (2, 7, 12). These later methods are similar to the current industrial standards for lignin fractionation in the pulp and paper industry of kraft and sulfite pulping (12, 13).

Lignin fractionation and refining is an important aspect for the overall production of lignocellulosic fuels and chemicals as it creates a by-product stream adding value to the process itself (2). Annually, 750,000 tons of lignin is used for industrial purposes namely to produce water-soluble lignosulfonates for complexing and emulsion stabilizing (14). Lignosulfonates from sulfite pulping act as dispersants, binders, complexing and emulsifying agents. Kraft pulping is a more often used chemical processes than sulfite pulping and results in the precipitation of black liquor which is typically burned for energy. Lignin has a heating value of 27 MJ/kg, consequently the conventional practice of burning spent liquor generates steam and electricity for the plant (15). The removal of the lignin fraction from lignocellulosic material for the improvement of downstream hydrolysis and fermentation is economically feasible in terms of refinery processes (13, 15).

Beyond the lignin polymer, enzymatic hydrolysis of plant cellulose and hemicellulose is inhibited by lignin-carbohydrate ester/ether linkages, thus preventing the activity of saccharafication enzymes from generating fermentable sugars (7, 13). As lignin-carbohydrate complexes (LCCs) are covalent bonds, they are energetically difficult to remove even after plant material is processed and sent through kraft pulping (16). No distinct enzymatic cleavage of lignin-carbohydrate complexes have been developed (17). There are known methods to cleave LCCs including ammonia recycle percolation to fractionate and remove the majority of lignin

but even under optimal conditions, 9.8% of lignin remains residually (7). While the extraction of lignin from plant material for sale as a by-product commodity does lower the levels of contaminating lignin in the material, the LCC lignin remains bonded to the structural carbohydrates to some degree even after delignification, potentially inhibiting saccharification (7).

Mainly, the structure of LCCs are ester or ether linkages formed between the α-carbon of a phenylpropane subunit of lignin and the primary hydroxyl group of a structural carbohydrate unit (17). Some LCCs are based on ether linkage formation between the C5 or even C3 attached hydroxyl group on an individual sugar unit and the α-carbon of a lignin phenylpropane unit but these variations are dependent upon plant species (10). Quinone methide intermediate groups naturally occur in lignin either during lignification or during H_2O elimination in mature plants (18). With the addition of carbohydrate units to quinine methide intermediates, benzyl ester and benzyl ether linkages are developed (10). In the formation of an LCC bond, the reacting hydroxyl group may be from glucose, xylose, mannose or other sugar monomers. Due to this, lignin can be covalently bonded to either of the structural polysaccharides, cellulose (a chain of glucose monomers) or hemicellulose (commonly a xylan chain of xylose monomers).

Cleavage of the ester-linked LCCs occurs under mild alkali or dilute alkali treatment often under normal room temperature conditions (10). On the other hand, ether-linked LCCs are resistant to mild alkaline hydrolysis. Multiple plant material pretreatment methods have been developed for cleaving LCCs in order to increase the efficiency of carbohydrate hydrolysis.

Of the multiple pretreatment methods which exist for enhancing the enzymatic saccharification of plant biomass, three main methods were examined in this study. Ammonia-

fiber expansion (AFEX) involves the exposure of plant biomass to ammonia in a heated/pressurized Paar reactor which is then quickly de-pressurized to expand the cellulose/hemicelluloses fibers to provide for greater enzymatic hydrolysis activity. Alkaline hydrogen peroxide (AHP) is a treatment for loading plant biomass with H_2O_2 increasing the pH with NaOH to break down the lignin bonds allowing for enhanced cellulose and hemicelluloses exposure to enzymatic hydrolysis. AHP treated biomass slurries then need to be acidified for enzymatic hydrolysis to occur. Extracted-ammonia fiber expansion (E-AFEX ™) is a similar process to AFEX with the additional step of extracting lignin from the plant biomass before the AFEX process.

One analysis for the comparison of pretreatments is the effect of the pretreatment on the amount of glucan that can be broken down to glucose or xylan to xylose without degradation of sugars given the same level of enzymes. As the matrixes of cellulose, lignin and hemicellulose form chemical and structural barriers to enzymatic hydrolysis, the efficiency of a pretreatment can be determined by the release of fermentable sugars. Enzyme loading is determined based on the glucan in raw plant biomass rather than the pretreated material and allows for comparisons across alternative pretreatment methods. The purpose of the work presented here is to compare the suitability of hydrolysate from different pretreatment methods as fermentation substrate for butyrate production.

Materials and Methods

AFEX-CS

Serum bottle batch fermentations were performed using Ammonia-Fiber Expansion pre-treated corn-stover received from the Dale lab which had been enzymatically hydrolyzed based

on a 6:1 ratio (weight (g) biomass/ volume (l) hydrolysate) 6% glucan loading and 18% solids loading. An analysis of this sample (HPLC – Aminex 87H): 70.985 g/L dextrose, 36.75 g/L xylose, 1.323 g/L acetic acid. This sample was diluted with MilliQ H_2O to 60 g/L dextrose; 31 g/L xylose so that fermentation runs could be comparable to the work performed previously on Reinforced Clostridial Media (RCM). The pH of the AFEX-CS hydrolysate was 4.9. A 3 treatment study was performed; treatment 1 was inoculated without pH adjustment, treatment 2 was pH adjusted with 5M NaOH to pH 7.5, treatment 3 was diluted 2x with ddH_2O and pH adjusted to 7.5 with 5M NaOH.

A 1-liter batch fermentation was performed with a sample of this material as well.

E-AFEX-CS

One liter of lignin extracted-ammonia fiber expanded corn stover (E-AFEX ™) hydrolysate was received from the Dale lab for fermentation to butyric acid. The initial glucose and xylose concentrations were 77.25 g/L and 22.65 g/L respectively. A three-treatment preliminary study was performed each with a 50ml working volume of the E-AFEX-CS hydrolysate in nitrogen sparged serum bottles kept at 36°C with 100 rpm shaking and inoculated with *Clostridia tyrobutyricum*. Treatment 1 was the E-AFEX-CS hydrolysate directly inoculated (starting pH 4.5). The 2nd treatment was run with the pH of the hydrolysate increased to 7.5 with 5M NaOH. The 3rd treatment was 25 ml of the E-AFEX-CS hydrolysate with 25 ml sterile MilliQ water (2 x dilutions) that had been pH adjusted to 7.5 with 5M NaOH.

AHP-CS

Alkaline Hydrogen Peroxide-pretreated corn stover hydrolysate obtained from the Hodge lab (MSU) was used for this study. Milled corn stover was treated with an H_2O_2 loading of 0.125 g/g biomass and then the pH of the slurry adjusted up to 11.5 with NaOH in order to break down lignin structures. The pH of the slurry was then brought back down to 4.5 for enzymatic hydrolysis performed in a similar manner as the AFEX-CS hydrolysate.

Specific Growth Rate (μ_{net})

Dry cellular weight (DCW) was used to determine the specific growth rate as described by Shuler *et al* (19). The DCW data points from the logarithmic growth phase were plotted on a semi-log graph to locate the period during that phase in which the culture experienced the fastest growth. These points were then used in the following equation: μ_{net} (1/h) = $(\ln(DCW_X/DCW_0))/(Time_X-Time_0)$, where DCW was measured in g/L and time in hours. DCW_X is the last point during the fasted logarithmic growth period and DCW_0 is the first point. $Time_X$ and $Time_0$ are described similarly.

Fermentations

One-liter batch fermentations were conducted in New Brunswick Bioflo 310 2.5 L working volume reactors under anaerobic conditions at 36°C. The AFEX-CS hydrolysate batch fermentation was conducted with 870 ml of the 6% glucan loaded AFEX-CS hydrolysate diluted with 80 ml sterile ddH_2O. For the diauxic control batch, 750 mL media of the following composition was used; 6 g yeast extract, 5 ppm (per 1 liter final solution) $FeSO_4$ 7 H_2O and 60g dextrose. To this media a 35g xylose solution desolved in 200ml ddH_2O sterilized separately was added after autoclaving both. Anaerobiosis was reached by sparging the vessel with nitrogen

prior to inoculation. The batches were inoculated with 50mL log phase *C. tyrobutyricum* cultures. The nitrogen sparging was maintained until logarithmic growth in the vessel was observed. Agitation was kept at 250 rpm and in order to maintain the *C. tyrobutyricum* cultures in acidogenic production, pH 6.0 was sustained with 5 M NaOH throughout the fermentation. Samples (10 mL) were withdrawn at regular intervals for analytical measurements.

Analytical Methods

Hydrolysate material and fermentation samples were analyzed for organic acids and sugars by HPLC (LC-20AT dual pump and 10A RI detector, Shimadzu) equipped with an ion exchange column (Aminex HPX-87H, 9 um, 7.8mm x 300mm, Bio-Rad) and a cation-H guard column (Micro-guard, 30mm x 4.6 mm) using 50 mM sulfuric acid as a mobile phase. The flow rate of the mobile phase was maintained at 1 mL/min during analysis with 20 μL of sample injected into the system with an auto-injector (SIL-20AHT, Shimadzu) with the column and guard maintained at 65°C in a column oven (CT0-20A, Shimadzu). Prior to analyses, samples were centrifuged at 10 000 rpm for 5 min in a micro-centrifuge (Microfuge 18, Beckman Coulter). Data for each sample was acquired with Shimadzu EZ Start 7.4 SP1 chromatography software using standards for glucose, xylose, butyrate, acetate and lactate.

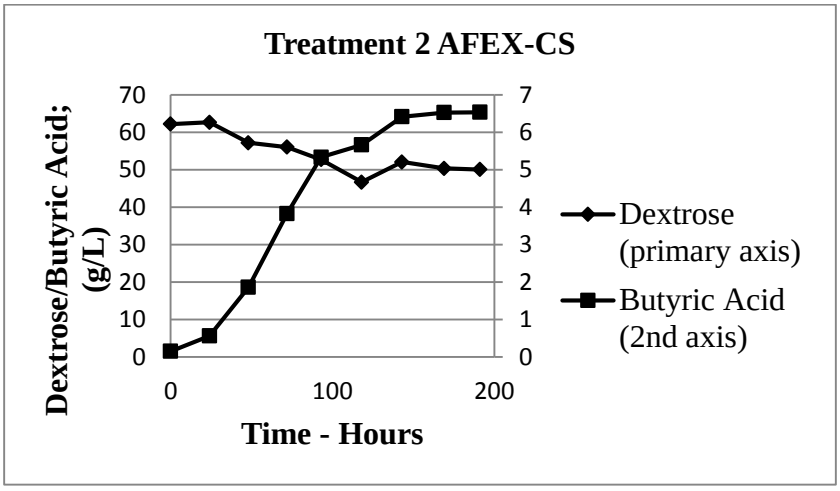
Results and Discussion

Several preliminary batch studies were performed to prove that agricultural residue hydrolysate can be utilized as a carbon source for *Clostridium tyrobutyricum* fermentation to produce butyrate. Un-adjusted AFEX-CS hydrolysate diluted with ddH_2O to approximate the same sugar concentrations (60.498 g/L dextrose; 30.938 g/L xylose) as previous Reinforced

Clostridial Media trials still proved too low of a pH for *C. tyrobutyricum* to ferment (data not shown). The pH of the pure hydrolysate is pH 4.9 and is likely too low for a healthy inoculum to begin a successful culture despite the low levels of initial acetate (0.88 g/L). Treatment 2, the pH adjustment to 7.5 of dilute AFEX-CS hydrolysate, was adequate for *C. tyrobutyricum* to use the hydrolysate for growth and the fermentation production of butyrate (Fig 10a). While no xylose was consumed during the treatment 2 fermentation, 11.85 g/L of dextrose was consumed (62.211 g/L dextrose initial reduced to 50.361 g/L dextrose after 169 hours) with the concomitant production of 6.528 g/L of butyrate.

Further dilution of AFEX-CS hydrolysate with pH adjustment proved equally utilizable as a fermentation substrate for *C. tyrobutyricum*. Treatment 3 began with 31.326 g/L dextrose which was consumed to 17.871 g/L dextrose after 96 hours, a 13.455 g/L difference (Fig 10b). The treatment 3 fermentation also demonstrated no consumption of xylose but did produce 5.693 g/L of butyrate demonstrating the ability of *C. tyrobutyricum* to ferment AFEX-CS hydrolysate.

a.



b.

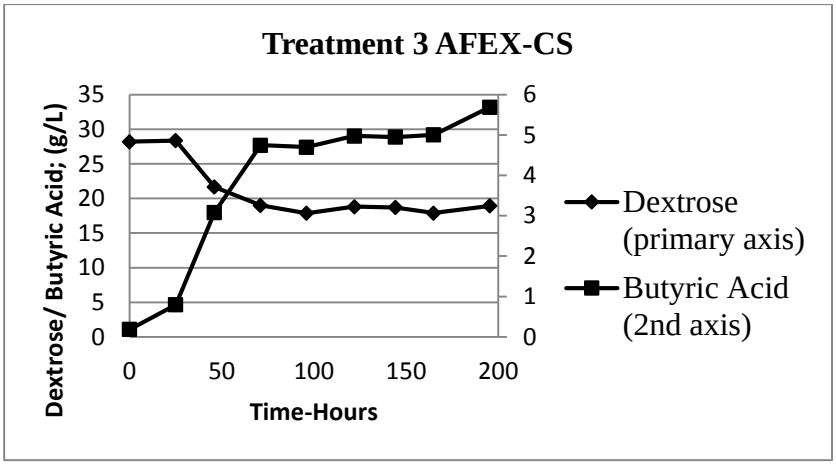


Figure 10a-b. *Clostridium tyrobutyricum* fermentation trials of pH adjusted AFEX-CS hydrolysate. **a)** Treatment 2 is AFEX-CS hydrolysate pH adjusted to 7.5 with 5M NaOH. **b)** Treatment 3 is AFEX-CS hydrolysate pH adjusted to 7.5 with 5M NaOH and further diluted 2x with ddH_2O. Batch fermentations were performed in sterile nitrogen sparged serum bottles at 50ml volume, 5 ml *C. tyrobutyricum* inoculums, 36°C and 50 rpm shaking.

As the degradation of lignin from biomass pretreatment leads to the generation of microbial inhibitors, lignin extraction is a method employed for the potential prevention of lignin degradation products from contaminating pretreated hydrolysate media. Serum bottle fermentations of *C. tyrobutyricum* were performed on lignin extracted-ammonia fiber expanded corn stover hydrolysate. No *C. tyrobutyricum* growth occurred on treatment 1, the direct inoculation of 50 ml of E-AFEX-CS with a 5 ml growth phase *C. tyrobutyricum* culture even after 300 hours of incubation. Despite the low initial acetate concentration of 0.370 g/L, the lack of growth was not surprising as the un-adjusted pH of the E-AFEX-CS hydrolysate was pH 4.5 and a similar low pH was seen to inhibit *C. tyrobutyricum* growth in AFEX-CS material.

After an extended lag phase of over 198 hours, treatment 2 of the E-AFEX-CS hydrolysate with pH adjusted to 7.5 was able to support *C. tyrobutyricum* fermentation (Fig. 11). The culture consumed 18.559 g/L dextrose (an initial concentration of 63.984 g/L dextrose reduced to 45.425 g/L after 366 hours) with the concomitant generation of 5.875 g/L butyrate with no consumption of the initial 20.56 g/L xylose. 1.389 g/L of acetate was also generated by the fermentation.

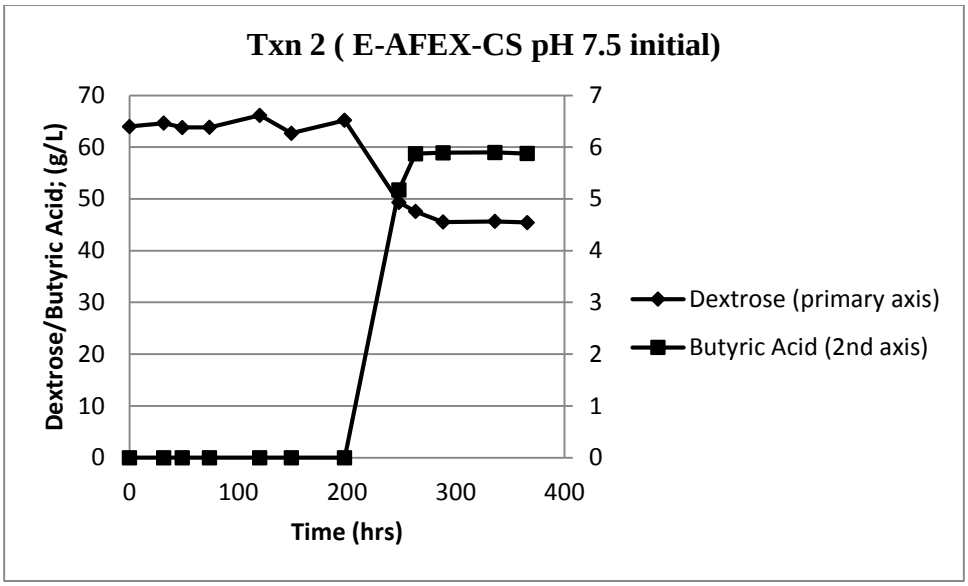


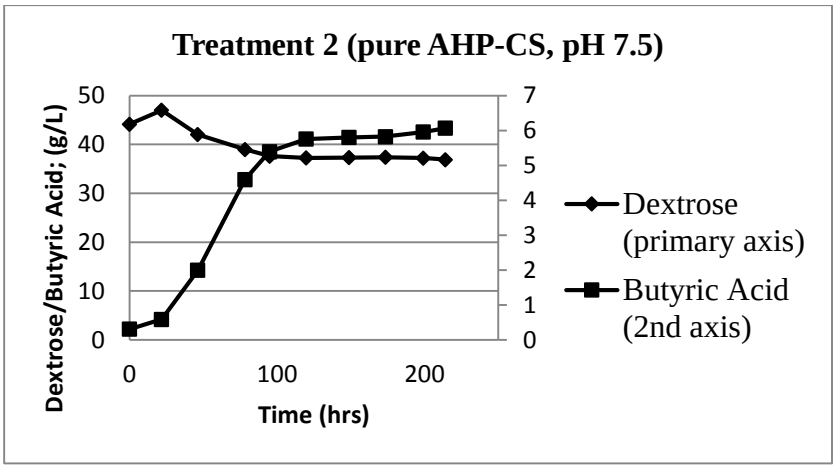
Figure 11. *Clostridium tyrobutyricum* fermentation trial of pH adjusted E-AFEX-CS hydrolysate. E-AFEX-CS hydrolysate batch fermentation was performed with 50 ml working volume in a nitrogen sparged serum bottle kept at 36°C with 100 rpm shaking and inoculated with 5ml of a *C. tyrobutyricum* culture $OD_{600nm} > 2$.

The third treatment of E-AFEX-CS hydrolysate was pH adjusted to 7.5 and the hydrolysate was diluted 2x with ddH_2O but no *C. tyrobutyricum* growth was observed even after 300 hours of incubation, similar to treatment 1.

Several serum bottle treatments were run of the alkaline hydrogen peroxide-pretreated corn stover and the direct fermentation of AHP-CS hydrolysate generated only a negligible amount of butyric acid which is likely caused by the low pH (pH 4.5) of the hydrolysate as it comes from the enzymatic treatment (data not shown). In treatment 2 the pH of the pure hydrolysate was adjusted up to 7.5 with 5M NaOH before inoculation and 5.8 g/L butyrate was produced after 100 hours of fermentation (Figure 12a). In treatment 5 the hydrolysate was pH

adjusted up to 7.5 and diluted in half with ddH_2O. The dilution was done to see if leftover H_2O_2 was having an inhibitory effect on the fermentation which could be reduced by dilution. Treatment 5 generated 5.1 g/L butyrate in less than 100 hours, similar enough to treatment 2 to conclude that residual H_2O_2 is not effecting AHP-CS fermentation (Figure 12b). As these were serum bottle studies there was no pH control on the fermentation so they both ended quickly as the pH dropped.

a.



b.

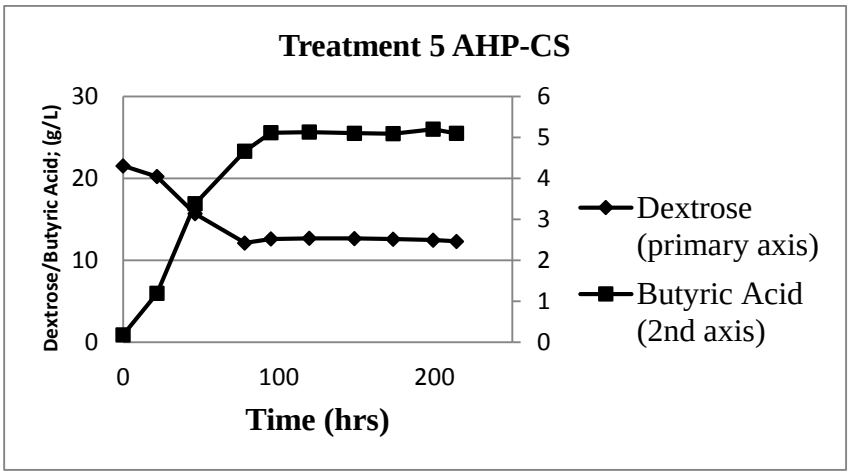


Figure 12a-b. Dextrose consumption and butyric acid production for *Clostridium tyrobutyricum* fermentation of Alkaline Hydrogen Peroxide pretreated corn-stover. Treatment 2 is pure hydrolysate and Treatment 5 is hydrolysate diluted 2x (ddH_2O). Hydrolysate was passed through a sterile filter (0.22 µm) prior to pH adjustment. Both treatments were initially pH adjusted to 7.5 with 5M NaOH. Fermentations were performed in sterile nitrogen sparged serum bottles at 50 ml volume, 5 ml *C. tyrobutyricum* inoculum, 36°C and 50 rpm shaking.

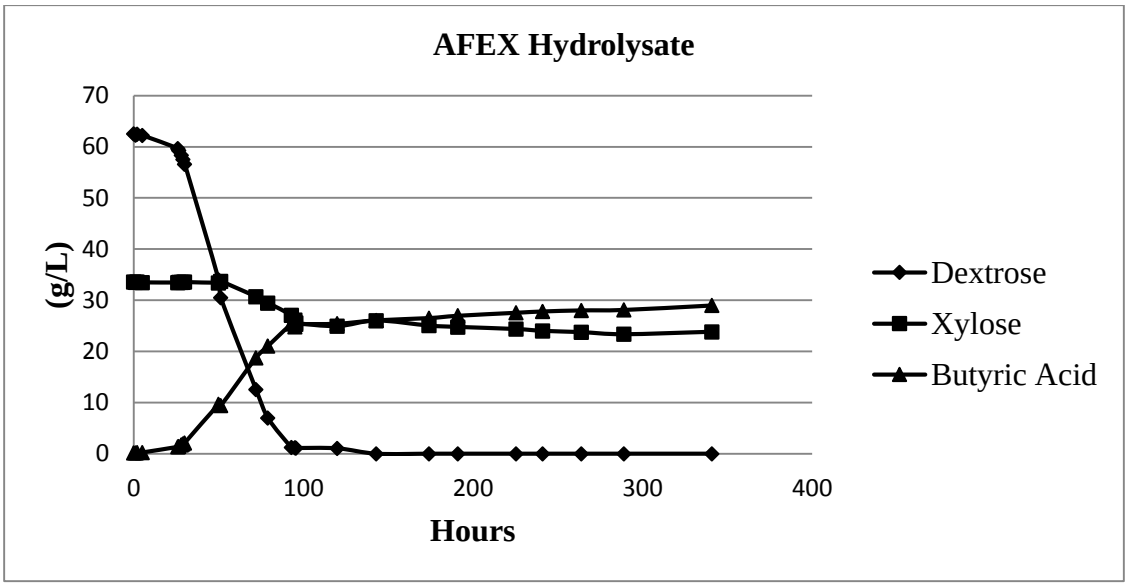


Figure 13. Fermentation kinetics for *Clostridium tyrobutyricum* fermentation of AFEX pre-treated corn stover hydrolysate. Batch was performed at 1L working volume under anaerobic conditions with 250 rpm agitation, 36 °C temperature control and pH 6.0 maintained with 5M NaOH. Component analysis was performed by HPLC on an Aminex 87H column.

The results indicate that such an agricultural waste product could be processed as a sustainable source of butyric acid as the final concentration of butyrate was 28.997 g/L. The initial AFEX-CS hydrolysate contained 62.561 g/L dextrose which was consumed to complete dryness, as well as an initial concentration of 33.552 g/L xylose, of which only 9.724 g/L was consumed before the fermentation ended (Fig 13).

A comparable control batch fermentation of *C. tyrobutyricum* was run in order to determine if the presence of microbial inhibitors from the degradation of lignin or sugar monomers effects the AFEX-CS fermentation. The diauxic control batch fermentation contained an initial concentration of 56.151 g/L dextrose and 40.16 g/L xylose.

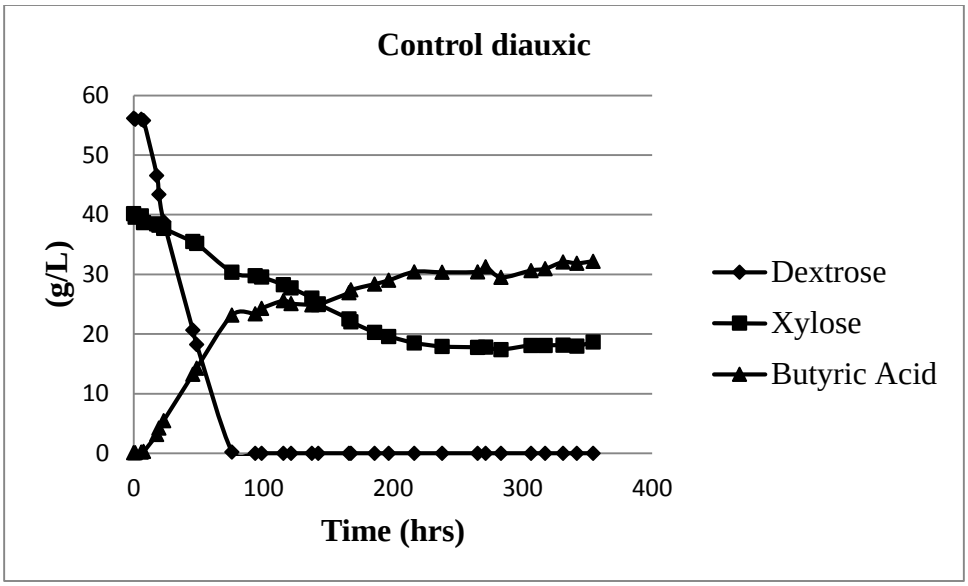
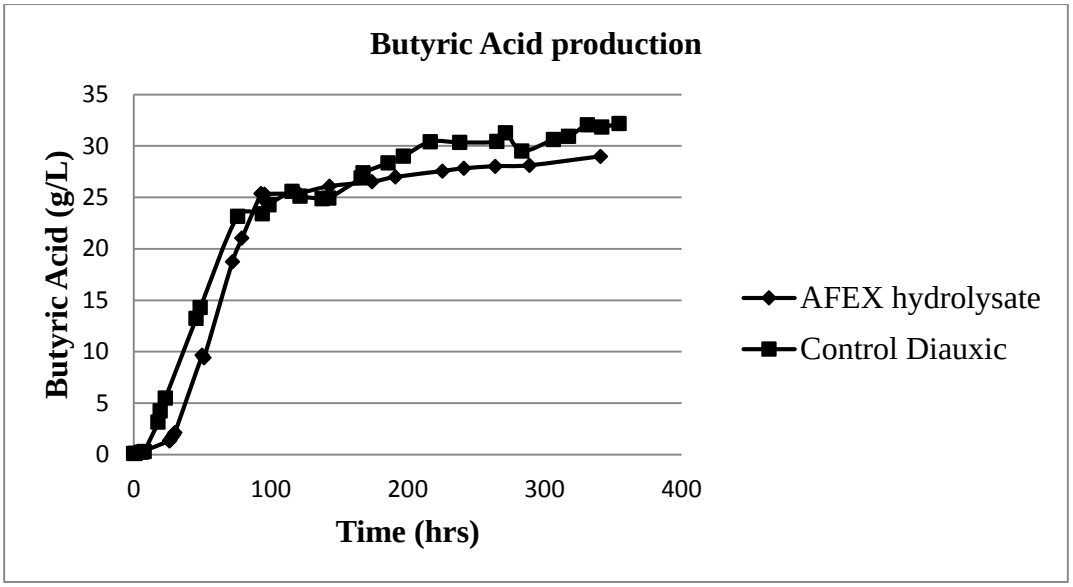


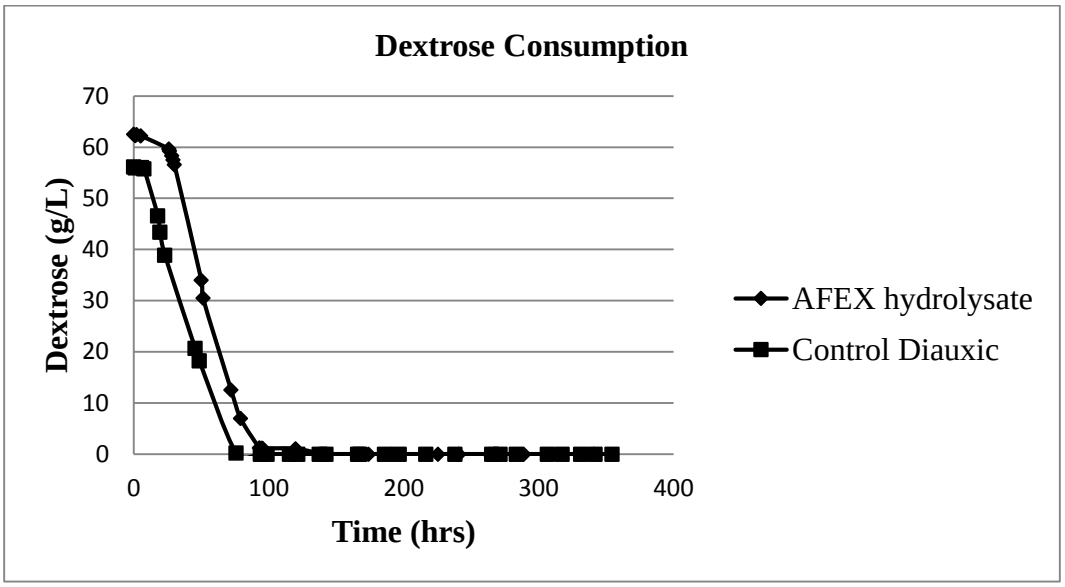
Figure 14. Fermentation kinetics for *Clostridium tyrobutyricum* fermentation of diauxic control media containing 56.151 g/L dextrose, 40.16 g/L xylose and 6 g/L yeast extract. Batch was performed at 1L working volume under anaerobic conditions with 250 rpm agitation, 36 °C temperature control and pH 6.0 maintained with 5M NaOH. Component analysis was performed by HPLC on an Aminex 87H column.

The diauxic control fermentation resulted in 32.2 g/L butyrate, with all of the dextrose being consumed to dryness but ending after 21.496 g/L xylose had been consumed (Figure 14). Although it performed better than the AFEX-CS hydrolysate, the comparable control fermentation also ended before all of the xylose had been consumed, meaning the culture is entering death phase before fully depleting the carbon source. This is possibly due to the toxic effects of butyrate acid which may be limiting the full potential of this type of batch fermentation. In the end, the fermentation kinetics of the AFEX-CS hydrolysate batch tracked closely to the control batch (Fig 15a-c**)**.

a.


Butyric Acid production
Butyric Acid (g/L)
35
30
25
20
15
10
5
0
0
100
200
300
400
Time (hrs)
AFEX hydrolysate
Control Diauxic

b.


Dextrose Consumption
Dextrose (g/L)
70
60
50
40
30
20
10
0
0
100
200
300
400
Time (hrs)
AFEX hydrolysate
Control Diauxic

c.

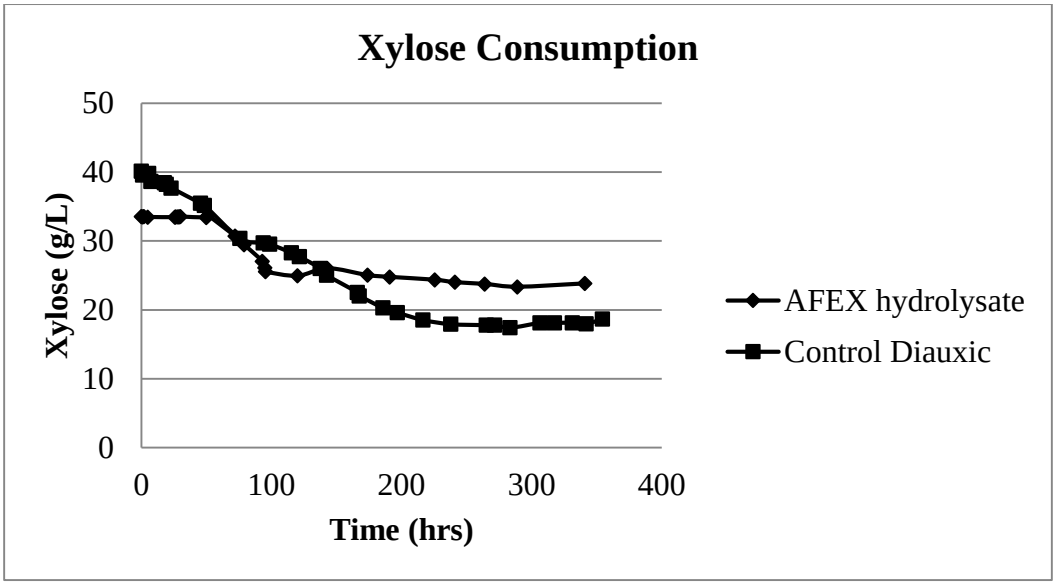


Figure 15a-c. Kinetics for *Clostridium tyrobutyricum* fermentation of AFEX pre-treated corn stover hydrolysate and a comparable control diauxic-fermentation (containing 6 g/L yeast extract). Each batch was performed at 1L working volume under anaerobic conditions with 250 rpm agitation, 36 °C temperature control and pH 6.0 maintained with 5M NaOH. Component analysis was performed by HPLC on an Aminex 87H column.

Fermentations conducted with lignocellulosic biomass hydrolysate generate artificially high optical densities as plant material has an additive effect to true cell culture density when determined using this method. This artifact of fermenting lignocellulosic hydrolysate occurred with the AFEX-CS batch (see Appendix B). Although this makes comparison of optical density and dry cellular weight data impossible between the AFEX-CS batch and the control batch, the specific growth rates can be determined as that formula is based upon rates of change in log-phase growth.

The fastest specific growth rate of the control batch was μ_{net} (1/h)= 0.19321 and occurred before noticeable xylose consumption had begun thus the *C. tyrobutyricum* were mainly consuming dextrose during their log phase of growth. The fastest specific growth rate of the 1 L AFEX-CS hydrolysate fermentation also occurred during dextrose consumption but the rate was μ_{net} (1/h)= 0.09029, half that of the control batch. The AFEX-CS hydrolysate batch did demonstrate 2x slower growth but on this same scale a xylose only control batch of *C. tyrbutyricum* exhibits an approximately 3x slower specific growth rate compared with dextrose only control batches (20). The lowered specific growth rate of the AFEX-CS hydrolysate batch is likely the result of inhibition rather than the lower energetic availability of xylose as primarily dextrose consumption occurred during the log phase of growth.

The advantage of AHP pretreatment over AFEX is that it can be performed at room temperature rather than 300°C. The disadvantage of AHP pretreatment compared to AFEX is that H_2O_2 is used which may drastically inhibit downstream bacterial fermentation. In terms of butyrate fermentation production, both pretreatment methods generated usable substrate from biomass (Fig. 10,12).

Conclusions

Ammonia-fiber expansion, alkaline hydrogen peroxide and extracted ammonia-fiber expansion pretreatment methods all generated usable *C. tyrobutyricum* fermentation substrate once the hydrolysate had been pH adjusted to a tolerable level for bacteria. Without continual pH maintenance throughout the fermentation none of the batch fermentations consumed enough dextrose to overcome diauxic inhibition and begin xylose consumption. Only the 1L batch fermentation with constant pH maintenance demonstrated the consumption of xylose substrate.

The vast majority of the fermentation work previously performed with pretreated hydrolysates has been for the yeast fermentation for bio-based ethanol production. As yeast tolerate lower pH and more severe growth conditions than bacteria, the adjustments of some growth conditions are necessary.

The 1L batch fermentation of AFEX-CS maintained at pH 6.0 generated 28.997 g/L butyrate, similar to the 32.2 g/L butyrate produced by the comparable control batch fermentation. The specific growth rate of the AFEX-CS batch was around half that (μ_{net} (1/h)= 0.09029) of the comparable control batch (μ_{net} (1/h)= 0.19321) indicating inhibition due to the use of the hydrolysate. While the slower growth rate would mean slower overall production utilizing the hydrolysate as compared to the control media, put into perspective, the difference in growth rate is not as large as that between utilizing purely dextrose control media versus control xylose batch fermentations.

APPENDIX

Table 11. Raw data used to generate Figures 13, 14 and 15a-c in Chapter 4. Table 11 includes OD and DCW data mentioned but not shown in the text. OD, optical density. DCW, dry cellular weight.

			g/L				
Substrate	Hours	Sample	Dextrose	Xylose	Acetic Acid	Butyric Acid	OD 600nm
AFEX-CS	0	T0(+)	62.561	33.552	1.265	0.132	0.61
AFEX-CS	1	T1	62.267	33.496	1.441	0.162	0.60
AFEX-CS	2	T2	62.499	33.565	1.463	0.199	0.60
AFEX-CS	5	T3	62.232	33.473	1.432	0.238	0.61
AFEX-CS	26	T4	59.687	33.448	1.798	1.341	2.70
AFEX-CS	26.5	T5	59.289	33.489	1.812	1.405	2.82
AFEX-CS	28	T6	58.338	33.513	1.883	1.702	3.18
AFEX-CS	29	T7	57.560	33.526	1.917	1.922	3.54
AFEX-CS	30	T8	56.604	33.543	1.982	2.130	4.04
AFEX-CS	50	T9	34.000	33.406	4.317	9.669	10.32
AFEX-CS	51.25	T10	30.496	33.688	4.193	9.410	10.48
AFEX-CS	72	T11	12.567	30.696	5.242	18.764	12.48
AFEX-CS	79	T12	6.991	29.470	5.176	21.046	13.36
AFEX-CS	93	T13	1.196	27.062	5.336	25.361	12.40
AFEX-CS	95	T14	1.131	26.136	5.140	24.784	12.08
AFEX-CS	95.5	T15	1.114	25.569	5.307	25.300	12.08
AFEX-CS	120	T16	1.048	24.932	5.277	25.426	11.92
AFEX-CS	143	T17	0.000	26.030	5.655	26.085	11.84
AFEX-CS	174	T18	0.000	25.039	5.785	26.527	11.52
AFEX-CS	191	T19	0.000	24.774	5.737	26.980	11.68
AFEX-CS	225.5	T20	0.000	24.363	5.630	27.567	11.60
AFEX-CS	241	T21	0.000	24.006	5.592	27.821	11.84
AFEX-CS	264	T22	0.000	23.755	5.505	28.034	11.60
AFEX-CS	289	T23	0.000	23.346	5.425	28.119	12.00
AFEX-CS	341	T24	0.000	23.828	5.750	28.997	11.84

Table 11 (cont'd)

			g/L				
Substrate	Hours	Sample	Dextrose	Xylose	Acetic Acid	Butyric Acid	OD 600 nm
Control	0.00	T0(+)	56.151	40.160	0.236	0.101	0.22
Control	1.00	T1	56.001	39.600	0.428	0.121	0.25
Control	5.75	T2	56.012	39.831	0.598	0.247	0.47
Control	7.50	T3	55.782	38.663	0.219	0.311	0.70
Control	17.75	T4	46.586	38.489	2.358	3.154	6.36
Control	19.33	T5	43.398	38.246	2.669	4.239	7.70
Control	23.00	T6	38.866	37.706	3.061	5.499	7.36
Control	45.50	T7	20.652	35.492	4.440	13.234	7.52
Control	48.50	T8	18.238	35.194	4.581	14.288	7.68
Control	75.75	T9	0.235	30.371	6.475	23.170	8.08
Control	93.75	T10	0.000	29.758	7.186	23.414	7.60
Control	98.75	T11	0.000	29.542	7.228	24.299	7.76
Control	115.50	T12	0.000	28.282	7.386	25.602	7.52
Control	121.50	T13	0.000	27.726	7.320	25.113	7.36
Control	137.50	T14	0.000	26.037	6.895	24.884	7.52
Control	142.50	T15	0.000	25.036	6.980	24.936	7.60
Control	166.00	T16	0.000	22.537	7.180	26.915	7.76
Control	167.58	T17	0.000	22.043	7.757	27.407	7.68
Control	185.75	T18	0.000	20.287	6.772	28.367	7.76
Control	196.83	T19	0.000	19.591	7.059	29.015	7.60
Control	216.50	T20	0.000	18.519	7.099	30.423	8.24
Control	238.00	T21	0.000	17.917	7.065	30.354	7.68
Control	265.25	T22	0.000	17.785	7.104	30.439	7.84
Control	271.50	T23	0.000	17.817	7.188	31.286	8.00
Control	283.50	T24	0.000	17.404	6.128	29.516	8.24
Control	306.50	T25	0.000	18.110	7.205	30.648	8.16
Control	317.50	T26	0.000	18.104	7.231	30.941	8.40
Control	331.25	T27	0.000	18.133	7.543	32.064	8.56
Control	341.83	T28	0.000	17.984	7.028	31.855	8.00
Control	354.50	T29	0.000	18.664	7.638	32.200	8.00

REFERENCES

REFERENCES

1. Werpy, T., and Petersen, G. (2004) Top Value Added Chemicals from Biomass Vol. 1: Results of Screening for Potential Candidates from Sugars and Synthesis Gas p. 76, United States Department of Energy, Springfield, VA

2. Chundawat, S. P. S., Bals, B., Campbell, T., Sousa, L., Gao, D., Jin, M., Eranki, P., Garlock, R., Teymouri, F., Balan, Venkatesh., Dale, Bruce E. (2013) Primer on Ammonia Fiber Expansion Pretreatment. In *Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals* (Wyman, C. E., ed) pp. 169-200, John Wiley & Sons, Ltd, Chichester, UK

3. Chandraraj Krishnan, L. d. C. S., Mingjie Jin, Linpei Chang, Bruce E. Dale, Venkatesh Balan (2010) Alkali-Based AFEX Pretreatment for the Conversion of Sugarcane Bagasse and Cane Leaf residues to Ethanol. *BIOTECHNOLOGY AND BIOENGINEERING* **107**, 441-450

4. Ezeji, T., Qureshi, N., and Blaschek, H. P. (2007) Butanol Production From Agricultural Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation. *Biotechnology and Bioengineering* **97**, 1460-1469

5. Sinnott, M. L. (2007) One-electron Chemistry of Carbohydrates. In *Carbohydrate Chemistry and Biochemistry* pp. 648-726, The Royal Society of Chemistry, Cambridge, UK

6. Banerjee, G., Car, S., Liu, T., Williams, D. L., Meza, S. L., Walton, J. D., and Hodge, D. B. (2012) Scale-Up and Integration of Alkaline Hydrogen Peroxide Pretreatment, Enzymatic Hydrolysis, and Ethanolic Fermentation. *Biotechnology and Bioengineering* **109**, 922-931

7. Kim, T. H., Lee, Y.Y. (2006) Fractionation of corn stover by hot-water and aqueous ammonia treatment. *Bioresource Technology* **97**, 224-232

8. Hodge, D. B., Karim, Nazmul M, Schell, Daniel J, McMillan, James D (2008) Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresource Technology* **99**, 8940-8948

9. Sakakibara, A. (1991) Chemistry of Lignin. In *Wood and Cellulosic Chemistry* (Hon, D. N. S., Shiraishi, Nobuo, ed), Marcel Dekker, New York, New York

10. Lai, Y.-Z. (1991) Chemical Degradation. In *Wood and Cellulosic Chemistry* (Hon, D. N. S., Shiraishi, Nobuo, ed) pp. 455-523, Marcel Dekker, Inc, New York

11. Chundawat, S. P., Vismeh, R., Sharma, L. N., Humpula, J. F., Sousa, L. d. C., Chambliss, K. C., Jones, D. A., Balan, V., and Dale, B. E. (2010) Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. *Bioresource Technology* **101**, 8429-8438

12. Pandey, M. P., Kim, Chang Soo (2010) Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chemical Engineering and Technology* **34**, 29-41

13. Helmerius, J. (2010) Integration of a Hemicelluloses Extraction Step into a Forest Biorefinery for Production of Green Chemicals. In *Chemical Engineering and Geosciences* Vol. Licentiate p. 34, Lulea University of Technology, Lulea Sweden

14. Nadji, H., Rodrigue, D., Benaboura, A., Bedard, Y., Stevanovic, T., Riedl,B. (2009) Value-Added Derivatives of Soda Lignin Alfa Grass (*Stipa Tenacissima*). II. Uses as Lubricants in Plastic Processing. *Journal of Applied Polymer Science* **114**, 3003-3007

15. Heiningen, A. v. (2006) Converting a kraft pulp mill into an integrated forest biorefinery. *Pulp & Paper Canada* **107**, 38-43

16. Henriksson, G., Lawoko, Martin., Martin, Maria Eugenia Eugenio., Gellerstedt, Goran. (2007) Lignin-carbohydrate network in woods and pulps: A determinant for reactivity. *Holzforschung* **61**, 668-674

17. Jeffries, T. W. (1990) Biodegradation of lignin-carbohydrate complexes. *Biodegradation* **1**, 163-176

18. Iversen, T. (1985) Lignin-carbohydrate bonds in a lignin-carbohydrate complex isolated from spruce. *Wood Science and Technology* **19**, 243-251

19. Shuler, M. L., and Kargi, F. (2002) How Cells Grow. In *Bioprocess Engineering Basic Concepts* (Amundson, N. R., ed) pp. 155-200, Prentice Hall PTR, Upper Saddle River, New Jersey

20. Adam M Jaros, U. R., Kris A Berglund (2013) Acetate adaptation of *Clostridia tyrobutyricum* for improved fermentation production of butyrate. *SpringerPlus* **2**, 1-8

CHAPTER 5: ADAPTATION TO SUBSTRATE INHIBITION FROM HIGH INITIAL GLUCOSE CONCENTRATION

Introduction

The utilization of lignocellulosic hydrolysates as the substrate for fermentation production is a sustainable method for generating butyrate. In regard to carbon efficiency, high conversion of cellulose to glucose and hemicellulose to xylose is optimal as sugar monomers are fermented into products. On the other hand, microbial fermentation production presents a unique challenge to exploiting lignocellulose hydrolysate substrates in that these hydrolysates contain total soluble sugar concentrations at levels inhibitory to bacterial growth. Substrate inhibition of microbial growth is analogous to and sometimes based on the inhibition of individual enzyme kinetics but is often so complicated that kinetic constants have no biological meaning (1). The issue of substrate inhibition is due in part to microbial stress from high osmotic pressure caused by sugar concentration (2). Such microbial stress during fermentation results in lowered product yields due to the increased energy diverted to cellular maintenance (3).

There are many process design options for the fermentation of hydrolysed lignocellulosic biomass into value-added products and each attend to the issue of substrate inhibition in different ways. The separate or sequential hydrolysis and fermentation (SHF) design separates the pretreated lignocellulosic biomass saccharification and fermentation steps into two different unit operations allowing for optimal temperature and pH to be used at each step. The optimal temperature for cellulase, xylanase and other hydrolytic enzymes is much higher than the optimal temperature for fermentation and is inhibitory for microbial growth (3, 4). The advantage to the SHF design is that by separating the fermentation stage from the saccharification stages, both

function at optimal rates unlike if they are coupled. The disadvantage to the SHF design is that the saccharification enzymes used in enzymatic hydrolysis are sugar inhibited so that without dilution or a method to remove the sugar product from the enzyme catalytic site, the saccharification stage is still subject to sugar inhibition (3). The enzymes used in saccharification such as cellulase and β-glucosidases originally developed in microbes with activity coupled with the subsequent uptake of the product (glucose) by the next metabolic enzyme (3). Due to this coupling, these individual enzymes did not evolve resistance to the presence of a high concentration of product and thus the separate saccharification stage generates inhibitory levels of sugar.

Alternatively, simultaneous saccharification and co-fermentation (SSF) process design lowers capital costs compared with SHF as both lignocellulosic saccharification and fermentations occur in the same volume. SSF process exhibits lower enzyme kinetics relative to SHF as saccharification must occur at a temperature and pH that fermenting yeast or bacteria can tolerate. Theoretically SSF designs overcome the issue of sugar inhibition both as an end product of saccharification and a substrate for fermentation as both processes occurring at the same time even at lowered reaction rates cause for utilization of sugar while it is being generated. Unfortunately, high amounts of solid lignocellulosic biomass (pretreated) loaded into the SSF system can inhibit mass transfer and prevent diffusion of sugar product away from enzymatic catalytic sites so both saccharification and the ultimate fermentation production is inhibited (3). This demonstrates an advantage of the separate hydrolysis and fermentation (SHF) system over SSF with a separate hydrolysis; solid materials can be removed and thus not interfere with the subsequent fermentation. Previous researchers have examined the levels of solid loadings which lead to significant glucose inhibition and concentrations of initial insoluble solids at 32% w/v

lead to lowered glucose generation from saccharification enzyme cocktails (Spezyme) compared with 30% initial insoluble solids loading (3). The limitation of the SSF design is based upon the concentration of biomass solids and ultimately on the sugar inhibition.

Bioethanol fuel plants conventionally utilize batch fermentation design with the transfer of yeast from previous batch and a high initial sugar concentration (5) . Some current operations in the bioethanol industry (from starch or sugar cane based substrate) attend to the issue of osmotic pressure from high sugar concentration by dilution of the media source in a fed-batch or continuous feed system (6). Yeast fermentations are conducted with media streams containing concentrations of sugar as high as 220 g/L but through a constant dilution stream the yeast are typically exposed to a maximum concentration of 70 g/L sucrose (2, 6). Overcoming osmotic pressure through augmentation of the microbe rather than process design is another possible solution to sugar inhibition.

Developing microorganisms with increased osmotolerance is a current research priority for the improvement of bioethanol fermentation (7). The ability of certain *Saccharomyces cerevisiae* strains to flocculate during fermentation imbues some of these strains with tolerance to high osmolality (7). Alternative strains such as *Schizosaccharomyces pombe* also may be used in bioethanol fermentation as they are tolerant to high osmotic pressures (7). High gravity fermentations (initial dissolved solids exceeds 200 g/L) with high substrate loads have been proposed as a method for increasing final ethanol concentrations and thus lower energy costs (7). Very high gravity (VHG) fermentations exceeding 300 g/L solids have also been proposed to concomitantly lower water usage and plant capacity necessary for fermentation (7, 8). These examples of high gravity fermentation medium are from starch hydrolysis or sugarcane

extraction but similarly high concentrations of sugar can be found in lignocellulosic hydrolysates.

Dilute sulfuric acid pretreated corn stover hydrolysates reached levels of 124.9 g/L total monomeric sugar concentration in undiluted slurries from the pretreatment (9). Similarly pretreated hydrolysate undergoing a high enzyme loading of (52 mg enzyme protein (Spezyme CP)/ g cellulose) achieved monomeric sugar concentrations approaching 200 g/L (9). Such high concentrations of soluble sugars are known to inhibit both hydrolysis enzymes and microbial growth. As high gravity concentrations of sugars are already being produced from pretreated lignocellulosic hydrolysis, a characterization of the tolerance of *Clostridium tyrobutyricum* to the osmotic pressure from high sugar concentrations is important to perform.

Overtly high initial concentrations of soluble sugars found in batch fermentations can reduce the butyrate production of *C. tyrobutyricum* fermentation. Varying the initial glucose concentrations during batch fermentations of *C. tyrobutyricum* from 5 to 60 g/L (27.8 to 333.6 mM) demonstrated an optimal initial glucose concentration of 40 g/L (222.4 mM) in terms of acetate and butyrate yield (10). Increasing the initial glucose concentrations from 5 to 40 g/L (27.8 to 222.4 mM) increased product yields from 0.105 mol acetate/mol glucose to 0.138 mol acetate/mol glucose and from 0.527 mol butyrate/mol glucose to 0.694 mol butyrate/mol glucose. *C. tyrobutyricum* batch fermentation at 60 g/L initial glucose (333.6 mM) dropped the acetate and butyrate yields to 0.048 mol acetate/mol glucose and 0.497 mol butyrate/mol glucose. While in this case the drop in product yields saw the concomitant increase in *C. tyrobutyricum* cell density, substrate inhibition still lowered productivity (10).

Industrial fermentation designs have focused on dilution of the media source as a means to overcoming the substrate inhibition caused by osmotic pressure. This solution requires increasing reactor capacity in order to maintain the same product recovery or lowered product yield both resulting in a negative impact on biorefinery economics. Instead, the work presented here characterizes the effects of high glucose concentration on fermentation by *C. tyrobutyricum* by examining the results of adapting a glucose consuming culture of *C. tyrobutyricum* to 150 g/L initial glucose concentration.

Methods

Adaptation Selection

A lyophilized stock culture of *Clostridium tyrobutyricum* (ATCC 25755) was re-hydrated under sterile anaerobic (nitrogen sparged) conditions in Reinforced Clostridial Media (RCM; Difco). Once the culture entered log phase, when the optical density (OD) at 600 nm was approximately 2.0, 5 ml of inoculum was transferred in duplicate to sterile anaerobic (nitrogen sparged) serum bottles containing 100 ml of RCM with 9.5 g glucose (100 g/L glucose total as RCM contains 5 g/L glucose). 100 g/L glucose challenged *C. tyrobutycum* cultures were incubated on a shaker at 36°C, 80 rpm for 24 hours. Duplicate cultures underwent a microscopic survey to check for positive cell growth and 5 ml of the most visually robust passaged to similar duplicate 100 ml RCM bottles with 125 g/L glucose and incubated under the same procedure. 24 hours later the same selection was used to inoculate duplicate sterile nitrogen sparged serum bottles containing 100 ml RCM with 150 g/L glucose incubated in the same manner as the previous selections. 24 hours after that, 50 ml of 150 g/L glucose challenged *C. tyrobutyricum* in log phase growth (OD at 600nm > 2.0) was used to inoculate the batch fermentation.

Fermentations

One liter batch fermentations were conducted in New Brunswick Bioflo 310 2.5 L working volume reactors under anaerobic conditions at 36°C. For each batch, 950 mL media of the following composition was used; 6 g/L yeast extract, 5 ppm $FeSO_4$ 7 H_2O, 450 mL ddH_2O and 500 mL glucose at 300 g/L sterilized separately. Anaerobiosis was reached by sparging the vessel with nitrogen prior to inoculation. The batches were inoculated with 50mL log phase *C. tyrobutyricum* 150 g/L glucose adapted or control (non-adapted ATCC 25755) cultures. The nitrogen sparging was maintained until logarithmic growth in the vessel was observed. Agitation was kept at 250 rpm and in order to maintain the *C. tyrobutyricum* cultures in acidogenic production, pH 6.0 was sustained with 5 M NaOH throughout the fermentation. Samples (10 mL) were withdrawn at regular intervals for analytical measurements.

Analytical Methods

Organic acids and residual sugar were analyzed by HPLC (LC-20AT dual pump and 10A RI detector, Shimadzu) equipped with an ion exchange column (Aminex HPX-87H, 9 um, 7.8mm x 300mm, Bio-Rad) and a cation-H guard column (Micro-guard, 30mm x 4.6 mm) using 50 mM sulfuric acid as a mobile phase. The flow rate of the mobile phase was maintained at 1 mL/min during analysis with 20 μL of sample injected into the system with an auto-injector (SIL-20AHT, Shimadzu) with the column and guard maintained at 65°C in a column oven (CT0-20A, Shimadzu). Prior to analyses, samples were centrifuged at 10 000 rpm for 5 min in a micro-centrifuge (Microfuge 18, Beckman Coulter). Data for each sample was acquired with Shimadzu EZ Start 7.4 SP1 chromatography software using standards for glucose, xylose, butyrate, acetate and lactate.

Dry Cellular Weight Determination

Cell growth was monitored during fermentation by measuring the optical density at 600 nm. The biomass from 40 mL cell suspension, removed in triplicate, was dried in an 80°C drier for 48 hours and the dry cell weight (DCW, g/L) determined. The optical densities were then converted to dry cell weight using the following equation: $DCW = 0.38(OD_{600})$. This optical density to dry cellular weight conversion formula was determined for the specific organism and media used in this study.

Specific Growth Rate (μ_{net})

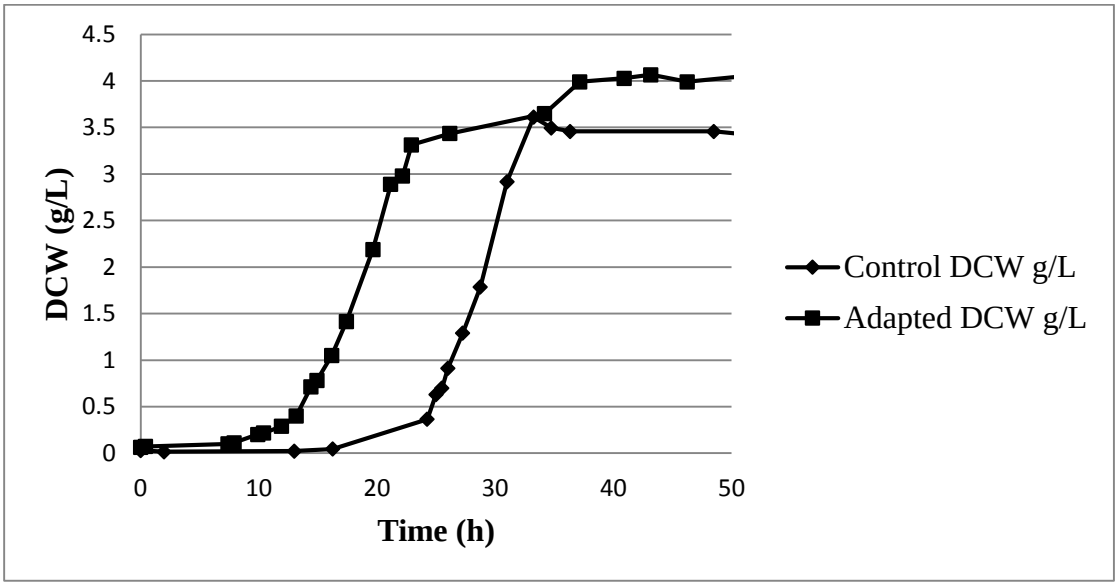
DCW was used to determine the specific growth rate as described by Shuler *et al* (1). The DCW data points from the logarithmic growth phase were plotted on a semi-log graph to locate the period during that phase in which the culture experienced the fastest growth. These points were then used in the following equation: $\mu_{net}\ (1/h) = (\ln(DCW_X/DCW_0))/(Time_X\text{-}Time_0)$, where DCW was measured in g/L and time in hours. DCW_X is the last point during the fasted logarithmic growth period and DCW_0 is the first point. $Time_X$ and $Time_0$ are described similarly.

Results and Discussion

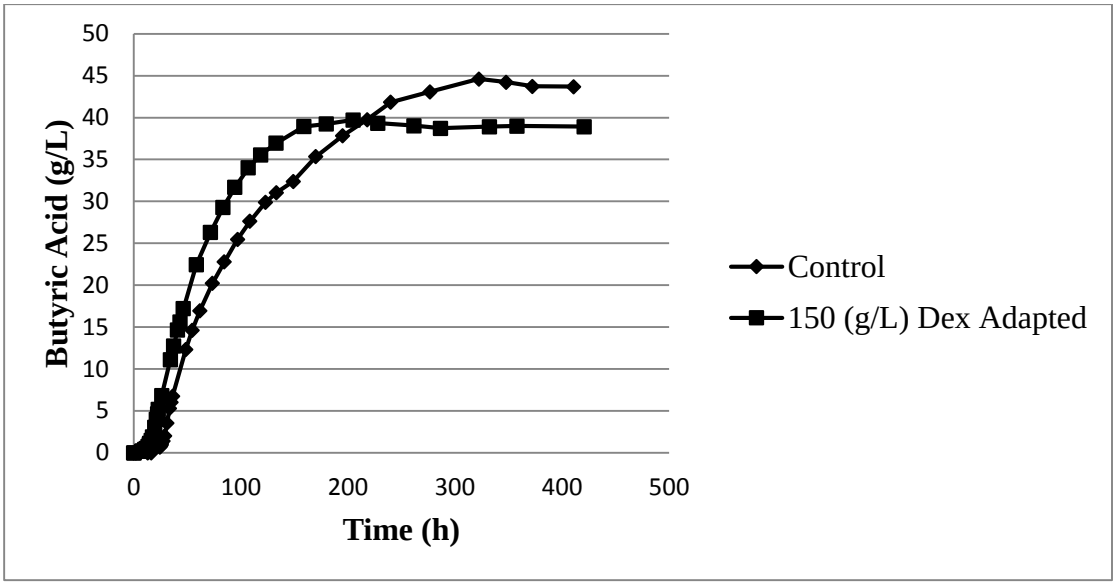
The selective adaptation of *C. tyrobutrycum* to a 150 g/L initial glucose re-inforced media resulted in a culture capable of tolerating the higher initial sugar load. As these results were positive, fermentation with a selectively adapted culture was performed as 1 liter batch fermentation on minimal media containing 150 g/L glucose as well as a comparative control fermentation with 150 g/L glucose but using a standard *C. tyrobutyricum* culture.

As seen in Figure 16, the high glucose adapted culture began log phase growth 10 hours after inoculation while the control culture required over 16 hours before beginning log phase. 150 g/L initial glucose presents a challenge to the microorganism in the form of high osmotic pressure thus a slight extension in lag phase growth is seen with the control culture (Fig. 16a). Despite the selectively adapted culture entering log phase sooner, no significant difference is demonstrated in the specific growth rates between the two cultures (i.e., the fastest rates of log phase growth were the same). μ_{net} (specific growth rate) of the 150 g/L selectively adapted culture challenged with 150 g/L glucose was 0.288 (1/h), the same as the control culture challenged with 150 g/L glucose.

a.



b.



c.

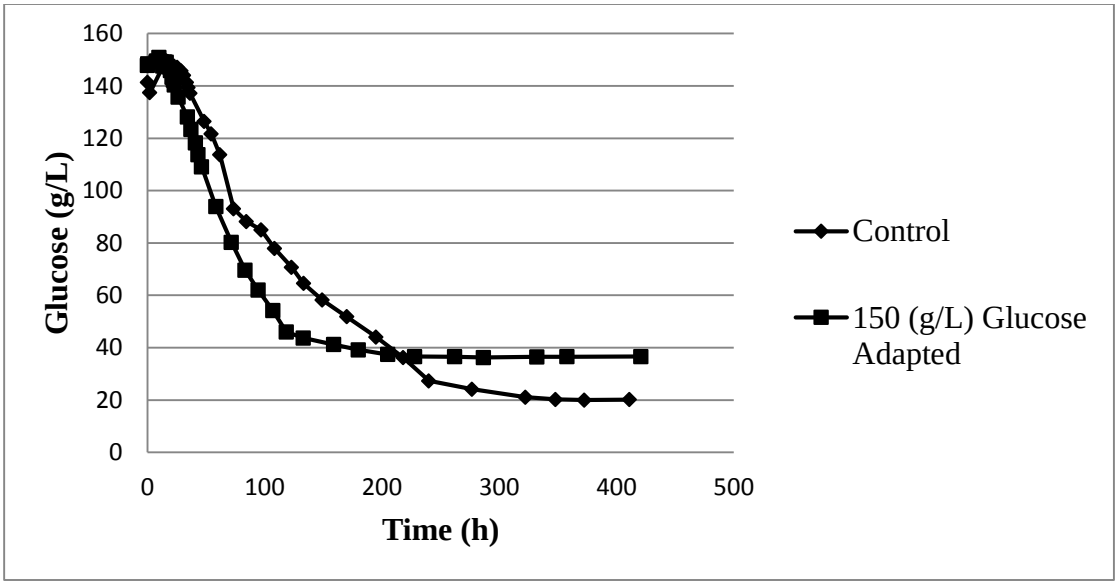


Figure 16a-c. *Clostridium tyrobutyricum* fermentation of control media (150 g/L glucose, 6 g/L yeast extract and 5 ppm $FeSO_4 \cdot 7H_2O$) at a 1 liter volume with pH controlled at 6.0 with 5M NaOH, 250 rpm agitation and a controlled temperature of 36°C. The selectively adapted batch

was inoculated with 50ml of a 150 g/L glucose adapted culture of *C. tyrobutyricum* and the control batch inoculated with 50ml of a standard culture. **a.** Dry Cellular Weight (DCW) **b.**Butyric acid production. **c.** glucose consumption.

High performance liquid chromatography performed on samples taken from the end of the two comparative fermentations resulted in concentrations of 43.695 g/L butyrate from the non-adapted culture and 38.931 g/L butyrate from the adapted culture. The residual glucose from the non-adapted *C. tyrobutyricum* was 20.135 g/L while the adapted culture left 36.605 g/L residual glucose. While the adapted strain was able to begin fermentation of the 150 g/L glucose much faster than the non-adapted strain (Fig 15.), yet the adaptation did not result in a high butyrate yield or sugar consumption.

Conclusions

A culture of *Clostridium tyrobutyricum* adapted to tolerate 150 g/L initial glucose in batch fermentations begins log phase growth several hours sooner than control culture, yet no increase is seen in specific growth rates (0.288 (1/h)). Similar glucose consumption and butyrate production was seen between both the adapted and control cultures with no net gain in butyrate production despite the adaptive tolerance to high glucose concentration.

APPENDIX

Table 12. Raw data used to generate Figures 16a-c in Chapter 5.

			g/L				
Culture	Hours	Sample	Dextrose	Acetic Acid	Butyric Acid	OD 600 nm	DCW g/L
Control	0.00	T0(+)	141.342	0.129	0.0	0.08	0.030
Control	2.00	T1	137.433	0.122	0.0	0.04	0.015
Control	13.00	T2	147.249	0.147	0.0	0.06	0.023
Control	16.25	T3	147.867	0.143	0.0	0.12	0.046
Control	24.25	T4	146.997	0.293	0.7	0.96	0.365
Control	25.00	T5	144.261	0.434	0.8	1.66	0.631
Control	25.50	T6	147.075	0.526	0.9	1.84	0.699
Control	26.00	T7	144.009	0.550	1.0	2.40	0.912
Control	27.25	T8	144.117	0.671	1.4	3.40	1.292
Control	28.75	T9	145.770	0.917	2.0	4.70	1.786
Control	31.00	T10	144.054	1.437	3.5	7.68	2.918
Control	33.25	T11	141.330	1.991	5.3	9.50	3.610
Control	34.75	T12	139.362	2.237	6.0	9.20	3.496
Control	36.33	T13	137.196	2.497	6.8	9.10	3.458
Control	48.50	T14	126.408	3.616	12.3	9.10	3.458
Control	54.50	T15	121.728	3.849	14.6	8.90	3.382
Control	61.75	T16	113.673	4.095	16.9	8.80	3.344
Control	73.50	T17	93.030	4.518	20.2	9.40	3.572
Control	84.50	T18	88.212	4.920	22.8	8.90	3.382
Control	97.00	T19	84.982	5.466	25.5	9.00	3.420
Control	108.50	T20	77.882	6.003	27.6	8.90	3.382
Control	123.00	T21	70.738	6.632	29.9	9.20	3.496
Control	133.17	T22	64.522	6.735	31.0	9.20	3.496
Control	149.00	T23	58.272	7.420	32.4	9.30	3.534
Control	170.00	T24	51.838	8.301	35.4	9.40	3.572
Control	195.00	T25	44.092	9.116	37.8	9.30	3.534
Control	218.00	T26	36.238	9.740	39.8	9.70	3.686
Control	240.00	T27	27.331	10.277	41.8	9.60	3.648
Control	276.75	T28	24.148	10.584	43.1	9.90	3.762
Control	322.33	T29	21.065	10.985	44.6	9.80	3.724
Control	348.00	T30	20.219	11.257	44.2	9.90	3.762
Control	372.50	T31	20.000	11.409	43.7	9.90	3.762
Control	411.00	T32	20.135	11.672	43.7	10.10	3.838

Table 12 (cont'd)

			g/L				
Culture	Hours	Sample	Dextrose	Acetic Acid	Butyric Acid	OD 600 nm	DCW g/L
Adapted*	0.00	T0(+)	147.783	0.000	0.000	0.17	0.065
Adapted	0.42	T1	148.419	0.000	0.000	0.19	0.072
Adapted	7.42	T2	149.151	0.149	0.183	0.26	0.100
Adapted	7.92	T3	149.550	0.149	0.198	0.29	0.112
Adapted	9.92	T4	150.936	0.191	0.319	0.53	0.201
Adapted	10.42	T5	149.550	0.216	0.400	0.57	0.217
Adapted	11.92	T6	149.313	0.300	0.532	0.76	0.289
Adapted	13.17	T7	147.552	0.396	0.728	1.05	0.399
Adapted	14.42	T8	149.022	0.539	0.990	1.88	0.714
Adapted	14.92	T9	149.097	0.610	1.116	2.06	0.783
Adapted	16.17	T10	149.079	0.770	1.474	2.76	1.049
Adapted	17.42	T11	147.438	1.029	1.906	3.72	1.414
Adapted	19.67	T12	145.875	1.600	3.039	5.76	2.189
Adapted	21.17	T13	143.349	2.027	3.985	7.60	2.888
Adapted	22.17	T14	142.203	2.272	4.629	7.84	2.979
Adapted	22.92	T15	140.319	2.426	5.163	8.72	3.314
Adapted	26.17	T16	135.789	3.006	6.827	9.04	3.435
Adapted	34.17	T17	128.166	4.351	11.103	9.60	3.648
Adapted	37.17	T18	123.327	4.711	12.731	10.50	3.990
Adapted	40.92	T19	118.266	5.222	14.647	10.60	4.028
Adapted	43.17	T20	113.727	5.553	15.625	10.70	4.066

* The *Clostridium tyrobutyricum* used to inoculate the adapted batch were tolerant to 150 g/L dextrose prior to inoculation.

Table 12 (cont'd)

			g/L				
Culture	Hours	Sample	Dextrose	Acetic Acid	Butyric Acid	OD 600 nm	DCW g/L
Adapted	46.25	T21	109.134	6.074	17.195	10.50	3.990
Adapted	58.42	T22	93.906	7.460	22.435	10.90	4.142
Adapted	71.67	T23	80.238	8.168	26.310	10.60	4.028
Adapted	83.42	T24	69.530	8.890	29.305	10.90	4.142
Adapted	94.42	T25	62.026	9.434	31.681	10.70	4.066
Adapted	106.92	T26	54.230	9.724	34.015	11.10	4.218
Adapted	118.42	T27	46.039	9.695	35.569	11.30	4.294
Adapted	132.92	T28	43.687	9.622	36.957	11.40	4.332
Adapted	158.92	T29	41.172	9.532	38.938	11.30	4.294
Adapted	179.92	T30	39.149	9.308	39.255	11.00	4.180
Adapted	204.92	T31	37.396	9.905	39.725	11.00	4.180
Adapted	227.92	T32	36.600	10.570	39.346	10.90	4.142
Adapted	261.92	T33	36.572	10.639	39.035	10.90	4.142
Adapted	286.67	T34	36.269	10.567	38.735	10.80	4.104
Adapted	332.25	T35	36.483	10.613	38.929	10.60	4.028
Adapted	357.92	T36	36.562	10.646	39.006	10.40	3.952
Adapted	420.92	T37	36.605	10.622	38.931	10.90	4.142

REFERENCES

REFERENCES

1. Shuler, M. L., and Kargi, F. (2002) How Cells Grow. In *Bioprocess Engineering Basic Concepts* (Amundson, N. R., ed) pp. 155-200, Prentice Hall PTR, Upper Saddle River, New Jersey

2. Luiz Carlos Basso, T. O. B., Saul Nitsche Rocha (2011) Ethanol Production in Brazil: The Industrial Process and Its Impact on Yeast Fermentation. In *Biofuel Production - Recent Developments and Prospects* (Bernardes, D. M. A. D. S., ed), InTech, Rijeka, Croatia

3. Hodge, D. B. (2005) Optimization of High Solids Lignocellulosic Biomass Conversion for Ethanol Production. In *Department of Chemical Engineering* Vol. PhD p. 308, Colorado State University Fort Collins

4. Guangya Zhang, H. G. (2012) Prediction of xylanase optimal temperature by support vector regression. *Electronic Journal of Biotechnology* **15**, 1-12

5. PAM Claassen, J. v. L., AM Lopez Contreras, EWJ van Niel, L Sijtsma, AJM Stams, SS de Vries, RA Weusthuis (1999) Utilisation of biomass for the supply of energy carriers. *Applied Microbiology and Biotechnology* **52**, 741-755

6. Simone Brethauer, C. E. W. (2010) Review: Continuous hydrolysis and fermentation for cellulosic ethanol production. *Bioresource Technology* **101**, 4862-4874

7. Oscar J. Sanchez, C. A. C. (2008) Trends in biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology* **99**, 5270-5295

8. FW Bai, W. A., M Moo-Young (2008) Ethanol fermentation technologies from sugar and starch feedstocks. *Biotechnology Advances* **26**, 89-105

9. Hodge, D. B., Karim, Nazmul M, Schell, Daniel J, McMillan, James D (2008) Soluble and insoluble solids contributions to high-solids enzymatic hydrolysis of lignocellulose. *Bioresource Technology* **99**, 8940-8948

10. Ji Hye Jo, D. S. L., Junhoon Kim, Jong Moon Park (2009) Effect of Initial Glucose Concentrations on Carbon and Energy Balances in Hydrogen-Producing Clostridium tyrobutyricum JM1. *Journal of Microbiology and Biotechnology* **19**, 291-298

CHAPTER 6: THE APPLICATION OF INDUCED WHOLE-CELL *PICHIA PASTORIS* IN A NATURAL BIOCONVERSION OF BUTANOL TO BUTYRALDEHYDE

Data in Chapter 6 has been submitted to the Brazilian Journal of Chemical Engineering

Adam M. Jaros[1,2], Ulrika Rova[1,2,*], and Kris A. Berglund[1,2]

[1]Luleå University of Technology, SE-971 87 Luleå Sweden, [2]Michigan State University, East Lansing MI 48824 USA

Data in Chapter 6 relates to the natural bio-oxidation production of butyraldehyde from butanol. The study presented here relates to the main work of the dissertation, natural butyrate production, in that butyric acid is commonly generated from the oxidation of *n*-butyraldehyde (see Chapter 1). A production pathway (bio-oxidation) for naturally generating butyraldehyde is presented here thus relating an alternative method for producing natural butyric acid.

Abstract

There is a demand for all-natural butyraldehyde and butyraldehyde derived flavor compounds creating a manufacturing potential for using bioconversion methods to generate butyraldehyde from butanol. In this study, the methylotrophic yeast *Pichia pastoris* was induced to generate alcohol oxidase enzyme (AOX) for the oxidation of butanol to butyraldehyde using a 1 L whole-cell bioconversion system. A typical buffer for maintaining physiological pH (0.1M potassium phosphate) was compared against a 0.5M Tris-borate chelating buffer as the amine is known to complex with the free aldehyde in the media and it was found that the amine buffer did prolong AOX activity (120 min compared to 96 min). Alternative product recovery methods were evaluated as well. An O_2 pressurized system maintained AOX activity for an extended period of time (966 min) and generated the most butyraldehyde compared to the other set-ups with 2.047 g.

Key words: *Pichia pastoris,* butyraldehyde, bioconversion, alcohol oxidase

Introduction

There is a strong market for all-natural food products with a labeling requirement that non-artificial flavor additives be used. Thus, flavor additives for such products must be generated through natural processes rather than be chemically synthesized. Bioconversion methods generating aldehydes utilizing the AOX enzyme of *Pichia pastoris* are of potential use as food additives for manufacturers developing natural products (1, 2). A major example of such an application is in the manufacture of orange essence and fresh fruit flavor, the primary aldehyde of which is acetaldehyde (3). Another practical bioconversion product from AOX is n-butyraldehyde, primarily used industrially as a starting material for preparing plasticizer

alcohols; it also is used to impart a butter or cheese flavor to a product (4, 5). Butyraldehyde can be used as a starting component to produce flavor compounds such as 2-ethyl butyraldehyde found in chocolate and cocoa or 2-methyl butyraldehyde indentified in swiss cheese flavor (6). With many applications in food, the development of an all-natural bioconversion process to obtain butyraldehyde is important to flavor manufacturers.

The AOX enzyme of the non-fermenting methylotrophic yeast, *Pichia pastoris* functions by performing a controlled oxidation of methanol to formaldehyde in order to generate energy (7). The *P. pastoris* culture must be induced with methanol to produce active AOX, once induction occurs; the AOX enzyme is able to oxidize the alcohol group on short chain (1-8 carbons) aliphatic alcohols to the corresponding aldehyde. This study examines the applicability of naturally synthesizing butyraldehyde using a whole-cell bioconversion method driven by the AOX enzyme of *P. pastoris.* There is a large body of work focusing on developing a process design for acetaldehyde production utilizing similar methods as those presented here but techniques for the specific production of butyraldehyde are less known. It is the goal of this work to elucidate the efficiencies and problems associated with different aspects of a butanol to butyraldehyde oxidation process design utilizing whole-cell *P. pastoris*. The specific methods evaluated here are the use of an alternate amine based buffering system, cell wall disruption and pressurization.

Background

Pichia pastoris AOX enzyme

*Candida, Hansenula,*and *Torulopsis* are the other genera of methylotrophic yeasts mentioned alongside *Pichia* in patents concerning a practical source of the AOX enzyme (8-10).

Although species within these genera do produce AOX enzymes, these versions have less of the enzymes desirable qualities relating to industrial application when compared with *P.pastoris* AOX. The AOX enzyme derived from *P. pastoris* has the widest substrate specificity of the AOX enzymes from any of the methylotrophic yeasts (11). This allows for the greatest range of potential aldehyde products to be produced from the non-specific enzyme, making it adaptable to many different product streams. An even more important feature for practical use is the fact that *P.pastoris* derived AOX has the highest alcohol to aldehyde conversion factor of the methylotrophic yeasts including other species of *Pichia* (12).

The main form of the alcohol oxidase enzyme in *P.pastoris* is coded from the AOX1 gene with repression/de-repression regulation of the AOX1 promoter and induction regulation of the AOX1 gene itself (13). Due to the carbon-source specific induction mechanism, transcription of the *P. pastoris* AOX1 gene requires the presence of methanol and absence of any other carbon sources (i.e., glycerol, glucose or ethanol) (14, 15). These genetic factors necessitate that cultures of *Pichia pastoris* be induced to produce the AOX enzyme in a dilute methanol fed vessel, and then once the cells are induced, they are transferred to a separate vessel for the butanol to butyraldehyde bioconversion to take place. *Pichia pastoris* is incapable of anaerobic fermentation, so as sparged air or pure oxygen is used to recover volatile aldehyde products it is also obligatory to maintain the dissolved oxygen at aerobic levels.

The Whole-Cell Bioconversion System

The whole-cell system also provides an efficient alternative in terms of process design over non-cellular AOX enzyme bioconversion systems requiring cellular disruption as well as purification of the enzyme (2). Within the peroxisome of the yeast, not only are cofactors and

coenzymes available but the optimal pH, ionic strength as well as hydrophilic to lipophilic ratio conditions are met and maintained by the cell (2). For extracted AOX to be used in a bioconversion system, these conditions would have to be managed by the operators, adding a level of complexity to an already overtly complex process.

In the whole-cell system, the peroxisome microbody of a fully methanol induced methylotrophic yeast sequesters the AOX enzyme which, in order to produce energy, oxidizes methanol to H_2O_2 and formaldeyhyde (CH_2O) in the first step of the dissimilatory pathway (16). This reaction and subsequent degradation of H_2O_2 to H_2O and oxygen by catalase all takes place in the peroxisome, where a far larger amount of catalase is present than the amount of alcohol oxidase (1). Thus, although the enzymatic inhibitor H_2O_2 is generated by the alcohol oxidase, the H_2O_2 is readily degraded as it is produced so there is reduced inhibition of AOX activity in the whole-cell system due to the entire reaction occurring within the peroxisome. Without the protection of peroxisomal catalase, purified AOX enzyme suffers reduced activity in the presence of H_2O_2 (17, 18). For these reasons, the subsequent analysis of butanol to butyraldehyde bioconversion was carried out with a whole-cell system except for one of the conversions which utilized a continuous cell-lyser to determine the effect of cellular disruption on the bioconversion.

One of the main causes of inhibition of AOX activity is the presence of aldehyde products generated during the bioconversion as small concentrations of aldehyde product present in the media can cause significant inhibition of AOX activity (19). Concentrations as low as 1 g/L acetaldehyde will generate an inhibitory effect on AOX while only 4 g/L may result in 50% inhibition (19). Rather than simply sparging out the volatile aldehyde products as they form, using a Tris pH buffering system which binds up free aldehyde as it is released from the cell can

greatly increase AOX activity (10). Utilizing a primary amine (Tris) buffer to readily chelate an aldehyde product during formation is a proven means of extending the enzyme activity while maintaining a physiological pH of 8.0 (10, 12). Lowering the pH to 6.0 then releases the aldehyde product from the complex and once removed allows for the re-use of the Tris buffer (3). The mechanism behind aldehyde-amine binding is not fully understood. Given the ease at which the complex is broken at a slightly neutral pH, the association was assumed to be hydrogen-bonding between the hydroxyl and amino groups of the Tris and the hydrated form of the aldehyde (3). More recent nuclear magnetic resonance spectroscopy studies indicate Tris and the aldehyde possibly undergo chemical bonding to form an imino-acetal although this may not occur until higher than physiological temperatures are reached (i.e., 130°C) (20).

Oxygen limitation and pressure elevation are other simple process design attributes which can be modified to increase oxygen transfer rate (OTR) and thus increase AOX activity (21). Higher pressure bioconversions increase the force of diffusion from the air bubble to the medium although the elevated pressure compresses the air bubbles reducing the volumetric oxygen transfer coefficient (21). The effect of high pressure is an overall increase in the OTR. Oxygen limitation by increasing methanol feed to an induced culture has also been shown to increase OTR but puts stress on the cells lowering productivity (21).

Materials and Methods

Preparation of Induced Pichia pastoris Cells

All of the *P. pastoris* cells used in these studies were wild-type, ATCC 28485. The media and induction method were followed from the Invitrogen *Pichia Fermentation Process Guidelines* (22). Inoculation cultures were grown up in Minimal Glycerol Medium (13.4 g Yeast

Nitrogen Base, 10 g glycerol, 2 ml 500x Biotin in 1000 ml ddH2O). The MGM was filter sterilized once all of the ingredients were in solution. 250 ml shake flasks containing 100ml MGM were inoculated with *P. pastoris* and vigorously shaken at 100 rpm at 30°C. Once the culture had an OD_{600}>2.0 it was ready for transfer to the induction fermentor.

Inductions and bioconversions were carried out in 2 liter working volume vessels of New Brunswick Bioflo 310's maintaining a temperature of 30°C and agitation at 900 rpm. The media used at the beginning of induction (glycerol batch phase) was 1 liter of 4% glycerol Fermentation Basal Salts (0.93 g Calcium sulfate, 18.2 g Potassium sulfate, 14.9 g Magnesium sulfate septa hydrate, 4.13 g Potassium Hydroxide, 40.0 g glycerol, 26.7 ml 85% Phosphoric acid to a total volume of 1 liter with ddH_2O). After autoclaving, the pH of the media was adjusted to 5.0 with 28% ammonium hydroxide and 4.35 ml of PTM (see Invitrogen recipe) trace salts were added (22). Glycerol fed-batch and methanol fed-batch phases were carried out as described in the Invitrogen Pichia Fermentation Process Guidelines with the exception that once the highest glycerol feed rate was established (18.5 ml/hr/L) the culture was left without a carbon source for 12-18 hours (22). This starvation period was found to greatly increase methanol consumption the next day when the methanol fed-batch phase was initiated. Induced *P. pastoris* were removed from the vessel and refrigerated at 4°C until use in the bioconversions.

Each bioconversion began with 100 ml of induced *P. pastoris* and 900 ml of the specified buffer. Bioconversions of induced *P. pastoris* were run at 30°C with 900 rpm agitation. For bioconversions requiring sparging, the condenser exhaust was attached to a 2 liter cold trap filled with a dry ice/ acetone mixture maintaining the temperature of the trap at less than -70°C. Cell extract for bioconversion was received with an in-line peristaltic pump passing whole-cell broth through a sterile micro-bead grinder. The pressurized bioconversion batch was not sparged as

any resulting overpressure in the vessel would have then caused for backflow up the sparging line. Instead, the sealed vessel was brought to 8 psi with pure O_2 by pressurizing the headspace above the liquid volume of the bioconversion.

Analytical Methods

Solvent substrates and aldehyde products were analyzed by HPLC (LC-20AT dual pump and 10A RI detector, Shimadzu) equipped with an ion exchange column (Aminex HPX-87H, 9 um, 7.8mm x 300mm, Bio-Rad) and a cation-H guard column (Micro-guard, 30mm x 4.6 mm) using 50 mM sulfuric acid as a mobile phase. The flow rate of the mobile phase was maintained at 1 mL/min during analysis with 20 μL of sample injected into the system with an auto-injector (SIL-20AHT, Shimadzu) with the column and guard maintained at 65°C in a column oven (CT0-20A, Shimadzu). Prior to analyses, samples were centrifuged at 10 000 rpm for 5 min in a micro-centrifuge (Microfuge 18, Beckman Coulter). Data for each sample was acquired with Shimadzu EZ Start 7.4 SP1 chromatography software using standards for glycerol, methanol, ethanol, butanol, formaldehyde, acetaldehyde and butyraldehyde.

AOX Assay

The colorimetric assay used to measure units of AOX activity is described by Prencipe et. al (23). 1 liter of an AOX buffered reagent was made containing 0.1M KPO_4, 0.7mM 4-aminophenazone, 1.7 mM Chromotropic acid di-sodium salt, 50 mg/L EDTA and 500 ml/L Triton X-100 and stored at 4°C until use. A volume of Horseradish peroxidase (HRPD) equivalent to 9 units of peroxidase activity (10 μL of 100x ddH_2O diluted stock) was added to 3 ml aliquots of AOX buffered reagent along with 10 μl of 4% ethanol (v/v) for substrate to create the final AOX solution with a limited stability of less than 8 hours. A standard curve of AOX activity for each

sample set was generated by adding a 100 µl volume of diluted AOX enzyme of a known activity at 5 different concentrations (0, 3.75, 7.5, 11.25, 15 Units AOX activity) to each 3.02 ml tube of the final AOX solution. These were vortexed and incubated at room temperature for 30 minutes until measuring absorbance at 600nm. Samples were measured by adding 100 µl of sample to the 3.02 ml final AOX solution and mixing, incubating and reading with the same method as the standards. The definition of a unit of AOX activity is that one unit will oxidize 1.0 µmole of methanol to formaldehyde per minute at pH 7.5 at 25°C.

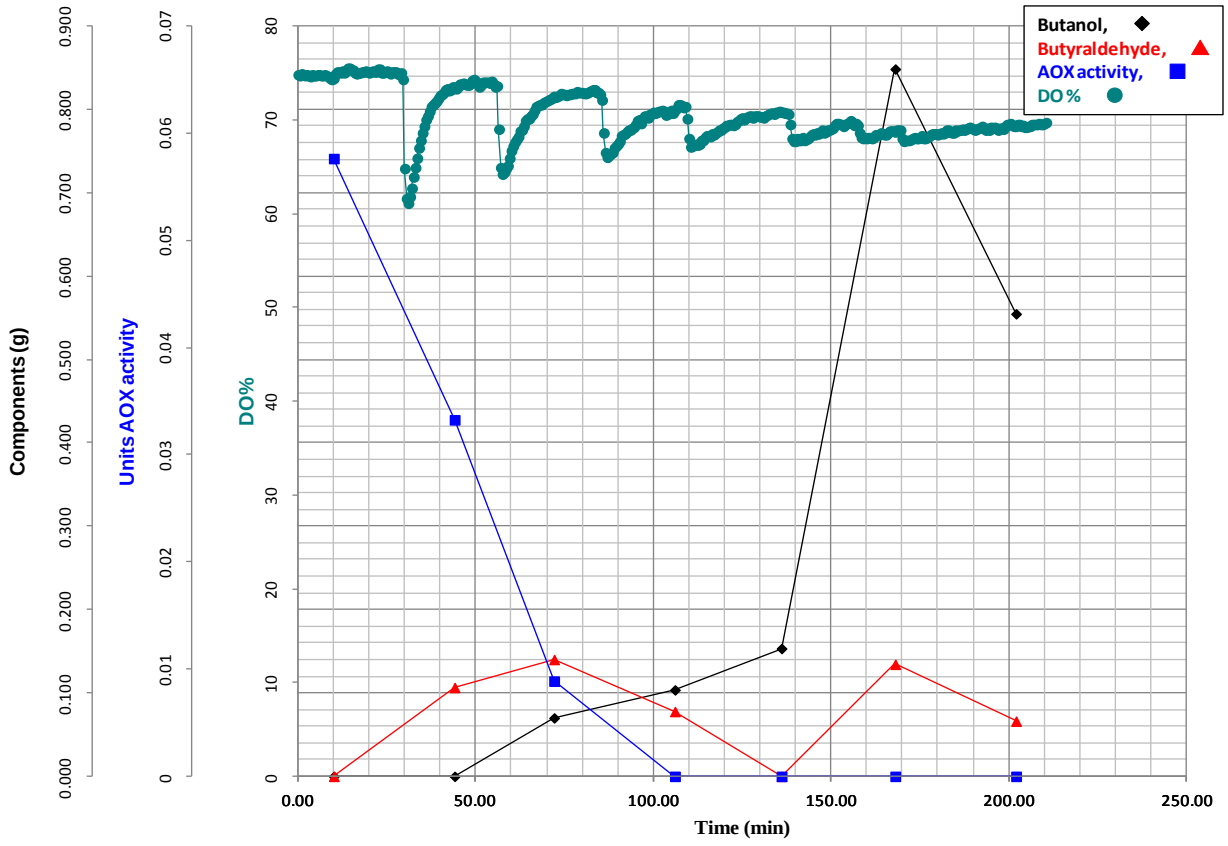
Catalase Assay

Catalase activity was determined using the Cayman Chemical Catalase Assay Kit (Item # 707002) following the procedure in the kits instruction manual (24). A standard curve was generated using the formaldehyde standards in the kit and measuring the absorbance at 540 nm. Samples were monitored for catalase activity using the same method. The definition of a unit of catalase activity is the decomposition of 1.0 µmole of H_2O_2 to oxygen and water per minute at pH 7.0 at 25°C with a substrate composition of 50 mM H_2O_2.

Results and Discussion

As a control, induced *P. pastoris* were used to run a whole-cell bioconversion with a 75:25 ratio of air to pure oxygen in the sparging stream. Having a lower level of the oxygen in the sparging line is known to trigger the effects of oxygen limitation in the induced cells which causes a release of more of the AOX1 enzyme and increases the alcohol substrate consumption rates (25). The 10 L/min sparging line of 75:25 air-O_2 never reached oxygen limitation and the dissolved oxygen remained high enough during the course of the study to make this bioconversion comparable to the other experimental setups (Fig. 17).

Figure 17. Butanol to Butyraldehyde Bioconversion using a whole-cell system. Induced *P. pastoris* 1 liter bioconversion took place at pH 7.25 to 7.4 using a 0.1 M potassium phosphate buffer and a 10 L/ min 75:25 air:O_2 sparging stream. The definition of a unit of AOX activity is that one unit will oxidize 1.0 µmole of methanol to formaldehyde per minute at pH 7.5 at 25°C. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

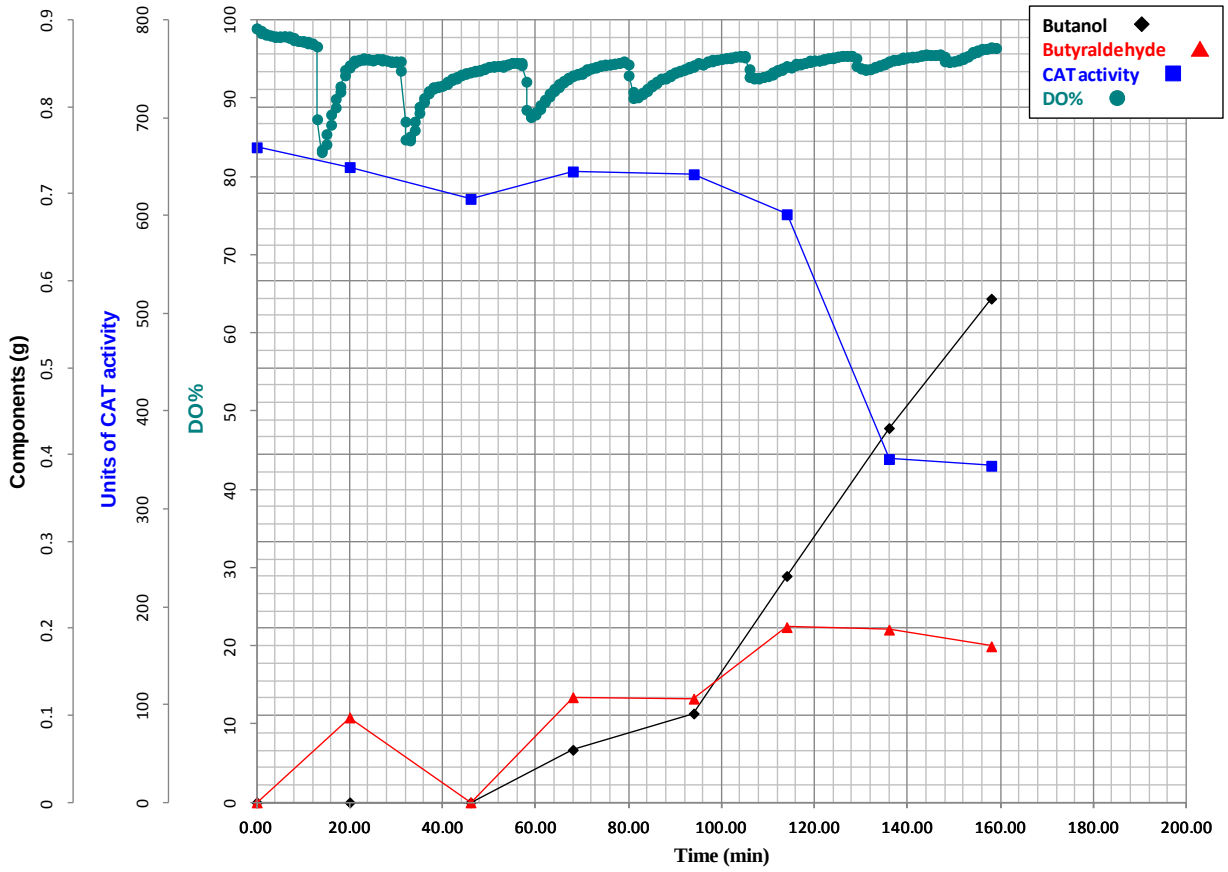


The control bioconversion was buffered with 0.1M potassium phosphate in order to maintain the whole-cells as well as the AOX enzyme. By weight, 2.08% of the 4.86 g of butanol added to the system was converted to butyraldehyde (Table 5). The live: dead ratio of induced *Pichia pastoris* cells were monitored and did not change over the course of the reaction. On the

other hand, AOX activity did fall precipitously during the first 100 minutes of the conversion and this phenomenon was potentially caused by the effects of product inhibition on the AOX enzyme (Fig. 17). 20 minutes after AOX activity reached lower than detectable levels (around 100 minutes), butanol began to accumulate significantly in the reaction vessel (Fig. 17).

A bioconversion was attempted utilizing a continuous cell lysing system as physical degradation of the yeast cellular membrane allows the activated AOX enzyme to directly contact the butanol and oxygen substrates to generate butyraldehyde. The use of a continuous micro-bead cell-lyser system resulted in 4.86 g of butanol converting to a total of 1.07 g butyraldehyde, 22.02% by weight **(**Table 5**)**. After 100 minutes the induced *P. pastoris* showed lowered oxidative conversion ability as can be seen with the DO% responding less readily to the additions of butanol (Fig. 18**)**. Also at 100 minutes, butanol began to more rapidly accumulate in the vessel with the protective catalase levels decreasing only after 115 minutes **(**Fig. 18**)**. With the cell-lyser running at 25°C and the bioconversion vessel at 30°C, once the cells were lysed the proteolytic enzymes were released from the cytoplasm and allowed to degrade the peroxisomal proteins including AOX and catalase since they were no longer protected within the microbody. Although the cell-lysing technique converted more butanol to butyraldehyde than the whole-cell system, the reduced DO% spikes after 115 minutes and rapidly increasing presence of butanol in the vessel indicate that the AOX activity quickly degenerated before 115 minutes of conversion (Fig. 18).

Figure 18. Butanol to Butyraldehyde Bioconversion using a continuous cell lysing system**.** Induced *P. pastoris* 1L bioconversion took place in pH 6.5 potassium phosphate buffer using a 10 L/min 50:50 air:O_2 sparging system. Cell-extraction and catalase assay described in the methods section. The definition of a unit of catalase activity is the decomposition of 1.0 µmole of H_2O_2 to oxygen and water per minute at pH 7.0 at 25°C with a substrate composition of 50 mM H_2O_2.

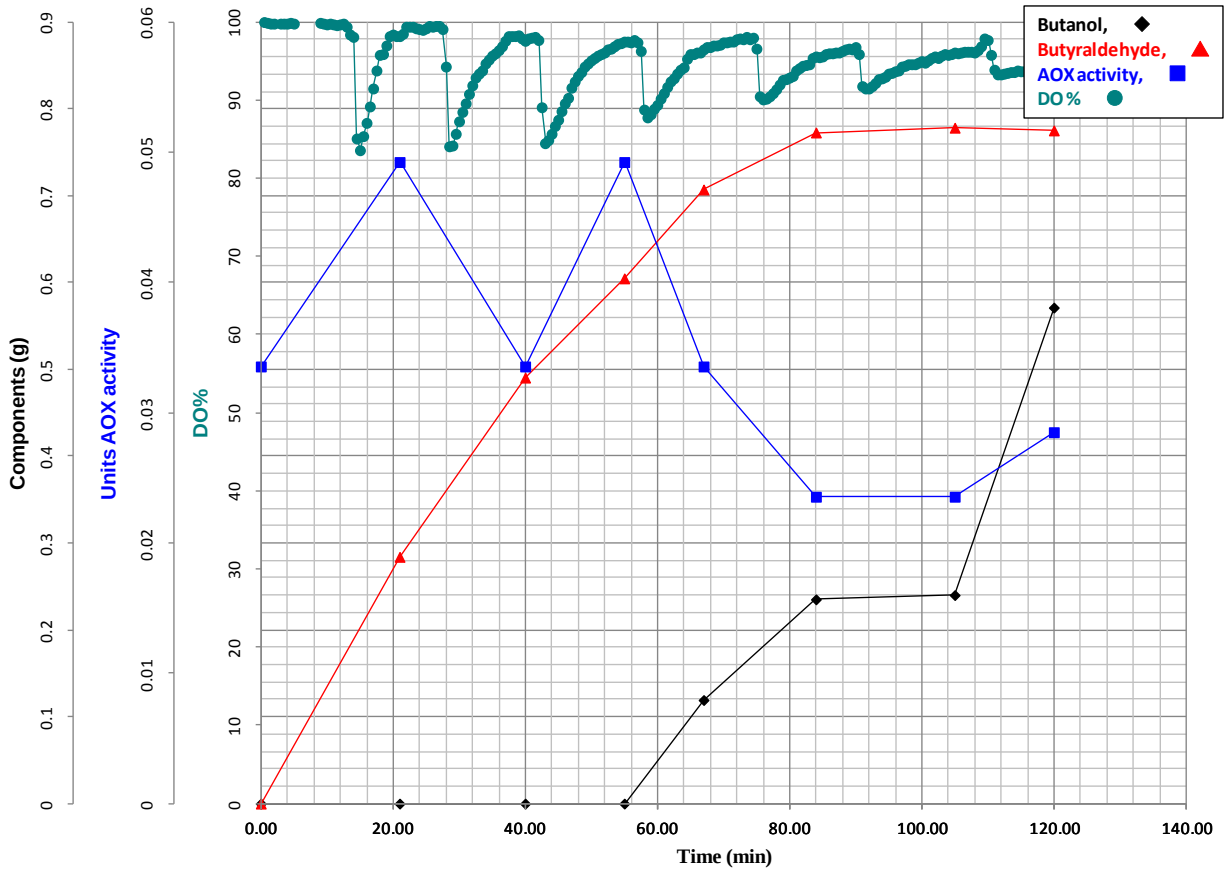


The next examination determined if product inhibition could be reduced and conversion time extended by preventing the interaction of the aldehyde product with the AOX enzyme. As the published data on product inhibition concerns acetaldehyde as the inhibitor and this study is based on producing butyraldehyde, the authors conducted a similar AOX activity study as

Murray et. al but to determine the effects of butyraldehyde (19). A 4% ethanol solution was used as the substrate in a 2M Tris-borate buffer and these tests demonstrated that 1.6 g/L butyraldehyde concentration in the reaction could inhibit AOX activity from 10 to 32%. 8.3 g/L butyraldehyde inhibited AOX activity from 20 to 61% (data not shown). These tests concluded that, similar to acetaldehyde, low levels of butyraldehyde in the reaction broth is responsible for product inhibition in AOX bioconversions.

The bioconversion utilizing a 0.5 M Tris-Borate pH buffer was able to provide a steady chelation of butyraldehyde as it was produced in the broth until the saturation level was reached around 80 minutes (Fig. 19). Comparing Fig. 19 to Fig. 17, the Tris-borate buffering system was most likely the cause of the reduced product inhibition and the much higher overall AOX activity in the 0.5M Tris-borate buffered bioconversion as compared to the 0.1M potassium phosphate buffer. The AOX activity in the 0.5M Tris-borate buffered conversion stayed above 0 for the entire bioconversion rather than dropping to 0 after 100 minutes as occurred in the potassium phosphate buffered conversion (Fig. 17). In terms of overall conversion the effects of reduced AOX inhibition due to sequestering butyraldehyde were positive as a 20.85% (g/g) conversion was seen in the 0.5M Tris-borate buffered reaction (Table 5). The pH was maintained at 8.3-8.4 for the duration of the reaction, higher than the potassium phosphate buffered conversion which maintained the pH at 7.25-7.4 (Table 5). Although the change in pH may cause for some difference in cellular health and thus effect the whole-cell conversion it is still within physiological limits and was necessary to demonstrate the differences between the buffering systems.

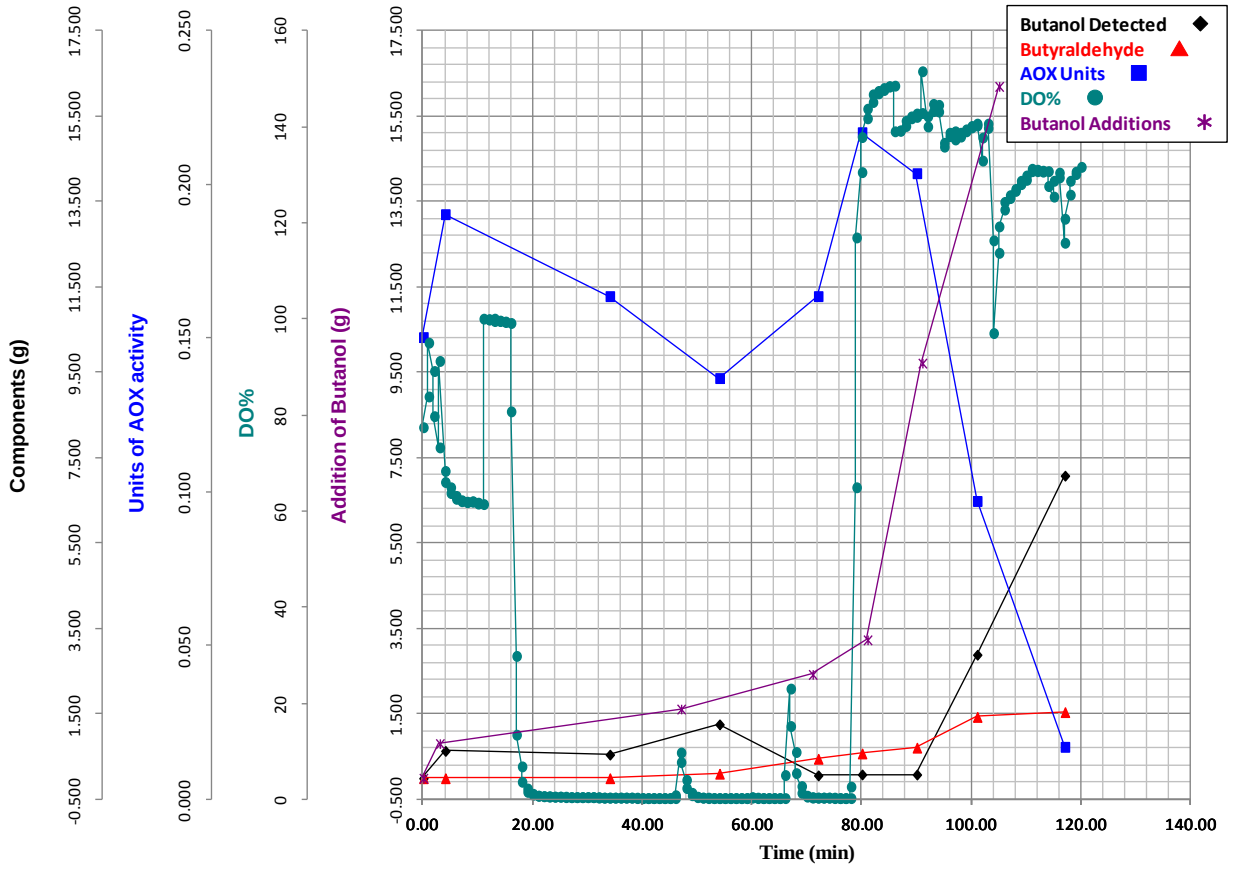
Figure 19. Effect of 0.5M Tris-Borate buffer binding butyraldehyde product during bioconversion. The 0.5 M Tris-Borate buffer maintained the pH at 8.4 during induced *P. pastoris* 1L bioconversion using a 10 L min^{-1} 50:50 air:O_2 sparging system. The definition of a unit of AOX activity is that one unit will oxidize 1.0 µmole of methanol to formaldehyde per minute at pH 7.5 at 25°C.



This study's AOX production inhibition data and that presented by Murray et al. provide evidence that even higher stoichiometric saturation limits of Tris-borate buffer to chelated aldehyde product are attainable, thus a 2M Tris-borate buffer was used in the next bioconversion (Fig. 20) (12). Most previous reports of product chelation as a means of reducing AOX inhibition

are demonstrated on ethanol to acetaldehyde bioconversions yet seem applicable to our work focused on butanol bioconversion to butyraldehyde.

Figure 20. Butanol to Buytraldehyde bioconversion pressurized to 8psi with pure O_2. Induced *P.pastoris* 1L bioconversion. This batch has no sparging occurring and is run with a 2M Tris-Borate buffer keeping the pH at 8.3. Agitation at 900 rpm. The definition of a unit of AOX activity is that one unit will oxidize 1.0 µmole of methanol to formaldehyde per minute at pH 7.5 at 25°C.



Another interesting method employed to increase productivity was demonstrated by Charoenrat et al. where using a pressurized system had increased the oxygen transfer rate into an active *Pichia pastoris* culture more than was found with oxygen limitation (21). For the next

bioconversion, instead of a sparging line, the vessel of the reaction was sealed and pure O_2 was used to pressurize the vessel to 8psi (Fig. 20). As the dissolved oxygen probe was calibrated to 0 and 100% oxygen saturation of the media at normal atmosphere, the probe gave some readings significantly higher than 100% DO during the pressurized bioconversion. The work reported here focuses on the bioconversion of butanol to butyraldehdye while reports from the literature concerning pressurization systems are usually applied to recombinant protein production (21).

The first 100 minutes of the 2M Tris-borate buffered pressurized bioconversion showed exceedingly high levels of AOX activity (over 0.1 units) compared that of the potassium phosphate buffered sparged conversion (maximum 0.058 units AOX activity) or the 0.5M Tris-borate sparged conversion (maximum 0.05 units AOX activity) (Fig. 17, 19, 20). Not shown here, the AOX activity dropped to almost nothing (0.004 units of activity for the next 846 minutes), but the bioconversion kept slowly taking place the entire time. In the pressurized vessel, of the 16.2 g of butanol added, 91.42% was converted to butyraldehyde in terms of the level of butanol remaining in the broth after the bioconversion.

The actual amount of butyraldehyde reclaimed from the pressurized bioconversion was 12.6% g/g (Table 5). This illustrates a common difficulty found in the production of aldehydes, the high volatility and low boiling point allows much of the product to pass through the exhaust of a cold trap or, as presumably occurred in this case, saturate the headspace of a pressurized vessel. Chiang et al. came across the same issue when they attempted to develop a method for large scale acetaldehyde production (1). In their work, they eventually switched to a liquid-nitrogen cold trap producing a condenser temperature of -75°C similar to the temperature of the cold trap in the bioconversions presented here. Even after the switch, Chiang et al. still found much of their product being lost due to its high volatility. Butyraldehyde has a much higher

boiling point, 74.8°C compared to acetaldehyde 20.2°C but from the strong scent given off by the bioconversion exhaust, much of it is still leaving the system un-reclaimed. Although oxygen pressurized design provides less of a recoverable % of converted butyraldehyde than either the cell-lyser or 0.5M Tris-borate buffered sparged set-ups, it provided for the largest amount of recovered butyraldehyde, 2.047 g from a 1L conversion volume.

Table 13: Summarization of data on Butyraldehyde production from 4 separate bioconversions.

	Trap	**Air:O_2**	**Buffer**	**pH**	**Time (min)**	**BuOH added (g)**
Whole-cell	Dry ice-acetone	75:25	0.1M Potassium Phosphate	7.25-7.4	192	4.86
Cell-Extract	Dry ice-acetone	50:50	0.1M Potassium Phosphate	6.5	158	4.86
Whole-cell	Dry ice-acetone	50:50	0.5 M Tris-Borate	8.3-8.4	120	5.67
Whole-cell Pressurized	None	0:100	2 M Tris-Borate	8.3	966	16.2

Table 13 (cont'd)

	Trap	Air:O_2	Buffer	BA in trap (g)	BA in broth (g)	% conversion (g/g)
Whole-cell	Dry ice-acetone	75:25	0.1M Potassium Phosphate	0.03	0.066	2.08
Cell-Extract	Dry ice-acetone	50:50	0.1M Potassium Phosphate	0.85	0.18	22.02
Whole-cell	Dry ice-acetone	50:50	0.5 M Tris-Borate	0.41	0.78	20.85
Whole-cell Pressurized	None	0:100	2 M Tris-Borate	NA	2.047	12.64*

* based on Butanol (g) added as a substrate and Butanol (g recovered) in broth, the % conversion (g/g) was 91.42% to butyraldehyde but this was lost in the headspace of the pressurized vessel.

Conclusion

A control whole-cell *P.pastoris* bioconversion using a 0.1M potassium phosphate buffer converted 2.08% of 4.86 g butanol to butyraldehyde. With the addition of cellular disruption to the same system, the conversion increased to 22.02%. The cell-lyser caused a rapid decrease in the catalase activity of the system after 120 minutes, reducing the ability of this system to degrade H_2O_2, a by-product of the conversion which inhibits AOX activity.

0.5 M Tris-borate chelating buffer in the whole-cell system lowered product inhibition and allowed for greater maintained AOX activity than the non-chelating buffer control. In this system, the butyraldehyde was bound in the buffer as it was formed allowing for 20.85% of the 5.67 g butanol added to be converted to butyraldehyde.

Sparging the active bioconversion with either air or oxygen is necessary to provide the AOX enzyme with oxygen substrate but the volatile nature of butyraldehyde makes it difficult to condense and collect in a cold trap. The recovery issue is partially solved by using an 8 psi pressurized vessel to provide the AOX with oxygen while preventing product inhibition with a strong chelator (2M Tris-borate) buffering the system and binding the butyraldehyde after conversion. Such a set-up resulted in a much longer conversion time (966 minutes of continued AOX activity) and of the 16.2 g of butanol fed into the vessel, 12.64% was recovered in the conversion broth. Given the small amount of unconverted butanol which was recovered from the broth, 91.42% must have been converted with much of it lost in the headspace of the pressurized vessel.

REFERENCES

REFERENCES

1. Chiang, H.-K., Foutch, G. L., and Fish, W. W. (1991) Biological Production of Acetaldehyde from Ethanol Using Non-Growing *Pichia pastoris* Whole Cells. *Applied Biochemistry and Biotechnology* **28/29**, 513-525

2. Duff, S. J. B., Murray, W. D., and Overend, R. P. (1989) Factors affecting the yeast-mediated conversion of ethanol to acetaldehyde in batch reactors. *Enzyme and Microbial Technology* **11**, 770-775

3. Raymond, W. R. (1984) Process for the Generation of Acetaldehyde from Ethanol. (Company, T. C.-C., ed), The Coca-Cola Company, United States of America

4. Kanand, J., Roper, M., Paciello, R., and Thome, A. (1999) Preparation of n-butyraldehyde and/or n-butanol. BASF Aktiengesellschaft, USA

5. Yolles, S. (1974) Controlled Release Flavor Compositions. Vol. 3,857,964, David E. Brook, United States

6. Ronzio, R. (2003) The Encyclopedia of Nutrition and Good Health. p. 737, Facts On File, Inc., New York, NY

7. Cregg, J. M. (2007) Distinctions between *Pichia pastoris* and Other Expression Systems. In *Pichia Protocols* (Cregg, J. M., ed) Vol. 389 pp. 1-10, Humana Press, Totowa, New Jersey

8. Giuseppin, M. L. F. (1987) Process for preparing a catalase-free oxidase and catalase-free oxidase-containing yeast, and use thereof. Unilever, Netherlands

9. Hartner, F., and Glieder, A. (2008) Mutant AOX1 Promoters. Technische Universitat Graz, USA

10. Murray(A), W. D., Duff, S. J. B., and Lanthier, P. H. (1989) Production of Natural Flavor Aldehydes From Natural Source Primary Alcohols C_2-C_7. p. 16, Canadian Patents & Development Limited, United States of America

11. Dienys, G., Jarmalavicius, S., Budriene, S., Citavicius, D., and Sereikaite, J. (2003) Alcohol oxidase from the yeast *Pichia pastoris* - a potential catalyst for organic synthesis. *Journal of Molecular Catalysis B: Enzymatic* **21**, 47-49

12. Murray(B), W. D., Duff, S. J. B., and Lanthier, P. H. (1989) Induction and stability of alcohol oxidase in the methylotrophic yeast *Pichia pastoris*. *Applied Microbiology and Biotechnology* **32**, 95-100

13. Cereghino, J. L., and Cregg, J. M. (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiology Reviews* **24**, 45-66

14. Inan, M., and Meagher, M. M. (2001) Non-Repressing Carbon Sources for Alcohol Oxidase (*AOX1)* Promoter of *Pichia pastoris*. *Journal of Bioscience and Bioengineering* **92**, 585-589

15. Cregg, J. M., Vedvick, T. S., and Raschke, W. C. (1993) Recent Advances in the Expression of Foreign Genes in *Pichia pastoris*. *Nature Biotechnology* **11**, 905-910

16. Ellis, S. B., Brust, P. F., Koutz, P. J., Waters, A. F., Harpold, M. M., and Gingeras, T. R. (1985) Isolation of Alcohol Oxidase and Two Other Methanol Regulatable Genes from the Yeast *Pichia pastoris*. *Molecular and Cellular Biology* **5**, 1111-1121

17. Couderc, R., and Baratti, J. (1980) Oxidation of Methanol by the Yeast, *Pichia pastoris*. Purification and Properties of the Alcohol Oxidase. *Agricultural and Biological Chemistry* **44**, 2279-2289

18. Kato, N., Omori, Y., Tani, Y., and Ogata, K. (1976) Alcohol Oxidases of *Kloeckera* sp. and *Hansenula polymorpha*: Catalytic Properties and Subunit Structures. *European Journal of Biochemistry* **64**, 341-350

19. Duff, S. J. B., and Murray, W. D. (1988) Production and Application of Methylotrophic Yeast *Pichia pastoris*. *Biotechnology and Bioengineering* **31**, 44-49

20. Bubb, W. A., Berthon, H. A., and Kuchel, P. W. (1995) Tris Buffer Reactivity with Low-Molecular-Weight Aldehydes: NMR Characterization of the Reactions of Glyceraldehyde 3-Phosphate. *Bio-organic Chemistry* **23**, 119-130

21. Charoenrat, T., Ketudat-Cairns, M., Jahic, M., Veide, A., and Enfors, S.-O. (2006) Increased total air pressure versus oxygen limitation for enhance oxygen transfer and product formation in a *Pichia pastoris* recombinant protein process. *Biochemical Engineering Journal* **30**, 205-211

22. Invitrogen, L. T. (2002) Pichia Fermentation Process Guidelines. In *http://tools.invitrogen.com/content/sfs/manuals/pichiaferm_prot.pdf* (Technologies, I. L., ed), Invitrogen Corporation, Online

23. Prencipe, L., Iaccheri, E., and Manzati, C. (1987) Enzymic Ethanol Assay: a New Colorimetric Method Based on Measurement of Hydrogen Peroxide. *Clinical Chemistry* **33**, 486-489

24. Cayman, C. C. (2009) Catalase Assay Kit: Catalog No. 707002. In *www.caymanchem.com* (Company, C. C., ed) Vol. 707002, Cayman Chemical Company, Ann Arbor, Michigan

25. Charoenrat, T., Ketudat-Cairns, M., Stendahl-Andersen, H., Jahic, M., and Enfors, S.-O. (2005) Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. *Bioprocess Biosyst Eng* **27**, 399-406

CHAPTER 7: CONCLUSIONS

The world's demand for butyraldehyde and butyric acid is increasing necessitating the development of methods for producing these chemicals using sustainable methods from renewable resources. While the industrial world currently depends on the oxo reaction of propylene to supply the needs of modern manufacturers, an economically feasible method of production of these chemicals utilizing agricultural or pulp and paper industry wastes provides a sustainable alternative. Xylose from hydrolyzed hemicellulose is a fermentable sugar utilized in the fermentation production of butyric acid by members of *Clostridium* spp.

The phenomenon examined in Chapter 2 was that the presence of an initial concentration of acetate during the batch fermentation production of butyrate by *C. tyrobutyricum* inhibits the culture and causes an extended growth-lag phase lowering the overall productivity rate. One of the main issues of utilizing a hemicellulose extracted xylose is the high levels of residual acetate, causing microbial inhibition in subsequent fermentations (Chapter 2). Despite lowered productivity, xylose consuming *C. tyrobutyricum* cultures challenged with high concentrations of acetate (17.6 g/L and 26.3 g/L) demonstrated higher butyrate yields than unchallenged cultures due to the bacteria's acetic acid re-uptake metabolic mechanism. Also, given similar concentrations of either carbon source, *C. tyrobutyricum* generates more butyrate by the fermentation of xylose under acetate challenged conditions than by fermenting glucose.

These results lead to the conclusion that hot water extracted hemicellulose from hardwood, a waste stream from the pulp and paper industry, is a cost-effective source of fermentable sugars as the majority of the monomeric sugar from this source is xylose. The

experimental data presented in Chapter 2 demonstrated that at every concentration of acetate inhibition (0, 4.4, 8.8, 17.6, 26.3 g/L), *C. tyrobutyricum* was able to eventually adapt to the acetate and after the lag-phase completely ferment all available sugar.

Chapter 3 concluded that the directed evolution of a *C. tyrobutyricum* culture to acetate tolerance is a means of reducing the lag-growth phase generated by a high concentration (26.3 g/L) of acetate in batch fermentation. Acetate is a microbial inhibitor and high concentrations of initial acetate induce extended lag periods in cellular growth as the non-tolerant culture adapts to the presence of free acetate in the media. The reduced lag-growth phase of the acetate tolerant culture increases the overall rate of butyrate production. The acetate tolerant culture fermenting glucose saw a complete negation of the high acetate induced lag-phase while the acetate tolerant xylose fermenting culture decreased lag-phase time by 75% compared with a common wild-type inoculum. The metabolic enzyme acetate kinase (AK) responsible for the acetyl-CoA conversion to acetic acid showed decreased activity in the presence of high concentrations of acetate whether or not the culture had been adapted to acetate or not. Decreased AK activity theoretically indicates that carbon is being fluxed into butyrate production rather than through the acetate generating branch of *C. tyrobutyricum* metabolism and this was demonstrated with acetate challenged batches generating higher butyrate yields than the un-challenged controls. Also, under high acetate conditions the evolutionarily directed acetate tolerant culture has a greatly reduced specific-growth rate compared to the non-tolerant culture. This data is interesting in that with the lower specific-growth rate the acetate tolerant culture has a much faster overall butyrate production rate, demonstrating the importance of a reduced lag-phase over specific growth rates for increasing productivity. Fortunately, drastic reduction of the extended lag phase caused by external acetate is achievable through selective adaptation of the *C.*

tyrobutyricum inoculation culture allowing for faster activation of the uptake mechanism (Chapter 3). Overall, the experimental data from both Chapter 2 and Chapter 3 lead to the conclusion that hemicellulose from pulp and paper industrial waste undergoing hot water extraction would be a suitable and cost-effective substrate for the fermentation production of butyric acid. The high levels of acetate typically released by hemicellulose hydrolysis are overcome to further increase fermentation productivity by the acetate up-take mechanism.

Chapter 4 examines the use of several pretreatment methods in the processing of farm-raised lignocellulosic hydrolysate material as a *C. tyrobutyricum* fermentation substrate. *C. tyrobutyricum* batch (serum bottle) fermentations were conducted with corn stover hydrolysates having undergone pretreatments of either ammonia-fiber expansion (AFEX), lignin extracted-ammonia fiber expansion (E-AFEX ™) or alkaline hydrogen peroxide (AHP) methods. The pretreated hydrolysates required pH adjustment in order to be appropriate for fermentation and each hydrolysate was adequately fermented to butyrate.

The hydrolysate fermentations were successful and subsequently 1 liter batch controlled temperature and pH (6.0) fermentations were performed with AFEX-CS hydrolysate and a comparative control batch with similar glucose and xylose concentrations. The AFEX-CS hydrolysate began with an initial concentration of 62.561 g/L glucose and 33.552 g/L xylose. After 143 hours of fermentation, the glucose had fermented to complete dryness and 9.724 g/L of the xylose was consumed resulting in 28.997 g/L butyrate. These results were similar to the 1 liter *C. tyrobutyricum* control batch run for comparison. The control batch started with 56.151 g/L glucose and 40.16 g/L xylose and resulted in 32.2 g/L butyrate with all of the glucose consumed and 21.496 g/L residual xylose. Substrate consumption and butyrate production rates were similar between the AFEX-CS and control batch. The main discernible difference between

the batches was that the fastest specific growth rate (μ_{net}) of the AFEX-CS fermentation was half the rate (μ_{net} (1/h)= 0.09029) of the control fermentation (μ_{net} (1/h)= 0.19321). It is the author's belief that the slower specific growth rate was the result of microbial inhibition due to inhibitors contained within AFEX-CS material.

Substrate inhibition based on the high osmotic pressure derived from high concentrations of sugars in fermentation media lower microbial growth and productivity. The study performed in Chapter 5 examines substrate inhibition on butyrate fermentation by selectively adapting cultures of *C. tyrobutyricum* to tolerate the presence of 150 g/L initial glucose in fermentation media. A high glucose (150 g/L) tolerant culture in log growth phase was used to inoculate a 1 liter controlled temperature and pH batch fermentation of 150 g/L glucose. A non-tolerant (original ATCC 25755 *C. tyrobutyricum*) 1 liter batch was also used to ferment 150 g/L glucose for comparison.

The high glucose adapted culture began fermentation of the 150 g/L glucose several hours sooner than the control batch demonstrating a reduction in the lag-phase due to substrate inhibition. Despite the shorter lag-phase, the high glucose adapted culture generated 38.931 g/L butyrate and left 36.605 g/L residual glucose by the time it stopped fermenting. The control *C. tyrobutyricum* culture generated 43.695 g/L butyrate and left a residual glucose concentration of 20.135 g/L. The log-phase specific growth rates of the cultures were identical $\mu_{net=}$ 0.288 (1/h). Adapting the *C. tyrobutyricum* culture to high glucose tolerance and did not imbue the adapted culture with faster specific growth rates, sugar consumption or higher final butyrate yield. The production advantage the high glucose culture had over the control culture was the ability to begin fermentation faster with a shortened lag-phase in growth.

In conclusion, data presented in this dissertation furthers the practical application of hydrolysed lignocellulosic biomass as substrate for the fermentation production of the value-added product butyrate. Namely, the acetate inhibition of *C. tyrobutyricum* concomitant with the use of xylose from hemicellulose can be overcome through an adaptive selection process. The adaptation of acetate tolerance imbues the culture with higher butyrate productivity upon exposure to acetate. Furthermore, pretreated lignocellulosic hydrolysates containing multiple forms of monomeric sugars are demonstrated usable as substrate for butyrate production. The use of alternative production methods outlined in this dissertation not only allow manufacturers to market products as non-synthetic for foods or flavors but also provides a sustainable means of producing butyrate acid for future generations.