

Differential Protein Expression and Butanol Production using
Clostridium beijerinckii

by

Aaron Esbenshade

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Biological Sciences

Program

YOUNGSTOWN STATE UNIVERSITY

December, 2012

**Differential Protein Expression and Butanol Production using *Clostridium
beijerinckii***

Esbenshade Aaron

I hereby release this thesis to the public. I understand that this thesis will be made available from the OhioLINK ETD Center and the Maag Library Circulation Desk for public access. I also authorize the University or other individuals to make copies of this thesis as needed for scholarly research.

Signature:

Aaron Esbenshade, Student

Date

Approvals:

Dr. Gary Walker, Thesis Advisor

Date

Dr. Douglas Price, Committee Member

Date

Dr. Jonathan Caguiat, Committee Member

Date

Peter J. Kasvinsky, Dean of School of Graduate Studies & Research

Date

Table of Contents

Acknowledgments	3
Abstract	4
1. Introduction	5
2. Background	7
2.1. Butanol	7
2.2. Butanol vs. Ethanol	7
2.3. ABE fermentation	9
2.4. Fermentable Carbon Sources	12
2.5. Lignocellulose	12
3. Specific Aims	14
4. Materials and Methods	15
4.1. Chemicals	15
4.2. Organism	15
4.3. Media	16
4.3.1. Glucose Media	16
4.3.2. Xylose Media	16
4.3.3. Hydrolysate YSU	16
4.4. Cell Collection	17
4.5. Cell preparation	17
4.6. Protein Analysis	18
4.7. Sugar and Solvent analysis	18
5. Results	19
5.1. HPLC Standards	19
5.2. Experimental HPLC	29
5.3. Protein Standards	39
5.4. Experimental Proteins	41
6. Discussion	42
7. References	50

Abstract

Current events demand new forms of renewable energy. Ethanol from fermentation has been the standard in biofuel for years, but not for long. It has become apparent that ethanol has serious drawbacks. Ethanol is more corrosive to engines and only has approximately 60% the energy content of gasoline per gallon. This problem may be remedied by the use of butanol. Butanol is far less corrosive than ethanol and holds 95% the energy of gasoline per gallon. Our research uses the bacterium *Clostridium beijerinckii* as the model for the fermentative production of butanol using discarded lignocellulose from biomass, specifically wood. The wood biomass (wood chip), is processed by treatment with high temperature and pressure, resulting in a hydrolysate consisting of free sugars and other breakdown products of cellulose and hemi-cellulose. We have been able to grow *C. beijerinckii* in media containing xylose or glucose as the principle sugars, both which are present in the wood extract. We had limited success growing bacteria in media whose only carbon source (sugar). is wood hydrolysate. Analysis has shown that the sugar concentrations in these wood extracts were too low to support significant growth. More highly concentrated versions of the hydrolysate appeared to kill the organism completely presumably due to the increased inhibitory compounds. We continued to study the bacterial proteome in efforts to identifying key proteins that play role in the overall process of butanol fermentation. In the future we hope to genetically engineer microorganisms to efficiently carry out butanol fermentation at commercially significant levels. We were also able to find that the scaled up version

of the reaction provided significantly higher production of butanol, presumably due to a more consistent growth environment.

Acknowledgments

I would like to thank my family, for their encouragement and support. I would also like to acknowledge my advisor Dr. Gary Walker and committee members Dr. Jonathan Caguiat and Dr. Douglas Price, all of which provided invaluable support and guidance last but not least I would like to express my thanks to all the undergraduate researchers who were invaluable during this endeavor.

1. INTRODUCTION

Every year as the world's population grows we demand ever more energy. Much of the energy required is in the form of gasoline to run our cars. In 2010 the United States alone consumed 9.8×10^{16} BTU's (British Thermal Units), of which 3.6×10^{16} or 36 quadrillion BTU's were in the form of petroleum (DOE, 2011). As this is a finite resource and we consume it at an ever increasing rate, consideration must be given to our eventual depletion of this resource. While there are many possible avenues that can be pursued one promising alternative is butanol produced via fermentation from renewable plant matter. As seen with ethanol, the current strategy is to use biofuel as an additive to our current petroleum products rather than substitutes for them. However the potential of butanol as a fuel is particularly high due to its chemical properties, high energy content and strong history as an industrially fermented solvent.

While butanol is currently produced almost exclusively from the refinement of crude oil in recent years, it was primarily produced via ABE (acetone-butanol-ethanol). Fermentation during the first half of the twentieth century until the early sixties (T. Ezeji, Qureshi, & Blaschek, 2007). ABE fermentation by *Clostridium acetobutylicum* ranks second only next to ethanol fermentation via *Saccharomyces cerevisiae* as the oldest fermentation process known (Ramey & Yang, 2004). However, due to the growing petrochemical industrial growth following the Second World War ABE fermentation methods failed to be economically viable and fell

completely to petrochemical methods of production. However, the energy crisis of the seventies sparked renewed interest in alternative fuels as well as sparking the green movement which has played a role during decades since to revive interest in bio-energy (Dürre, 1998, 2008)

In more recent years, the demand for biofuel has been driven by government mandates both in the United States and the European Union (Hertel, Tyner, & Birur, 2010). While these regulations and their pursuant subsidies have done much to jump start a revival of the biofuel industry, it is not without its consequences. With the growth of the biofuel industry so has the demand for substrates that can be fermented as in corn, sugar cane, rapeseed oil, palm oil and soybeans. The inability of the agricultural industry to meet the demand of these biofuel substrates has had the unintended consequence of tightening the already insufficient world food supply (Boddiger, 2007). This has driven the pursuit of alternative carbon sources for the production of these fuels. The most abundant renewable source of carbon is lignocellulose, which does not need to compete with the food source as it can be found in all manner of inedible agricultural residues and various other inedible plant byproducts from many industrial processes (T. Ezeji et al., 2007). This source however offers its own difficulties due to its partial composition of inhibitory compounds, mainly furans and weak acids (Almeida et al., 2007). Additionally the sugars found in these residues are not easily accessible and most often need to be prepared by some form of hydrolysis of polysaccharides.

2. BACKGROUND

2.1 Butanol

1-butanol (CAS no. 71-36-3). is a linear four carbon primary alcohol with the molecular formula C_4H_9OH . Butanol is a liquid at room temperature and is not miscible in water. Some similar molecules include methanol, and ethanol, which have one and two respectively. These molecules however are dissimilar in their miscibility in water. Butanol has minimal solubility in water and an energy density of 29.2 MJ/L. These properties, along with its non-corrosive nature, mean that it can be used as a direct replacement for gasoline, and can be used without any modifications to the current infrastructure (Lee et al., 2008).

2.2 Butanol vs. Ethanol

While the vast majority of biofuel produced in the last decade has been in the form of ethanol produced via corn starch, a renewed interest is being given to butanol. Butanol more closely resembles gasoline in energy density and in heat of vaporization (Lee et al., 2008, p. 2).

One of the many problems with ethanol is its hydroscopic nature making it readily absorb water from the atmosphere. This can cause corrosion to engines and pipelines and prohibits the transportation of it through existing pipelines. Therefore, must be transported by truck or train, which greatly reduce the efficiency of transportation (Ramey, 2007).

As shown in the table below, both butanol and gasoline also have a much higher energy density than ethanol. This is important to note when doing a cost analysis of one versus the other.

	Cost/Gallon	Miles/Gallon	Cost/Mile
E85	\$3.29	17.6	\$0.19
Gasoline	\$3.88	22	\$0.18
Butanol from :			
Petroleum	\$5.23	25	\$0.21
Corn	\$5.10	25	\$0.20
Sugar Cane	\$4.98	25	\$0.20
Wood	\$3.40	25	\$0.14
Bagasse	\$2.76	25	\$0.11
Glycerol	\$1.62	25	\$0.06

Table 1. Cost comparison of butanol vs E85 and Gasoline(Ramey, n.d.; Wilson, 2011)

As shown in table 1 not only can butanol outperform the current ethanol based E85 biofuel, but due to a more complete and clean burning combustion, butanol actually outperforms gasoline as well in miles per gallon. However, even with this increased efficiency butanol still falls slightly short of gasoline and E85 in cost per mile when derived from traditional methods. More recently attention has been given to less expensive carbon substrates and as seen again in the previous table this allows butanol to not only compete with subsidized ethanol but to outright beat both E85 and gasoline by significant margins. Bagasse is the left-over lignocellulose from sugar cane and sorghum which along with wood do not directly compete with food production. While glycerol is used to a limited degree as a sweetener and preservative it is not vital as a staple food like corn is. For that

matter butanol itself is approved under current laws to be used as an artificial flavoring for food (FDA, 1996).

Butanol however is not without its problems. The production of butanol requires a more controlled approach. Where yeast is successful at overcoming non sterile environments, *Clostridium* are much more sensitive and require prior sterilization of the fermentors. At the same time current research was finding that butanol might be a more viable biofuel an analysis of then current industrial techniques found the cost to produce a kilogram of ethanol to be \$0.55 and a kilogram of butanol to be \$2.34 (Pfromm, Amanor-Boadu, Nelson, Vadlani, & Madl, 2010). Over eighty two percent of this cost is in the cost of additional feedstock. But even as we have found that more recent techniques have been developed to produce nearly the same quantity of both fuels from the same feedstock, with butanol containing just over sixty percent more energy per unit volume it has become a much more attractive alternative fuel. Additionally *Clostridium beijerinckii* BA101, a hyper-butanol producing strain, has been shown capable of utilizing several different sugars present in cellulose and hemi-cellulose simultaneously from their respective polysaccharides (Thaddeus C Ezeji, Qureshi, & Blaschek, 2004; T. Ezeji et al., 2007). This Additionally there are many chemical by-products that can be separated from the fermentation for prices far surpassing the price of gasoline or diesel (Wilson, 2011)

2.3 ABE Fermentation

ABE (Acetone Butanol Ethanol), fermentation was the primary source of butanol until the early 1960's due to competition from the growing petrochemical industry (Thaddeus C Ezeji et al., 2004). This however was only true so long as oil was relatively cheap and abundant. More recently however our knowledge of this fermentation process has grown significantly while at the same time ever increasing demand and instability in the oil markets has caused petrochemical routes of production to become much more expensive.

This increased expense has given rise to investigation into the use of biomass, and more specifically lignocellulose derived from the biomass after the initial food stuffs has been removed and only the non-edible portion of the biomass remains. In this series of experiments we have chosen to concentrate on wood as our carbon source. This is due to the many tons of hemicellulose that is disposed of each year as a by-product of the pulp and paper industry as they purify the cellulose in order to make all manner of paper and wood products.

The primary polysaccharide in the resulting extract is xylan, which upon hydrolysis consists of many individual xylose sugars. This hydrolysis can be accomplished by a few systems. Industrial separation generally involves high heat and pressure or else a lower heat in the presence of an acid. This particular species of *Clostridium* was chosen due to its ability tolerate higher levels of solvents, as well as its suspected possession of an enzyme called 1,4-beta-xylosidase. This enzyme if

present would allow this particular organism to break the bonds between the 1 and 4 carbon atoms of adjacent pentose sugars in the xylan chains of the hemi-cellulose. If this protein could be expressed in substantial enough quantities it would allow direct fermentation of xylan into butanol without intermediate industrial hydrolysis.

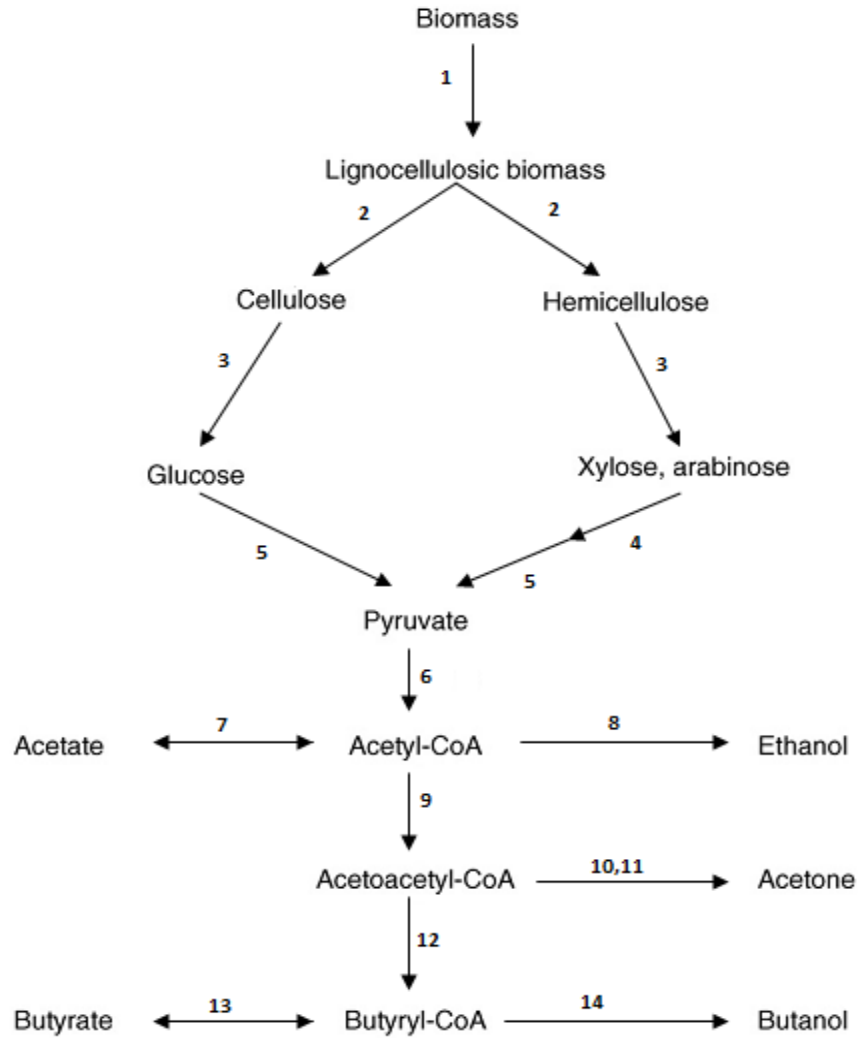


Figure 1. 1, crop preparation; 2, hot separation of cellulose and hemicellulose; 3, hydrolysis of polysaccharides by either industrial or biological methods; 4, transketolase-transaldolase sequence producing fructose 6-phosphate and glyceraldehydes 3-phosphate; 5, EMP pathway; 6, pyruvate-ferrodoxin oxidoreductase; 7, phosphate acetyltransferase and acetate kinase; 8, acetaldehyde dehydrogenase and ethanol dehydrogenase; 9, thiolase; 9, 3-hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase; 10, acetoacetyl-CoA:acetate/butyrate:CoA transferase; 11, acetoacetate decarboxylase; 12, 3-hydroxybutyryl-CoA dehydrogenase, crotonase and butyryl-CoA dehydrogenase; 13, phosphate butyltransferase and butyrate kinase; 14, butyraldehyde dehydrogenase and butanol dehydrogenase (Thaddeus Chukwuemeka Ezeji, Qureshi, & Blaschek, 2007; Haus et al., 2011)

2.4 Fermentable Carbon Sources

Until the early sixties the primary carbon source used for ABE fermentation was Molasses (Dürre, 2008). Since then, many other carbon sources have been used, including but not limited to: maize, sugarcane, soybeans, rapeseed, and palm oil (Boddiger, 2007). A growing interest is more recently being given to sources that do not compete with our food supply. These include things such as switch grass, woodchip extract, and dried corn stalk fiber (Boddiger, 2007; Koukiekolo et al., 2005; Sun & Liu, n.d.). The portion of interest in all of these sources is their lignocellulose. Various strains of butanol fermenting *Clostridium* are of particular interest in this area due to the nature of the lignocellulose. This is because the primary sugar sugars found in lignocellulose are the hexose and pentose sugars glucose and xylose respectively. As you can see in figure one the ABE fermentation pathway in *Clostridium beijerinckii* already possesses the required pathway for fermentation of both sugars. Alternatively we have yet to find a naturally occurring pathway for the fermentation of ethanol directly from pentose sugars (Galbe, Gorwa-Grauslund, Zacchi, & others, 2006). Fermentation of pentose sugars is particularly desirable due to nature of hemi-cellulose being easier to hydrolyze as compared to cellulose.

2.5 Lignocellulose

Lignocellulose comes from a variety of sources and is the most abundant renewable carbon source on the planet (T. Ezeji et al., 2007). About 25 – 30 percent of the lignocellulose biomass is in the form of hemicellulose making it the second most abundant source, and of the hemicellulose the most abundant group is xylan (Koukiekolo et al., 2005).

While as high as 67% of lignocellulose can be chains of these fermentable sugars, it is not all readily hydrolyzed by current butanol producing organisms (T. Ezeji et al., 2007). Because of this the lignocellulose must first undergo a dilute sulfuric acid hydrolysis (Qureshi et al., 2008). This hydrolysis will allow the production of several sugars including: glucose, xylose, arabinose, galactose, mannose, and rhamnose. If the hydrolysis is taken too far, these sugars break down into two main groups of inhibitory compounds. The first group furans can be further divided into phenols, furfural, and HMF (hydroxymethylfurfural). The second group being weak acids, primarily, acetic, formic, and levulinic acid (Almeida et al., 2007)

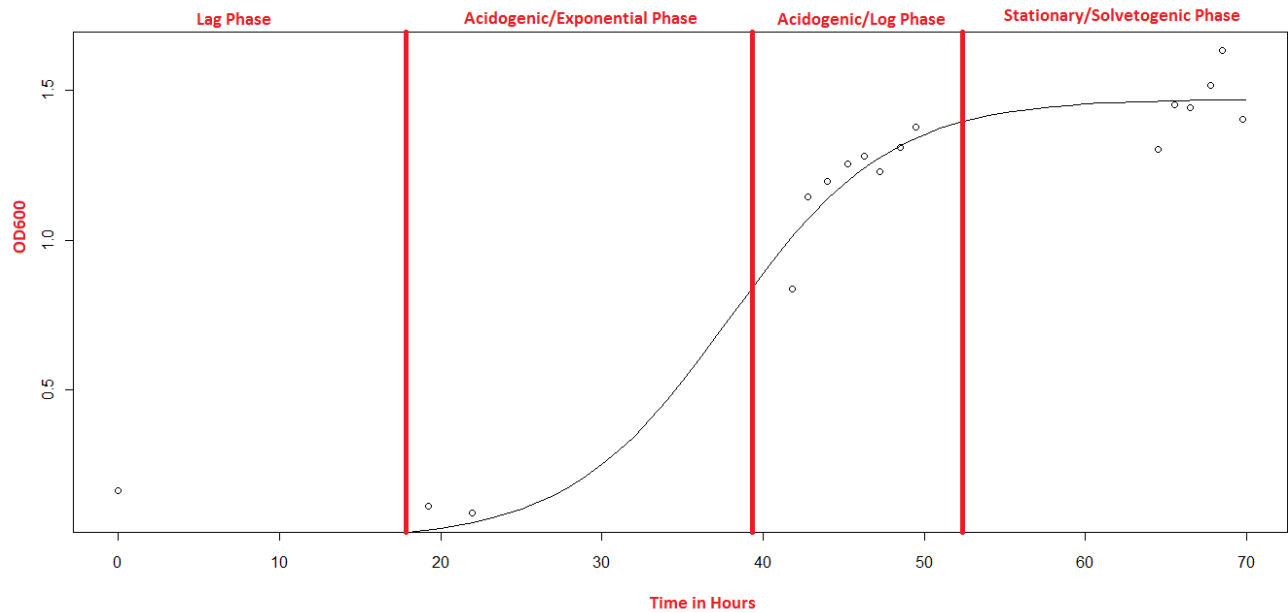


Figure 2. Cell growth of *Clostridium Beijerinckii* grown on standard glucose media in 1 liter reactor at 37 degrees Celsius.

Figure 2 gives an example of our baseline growth curve using our organisms proffered carbon source glucose. The areas of the predicted acidogenic and solventogenic phases are labeled. This will be useful later in comparing the growth under experimental conditions

3. Specific Aims

The aim of this project is to analyze differences in the proteome of *Clostridium beijerinckii* during utilization of wood hydrolysate as a carbon source, and to determine solvent production rates and sugar consumption rates using HPLC (Tsuey, Ariff, Mohamad, & Rahim, 2006). It is believed that removal of the inhibitory compounds in the wood hydrolysate using a combination of acid hydrolysis and over liming will allow us to conduct ABE fermentation using *Clostridium beijerinckii*, with the end goal being analysis of differential protein expression between growth on wood hydrolysate and that of growth in glucose, xylose and glycerol media. These pure carbon sources were chosen in order to demonstrate the proteins used in the digestion of 6, 5, and 3 carbon sugars respectively. Additionally it is expected that the proteome will change between the acidogenic and solvent phases as well and these changes will be noted as they relate to the different carbon sources (Mao et al., 2010)

4. Materials and Methods

4.1 Chemicals

The following chemical substrates were obtained from the following locations for preparation of our media: D-glucose, Amresco in Solon, OH; D-(+)-xylose, Sigma Aldrich St. Louis MO; Glycerol, Amresco Solon, OH; yeast extract bacteriological, Amresco Solon, OH; K_2HPO_4 anhydrous, Amresco Solon, OH; KH_2PO_4 , Fisher Science Education Hanover Park, IL; $MgSO_4 \cdot 7 H_2O$, Amresco Solon, OH; $FeSO_4 \cdot 7 H_2O$, Merck & Co., Inc. Rahway, NJ; 4-aminobenzoic acid, 99% Sigma Aldrich, MO; ammonium acetate crystalline, Mallinckrodt Paris, KY; Water obtained from Millipore water filtration system

4.2 Organism

The organism that was used had been obtained from American Type Culture Collection (ATCC) bioresource center and has been designated as *Clostridium beijerinckii* ATCC 35702. The bacteria arrived lyophilized in a sealed glass vial. They were seeded into a 15ml tube containing 10ml Glucose media.

4.3 Media

4.3.1 Glucose Media

60g glucose, 5g yeast extract, 1g $K_2PO_4 \cdot 3H_2O = .683999$ grams K_2PO_4 anhydrous, 1g KH_2PO_4 , 1g $MgSO_4 \cdot 7H_2O$, .5g $FeSO_4$, .1g amino benzoic acid, and 3g ammonium acetate. The flask was then filled to 1L with deionized water

4.3.2 Xylose Media

Initially cultures were grown in 15ml screw top tubes containing 5ml of a nutrient solution containing 60 Xylose, 5 g yeast extract, 1 g $KH_2PO_4 \cdot 3H_2O$, 1 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, 0.5 g $FeSO_4$, 0.1 g amino benzoic Acid, 3 g ammonium acetate per liter. Allowing this culture to grow for forty eight to sixty two hours allowed the bacteria to reach growth phase. This phase of growth was expected to be the optimal condition for the bacteria to acclimate to the new growth media.

4.3.3 Hydrolysate YSU

Sawdust from maple trees was heated under pressure at a 7% wood to liquor ratio to a temperature of 160 °C for the duration of 120 minutes. This has been

shown to be the optimal temperature for sugar extraction (Hu, Lin, Liu, & Liu, 2010). This liquid was then extracted from the spent wood and this process will be repeated with new wood up to three times. This hot water extract was then used as a supplement to our standard xylose media in an attempt to induce differential protein expression as compared to our control growth media of pure xylose.

4.4 Cell Collection

The cells were collected from the 500 ml flasks by opening and flaming the flasks before a 15ml sample was poured into a 15ml pre-weighed sample collection tube. 1ml of this sample was used to measure the cell growth via OD600 reading using a Bio-Rad smartspec plus. The supernatant collected from each time point was then used to test for acid and solvent production at these time points as well. Additionally a 7 liter Bioflo 110 bioreactor was used. Samples collected from this were able to be collected through a sample valve. These samples were 50 ml and were handled the same as the smaller samples.

4.5 Cell preparation

The cells separated from the media via centrifugation at 8000 x g for 15 minutes. The supernatant was then transferred to clean 15 ml tubes and frozen at -20 °C . The pellet was then re-suspended in TBS buffer and centrifuged again at

8000 x g for 15 minutes to wash away any contaminants and were then frozen.

4.6 Protein Analysis

The pelleted cells were later re-suspended in SDS loading buffer containing 250 mM tris-HCL, pH 6.8; 0.02% SDS; 10% Glycerol; 20 mM DTT; 0.01% Bromophenol Blue and lysed using a small sonicator. These proteins were then be loaded onto a 12% SDS-page 7 cm gel and run in a in a Bio-Rad Criterion cell. Next the gel was soaked in Coomassie for 1 hour, this was followed by two one hour washes with high destain, and then left to sit overnight in low destain. The next morning an image was taken using a PharosFX molecular imager. This image was then used to evaluate load volumes based on visual intensity of the banding. These samples where then assigned a load volume of either 10, 20 or 30 micro liters. Having established proper load volume these protein samples where then run on an 11cm criterion gel with a density gradient of 8-16% acrylimide.

4.7 Sugar and Solvent analysis

Analysis of sugar and solvent concentrations will be measured from media samples both before and after fermentation. For this Waters 1515 high pressure liquid chromatograph (HPLC). with a Waters 2414 refractive index (RI) detector

was used. In order to ensure consistency of sample loads a Waters 717plus auto sampler will also be used. Samples will be run using an auto sampler in order to ensure consistency of sample injection. The column used was a Rezex ROA-Organic Acid H+ 8%. This particular column is uniquely suited to this experiment due to its ability to resolve the sugars and alcohols in one run. The column was heated to 80 °C, and the flow rate was set to 0.8ml/min and the mobile phase used was 3 mM H₂SO₄ with a sample volume of 10 µL. This configuration has been found to increase the differentiation between the peaks for butyric acid, acetone and ethanol (Tsuey et al., 2006). While the primary sugar of interest will be xylose due to the interest in its derivation from xylan using the xylan 1,4-beta-xylosidase. Additionally the primary solvent of interest will be butanol, and the retention times of both of these are sufficiently distant to be easily resolved. However the increased resolution could be beneficial for any additional analysis of this experiment, or for further studies. Determination of peaks and the area underneath them will be conducted using breeze software.

5. Results

5.1 HPLC Standards

For the first experiment, we added 50mL of dormant cell solution that had been grown overnight in glucose media then refrigerated to induce dormancy. For

our initial experiments we used a 1 liter fermentor which was then filled with 500ml of the glucose media and 50ml of dormant cell solution. The media was filter sterilized. And the heated magnet stir plate was used to ensure a constant temperature of 37 degrees was maintained. This allowed us to establish a control which showed that our media was sufficient for growth, and provide a baseline growth rate for future trials.

Having established a baseline as seen in figure 2 for growth we were able to determine the time points needed in order to collect samples from the lag phase, acidogenic phase, solventogenic phase and terminal phase. This allowed us to target our samples during these specific events. The next step having established reliable OD600 growth data we proceeded to establish HPLC control samples for xylose (fig 3), acetic acid (fig 4), butyrate (fig 5), acetone (fig 6), and butanol (fig 7) in order to construct a calibration curve. In this way we were able to measure their accumulation in grams per liter at the sample time points. We expected to observe butanol production during the time labeled in figure 2 as the solventogenic phase.

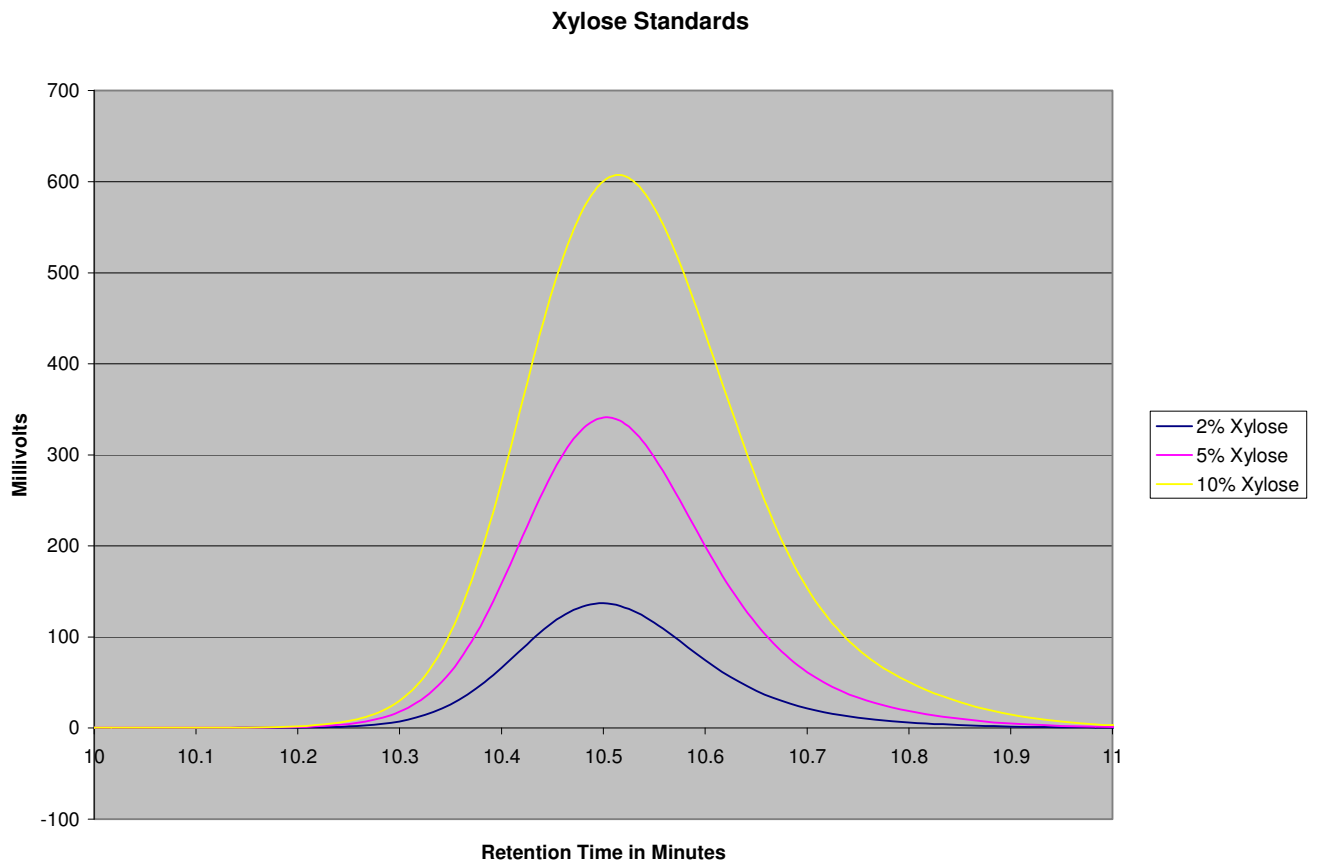


Figure 3. Xylose standards taken on June 4, 2012 using Waters HPLC with RI detector

Figures 3 through 7 are all produced from our HPLC controls data and represent quantities of a particular compound exiting the column as a function of time. The X axis indicates how many minutes it took from the injection of the sample for a particular molecule to leave. The Y axis indicates the increased voltage across the RI detector as the quantity of a given molecule increases to refract more light towards the sensor.

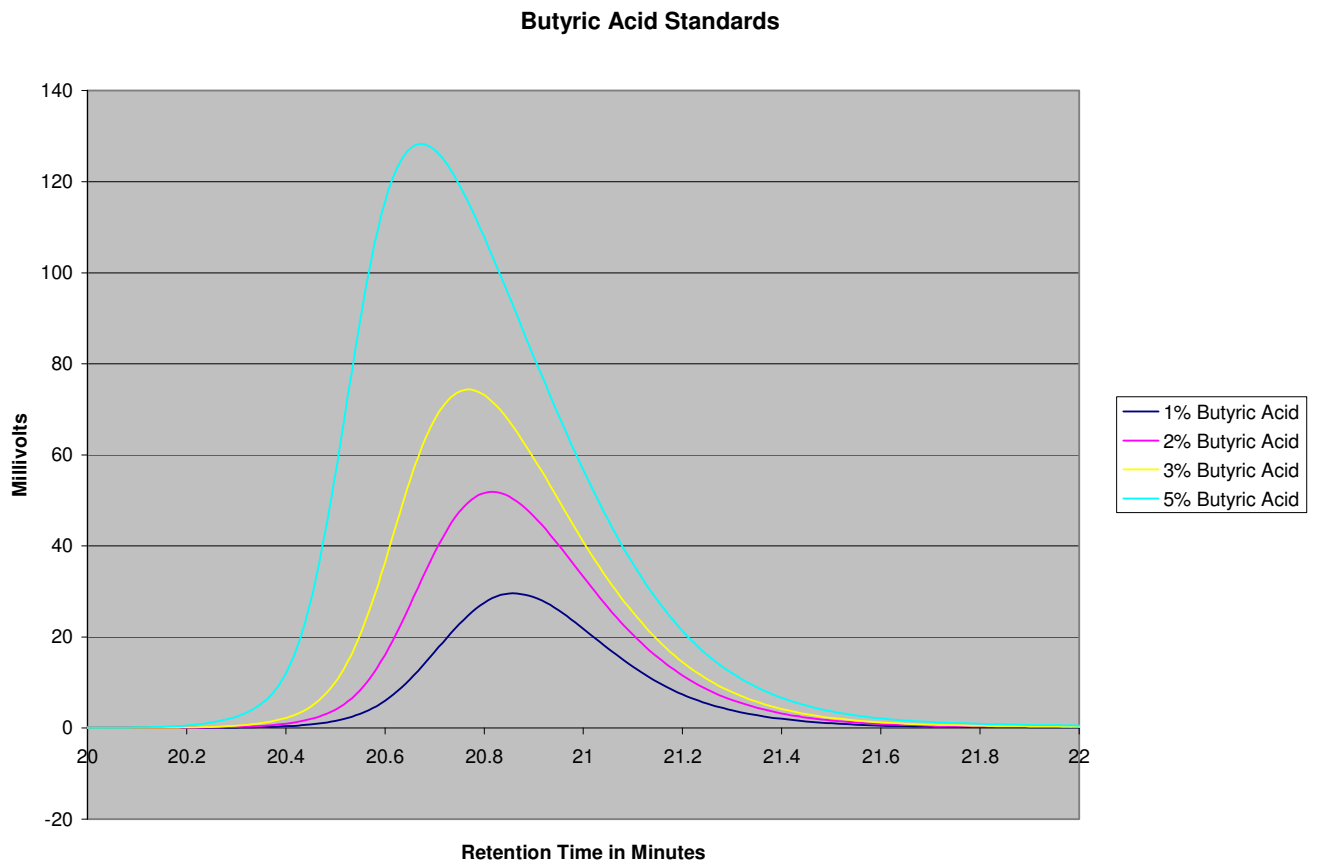


Figure 4. Butyric acid standards taken June 11, 2012 using waters HPLC with RI detector

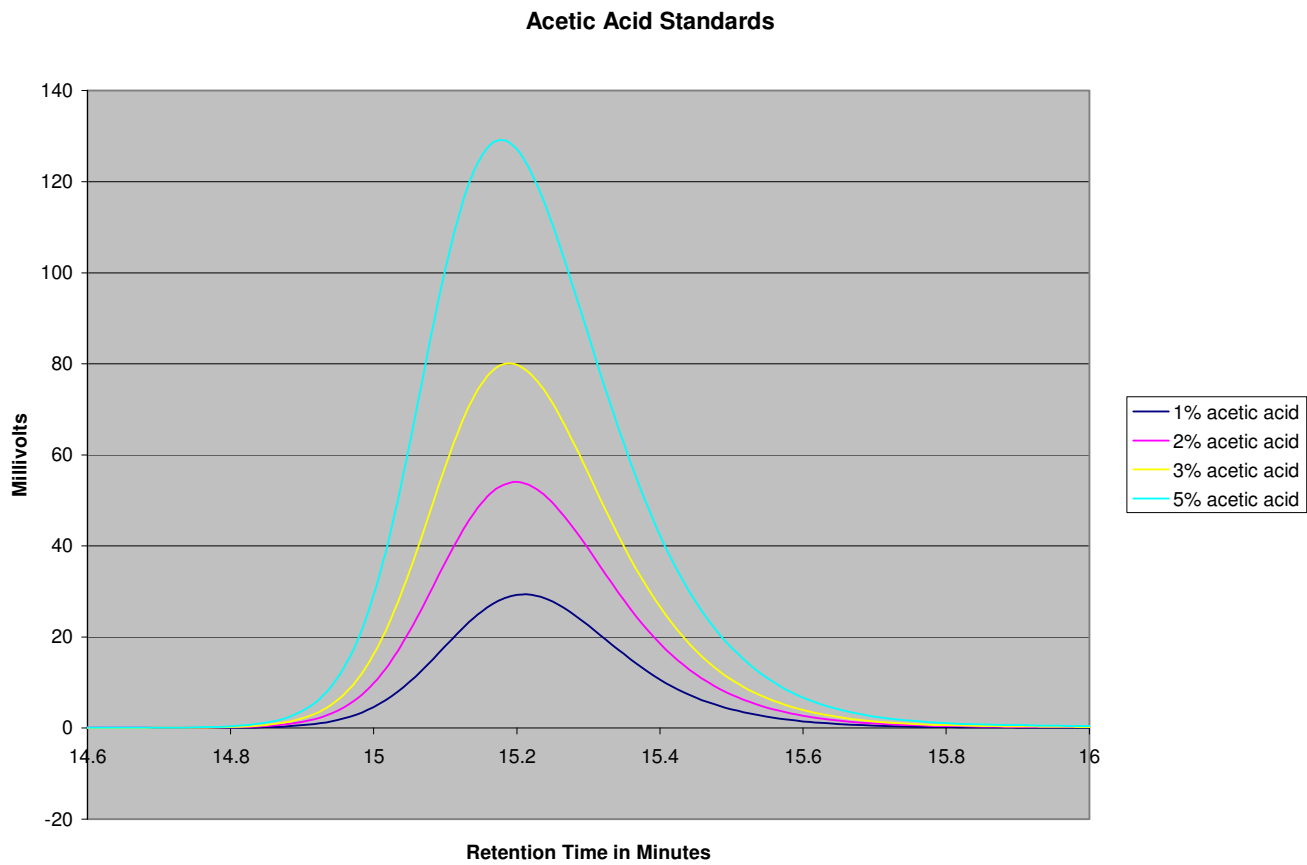


Figure 5. Acetic Acid Standards taken June 11, 2012 using Waters HPLC with RI detector

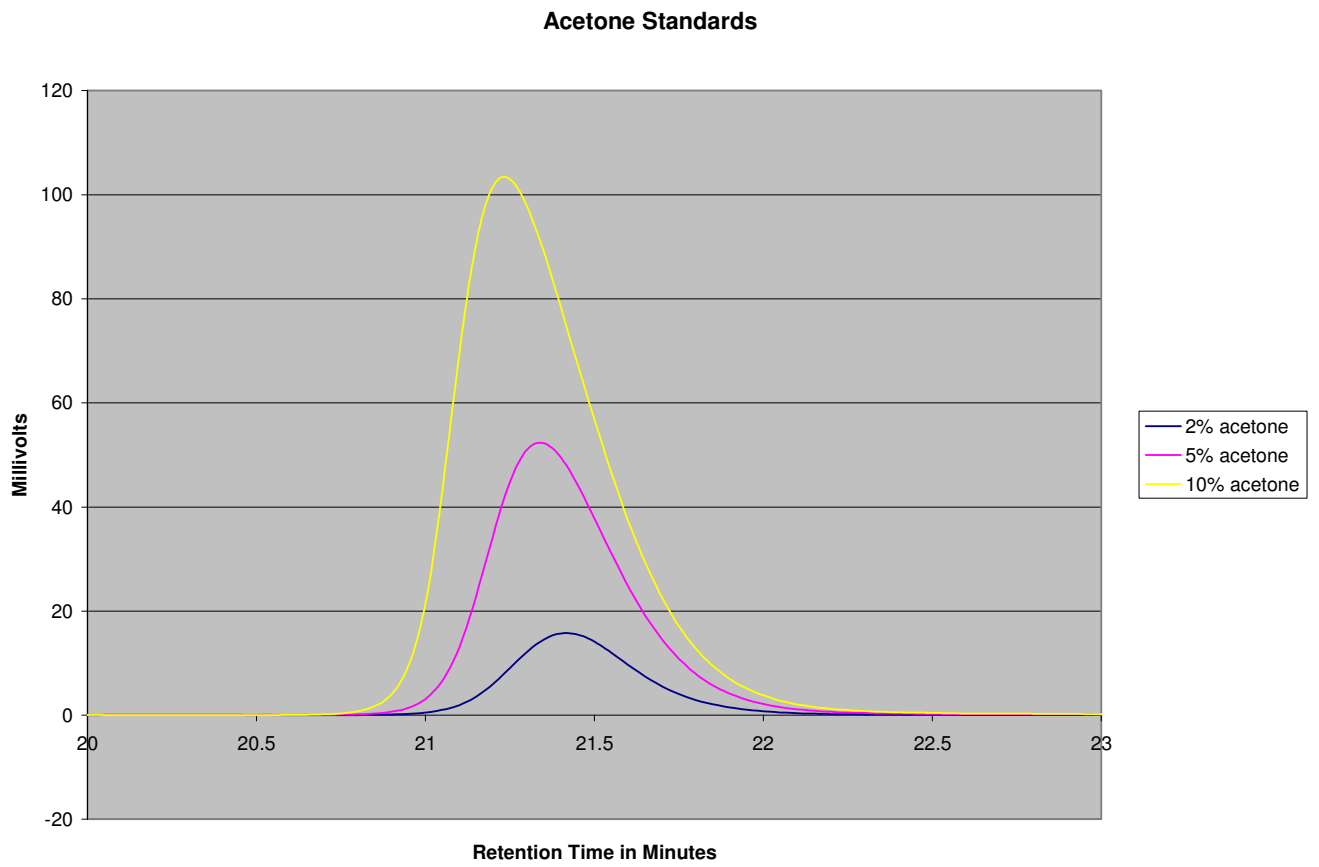


Figure 6. Acetone standards run on June 11, 2012 using Waters HPLC with RI detector

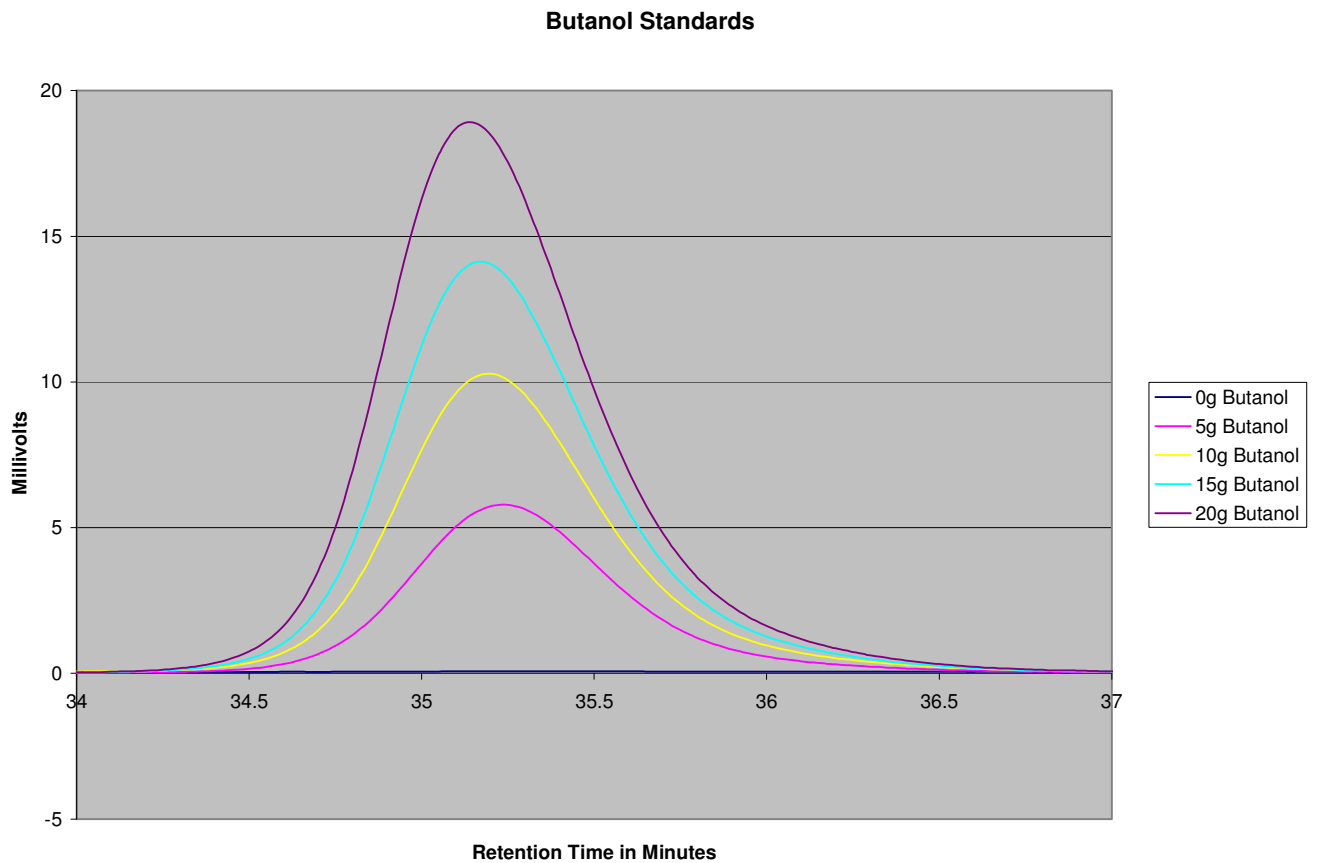


Figure 7. Butanol standards where run on June 4, 2012 using Waters HPLC with RI detector

As can be observed in figures 3, 4, 5, 6, and 7 higher concentrations of a particular substance within the media caused the peak to be proportionally larger in area due to an increased refractive index in the mobile phase as it pas through the RI detector. Additionally it can be noted in figures 4 and 6 that higher concentrations of acetone and butyric acid within the media caused the peak to be shifted left indicating that it pushed through the column a little more quickly with increased concentration. This shift is predictable based on concentration and can be accounted for and predicted based on the shift seen in the standards. Figure 8 has been plotted to show the correlative linear progression of the change in retention time as it relates to quantity.

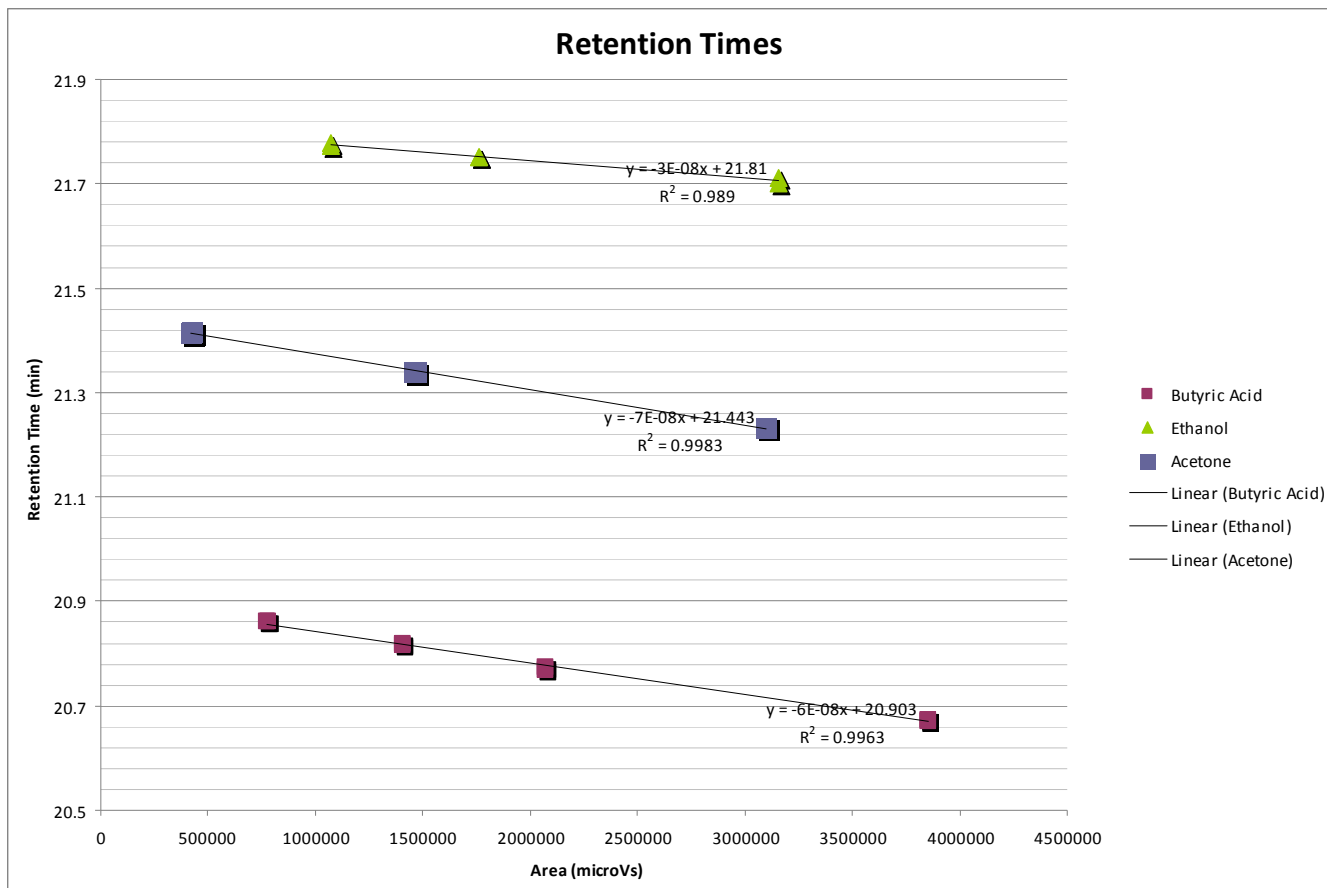


Figure 8. linear fit modeling expected retention time as it relates to area under the curve.

As can be seen in the R squared values all over .098 it can be safely concluded that this model is significant and dependable to predict the relative position of the retention time based on the area under the curve, in this way we have a dependable confirmation that both the experimental and control peaks correlate to the same compound.

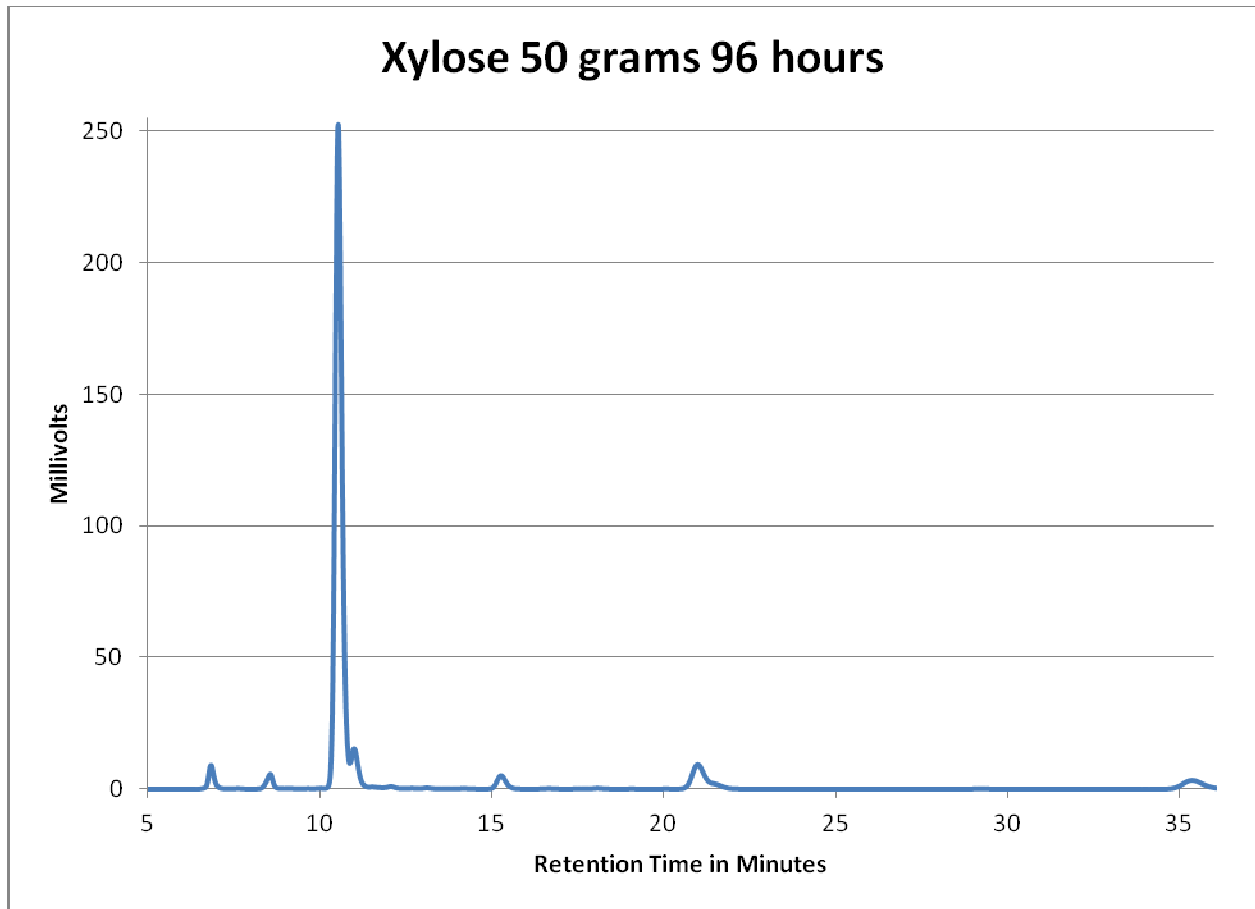


Figure 9. Raw HPLC data from experimental suupernatant June 18, 2012

Figure 9 is a good representation of a typical experimental data output from our Waters HPLC. As you can see compared to the retention times predicted based on our model the major peak over 250 mill volts correlates to xylose and one of the minor peaks out near 36 minutes correlates to butanol. Acetic acid is also distinguishable about 16 minutes. Ideally we would have monitored butanol concentration as it correlated to butyric acid concentration and acetone concentration as it correlated to acetic acid concentration. However, the column retained butyric acid and acetone between 21 and 22 minutes, making it difficult to distinguish between these two components for the low levels of concentration seen

in the 500 ml flasks. Because of this we proceeded with the analysis of the correlation between butanol and xylose.

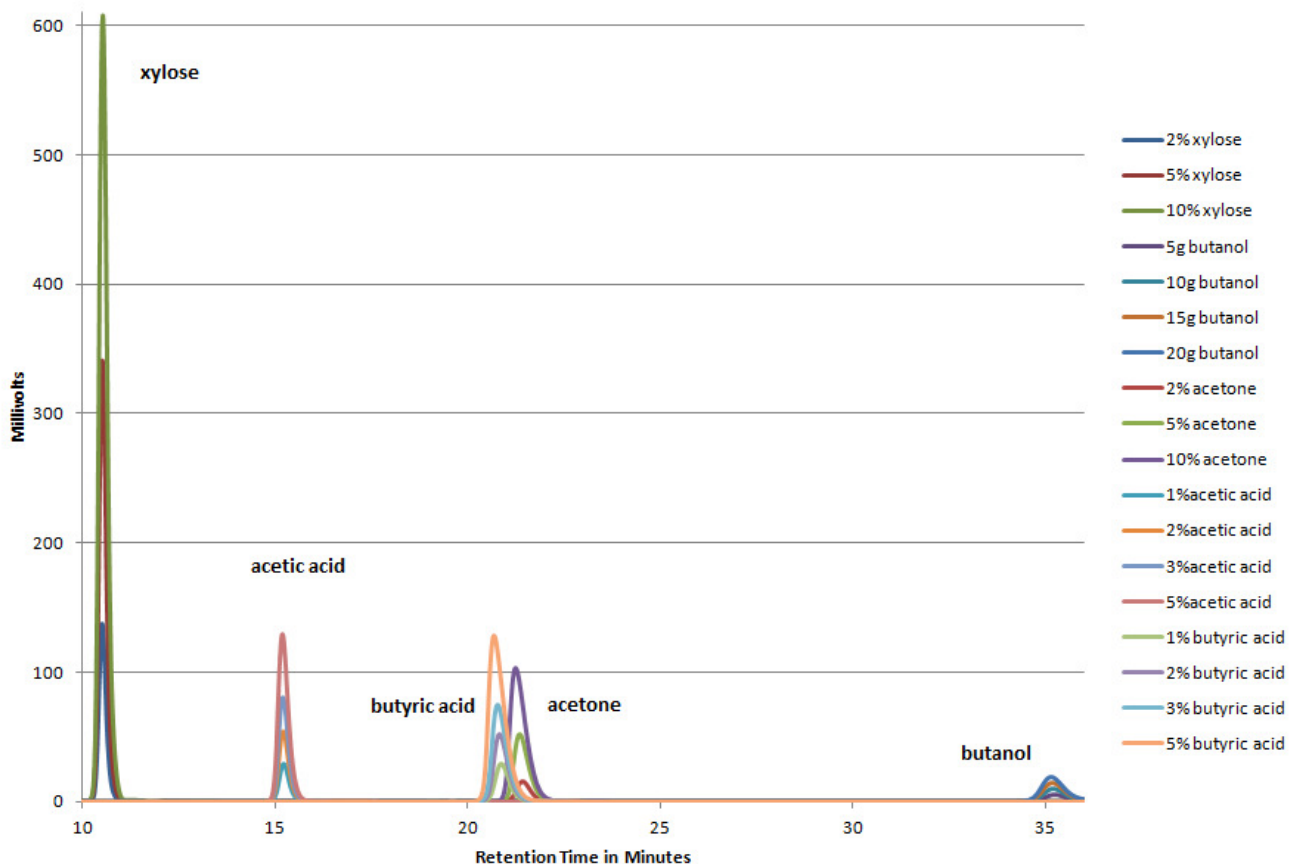


Figure 10. Labeled plot of all HPLC standards raw data plotted together

Figure 10 can be compared to figure 9 to show how our experimental peaks correlated with our controls. It can be seen that all standards can be accounted for as peaks in figure 9 except for acetone which can be seen only as a shoulder on the right hand side of the butyric acid peak.

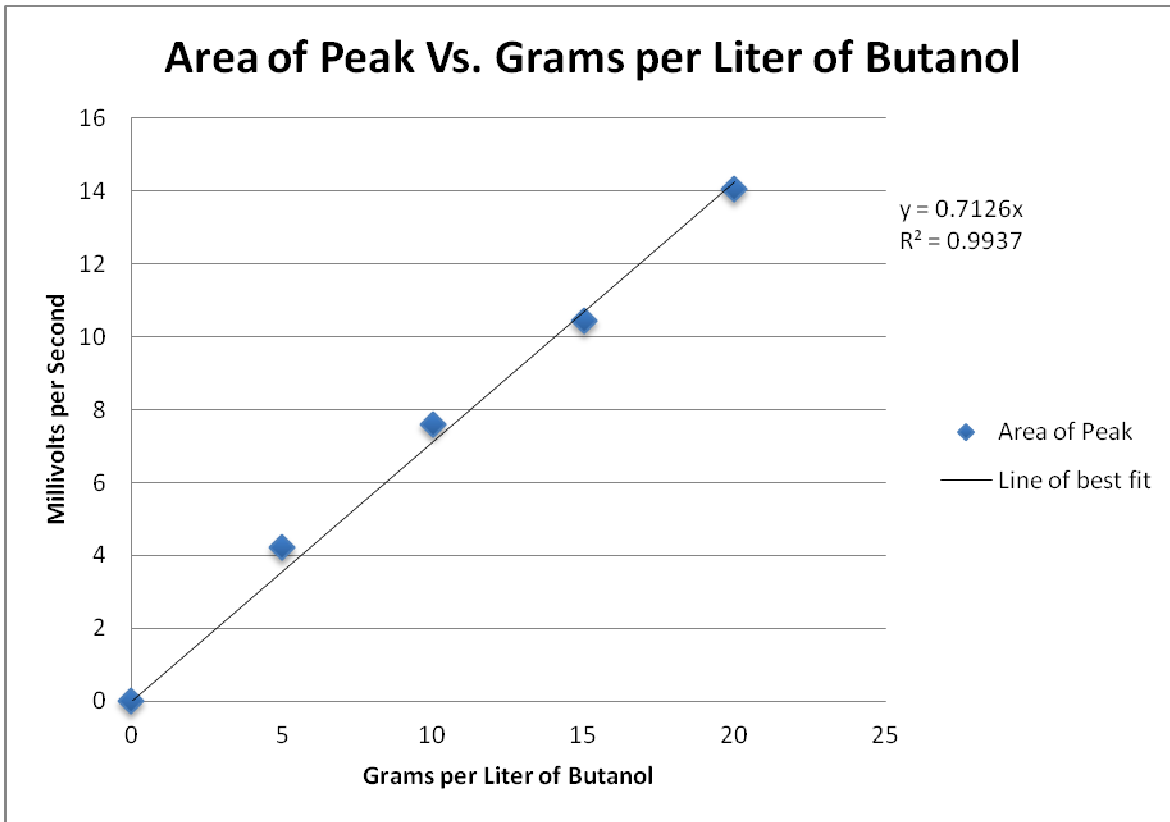


Figure 11. Correlation between area and grams of butanol as calculated using the standards in figure 8

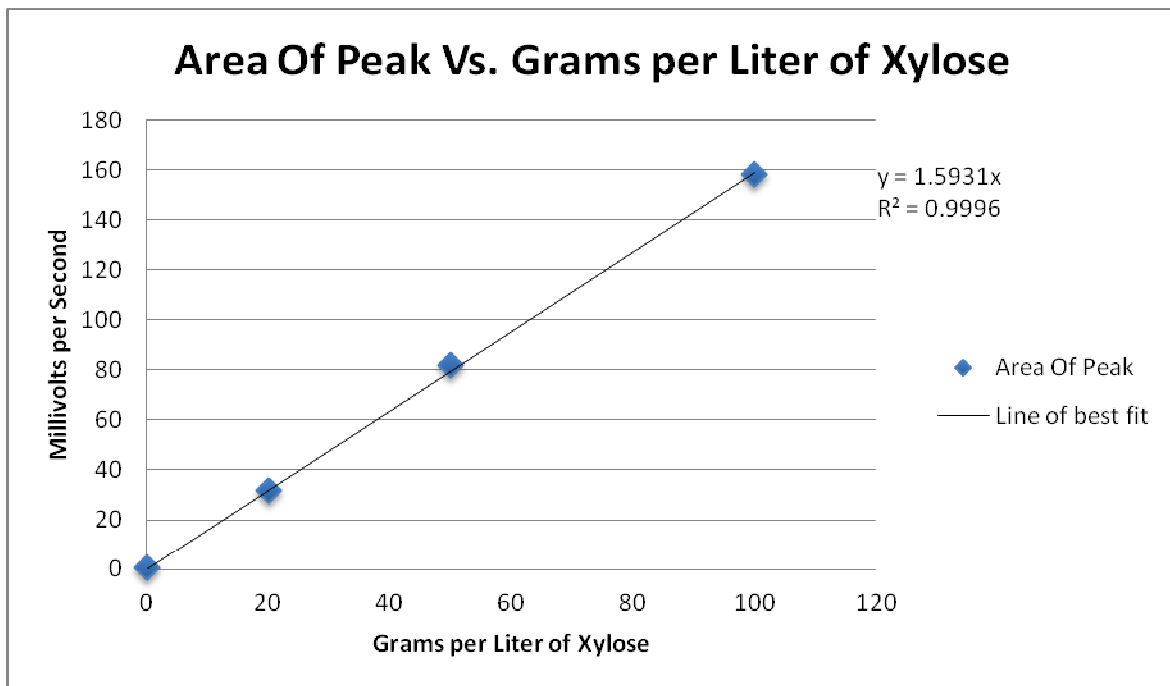


Figure 12. Correlation between area and grams of Xylose as calculated using the standards in figure 4

Figures 10 and 11 demonstrates a plot of our know standards of several known concentrations plotted against the area under the curve as derived using Breeze software. We where then able to determine a best fit line as well as R^2 value using Microsoft Excel. Using this equation for our best fit line, we where then able to determine with high accuracy a concentration value for these substances in our experimental supernatants.

5.2 Experimental HPLC

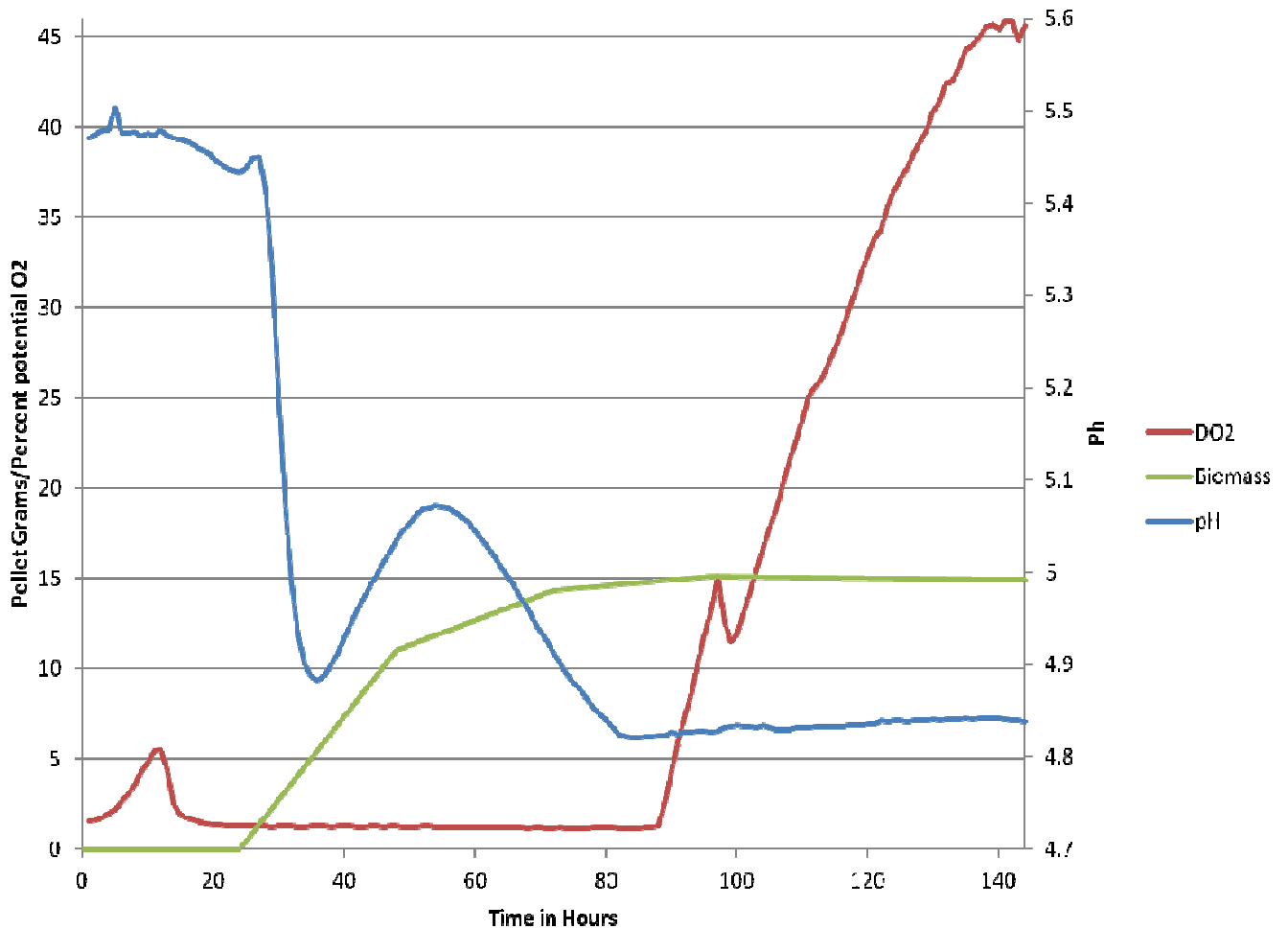


Figure 13. Plot of Data for Biomass, pH and DO2 (dissolved oxygen) xylose media 7 liter Bioflo 110

As can be seen in figure 12 our growth curve based on pellet weights indicating biomass fits with our earlier glucose growth curve. However, solvent production appears to begin earlier than expected as can be seen in the pH beginning to rise slightly before 40 hours.

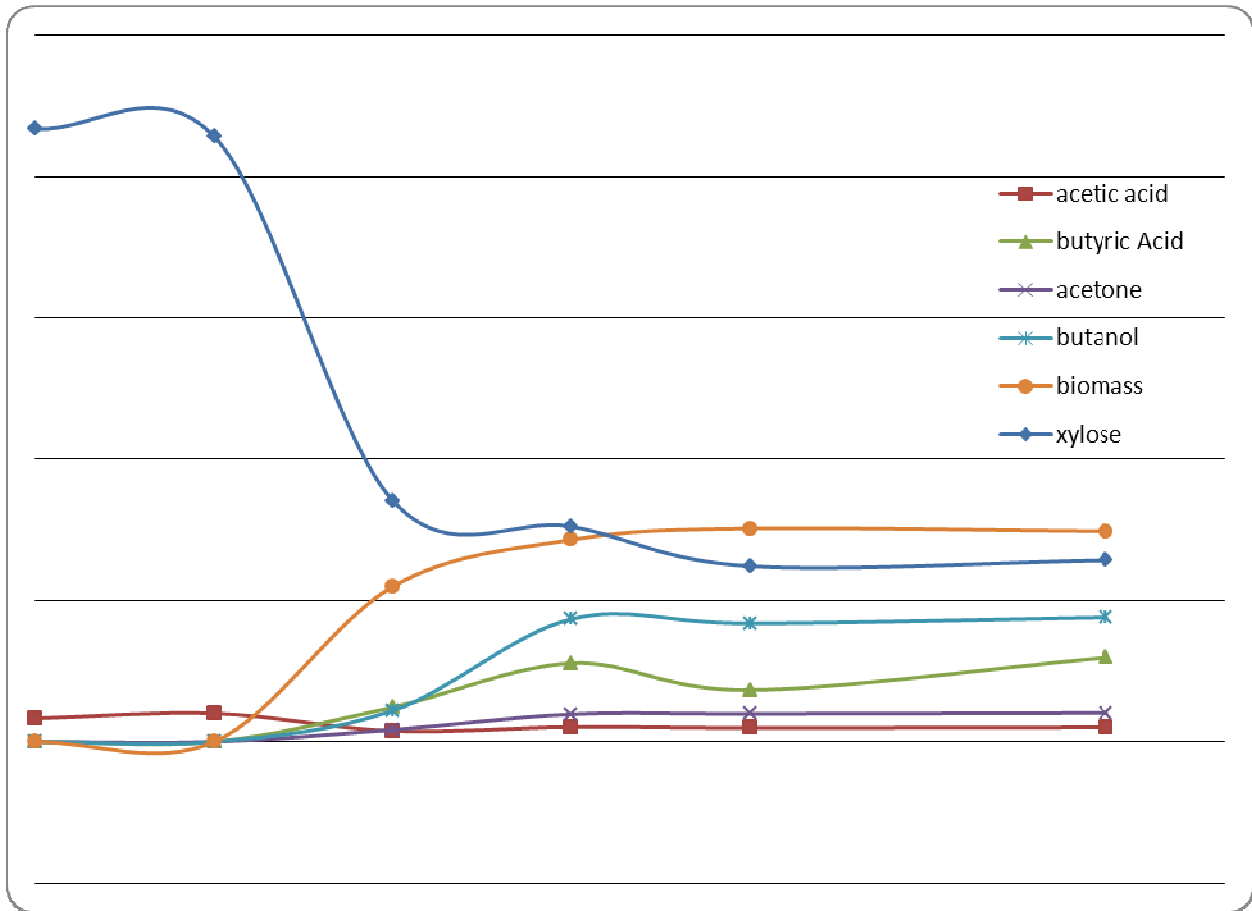


Figure 14. Solvents, acids and sugars in g/L and biomass measured in wet weight of cells pelleted from samples from Bioflo 110 bioreactor with 4 liters of 40 per liter xylose media.

In figure 14 the X axis shows us time from the beginning of the culture. The left Y axis shows gram per liter of the butanol and acetone as well as butyric and acetic acid, this axis also measures the wet weight in grams of the pelleted cells.

The right Y axis shows us the concentration of xylose in grams per liter. This allows us to see that our solvent production did begin earlier than predicted and leveled off about the same time the culture stopped growing. It can also be seen that final butanol concentration in figure 14 of almost 9 grams per liter of butanol achieved in the Bioflo 110 fermentor is significantly higher than the batches grown in 500 ml which produced no higher than 5 grams per liter final volume as seen in figures 17, 20, 23 and 27. Additionally xylose depletion as compared between the Bioflo 110 bioreactor as seen in figure 14 versus the depletion of xylose seen in the comparable 40 gram per liter xylose batches grown in 500 ml flasks seen in figure 16 was significantly greater in the Bioflo 110 bioreactor. It should also be noted the pellet weights of the samples from the 500 ml flasks, figures 18, 21, 24 and 27 where roughly the same weight as the pellets from the Bioflo 110 bioreactor.

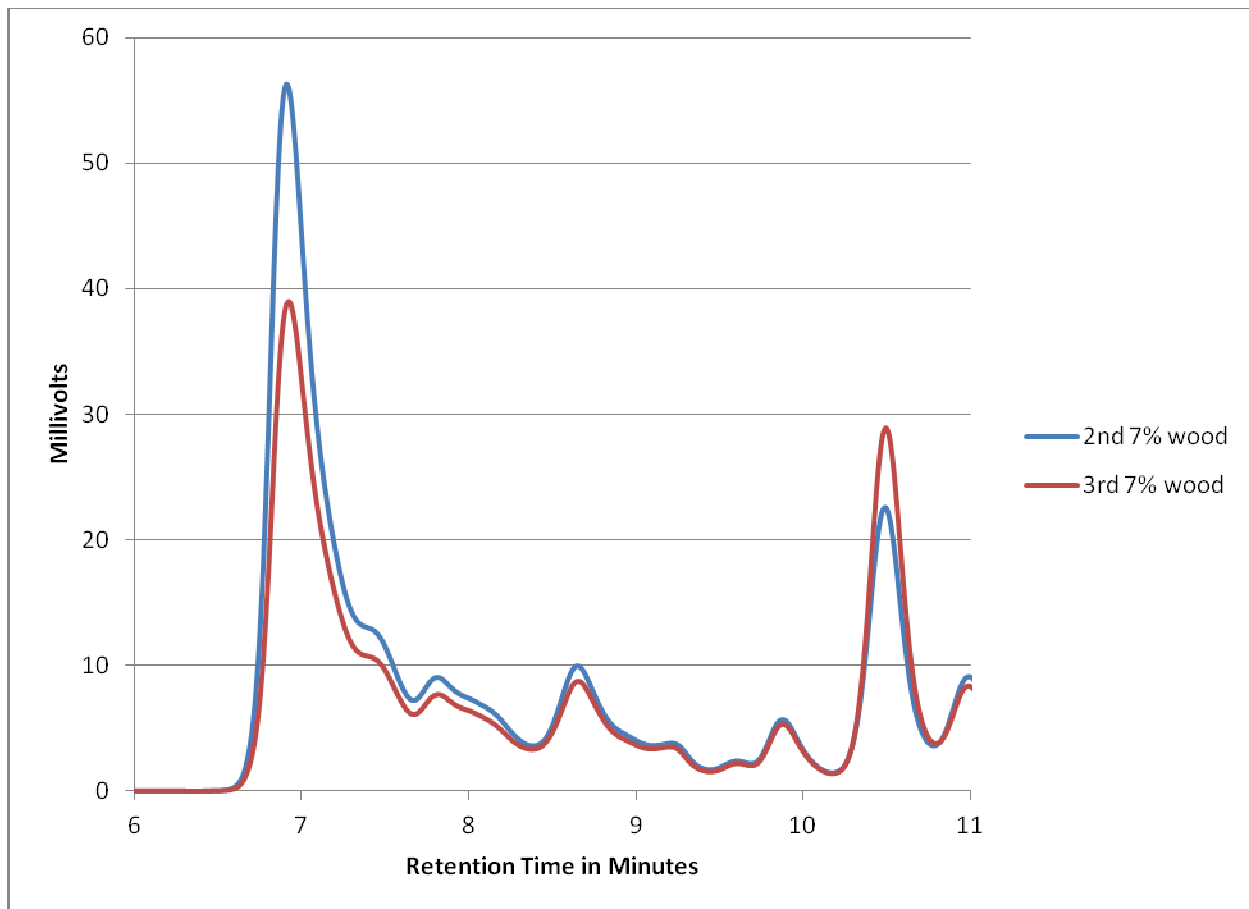


Figure 15. Waters HPLC data from Wood extracts June 4, 2012

Our HPLC analysis of the wood shown in figure 13 indicates that second and third repetitions of our hot water wood extraction yielded higher levels of xylose with successive repetitions. Also in figure 13 we can see the xylose at time 10.5 minutes, but we also see a significant increase of compounds at 7 minutes. Seven minutes is the first point at which we see a peak, this may indicate a conglomeration of all substances present in the extract that do not interact with the column and therefore are not slowed down and separated. However we can see in figure blank that even with substantial xylose present in the wood extract

subsequent experiments using this extract as a media supplement proved fruitless as no detectable growth or solvent production occurred. Cultures were however able to be grown under various concentrations of xylose in our 500 ml culture flasks.

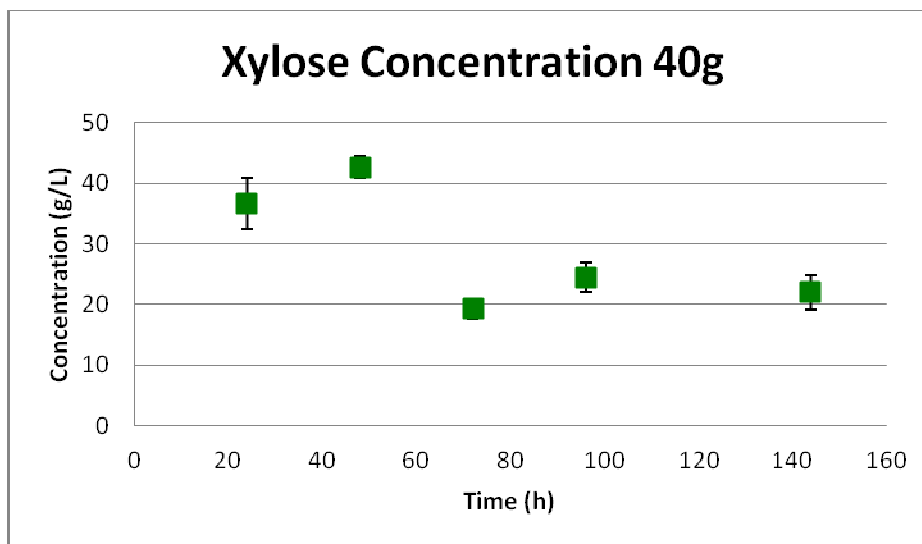


Figure 16. average xylose concentration of 250 ml fermentation batches grown in 40g per liter xylose.

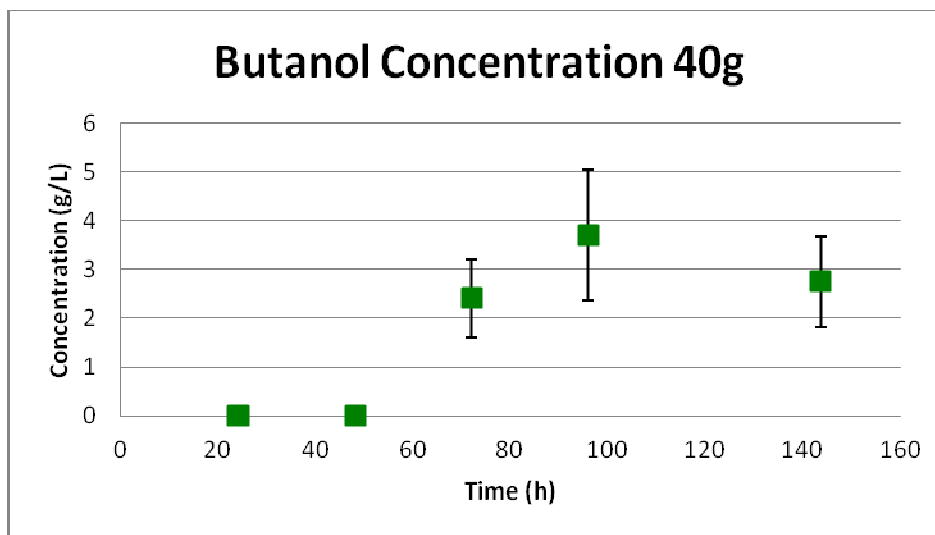


Figure 17. average butanol concentration of 250 ml fermentation batches grown in 40g per liter xylose.

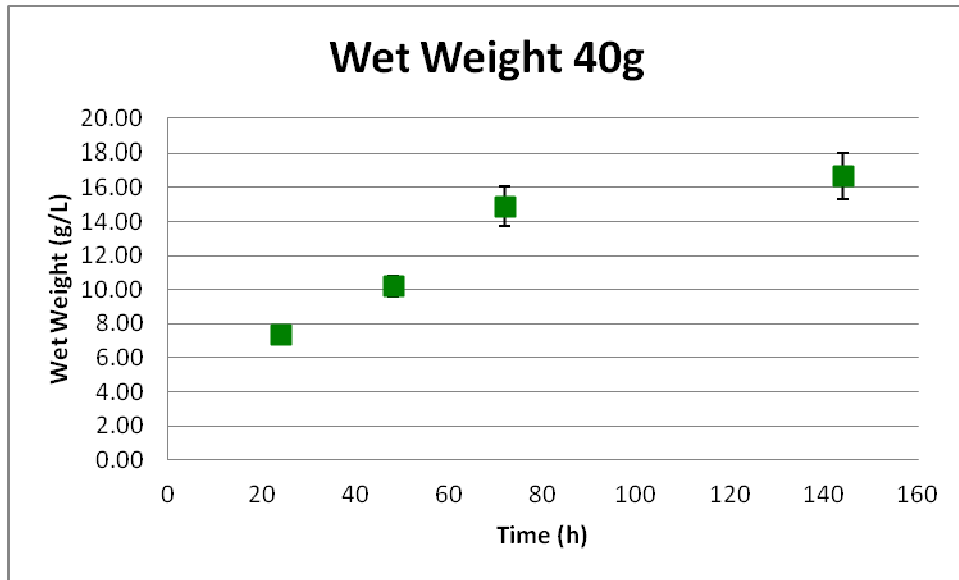


Figure 18. average pellet weight of 250 ml fermentation batches grown in 40g per liter xylose.

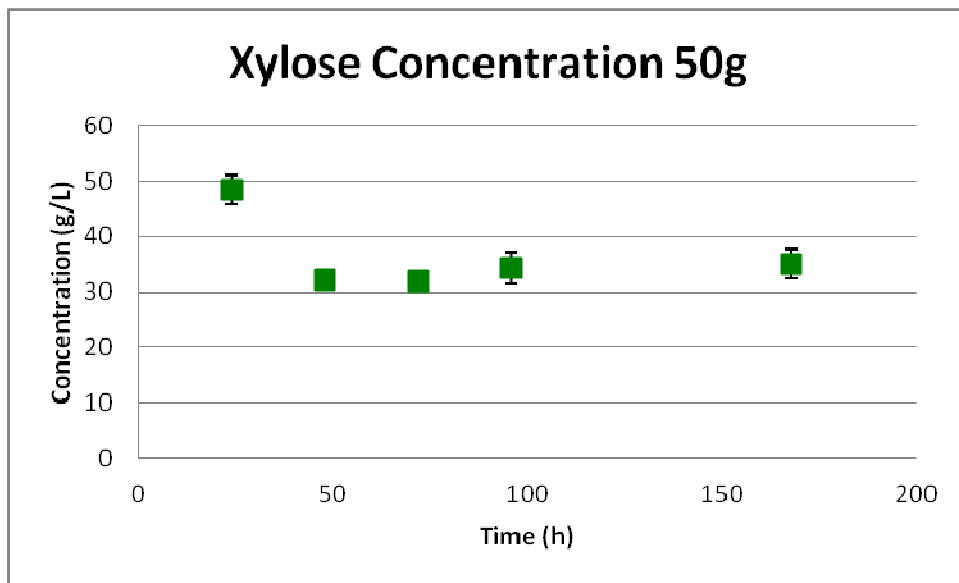


Figure 19. average xylose concentration of 250 ml fermentation batches grown in 50g per liter xylose.

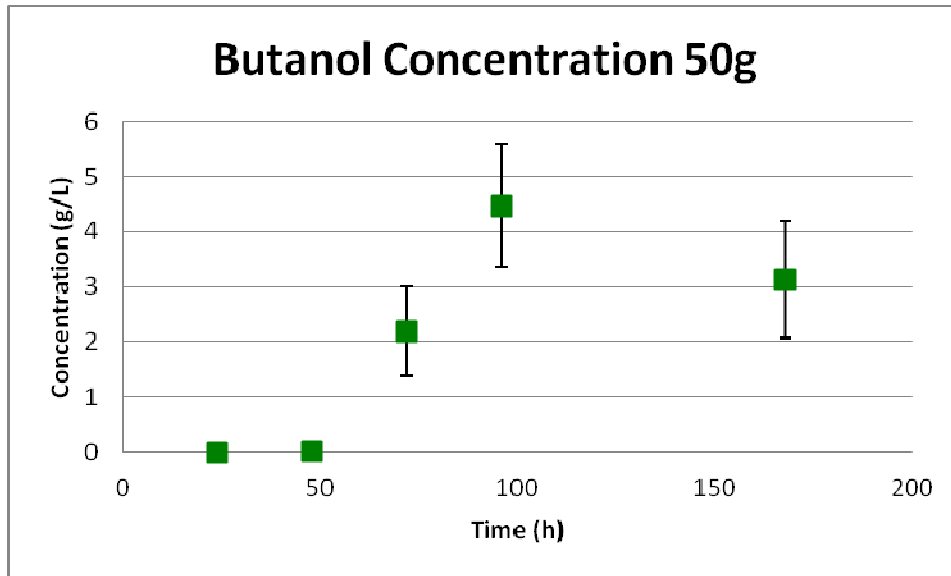


Figure 20. average butanol concentration of 250 ml fermentation batches grown in 50g per liter xylose.

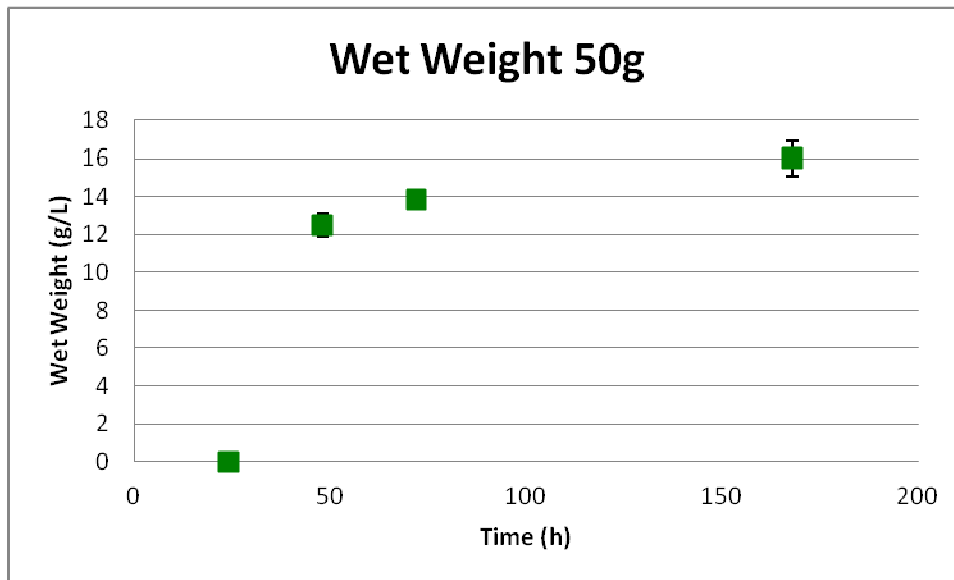


Figure 21. average pellet weight of 250 ml fermentation batches grown in 50g per liter xylose.

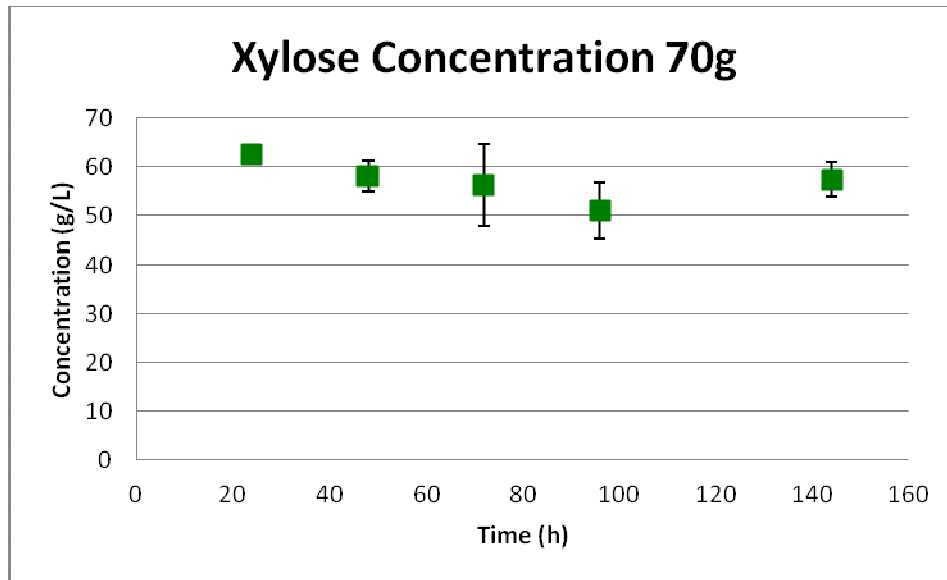


Figure 22. average xylose concentration of 250 ml fermentation batches grown in 70g per liter xylose.

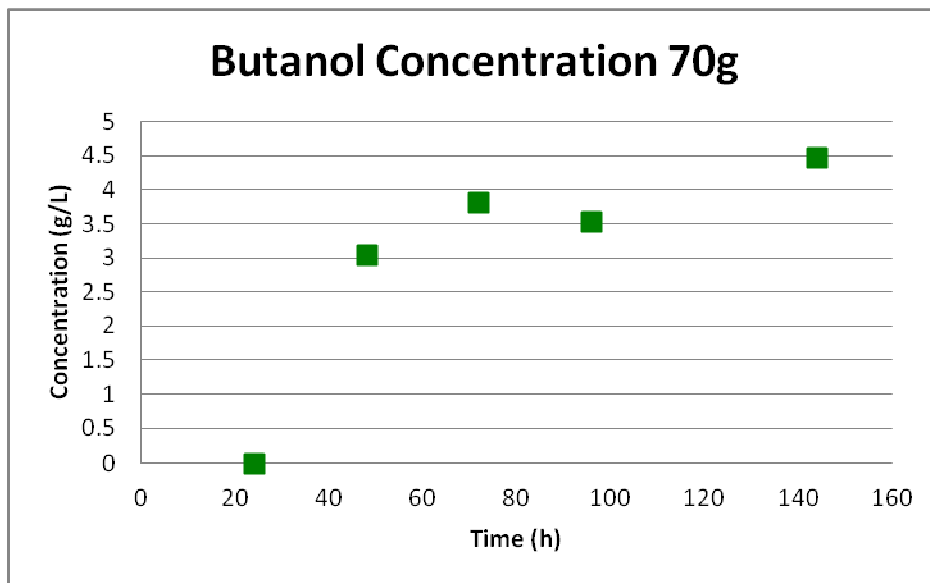


Figure 23. average butanol concentration of 250 ml fermentation batches grown in 70g per liter xylose.

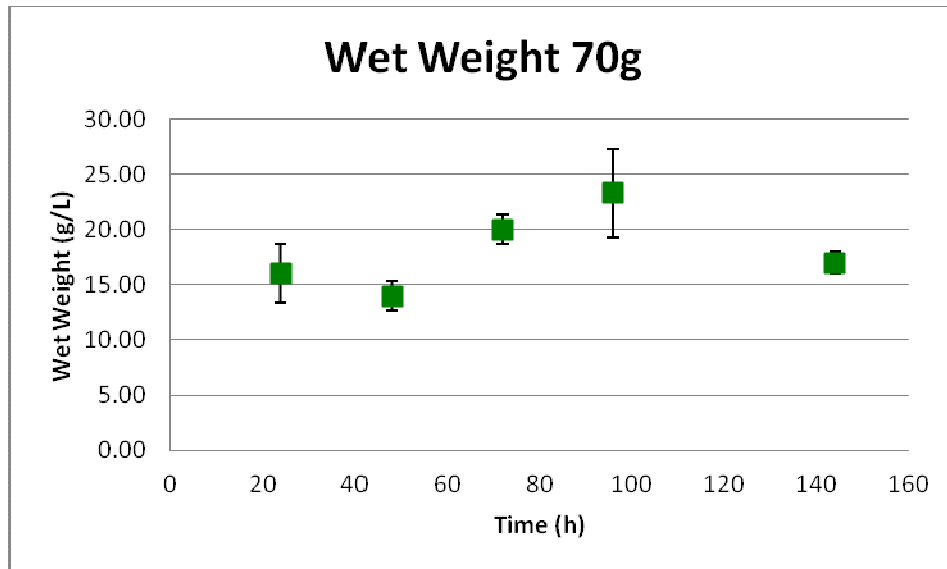


Figure 24. average pellet weight of 250 ml fermentation batches grown in 70g per liter xylose.

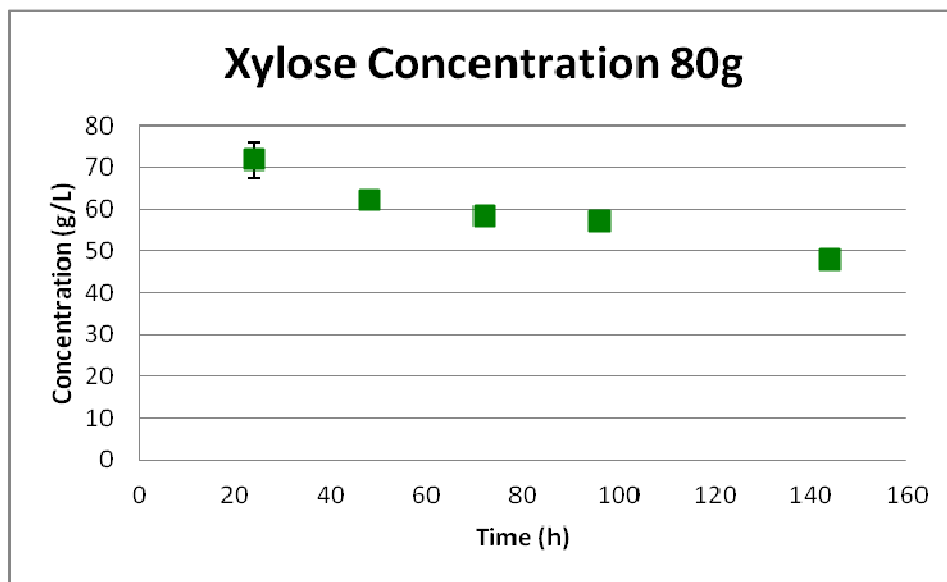


Figure 25. average xylose concentration of 250 ml fermentation batches grown in 80g per liter xylose.

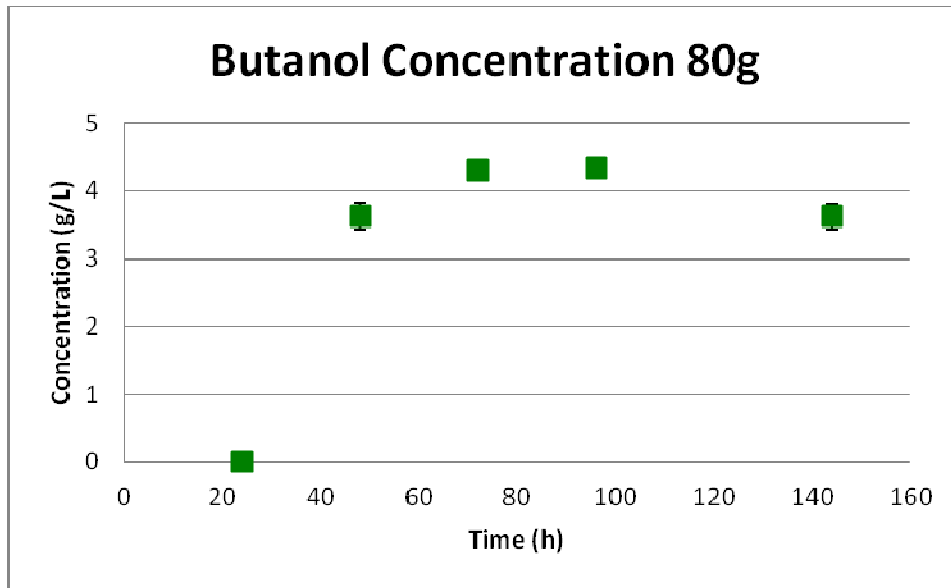


Figure 26. average butanol concentration of 250 ml fermentation batches grown in 80g per liter xylose.

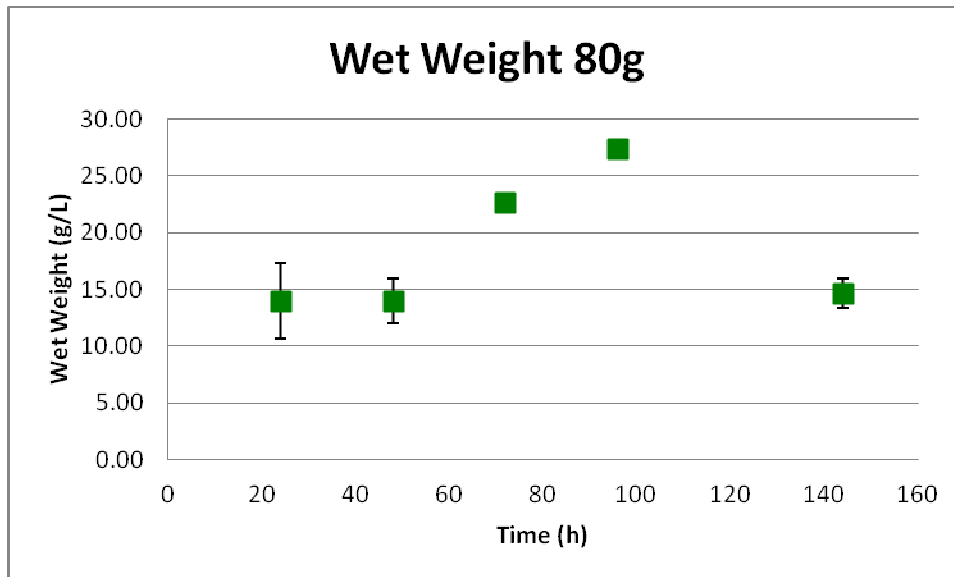


Figure 27. average pellet weight of 250 ml fermentation batches grown in 80g per liter xylose.

Figures 16 through 27 provide the averaged data with error bars from all of our runs using the 500 ml flasks containing four different concentrations of xylose

media. The X axis for figures 16 through 27 show the number of hours from the start of the batch that the sample was taken. In figures 16, 19, 22 and 25 the Y axis shows grams per liter of xylose. Figures 17, 20, 23, 27 have a Y axis representing grams per liter of butanol, and finally figures 18, 21, 24, 27 have a Y axis that represents wet weight in grams of the cells pelleted from the samples.

5.3 Protein Controls

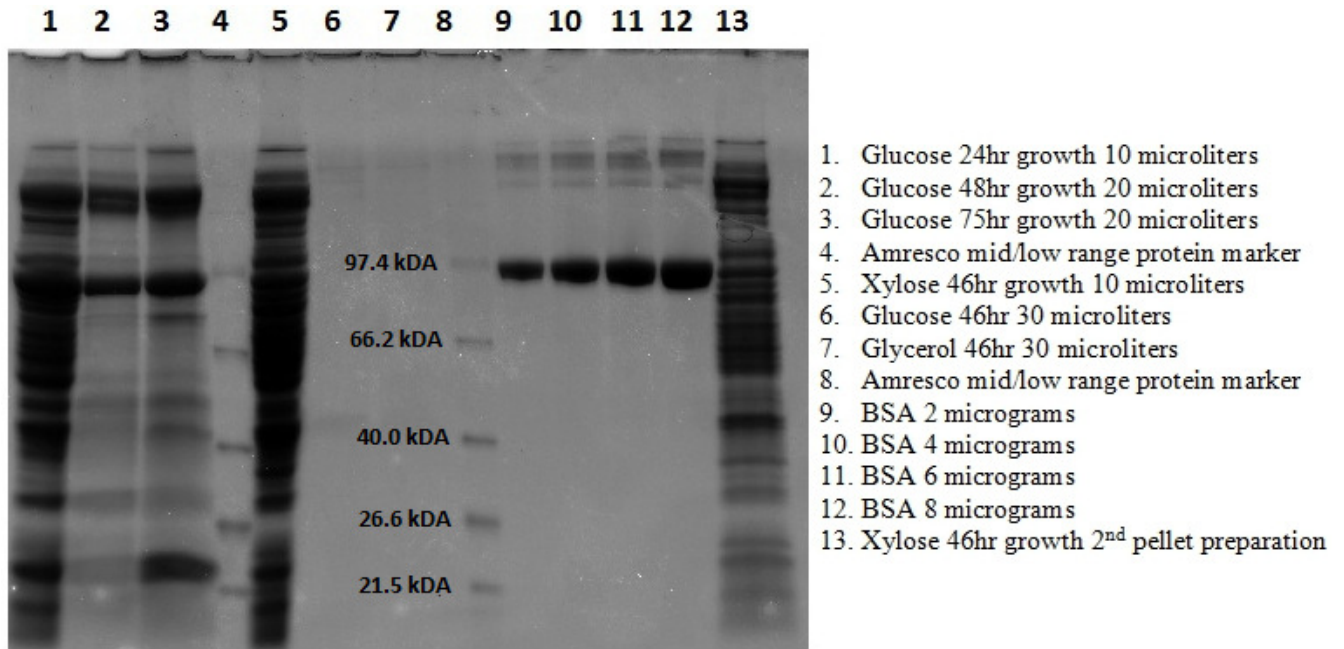


Figure. 28 One dimensional criterion gel containing fermentation proteins and BSA standards and weight marking standards

In figure 28 we can see the obvious banding differences of the organism at various time points as well as the differences between lanes 1,2 and 3 containing proteins from fermentation batches grown in glucose as compared to lanes 5 and 13 grown in xylose.

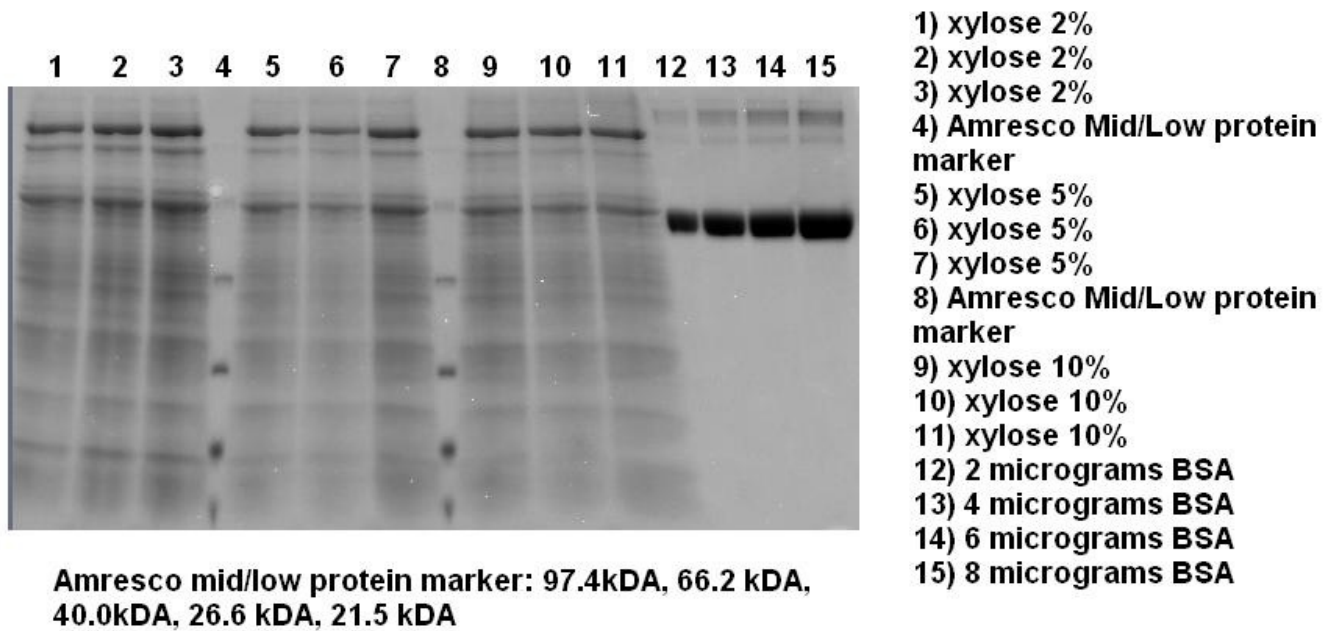


Figure 29. One dimensional gel of proteins from *Clostridium Beijerinckii* grown for 48 hours in three different concentrations of xylose

Figure 29 allows us to see that protein expression appears to be consistent at this time point regardless of xylose concentration.

5.4 Experimental Proteins

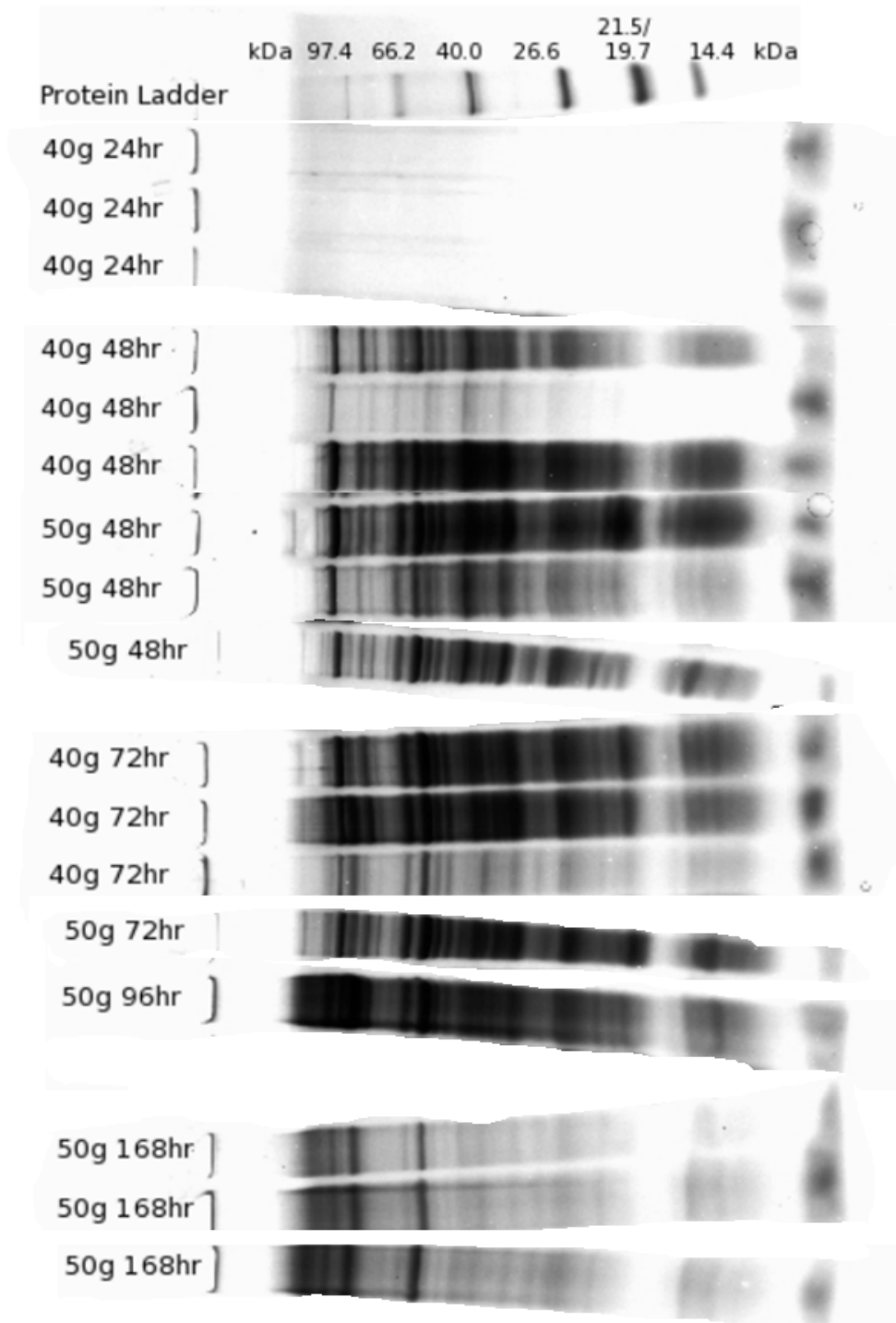


Figure 30. One dimensional criterion gel of *Clostridium beijerinckii* grown on 40 and 50 grams per liter of xylose at various time points

In figure 30 we can see that growth after 24 hours was insufficient to produce enough cells for protein analysis. Additionally in figure 30 we can see at the 96 hour mark and up an increased level of expression of proteins found to be larger than 97 kDA.

6. Discussion

The initial hypothesis that proteins would be differential expressed at different time points during the fermentation was consistent with our result, however our hypothesis that these proteins would differ from fermentations conducted using wood hydrolysate as a carbon source was unable to be tested due to the toxicity of the hydrolysate itself.

Additionally it was noted the larger Bioflo reactor appeared to provide a more favorable environment for the fermentation process. There are many possibilities for why this could be. I believe that the biggest reason was the periodic removal from the incubator and subsequent introduction of some oxygen to the environment every time that a sample was taken. It appears that the sampling points may have killed significant quantities of cells which continued to show up as biomass in the pellet weights but failed to produce the normal quantities of solvents before dying.

If this research were to be taken further it would be prudent to attempt fermentation of purified xylan. This would allow one to determine this organisms expression level of 1,4-beta-xylosidase without the interference of inhibitory

compounds. While there are other ways to break down these crystalline sugar chains most focus today is going into enzymatic methods of hydrolysis, since this gives us the most suitable product for subsequent fermentation of biofuel (Galbe et al., 2006).

This however has proven difficult due to the high costs generally associated with enzyme production (Merino & Cherry, n.d.). So clearly avoiding both industrial processing of the carbon source as well as production of enzymes from an extracellular source could prove very beneficial in the quest for cost efficient biofuel. There would also be a potential benefit in attempting utilization of different sources of lignocellulose. For example in fermentation of ethanol, pine wood has been found to provide a relatively easy to ferment source after it has been saturated with SO₂ and steamed (Galbe et al., 2006). Perhaps this would also be a viable option with butanol. Additionally one strain in particular has been developed called *Clostridium beijerinckii* BA101 has been developed, which appears to be stimulated by both furfural and hydroxymethylfurfural under experimental conditions, and additionally it has been shown to produce notably higher levels of solvents (T. Ezeji & Blaschek, 2008). One drawback however is the fact that this organism is still in the early stages of characterization and little is known about its chemical pathways. An improved understanding might allow us to determine the best carbon sources for this organism and its future potential in solvent production from lignocellulose. Additionally a proprietary strain of *Clostridium cellulolyticum* has been modified to

produce butanol directly from cellulose sources. This however is unavailable due to current litigation concerning patent infringement between Gevo and Butamax (Fairley, 2011).

Another known issue in many strains is the preference of glucose over xylose and other pentose sugars in the metabolic pathways solvent producing clostridia (T. Ezeji & Blaschek, 2008). More recent research found at least one rate limiting step to the pentose utilization pathway to be under production of transaldolase coded for by the *talA* gene in *Escherichia coli*. This gene was successfully expressed via plasmid in *Clostridium acetobutylicum* (Gu et al., 2009). This is of significance since xylose is much easier to hydrolyze than cellulose.

While xylose is easier to hydrolyze by industrial methods, it is known that *Clostridium thermocellum* is capable of fermenting cellulose directly. This process produces ethanol, hydrogen, acetic acid and butyric acid (Weimer & Zeikus, 1977). While ethanol and hydrogen are directly useful, the butyric and acetic acid are known precursors to butanol and acetone that are normally produced during the acidogenic phase of ABE fermentation. Perhaps a concurrent culturing of these two organisms would allow for immediate fermentation of cellulose to butanol.

Three alternative methods of measuring intermediates that were explored were the use of mass spectrometry, gas chromatography and NMR (nuclear magnetic resonance). It was decided not to use mass spectrometry due to the possibility that salt ions from our growth media would build up in the ion trap and thereby damage the machine. Gas chromatography was discarded as the

temperature required to gasify our components of interest was exceptionally high. Finally NMR was found to contain high levels of noise in the data due to the large number of molecules in the sample, as well as its inability to separate out the individual components of the sample.

Protein analysis might prove fruitful if 2 dimensional gels would be run in order to more precisely isolate differentially expressed proteins. These might then be analyzed for possible functions. I believe it plausible that the large proteins expressed after 96 hours could very well be related to the production of endospores formed around the cells once the environment had become too inhospitable for continued cell growth. Additionally some of the proteins present in these late samples could be associated with proton pumps or various other stress proteins associated with cell survival under acidic conditions or solvent tolerance.

In conclusion, our research indicates that there is potential in the production of butanol by the xylose fermentation pathway of *Clostridium beijerinckii* 35702. However we also found that xylose derived from hot water hydrolysis of hemi-cellulose from maple wood comes with a large enough concentration of inhibitory compounds that it doesn't appear to be directly usable as a carbon source for butanol production using this particular organism. There are however many alternative avenues and organisms that show great potential as butanol is becoming more widely accepted as a potential renewable fuel source. We also found that this organism does seem to greatly prefer the more highly controlled environment of the Bioflo 110 bioreactor.

8. References

- Almeida, J. R., Modig, T., Petersson, A., Hähn Hägerdal, B., Lidén, G., & Gorwa Grauslund, M. F. (2007). Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *Journal of Chemical Technology and Biotechnology*, 82(4), 340–349.
doi:10.1002/jctb.1676
- Boddiger, D. (2007). Boosting biofuel crops could threaten food security. *The Lancet*, 370(9591), 923–924. doi:10.1016/S0140-6736(07)61427-5
- DOE, U. S. (2011, October 19). EIA - Total Energy - Energy Perspectives. Retrieved February 9, 2012, from
<http://www.eia.gov/totalenergy/data/annual/perspectives.cfm>
- Dürre, P. (1998). New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. *Applied Microbiology and Biotechnology*, 49(6), 639–648. doi:10.1007/s002530051226
- Dürre, P. (2008). Fermentative Butanol Production. *Annals of the New York Academy of Sciences*, 1125(1), 353–362. doi:10.1196/annals.1419.009
- Ezeji, T., & Blaschek, H. P. (2008). Fermentation of dried distillers grains and soluble (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia. *Bioresource Technology*, 99(12), 5232–5242.

- Ezeji, T., Qureshi, N., & Blaschek, H. P. (2007). Butanol production from agricultural residues: Impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. *Biotechnology and Bioengineering*, 97(6), 1460–1469. doi:10.1002/bit.21373
- Ezeji, Thaddeus C, Qureshi, N., & Blaschek, H. P. (2004). Butanol fermentation research: upstream and downstream manipulations. *The Chemical Record*, 4(5), 305–314. doi:10.1002/tcr.20023
- Ezeji, Thaddeus Chukwuemeka, Qureshi, N., & Blaschek, H. P. (2007). Bioproduction of butanol from biomass: from genes to bioreactors. *Current Opinion in Biotechnology*, 18(3), 220–227. doi:10.1016/j.copbio.2007.04.002
- Fairley, P. (2011, March 22). Bug Creates Butanol Directly from Cellulose. *Technology Review*. Retrieved August 3, 2012, from <http://www.technologyreview.com/news/423373/bug-creates-butanol-directly-from-cellulose/>
- FDA, F. and D. A. (1996, April 1). 21 CFR 172.515 - Synthetic flavoring substances and adjuvants. Retrieved April 4, 2012, from <http://www.gpo.gov/fdsys/search/pagedetails.action?collectionCode=CFR&searchPath=Title+21&granuleId=CFR-1996-title21-vol3-sec172-515&packageId=CFR-1996-title21-vol3&oldPath=Title+21&fromPageDetails=true&collapse=true&bread=true>

- Galbe, M., Gorwa-Grauslund, M. F., Zacchi, G., & others. (2006). Bio-ethanol-the fuel of tomorrow from the residues of today. *TRENDS in Biotechnology*, 24(12), 549–556.
- Gu, Y., Li, J., Zhang, L., Chen, J., Niu, L., Yang, Y., Yang, S., et al. (2009). Improvement of xylose utilization in *Clostridium acetobutylicum* via expression of the talA gene encoding transaldolase from *Escherichia coli*. *Journal of biotechnology*, 143(4), 284–287.
- Haus, S., Jabbari, S., Millat, T., Janssen, H., Fischer, R.-J., Bahl, H., King, J., et al. (2011). A systems biology approach to investigate the effect of pH-induced gene regulation on solvent production by *Clostridium acetobutylicum* in continuous culture. *BMC Systems Biology*, 5(1), 10. doi:10.1186/1752-0509-5-10
- Hertel, T. W., Tyner, W. E., & Birur, D. K. (2010). The Global Impacts of Biofuel Mandates. *Energy Journal*, 31(1), 75–100.
- Hu, R., Lin, L., Liu, T., & Liu, S. (2010). Dilute sulfuric acid hydrolysis of sugar maple wood extract at atmospheric pressure. *Bioresource technology*, 101(10), 3586–3594.
- Koukiekolo, R., Cho, H.-Y., Kosugi, A., Inui, M., Yukawa, H., & Doi, R. H. (2005). Degradation of Corn Fiber by *Clostridium cellulovorans* Cellulases and Hemicellulases and Contribution of Scaffolding Protein CbpA. *Applied and Environmental Microbiology*, 71(7), 3504–3511. doi:10.1128/AEM.71.7.3504-3511.2005

- Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S. (2008). Fermentative butanol production by clostridia. *Biotechnology and Bioengineering*, *101*(2), 209–228. doi:10.1002/bit.22003
- Mao, S., Luo, Y., Zhang, T., Li, J., Bao, G., Zhu, Y., Chen, Z., et al. (2010). Proteome reference map and comparative proteomic analysis between a wild type *Clostridium acetobutylicum* DSM 1731 and its mutant with enhanced butanol tolerance and butanol yield. *Journal of proteome research*, *9*(6), 3046–3061.
- Merino, S. T., & Cherry, J. (n.d.). Progress and Challenges in Enzyme Development for Biomass Utilization. In L. Olsson (Ed.), *Biofuels* (Vol. 108, pp. 95–120). Berlin, Heidelberg: Springer Berlin Heidelberg. Retrieved from <http://www.springerlink.com/content/dk87831678t562v7/>
- Pfromm, P. H., Amanor-Boadu, V., Nelson, R., Vadlani, P., & Madl, R. (2010). Bio-butanol vs. bio-ethanol: A technical and economic assessment for corn and switchgrass fermented by yeast or *Clostridium acetobutylicum*. *Biomass and Bioenergy*, *34*(4), 515–524. doi:10.1016/j.biombioe.2009.12.017
- Qureshi, N., Ezeji, T. C., Ebener, J., Dien, B. S., Cotta, M. A., & Blaschek, H. P. (2008). Butanol production by *Clostridium beijerinckii*. Part I: Use of acid and enzyme hydrolyzed corn fiber. *Bioresource Technology*, *99*(13), 5915–5922. doi:10.1016/j.biortech.2007.09.087
- Ramey, D. (2007). Butanol: The Other Alternative Fuel. *NATIONAL AGRICULTURAL BIOTECHNOLOGY COUNCIL REPORT*, 37.

- Ramey, D., & Yang, S. T. (2004). Production of butyric acid and butanol from biomass. *final report to the US Department of Energy, Contract No.: DE-F-G02-00ER86106.*
- Sun, Z., & Liu, S. (n.d.). Production of n-butanol from concentrated sugar maple hemicellulosic hydrolysate by *Clostridia acetobutylicum* ATCC824. *Biomass and Bioenergy*. doi:10.1016/j.biombioe.2010.07.026
- Tsuey, L., Ariff, A., Mohamad, R., & Rahim, R. (2006). Improvements of GC and HPLC analyses in solvent (acetone-butanol-ethanol) fermentation by *Clostridium saccharobutylicum* using a mixture of starch and glycerol as carbon source. *Biotechnology and Bioprocess Engineering*, 11, 293–298.
- Weimer, P. J., & Zeikus, J. G. (1977). Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence of *Methanobacterium thermoautotrophicum*. *Applied and Environmental Microbiology*, 33(2), 289–297.
- Wilson, R. (2011, March 16). “Why make a \$2 fuel when you can make a \$5 chemical” – Cobalt CEO Rick Wilson. *Biofuels Digest*. Retrieved March 27, 2012, from <http://www.biofuelsdigest.com/bdigest/2011/03/16/why-make-a-2-fuel-when-you-can-make-a-5-chemical-cobalt-ceo-rick-wilson/>