Sustainable Biofuels Production Through Understanding Fundamental Bacterial Pathways Involved in Biomass Degradation and Sugar Utilization

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Sustainable biofuel production through understanding fundamental bacterial pathways involved in biomass degradation and sugar utilization

A Thesis Presented

by

JAMES CM HAYES

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
September 2013

Molecular and Cellular Biology Graduate Program
Sustainable biofuel production through understanding fundamental bacterial pathways involved in biomass degradation and sugar utilization

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JAMES CM HAYES

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ABSTRACT

Sustainable biofuels production through understanding fundamental bacterial pathways involved in biomass degradation and sugar utilization.

September 2013
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Directed by: Professor Jeffrey L. Blanchard

Genomic analysis and physiological experiments conducted on the lignocellulosic biomass degrading bacterium *C. phytofermentans*, indicates that it can degrade and utilize a wide-range of carbohydrates as possible growth substrates. Previous experiments characterized the expression of the degradation and transport machinery using custom whole genome oligonucleotide microarrays. The results indicated that *C. phytofermentans* utilizes ATP-binding cassette (ABC) transporters for carbohydrate uptake and does not use the sole phosphoenolpyruvate-phosphotransferase system (PTS) for any of the tested substrates. While some ABC transporters are specific for a single carbohydrate, the expression profiles indicate that others may be capable of transporting multiple substrates. Distinct sets of Carbohydrate Active Enzymes (CAZy) genes were also up-regulated on specific substrates indicative of *C. phytofermentans* ability to selectively degrade plant biomass. We also identified a highly expressed cluster of genes which includes seven extracellular glycoside hydrolases and two ABC transporters with unknown specificity. These results lead to the hypothesis that when grown on plant biomass, *C. phytofermentans* is capable of degrading and transporting all major carbohydrate components of the plant cell. To test this, *C. phytofermentans* was grown on three different plant biomass substrates (*Brachypodium distachyon*, Cornstover, and Switchgrass). Results from this expression data indicated that *C. phytofermentans* may be utilizing multiple substrates. This can be seen through the expression of multiple sugar ABC transporter clusters, glycoside hydrolases, and
sugar utilization pathways being expressed. To further test the sugar utilization pattern, growth studies of *C. phytofermentans* were performed on individual saccharides (glucose, cellobiose, xylose, and fucose) as well as a combination of all these sugars. From these studies we determined that *C. phytofermentans* does not show a characteristic diauxic shift indicative of preferential sugar utilization. This result was supported further by HPLC analysis indicating that co-utilization of sugars was occurring, however rates of consumption were different for some of the sugars. Expression analysis of dual sugar combinations of glucose/cellobiose, glucose/xylose, and glucose/fucose also shows that genes involved in the transport and utilization of each sugar are expressed. The results from this study indicate that *C. phytofermentans* does not undergo carbon catabolite repression when grown on a mixture of sugars and can utilize multiple sugars simultaneously.
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CHAPTER I

INTRODUCTION

Lignocellulosic biomass has been described as being 'recalcitrant' due to the resistance of this substrate to degradation without the use of costly pretreatment steps (Himmel, 2008). This property makes it very difficult for microbial species to utilize the two main sugar components of lignocellulosic biomass, cellulose and hemicellulose. Cellulose is composed of long chains of glucose molecules with a β(1-4)-linkage. These long chains of glucose can then form more highly ordered structures called microfibrils (Kroon-Batenburg and Kroon, 1997). These structures are formed with other chains through hydrogen bonding and Van de Waal interactions which contributes to its recalcitrant nature (Kroon-Batenburg and Kroon, 1997). The hemicellulose component is more heterologous in nature. It is composed of a group of polysaccharides comprised of several 5- and 6-carbon sugars which include xylose, glucose, galactose, mannose, fucose, rhamnose and arabinose. These chains are typically shorter than cellulose chains and are most often branched (Warren, 1996). Hemicellulose chains then bind to cellulose microfibrils to help prevent aggregation of the cellulose and to provide flexibility to the cell wall (Gomez, Steele-King, and McQueen-Mason, 2008). These cellulose and hemicellulose structures are then combined with the polyphenolic compound lignin to form the main structure of lignocellulosic biomass. Microfibrils are then combined to produce macrofibrils and it is these macrofibrils that form the plant cell wall (Fig. 1-1).
Figure 1-1 Structure of Lignocellulosic Plant Biomass. (adapted from Tomme et al., 1995)
Lignocellulosic biomass has been proposed as a sustainable feedstock for biofuel and other chemical production due to its low cost, abundance, and the fact that it not used for human consumption (Perlack RD, et al., 2005). Current commercial production of biofuels from lignocellulosic biomass requires four main steps (Lynd, 1996). These four steps are: saccharolytic enzymes production, chemical and enzymatic hydrolysis of biomass, hexose fermentation, and pentose fermentation. However, problems exist due to inefficient fermentation of pentose sugars present in hemicellulose (i.e. xylose) and the high cost of saccharolytic enzymes production (Banerjee, et al., 2010; Jin, et al., 2010). It has been proposed that consolidated bioprocessing (CBP), in which the lignocellulosic biomass is directly converted to ethanol in a single reactor could be used to mitigate these issues since all four events occur simultaneously (Lynd, 1996). Previous attempts to combine these steps using genetically engineered yeast and E. coli strains have given some promising results, however problems still persist in the efficiency of this process. Two of the most pressing issues still to be addressed are related to the enzymatic breakdown of the biomass and the utilization of the various pentose and hexose sugars.

One possible candidate microbe for CBP is the bacterium Clostridium phytofermentans (C. phytofermentans). C. phytofermentans is a gram-positive, mesophilic, obligate anaerobe, isolated from soil samples from the Quabbin Reservoir in central Massachusetts (Warnick, et. al. 2002). C. phytofermentans is unique in that it can degrade and ferment plant biomass, such as AFEX-treated corn stover (Jin, Balan, Gunawan, & Dale, 2011), switchgrass, and the model grass Brachypodium distachyon (Lee, et. al., 2012) to ethanol as the primary end product. This bacterium has also been shown to be genetically tractable (Tolonen, et. al. 2009) which makes genetic engineering a possibility. Previous experiments on purified substrates were conducted to characterize the degradation and transport machinery using custom whole genome cDNA microarrays (Petit, et. al., 2013). C.
phytofermentans was grown on individual substrates found in lignocellulosic plant biomass and gene expression analysis was conducted. The substrate included mono-saccharides (Glucose, L-Arabinose, Mannose, Xylose, Fucose, Galactose, and Rhamnose), a disaccharide (Cellobiose), and oligo-saccharides (Cellulose, Xylan, Pectin, and Laminarin). Distinct sets of Carbohydrate Active Enzymes (CAZy) genes were up-regulated on specific substrates indicative of C. phytofermentans ability to selectively degrade lignocellulosic biomass. These results lead to the hypothesis that C. phytofermentans utilizes its large repertoire of degradation enzymes in order to breakdown and utilize lignocellulosic biomass.

The CAZy database (http://www.cazy.org/) was created in order to help classify the enzymes involved in the degradation, modification, or formation of glycosidic bonds (Cantarel, et. al. 2009). In terms of degradation there are four main classes Glycoside Hydrolases (GH), Glycosyltransferases (GT), Polysaccharide Lyases (PL), and Carbohydrate Esterases (CE). These classes indicate the type of chemical reaction which is used to break or modify the bond. For example the Glycoside Hydrolases cleave glycosidics bonds through hydrolysis. In C. phytofermentans, a total of 116 GHs have been predicted to be present in the genome (Petit, et. al., manuscript accepted). This large repertoire of hydrolases helps to explain the ability of this bacterium to breakdown and utilize a complex substrate such as lignocellulosic biomass.

Along with this large collection of hydrolases for the degradation of lignocellulosic biomass, C. phytofermentans also contains a large number of ATP-binding cassette (ABC) transporters (Petit, et. al., manuscript accepted). These transporters are believed to be used in the uptake of the various different sugar components produced during the degradation of lignocellulosic biomass. Previous results also indicate that C. phytofermentans utilizes these ABC transporters for carbohydrate uptake and does not use the sole phosphoenolpyruvate-phosphotransferase system (PTS) for any of the tested substrates. In other microbial organism, sugar uptakes systems have been well studies and characterized. The
genomes for several anaerobic, mesophillic Clostridial species indicates a preference for PTS for the uptake of sugars (Mitchell & Tangney, 2005). This contrasts with anaerobic thermophillic bacteria such as Thermotoga maritima, Carboxydothermus hydrogenoformans, and Thermoanaerobacter tengcongensis, whose genomes indicate a preference for ABC transporters (Vanfossen, Verhaart, Kengen, & Kelly, 2009). Upon examination of C. phytofermentans' genome, 49 putative sugar ATP-binding cassette (ABC) transporters and a single PTS sugar transporter were identified making C. phytofermentans unique from other sequenced mesophillic Clostridial species. This is of interest because the sequential utilization of sugars, termed carbon catabolite repression (CCR), in a mixed solution has been linked to PTS transporters (Kovárová-Kovar & Egli, 1998; Sasaki, Jojima, Inui, & Yukawa, 2008). Overcoming CCR to more efficiently transport and utilize the sugars commonly found in lignocellulosic plant biomass is therefore a desirable trait for any CBP bacteria.
CHAPTER II
MATERIALS & METHODS

2.1. Growth of *C. phytofermentans* on Plant Biomass

The anaerobic techniques of Hungate (Hungate, 1969) were used in all media preparation and transfers. Seed cultures of *C. phytofermentans* grown on modified GS-2 media with 0.6% cellobiose were used. Cultures for time points were started by inoculating 75 ml samples of modified GS-2 media and 10% salt solution each with 1 g of pebble-milled plant biomass. Cultures were incubated at 30°C. Cultures were collected at day 5 for mRNA extraction and HPLC.

Modified GS-2 media (Petit, et. al. 2013) was prepared as follows: For 1.0 L: KH₂PO₄, 4 g; Na₂HPO₄, 6.5 g; Urea, 2.10 g; Sodium Citrate, 3.0 g; L-cysteine HCl, 2.0g; 0.1% Resazurin, 1.0ml; 10% switchgrass slurry (see below for preparation), 100 ml, milliQ water, 870 ml. The pH was adjusted to 7.0 using 6N KOH. Switchgrass was a homogenate of mixed, chopped stem and leaf material grown and harvested as hay at the University of Massachusetts, South Deerfield farm facility in 2009. *Brachypodium distachyon* was provided by the Hazen Lab at the University of Massachusetts - Amherst. The Cornstover was provided by the Leschine Lab at the University of Massachusetts - Amherst. The biomass was further dried in a 50°C oven for two days, and ball milled at 20 Hz for 2 minutes. This ball milled biomass was then pebble milled in milliQ water for 5 days to produce a 10% switchgrass slurry. Salt solution was prepared as follows: for 100 ml: MgCl₂×6H₂O, 1.0 g; CaCl₂×2H₂O, .15 g, FeSO₄×7H₂O, 0.00125 g, autoclaved for 20 minutes.

2.2. Biomass Grown Sample Collection

At the desired time point, the 75 ml cultures were sacrificed. Each 75 ml culture was split into two (2) 50 ml Falcon tubes. These tubes were centrifuged at 3000 g for 20 mins. A 1.5 ml sample of the supernatant was taken for metabolic output and frozen at -20°C until needed. The rest of the
supernatant was decanted and the pellet frozen at -80°C.

2.3. Growth of C. phytofermentans on Soluble Sugars

The anaerobic techniques of Hungate (Hungate, 1969) were used in all media preparation and transfers. Seed cultures of C. phytofermentans grown on defined M6 media with 0.3% concentrations of the sugar or combinations of sugars. Cultures were started by inoculating 10 ml samples of defined M6 media and 5% CPV4 vitamin mix solution with 3 g/L of each desired sugar. Cultures were incubated at 30°C. Two cultures were sacrificed at each time point for HPLC and mRNA extraction.

Defined M6 media was prepared as follows: For 1.0 L: NaH₂PO₄, 2.0g; K₂HPO₄,10.0g; (NH₄)₂SO₄, 0.10g; Cysteine HCl, 1.0g; AA1 Solution (see below preparation), 10 ml; XT2 Solution (see below preparation), 40 ml; BTE Solution (see below preparation), 10ml; milliQ water, 870 ml; 0.1% Resazurin, 1.0ml; CPV4 vitamin mix, 20 ml; 10% filter sterilized Glucose, 30 ml; 10% filter sterilized secondary sugar, 30 ml. Note that CPV4 vitamin mix and sugar solutions are added after the media has been autoclaved.

AA1 solution: Alanine, 0.5g; Arginine, 0.5g; Histidine, 0.5g; Isoleucine, 0.5g; Leucine, 0.5g; Methionine, 0.5g; Proline, 0.5g; Valine, 0.5g; milliQ water, 100ml. Autoclave for 20 mins.

Modified Balch’s Trace Elements (BTE): Nitriolotriacetic acid, 1.50g; MgSO₄.7H₂O, 3.00g; MnSO₄.4H₂O, 0.50g; NaCl, 1.00g; FeSO₄.7H₂O, 0.10g; CoCl₂.6H₂O, 0.10g; CaCl₂, 0.10g; ZnSO₄.7H₂O, 0.10g; CuSO₄.5H₂O, 0.01g; AlK(SO₄)₂.12H₂O, 0.01g; H₃BO₃, 0.01g; Na₂MoO₄.2H₂O, 0.01g; NiSO₄.6H₂O, 0.03g; Na₂SeO₃, 0.02g; Na₂WO₄.2H₂O, 0.02g; milliQ water, 1000ml. To dissolve, add Nitriolotriacetic acid to 500ml water, adjust pH to 6.5 with KOH. Add remaining salts one at a time. Bring final volume to 1 L with water. Autoclave for 20 mins.

XT2 Solution: Xanthine, 0.25g; Thymine, 0.25g; milliQ water, 99ml; 6N NaOH, 1ml. Autoclave for 20 mins.
CPV4 Vitamin Mix: p-Aminobenzoic Acid, 4.0mg; Biotin, 0.1mg; Folinic Acid, 0.6mg; Nicotinamide, 8.0mg; Pantethine, 0.5mg; Pyridoxal HCl, 0.4mg; Riboflavin, 3.0mg; Thiamine, 1.0mg; milliQ water, 100ml. Dissolve in 95ml water, adjust pH to 7.0 with 1N NaOH. Heat the solution almost to boiling to dissolve, adjust volume, and filter sterilize.

2.4. OD Measurements and Soluble Sugar Sample Collection

The optical density (OD) of the samples were followed at 600 nm. At desired time points, two (2) 10 ml cultures were sacrificed. Each 10 ml culture was transferred into a 15 ml Falcon tube. These tubes were centrifuged at 3000 g for 20 mins. A 1.5 ml sample of the supernatant was taken for HPLC and frozen at -20°C until needed. The rest of the supernatant was poured off and the pellet frozen at -80°C until needed for mRNA extraction.

2.5. Metabolic Output and Residual Sugars

At the desired time points, concentrations of metabolites (acetate, ethanol, lactate, formate, propionate, butyrate, and propanol) and sugars (glucose, cellobiose, xylose, and fucose) from centrifuged, filtered culture supernatant were measured by HPLC (High Performance Liquid Chromatography) using a BioRad Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 55°C with 0.005 M H₂SO₄ as the running buffer.

2.6. mRNA extraction

Frozen pellets in 50 ml or 15 ml Falcon tubes were thawed on ice. To thawed pellets 5ml of Trizol reagent (Invitrogen) was added and homogenized using a vortex. The samples were spun down at 3000x g for 20 mins. The Trizol reagent was removed and the pellet saved for plant biomass degradation assay. The resulting Trizol reagent was processed following the standard Trizol procedure
and cleaned up using the RNEasy kit (Qiagen). mRNA integrity was checked by gel electrophoresis.

2.7. Microarray Hybridization and Processing

mRNA samples were sent to the Genomic Core Facility at the University of Massachusetts Medical Center for microarray processing. The resulting expression files were normalized using custom scripts in R and analyzed as Excel spreadsheets.
CHAPTER III
PATHWAYS INVOLVED IN PLANT DECOMPOSITION IN C. PHYTOFERMENTANS

Initial experiments were performed to test the growth of *C. phytofermentans* on the model grass *Brachypodium distachyon* (*Brachy*). Using substrate degradation, light microscopy, supernatant protein assays, and HPLC analysis, we determined that *C. phytofermentans* was capable of growing on plant biomass (Fig. 3-1). From this data we decided that samples would be collected after 5 days of growth, since this is during mid-exponential growth. We also expanded our substrates to include two industrially relevant feedstocks, switchgrass and corn stover. Previous attempts to collect sufficient mRNA from insoluble substrates using a lysozyme protocol were hindered by low yields (~1 μg of RNA per tube). To correct this, a new extraction protocol utilizing Trizol reagent was developed which gave much better yields (4-5 μg of RNA per tube).

Using this new protocol, we collected mRNA from *C. phytofermentans* growing on our three (3) lignocellulosic biomass samples; switchgrass, corn stover, and *Brachy*. Samples were collected after 5 days of growth, the mRNA extracted, checked for integrity, and sent for microarray processing. Supernatant samples were also collected for HPLC analysis of metabolic output.

Comparing these results to previous experiments on purified substrates, a number of interesting conclusions can be drawn. First is the expression of the glycoside hydrolases used by *C. phytofermentans* to degrade the plant biomass. We see that the response between the three substrates is similar to that observed on cellulose (Fig. 3-2) with the exception of a xylanase (*C.phytofermentans_2105*) and a mannanase (*Cphy_1071*). This glycoside hydrolase response is also observed on a number of monosachrhides such as arabinose, fucose, rhamnose, and xylose (Data not shown). These results indicate that this hydrolase expression could be a general response to the presence of plant biomass. Previous growth experiments on xylan show that *C. phytofermentans* can
efficiently degrade and utilize this substrate faster than glucose, which could indicate a preference for this substrate (Tolonen, et. al. 2011). From the glycoside hydrolase response on plant biomass, we see that *C. phytofermentans* is not expressing the majority of the enzymes which are expressed during degradation of this substrate (Fig. 3-2). However, a previous experiment using a *C. phytofermentans* strains incapable of degrading cellulose still showed growth on plant biomass (Data not shown). It would be interesting to investigate the glycoside hydrolase response in this strain to see if the same hydrolase profile is expressed as the wild-type or if a response closer to xylan degradation is observed.

A second conclusion is that *C. phytofermentans* may be utilizing multiple sugars simultaneously. This can be seen from the expression of putative transporters for celllobiose/xylose/xylan (Fig. 3-3) and the genes involved in the pentose-phosphate pathway (Fig. 3-4). Also the presence of proprionate in the supernatant of samples grown on corn stover grown cultures (Fig. 3-5) indicates the utilization of fucose and/or rhamnose since this product is only observed during growth on this substrate (Petit. et. al., 2013).
Figure 3-1. Growth of C. phytofermentans on plant biomass. (A) Comparison of residual plant biomass after 5 days of growth on Brachy. The tube on the right was prepared identically to the inoculated tube on the left. (B) Metabolic output of 5 day old tubes used for gene expression analysis for Brachy grown C. phytofermentans cultures. Samples for panel B indicate the averaged metabolic output from samples used for Microarray sample 1 and Microarray sample 2. Error bars indicate the Standard Deviation of the averaged samples.
Figure 3-2. Glycoside hydrolase response curves from gene expression analysis of C. phytofermentans grown cultures on (A) Brachypodium, (B) Corn Stover, (C) Switchgrass compared to Cellulose and (D) Switchgrass compared to Xylan. CS - Cornstover, SG - Switchgrass, Cel - Cellulose.
Figure 3-3. Heatmap showing the relative gene expression of putative sugar ABC transporters during growth on purified and plant biomass substrates. All values are shown in Log2 relative to Glucose with red indicating a higher expression, black similiar expression, and green a lower expression than on glucose. Labels on the right indicate the putative transporter clusters for the sugar shown. Ard - D-arabinose, Arl - L-arabinose, Ceb - Cellobiose, Cel - Cellulose, Fuc - Fucose, Gal - Galactose, Lam - Laminrin, Man - Mannose, Pec - Pectin, Rham - Rhamnose, Xyn - Xylan, Xyo - Xylose, Brachy - Brachypodium distanion, CS - Cornstover, SG - Switchgrass.
Figure 3-4. Heatmap showing the relative gene expression of the putative Pentose Phosphate pathway during growth on plant biomass compared to cellulose, xylan, and xylose. All values at top of figure are in Log2 relative to Glucose with red indicating a higher expression, black similar expression, and green a lower expression than on glucose. Labels on the right indicate the NCBI gene annotation for the putative pentose phosphate genes.
Figure 3-5. Metabolic output of C. phytofermentans cultures (n = 6) grown on corn stover used for microarray analysis after 5 days of growth on corn stover. Values on the y-axis are in (mM) while various metabolites are shown on the x-axis. Error bars indicate the standard deviation between the samples.
CHAPTER IV

SIMULTANEOUS SUGAR UTILIZATION BY C. PHYTOFERMENTANS

The potential ability to grow on multiple sugars simultaneously was further investigated in the next experiment. *C. phytofermentans* was grown on individual sugars, sugar pairs, and as a mixture of four sugars and a combination or growth experiments, HPLC analysis, and gene expression analysis were conducted. From the growth on the four sugar mixture (Fig. 4-1) we observe no diauxic shift, which is a classical indication of sequential sugar utilization and carbon catabolite repression (CCR).

While no diauxic shift was observed, possibly indicating simultaneous sugar utilization, no increase in growth rate in the mixtures compared to the single sugars was seen (Table 4-1). Interesting is the lower observed growth rate on cellobiose compared to all the other sugars.

In order to further investigate if *C. phytofermentans* is able to simultaneously utilize multiple sugars, HPLC analysis of the cultures in Fig. 4-1 was conducted. These experiments clearly indicate that *C. phytofermentans* is utilizing all 4 sugars at the same time (Fig. 4-2). However, while all four sugars are being utilized simultaneously, there does appear to be a preference for cellobiose and glucose over xylose and fucose (Fig. 4-2). By 48 hrs. all of the cellobiose has been utilized, at 72 hrs. all of the glucose has been utilized, and at this same 72 hrs. time point xylose and fucose are still present in the media. These results agree with our hypothesis that *C. phytofermentans* can utilize multiple sugars simultaneously. We can also determine that fucose is being utilized and not only transported into the cell since we observe propionate and propanol in the solutions (Fig. 4-3B). These two products are not produced during the metabolism of glucose, cellobiose, or xylose, and are unique for this sugar in the mixture (Petit, et. al. 2013). Of further interest is the formation of 1,2-propanediol (Fig 4-3B). It is believe that fucose is metabolized to 1,2-propanediol where it is then utilized further in a bacterial microcompartment (BMC) to produce propionate and propanol (Petit, et. al. 2013). The formation of 1,2-propanediol is not observed during growth on fucose or rhamnose individually and
has only been detected when other sugars are present. This had led to the hypothesis that *C. phytofermentans* may excrete this product during growth with other sugars and could possibly be utilized after these other sugars have been exhausted.

Gene expression analysis was also conducted on these samples. From these results we noticed the repression of the highly expressed cellulases (*Cphy_3367* and *Cphy_3368*) and xylanase (*Cphy_2105*) which is usually observed during growth on xylose and fucose (Fig. 4-4) but not on glucose or cellobiose. This seems to indicate that the presence of glucose is repressing the expression of these gene. Since one of these genes (Tolonen, et. al. 2009) has been shown to be required for cellulose degradation, this could mean that in the presence of glucose no cellulose will be degraded.
Figure 4-1. Lack of diauxic shift during growth of C. phytofermentans cultures (n=3) on a four sugar mixture. Sugar mixture was glucose, cellobiose, xylose, and fucose. Error bars indicate the standard deviation between tubes.
Figure 4-2. Sugar utilization during growth of C. phytofermentans cultures grown on a four sugar mixture. At each time point two cultures were sacrificed and the residual sugar concentration was determined by HPLC. Diamonds - cellobiose, square - glucose, triangle - xylose, and circle - fucose. Error bars indicate the difference of the sugar concentration between the two samples at that time point.
Figure 4-3. Metabolic output during growth of C. phytofermentans on a four sugar mixture. At each time point two cultures were sacrificed and the residual sugar concentration was determined by HPLC. (A) Total production formation. Diamond - Ethanol, Square - Acetate, Diamond - Formate. Note: Lactate is formed but overlaps with Acetate. (B) Insert of graph (A) removing all products except Propionate, Propanol, and 1,2-Propanediol. Error bars indicate the difference of the sugar concentration between the two samples at that time point.
Figure 4-4. Gene expression of two cellulase (Cphy_3367 and Cphy_3368) and a xylanase (Cphy_2105) from C. phytofermentans from different growth substrates. Y-axis is absolute signal intensity from microarray analysis in which a higher number indicates higher expression. Columns indicate the growth substrate for each microarray analysis (n =2). Samples are Brach, Corn stover, Switchgrass, Ceb - Cellobiose, Glu/Ceb - mixture of Glucose and Cellobiose, Fuc - Fucose, Glu/Fuc - mixture of Glucose and Fucose, Xyo - Xylose, Glu/Xyo - mixture of Glucose and Xylose, Glu - Glucose, and Xylan.
CHAPTER V
CONCLUSIONS

To date the full industrial potential for ethanol biofuel production from *C. phytofermentans* still remains to be seen. However in order to better understand and exploit this potential a greater understanding of the fundamental pathways and processes involved in biomass degradation and sugar utilization must be examined. The research presented here helps to expand on that knowledge through characterization of the gene expression on plant biomass and sugar utilization patterns in this bacterium.

Gene expression experiments conducted on three different plant biomass substrates showed a much smaller glycoside hydrolase response than hypothesized. From similar experiments conducted on xylan (Petit, et. al. 2013), we observed a broad expression of glycoside hydrolases potential involved in xylan/xylose utilization. However even though plant biomass contains ~30% hemicellulose, which contains xylan, the same response in not observed and only a single xylanase (Cphy_2105) is highly expressed. This could indicate that this xylanase is sufficient for hemicellulose utilization. We also noted the expression of transporters and pathways involved in the utilization of cellobiose, xylan, and xylose, along with the production of propionate indicated a potential for simultaneous sugar utilization.

Analysis of growth and sugar/production profiling during growth on sugar mixtures indicates that *C. phytofermentans* can utilize multiple sugars simultaneously, however there does seem to be a preference (in order) for cellobiose, glucose, xylose, fucose. This is in contrast to most other bacteria that have a strict pattern of sugar utilization. However we hypothesize that the utilization of ABC sugar transporters instead of PTS transporter which is involved in CCR could explain this. While *C. phytofermentans* does contain a putative sugar PTS, it does not to be expressed under any of the conditions tested to date.
During growth on these sugar mixtures we also observed some interesting results. First is the repression of the cellulases and xylanase which are normally highly expressed during growth on xylose and fucose. This repression could lead to lower levels of biomass degradation if glucose is also present in the culture. However recent research seems to refute this hypothesis (Jin. et. al., 2012). In this experiment \textit{C. phytofermentans} was grown on AFEX-treated corn stover with or without supplementation. The supplementation was the water extract following washing of the AFEX-treated corn stover and contained monomers such as glucose and xylose and also longer oligomers of both sugars. There was a higher conversion of the biomass, both the cellulose and hemicellulose component, in the presence of the supplementation. This would seem to indicate that under these conditions there is no repression. The presence of the oligomers of glucose or the corn stover could activate these enzymes by another mechanism. The second observation is the accumulation of 1,2-propanediol in the culture media during growth on fucose and other sugars. This would seem to indicate that \textit{C. phytofermentans} might not require the full metabolism of this sugar when other sources of energy are also present.
BIBLIOGRAPHY


