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Engineering Stable Anaerobic Consortia by Understanding the Genomic Basis for Stable Interaction

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Chemical Engineering

by

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December 2022

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- X. Peng, S. E. Wilken, T. Lankiewicz, S. Gilmore, **J. Brown**, J. Henske, C. Swift, A. Salamov, K. Barry, I. Grigoriev, M. Theodorou, D. Valentine, M. O'Malley. Genomic and functional analyses of fungal and bacterial consortia that enable lignocellulose breakdown in goat gut microbiomes. ***Nature Microbiology.*** (2021).
- S. Gilmore‡, T. Lankiewicz‡, S. E. Wilken, **J. Brown**, J. Sexton, J. Henske, M. Theodorou, D. Valentine, M. O'Malley. Top-down enrichment guides in formation of synthetic microbial consortia for biomass degradation. ***ACS Synthetic Biology***. (2019).
- C. Swift, **J. Brown**, S. Seppälä, M. O'Malley. Co-cultivation of the anaerobic fungus *Anaeromyces robustus* with *Methanobacterium bryantii* enhances transcription of carbohydrate active enzymes. ***The Journal of Industrial Microbiology & Biotechnology***. (2019). *Invited publication*.
- I. Podolsky‡, S. Seppälä‡, T. Lankiewicz, **J. Brown**, C. Swift, M. O'Malley. "Harnessing nature's anaerobes for biotechnology and bioprocessing," ***Annual Review of Chemical & Biomolecular Engineering***. (2019). ‡ equal author contributions
- U. de Silva, **J. Brown**, Y. Lapitsky. "Poly(allylamine)/tripolyphosphate coacervates enable high loading and multiple-month release of weakly amphiphilic anionic drugs: an *in vitro* study with ibuprofen". ***RSC Advances***. (2018).

PLANNED PUBLICATIONS

- **Submitted**: T. Lankiewicz, H. Choudhary, Y. Gao, B. Amer, S. Lillington, P. Leggieri, **J. Brown**, C. Swift, A. Lipzen, H. Na, M. Amirebrahimi, M. Theodorou, E. Baidoo, K. Barry, I. Grigoriev, V. Tomhokin, J. Gladden, S. Singh, J. Mortimer, J. Ralph, B.

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- **In review: J. Brown**, C. Swift, M. Perisin, M. Benyamin, V. Singan, Christa Pennacchio, I. Grigoriev, M. O'Malley, "Co-cultivation of anaerobic fungi with *Clostridium acetobutylicum* bolsters butyrate and butanol production from cellulose and lignocellulose". ***The Journal of Industrial Microbiology & Biotechnology***. (2022).
- **In progress**: **J. Brown**, T. Gierke, L. Butkovich, C. Swift, V. Singan, C. Daum, K. Barry, I. Grigoriev, M. O'Malley. "High-quality RNA extraction and regulation of carbohydrate active enzymes is correlated with stage of growth in anaerobic fungi". ***Frontiers in Fungal Biology***. (2022).

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- **J. Brown,** C. Swift, M. Perisin, X. Peng, S. Seppälä, M. O'Malley. "Engineering Stable Anaerobic Consortia by Understanding the Genomic Basis for Syntrophic Interactions". UCSB Bioengineering Student Seminar Series, June 2020. (oral presentation)
- **J. Brown,** X. Peng, S. Gilmore, J. Henske, M. O'Malley. "Engineering Stable Anaerobic Consortia by Understanding the Genomic Basis for Syntrophic Interactions". American Chemical Society 257th National Meeting and Exposition, Orlando, FL, March 2019. (rapid fire poster presentation and poster presentation)
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- **J. Brown**, X. Peng, S. Gilmore, J. Henske, M. O'Malley. "Engineering Stable Anaerobic Consortia by Understanding the Genomic Basis for Syntrophic Interactions". American Chemical Society 255th National Meeting and Exposition, New Orleans, LA, March 2018. (poster presentation)
- **J. Brown**, X. Peng, S. Gilmore, J. Henske, M. O'Malley. "Engineering Stable Anaerobic Consortia by Understanding the Genomic Basis for Syntrophic Interactions". Joint Genome Institute User Meeting, San Francisco, CA, March 2018. (poster presentation)

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ABSTRACT

Engineering Stable Anaerobic Consortia by Understanding the Genomic Basis for Stable Interaction

by

Jennifer L. Brown

Waste management and sustainable energy production are two major concerns of modern society. The use of microbial consortia for waste treatment has the potential to address both of these concerns simultaneously, since consortia possess the ability to convert crude biomass into biofuels and bio-based chemicals. The organic fraction of municipal solid waste (OFMSW) or food waste is an abundant and inexpensive carbon substrate that can be utilized by microbial systems to generate useful products. Anaerobic consortia containing fungi, bacteria, protozoa, and methanogenic archaea capable of converting wet waste materials into valuable substances already exist in nature and have been isolated from the guts of herbivores. Although bioreactors utilizing undefined natural consortia to digest wet waste and generate biogas have been constructed, the failure rate is high due to instability and death of the microbial community. The development of biotechnology capable of handling variable input, recovering from environmental disturbances, and producing consistent products is dependent upon engineering stability and robustness among consortia members. To achieve this, it is necessary to understand the genomic basis for stable interaction between members of these microbial communities.

In this work, transcriptional and metabolic changes induced by methanogen co-culture were evaluated in the anaerobic fungal strain *C. churrovis* across a variety of substrates to identify mechanisms that impact biomass breakdown and sugar uptake. Co-culture with the methanogen increased overall transcription of carbohydrate active enzymes (CAZymes), carbohydrate binding modules, and dockerin domains in co-cultures grown on both lignocellulose and cellulose. Next, a system for simultaneous and sequential co-cultivation of the anaerobic fungus *Anaeromyces robustus* and the anaerobic bacterium *C. acetobutylicum* was established based on lactate cross-feeding to produce butyrate and butanol from lignocellulose. Higher levels of butyrate and butanol in fungal and *C. acetobutylicum* cultures reveal that creating consortia that include these two microbes could be a promising future avenue of industrial bio-butyrate and biobutanol production. Finally, a method to extract high-quality RNA from anaerobic fungi at multiple timepoints in the fungal growth phase was developed to fully characterize differential expression in both fungal monocultures and fungal-methanogen co-cultures. The fungal strain *Anaeromyces robustus* co-cultivated with the methanogen *Methanobacterium bryantii* upregulates genes encoding fungal carbohydrate active enzymes and other cellulosome components relative to fungal monocultures when grown on a cellulose substrate, but expression patterns changed at 24-hour intervals throughout the fungal growth phase. Overall, this work indicates that anaerobic fungi can be successfully combined with non-native microbes in consortia capable of converting low-cost biomass substrates into value-added products.

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1 Introduction

1.1 Motivation

Consortia-based bioprocessing

Industrial biotechnology seeks to process natural resources to produce fuels, chemicals, and materials through biological means by applying engineering principles [1]. Currently, most of these biotechnological products are produced using cultures consisting of a single organism or cell type. Very few bioprocesses use cultures of mixed microbial populations [2]. Multiple interacting microbial populations, known as consortia, can offer distinct advantages over pure-culture bioprocessing [3]. Distributing a metabolic pathway among a microbial consortium enhances production [4]. The metabolic load of individual organisms is reduced. This allows consortia to perform complicated functions that individual populations are not able to, since metabolic imbalance in the pure population host cells often leads to slowed growth and decreased production. The number of exogenous elements that can be cloned and optimized in a single cell is limited, but the number of exogenous elements that can be introduced in a consortium is greatly expanded due to the diversity of the microbial community [5]. Consortia can be more robust to environmental fluctuations than individual populations due to the diverse metabolic modes present among the members of the microbial community [6]. Instead of being limited to a single substrate, consortia are capable of using a mixed substrate to produce a narrow spectrum of desired products [7]. Mixed-culture bioprocesses also have the potential to mitigate byproducts, which can be toxic to cell growth through degradation or conversion, resulting in increased product yield and substrate utilization [2,8].

At present, consortia-based bioprocessing is used for biogas production, wastewater treatment, food and beverage production through natural fermentation, and biological soil remediation [9–12]. One application of consortia-based bioprocesses is the anaerobic digestion of waste, the focus of this project. The organic fraction of municipal solid waste (OFMSW) is an abundant and inexpensive carbon substrate that can be utilized by microbial systems to generate useful products, such as methane and ethanol. The OFMSW waste streams are typically made up of food waste, paper/cardboard, wood, and plastics; in addition to having high water content [13]. The typical proportions of these components (subject to variation) are shown in Table 1.1.

Table 1.1 Organic Fraction of Municipal Solid Waste Components

32% food waste (sugar & pectin)
25% paper/cardboard (cellulose)
14% wood (lignocellulose & hemicellulose)
14% plastics
high water content

A significant portion of the OFMSW is lignocellulosic biomass. This recalcitrant substrate generally requires an energy-intensive pre-treatment step using conventional methods of biofuel production [14,15]. By taking a novel approach of using consortia-based bioprocessing containing anaerobic fungi, cellulase production, biomass hydrolysis, and sugar fermentation can be consolidated, providing an alternative approach to both conventional methods and classical consolidated bioprocessing (CBP), which aims to accomplish this using only one organism. Enrichment techniques have allowed for the isolation of syntrophic pairs of fungi and methanogens from naturally-occurring consortia found in the guts of herbivores [16]. The fungi possess both cellulosomes that break down a wide range of plant matter into

fermentable sugar, and hydrogenosomes that, in turn, play a key role in converting the sugars into hydrogen gas after they have undergone glycolysis in the cytoplasm [17–19]. The hydrogen gas produced by the fungi can then be used by methanogenic archaea in the consortia to produce methane [20]. Co-cultures of fungi and methanogens have been shown to accelerate biomass degradation, while providing methane as a product and simultaneously removing the metabolic end products of the fungi, such as hydrogen, that may result in inhibition of fungal growth and function if allowed to accumulate [21–25].

1.2 Current challenges in consortia-based bioprocessing

While consortia can be more robust to environmental fluctuations than individual populations, further improvements in the operational stability of consortia-based bioprocessing are required before anaerobic digestion can be widely commercialized [26,27]. Despite the use of metagenomic sequencing to determine putative functions of interacting members of microbial consortia [28,29], many questions still remain regarding nutrient flow, nutrient exchange, and the dynamics and interplay of members of the microbial community, which are difficult to measure. Many of the microbes found in naturally occurring consortia have not been cultured in the laboratory [30–32], resulting in a lack of knowledge regarding community structure and the effects of environmental perturbations [33,34]. As a result, although bioreactors using natural consortia have been constructed to digest combined food and wet waste to capture biogas, the failure rate is high due to instability and death of the undefined consortia, resulting in unpredictable fermentation byproducts.

Currently, anaerobic digesters are operated by seeding with substances containing natural anaerobic consortia, and maintained using operating conditions determined through

empirical observation rather than a true understanding of the microbial interactions [35]. In order to engineer robustness into the relationship between consortia members and build stable consortia, a thorough understanding on a genomic level of the functioning of anaerobic consortia is required; particularly an understanding of what metabolic processes are responsible for stability and production [36].

1.3 Strategies to engineer stability and robustness

Systems biology aims to derive and predict the emergent properties of complex systems such as anaerobic consortia from knowledge of their individual parts [37]. Microbial interactions are particularly challenging to study since their number grows exponentially with increasing diversity of the community [38,39]. While efforts have been made to characterize strains individually, this approach neglects to account for genes that may only be expressed in a community setting [40]. New experimental and computational tools for screening and predicting community behavior have been developed that allow ecosystem stability and dynamics to be examined [41].

For this project, I sought to examine the genomic basis for syntrophic interactions, with the goal of analyzing and optimizing anaerobic cellular networks for application in the development of stable and robust microbial communities and bioprocesses [1,42]. Transcriptomics provided insight into the metabolism and biomass-degrading activity of the fungal population by studying global gene expression as a function of different conditions via RNAseq [22].

2 Co-cultivation of the anaerobic fungus *Caecomyces churrovis* with *Methanobacterium bryantii* enhances transcription of carbohydrate binding modules, dockerins, and pyruvate formate lyases on specific substrates

2.1 Introduction

Anaerobic fungi are efficient degraders of recalcitrant lignocellulosic biomass that are found in the guts of herbivores. The high number of CAZymes (carbohydrate active enzymes) that anaerobic fungi produce has driven efforts to collect genomic and transcriptomic data for a variety of emerging anaerobic fungal species, with a focus on the differential transcriptional response of anaerobic fungi to complex carbohydrates versus monomeric sugars [18,22,43–46]. Gut fungi function within a community of biomass-degrading bacteria, protozoa, and methanogenic archaea linked by complex metabolic interactions and functional redundancy [47]. Isolating individual members of these natural consortia is one approach to develop a more detailed understanding of microbial interactions, which can then be used to design

optimized consortia for biotechnological applications to break down lignocellulose-rich waste. These microbes can be selected through "top-down" enrichment techniques such as serial cultivation or antibiotic treatment to isolate syntrophic pairs of fungi and methanogens from naturally-occurring consortia. Alternatively, communities can be formed using "bottom up" methods mixing separate axenic cultures of these microbes to create synthetic pairings linked by metabolic dependency [16,47,48].

Fungal-methanogen co-cultures have been extensively studied due to the mutually beneficial relationship between the two organisms resulting from their complementary metabolism – fungi produce hydrogen (H_2) as an unwelcome byproduct of their own metabolism, which methanogens use in the biosynthesis and release of methane [48–54]. Many previous studies report that co-cultivation of anaerobic fungi with methanogens can enhance biomass breakdown, but the metabolic mechanisms responsible for this outcome are unclear and not uniformly reproducible [25,53,55–57]. For example, a recent study concluded that the removal of fungal metabolites by methanogens does not increase the rate of gas production or the rate of substrate deconstruction by a synthetic community of fungi and methanogens relative to fungal monocultures [48]. It has also been hypothesized that co-cultivation of fungi and methanogens results in increased sugar utilization and flux through the fungal hydrogenosome through increased transport and carbon conversion [54,58]. Additionally, we recently reported that *M. bryantii* enhances the transcription of genes encoding ABC transporters, MFS transporters and G-protein coupled receptors (GPCRs) in the fungus *Anaeromyces robustus*, indicating that co-cultivation may increase the rate of sugar utilization through the increased expression of sugar transporters [49]. Although many studies have been conducted to determine how co-cultivation with methanogens affects fungal

metabolism and biomass breakdown, none have characterized transcriptional and metabolic outcomes across a variety of relevant substrates, which is critical to detangling competing effects of substrate response [49,50].

Here, we present the first genome of an anaerobic non-rhizoid forming fungus of the *Caecomyces* genus, and further examine its transcriptional response to the presence of methanogens in multiple synthetic co-cultures supported on lignocellulose, hemicellulose, cellulose, and sugars. *Caecomyces churrovis* lacks the extensive rhizoid network formed by other previously sequenced anaerobic gut fungi to aid in biomass breakdown. Improvements in long-read sequencing technologies enabled assembly and annotation of CAZymes and associated cellular machinery despite the complex fungal physiology, unknown ploidy, AT-content, and repeat-richness. By combining RNA-Seq with growth and chemical data, we determine how the fungus responds to co-cultivation with a non-native methanogen in synthetic co-culture. The ability to pair two microbes based on complementary metabolism alone presents the opportunity to combine non-native microbes in a desired technological application without the constraint of naturally developed syntrophy. While other studies have examined global transcriptomic response and CAZyme regulation in anaerobic fungi cultivated with methanogens on a single substrate, none to date have explored regulation across a range of substrates or differences occurring in transcriptional regulation between multiple fungal strains on the same substrate [49,50]. Through a combination of genomic, transcriptomic, and metabolomic data we found that the *C. churrovis* genome possesses an abundance of both CAZymes and carbohydrate binding modules as shown in Figure 2.1. Co-culture of *C. churrovis* with a non-native methanogen enhances transcription of gene sets associated with fungal substrate binding and fungal-methanogen interactions such as

carbohydrate binding modules in families 1 and 18, pyruvate formate lyase (PFL) function in the cytosol or possibly the hydrogenosome, and enzymes that are potential bottlenecks for sugar utilization in fungi across multiple substrates. Overall, understanding how methanogen co-culture influences the fibrolytic and metabolic behavior of anaerobic fungi aids in the design of new strategies for conversion of lignocellulose to fermentable sugars and value-added products, and reveals the genetic mechanisms that underpin fungal-methanogen interactions.

2.2 Results and Discussion

2.2.1 The Caecomyces churrovis *genome encodes an abundance of CAZymes and carbohydrate binding modules*

Anaerobic fungi are emerging platforms for hydrolysis of crude lignocellulose, as they produce powerful CAZymes and mechanically associate with and often penetrate plant cell walls [45,59,60]. The first high quality genome of a non-rhizoid forming anaerobic fungus from the *Caecomyces* genera was sequenced with PacBio SMRT sequencing using high molecular weight DNA fragments, a method that is critical to high-quality genome assemblies for anaerobic fungi [18,20,61]. Previously, we assembled a *de novo* transcriptome of *C. churrovis* by pooling RNA from batch cultures grown on glucose, cellobiose, cellulose, and reed canary grass, obtaining an inclusive set of expressed genes for these substrates [45]. The acquisition of the *C. churrovi*s genome now enables more detailed investigation of genetic regulatory mechanisms, splicing, ploidy, and comparative genomics that cannot be accomplished with a sole transcriptome. Based on genome sequencing, 15,009 genes were annotated/identified, compared to the predicted 33,437 genes based on the sequenced

transcriptome (predicted by taking into account the number of transcripts less isoforms); this difference in gene number prediction between transcriptomes and genomes is consistent across anaerobic fungi and likely reflective of ploidy [18,45]. This discrepancy is largely explained by our observation that this strain of *Caecomyces* is likely a diploid (or dikaryon), as we detected ~10k gene models on smaller scaffolds in regions that were >95% identical to regions on larger scaffolds. These scaffolds were designated as secondary scaffolds and these secondary models/alleles were not included in further analyses but are available from MycoCosm [62]. Table 2.1 depicts genomic features for high-resolution sequenced anaerobic fungi, as reported by the JGI MycoCosm pipeline [62].

Table 2.1: Overview of sequenced anaerobic fungal genome features and statistics[18,43,44]

	Caecomyces churrovis	***Anaeromyces robustus***	***Neocallimastix californiae***	***Neocallimastix lanati***	***Piromyces finnis***	***Pecoramyces ruminantium***
Genome size (Mbp)	165.50	71.69	193.03	200.97	56.46	100.95
No. scaffolds	7737	1035	1801	970	232	32574
% GC content	19	16	22	18	21	17
Scaffold L50 (Mbp)	0.03	0.14	0.44	1.03	0.75	0.00
No. of gene models	15,009	12,832	20,219	25,350	10,992	18936
Gene % CAZymes	7.22	6.73	7.23	7.05	6.45	5.67
No. of DDPs*	389	276	422	586	227	318
No. of scaffoldins	36	26	55	93	14	83
No. of diploid gene pairs	10972	147	1154	497	146	3,113

***DDPs=dockerin domain proteins.**

As noted in Table 2.1, the *C. churrovis* genome is GC depleted on the same order of magnitude as the other sequenced anaerobic fungal strains. Such extreme codon biases have

made it challenging to heterologously express and evaluate the function of anaerobic fungal genes (like CAZymes) in model systems [63–65]. Homopolymeric runs of amino acids are found in the *C. churrovis* genome, which are common in the CAZyme machinery of anaerobic fungi, and could serve as glycosylation sites that prevent proteolytic cleavage [65]. Collectively, the function of such features needs to be better characterized if gut fungal CAZymes from strains such as *C. churrovis* are to be heterologously produced in a model organism [65].

Anaerobic gut fungi possess an abundance of CAZymes with diverse functions, and are particularly rich in hemicellulases (especially glycosyl hydrolase 10 family) and polysaccharide deacetylases [60]. Some CAZymes are anchored by non-catalytic fungal dockerin domains (NCDDs) to cohesin domains on large scaffoldin proteins to form enzymatic complexes called fungal cellulosomes [18]. The high-resolution genome presented here enabled a Hidden Markov Model (HMM) analysis of the *C. churrovis* genome, which annotated 36 genes as fungal scaffoldins, compared to the 38 transcripts predicted based on tblastn alignment of the previously sequenced transcriptome [66]. The quantity of predicted proteins identified as cellulases, hemicellulases, and other accessory enzymes along with the total number of CAZymes for each of the 6 sequenced fungal strains are listed in Supplementary Table 7.1.1. Fewer total CAZymes in the above categories were identified using predicted proteins found in the sequenced genome (338) than were identified by counting the number of transcripts in the sequenced transcriptome (512), which did not take ploidy into account. The highest abundance accessory enzymes identified in the genome were pectin lyases (15.7% of all CAZymes), in contrast to the transcriptome, in which carbohydrate esterases containing SGNH (defined by four invariant residues – serine, glycine, asparagine,

and histidine) hydrolase domains were identified as the most abundant (Supplementary Table 7.1.1) [67,68]. However, the *C. churrovis* genome also contains the smallest number of polysaccharide lyase domains (PLs) of any of the 6 fungal genomes characterized (Fig. 2.1).

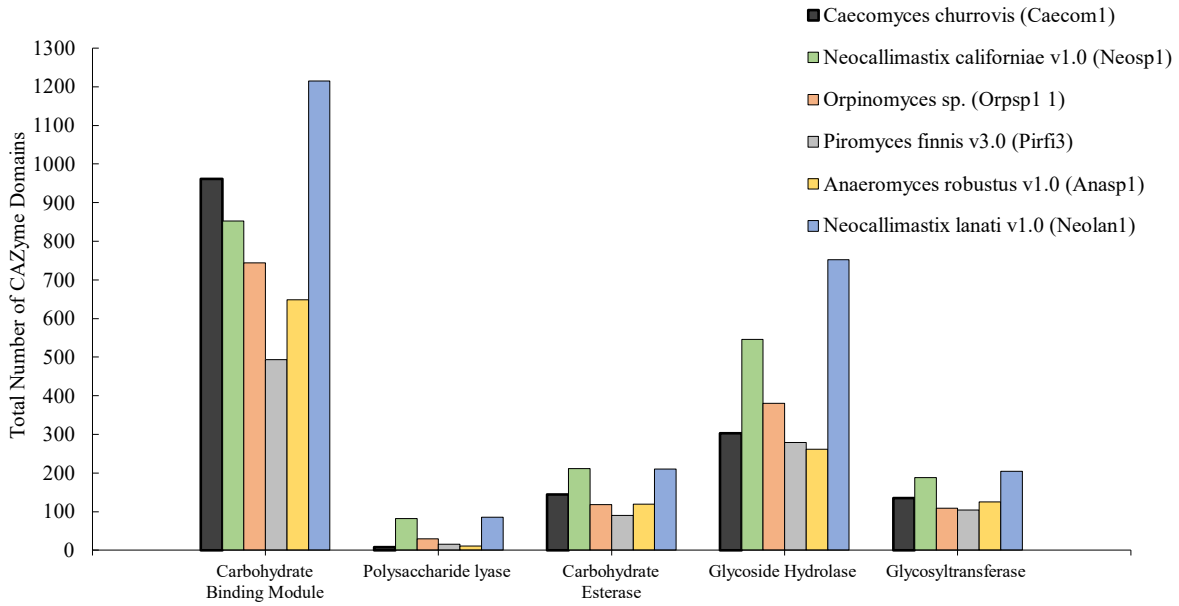


Figure 2.1. Number of different types of CAZyme domains in six sequenced anaerobic fungi. *C. churrovis* has the highest number of domains annotated as carbohydrate-binding modules compared to most other sequenced anaerobic fungi. Annotation data for these strains can be found at https://mycocosm.jgi.doe.gov.

Proteins containing non-catalytic fungal dockerin domains (NCDDs) were also identified and found to be relatively consistent across strains, in agreement with what was observed for transcript counts (Table 2.1). However, in contrast to the observation that *C. churrovis* NCDD containing transcripts represented only 15% of all CAZyme transcripts in comparison to 27.9-31.4% for the three other fungal strains examined, the number of NCDD containing proteins represented 35.9% of all CAZyme proteins for *C. churrovis*, similar to the other three fungal strains (Table 2.1). This suggests that while *C. churrovis* may place greater emphasis on secreted un-complexed, free enzymes to attack plant biomass and release fermentable sugars compared to rhizoid-forming anaerobic fungi based on previously

collected transcriptional data, its genome still contains a proportion of NCDD proteins similar to that observed in the genomes of rhizoid-forming anaerobic fungal genera. *C. churrovis* also has the second highest number of carbohydrate binding module domains (CBMs) compared to five other high-quality anaerobic fungal genomes (Figure 2.1). Further analysis revealed that of these genes, *C. churrovis* also possessed the highest number of CBM family 18 domains among anaerobic fungi sequenced to date (Supplementary Figure 7.1.1).

It was previously reported that N6-methyldeoxyadenine (6mA) is associated with transcriptionally active genes in early-diverging fungal lineages in a study using single-molecule long-read sequencing to determine which adenines were methylated [69]. Of the 6,692 genes that were methylated when the *C. churrovis* genome was sequenced, 4,063 had KOG annotations, 1,002 had KEGG annotations, 3,450 had GO annotations, and 401 were annotated as CAZymes. Almost 1% of all adenines are methylated, and 93% of modifications are at AT dinucleotides, as shown in Supplementary Figure 7.1.2A. Very few symmetric runs were present, consistent with avoidance of TAT/ATA reported previously [69]. Modifications are primarily at the start of genes, specifically ramping up in presence at the start of transcription (Supplementary Figure 7.1.2B). 6mA was rare in repetitive regions of the genome (Supplementary Figure 7.1.2C) and a large proportion of total 6mA was restricted to genic space (Supplementary Figure 7.1.2D).

These results agree with the trends observed for other anaerobic fungal species, further serving to identify 6mA as a widespread epigenetic mark in early-diverging fungi that is associated with transcriptionally active genes [69]. Note that only ~6% of methylated genes in the genome are annotated as CAZymes, indicating that these genes are not always highly transcribed, but rather the majority of CAZymes are transcribed as needed in response to

external stimuli, such as co-culture, growth substrate, etc. Nevertheless, association of gene expression with adenine methylation is necessary to understand and develop transformation techniques, which has proven difficult in anaerobic fungi and other non-model eukaryotic systems to date [60,70]. Accounting for methylated adenine cluster (MAC) positioning and other epigenetic features could help achieve the methylation required to sufficiently overexpress target genes, such as the CAZymes involved in applications requiring biomass breakdown in both fungal monoculture and in anaerobic biomass-degrading consortia [69].

2.2.2 Synthetic co-cultures of C. churrovis *with methanogen* M. bryantii *produce methane*

Establishing synthetic co-cultures of anaerobic fungi with methanogens is a valuable tool to probe the impact of co-culture on plant biomass breakdown, substrate uptake, and growth of the individual microbes [48]. Once plant biomass has been broken down into its constituent sugars by fungal CAZymes, they are catabolized by the fungi and other organisms in the native rumen environment [59]. Sugars consumed by the fungi undergo glycolysis in the fungal cytoplasm, and the resulting malate and pyruvate are taken up by the fungal hydrogenosome, where they are converted to H_2 and formate via hydrogenase and pyruvate formate lyase, respectively [17–19]. The hydrogen and formate produced are then exported and available to neighboring methanogens, which assimilate these products and ultimately generate methane [20]. As such, the metabolic exchange between anaerobic fungi and methanogens benefits both microbes, since it is hypothesized that fungal metabolic end products such as H_2 and formate may inhibit fungal growth and function if allowed to accumulate, while the methanogens are provided with their required growth substrates [71].

Figure 2.2A summarizes the design of this experiment. Cumulative pressure was measured daily (as a proxy for microbial growth) in order to determine when mid-log growth phase had been reached, at which time the cultures were harvested for RNA extraction as shown in Figure 2.2B and C [48]. Gas chromatography was used to determine the concentration of methane and hydrogen in the headspace gas of synthetic co-cultures and fungal monocultures on each substrate prior to harvest for RNA extraction at mid-log growth phase. No significant amount of hydrogen was detected in the co-cultures, and no methane was detected in the fungal monocultures, in agreement with *M. bryantii*'s H_2/CO_2 requirement for methane production [72], as shown in Supplementary Figure 7.1.3. The absence of hydrogen in the co-cultures indicates that stable pairings of the fungus and methanogen were formed on all substrates (Fig. 2.2D), which is consistent with previous observations for the *N. californiae* and *A. robustus* anaerobic fungal strains paired with the same methanogen and grown on cellulose and lignocellulosic reed canary grass [48,49]. Subsequently, transcriptional regulation coupled with HPLC analysis was used to determine the impact of co-cultivation on fungal sugar utilization, hydrogenosome function, secondary metabolite production, and membrane protein regulation in stable, non-native fungal-methanogen co-cultures.

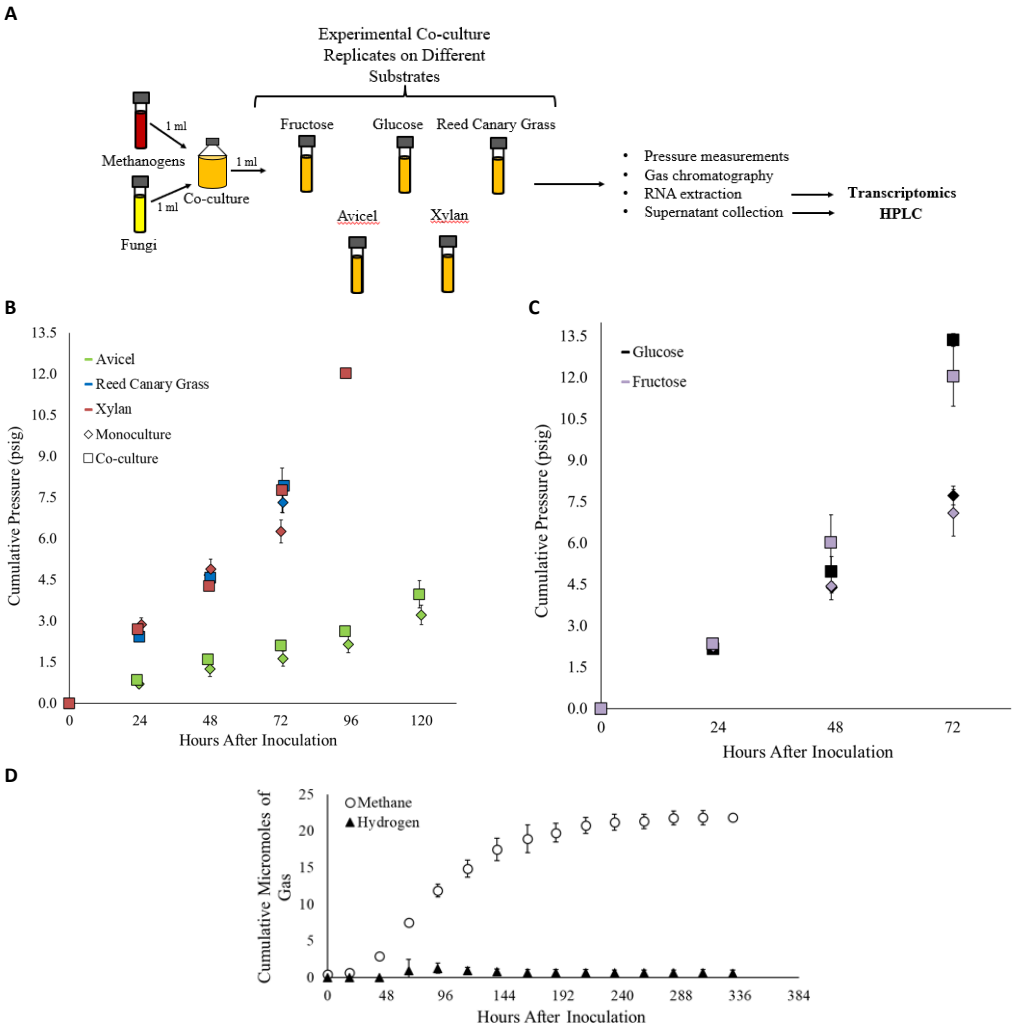


Figure 2.2. Monocultures and co-cultures were harvested at mid-log growth phase as determined by cumulative pressure. (A) Schematic of the experimental process of cultivating and harvesting co-cultures. A similar process was followed for cultivating and harvesting monocultures, except the seed culture was inoculated with 1 mL of fungus only. (B and C) Cultures were harvested at pre-determined pressure ranges indicative of the mid-log growth stage for each culturing condition. Cumulative pressure (psig) is plotted versus hours after inoculation for co-cultures and monocultures grown on biomass and components of biomass - reed canary grass, Avicel®, and xylan – in Figure B. Cumulative pressure (psig) is plotted versus hours after inoculation for co-cultures and monocultures grown on soluble sugars – glucose and fructose – in Figure C. Pressure readings for co-cultures are indicated by squares and pressure readings for monocultures are indicated by diamonds. Each substrate is color coded according to the key on the plot. Cultures were harvested at the mid-log growth phase, as indicated by the final pressure time point for each sample. (D) Longterm methane and hydrogen data produced by co-cultures of the anaerobic fungus *C. churrovis* and the methanogen *M. bryantii* on a reed canary grass substrate. Cultures were grown in a complex media formulation, in contrast to cultures harvested for RNA extraction which were grown on MC-. Low levels of accumulated hydrogen indicate stable co-culture over the course of fungal growth.

2.2.3 Co-culture with a methanogen enhances production of fungal carbohydrate binding modules and fungal dockerins across multiple substrates

Changes in the transcriptional regulation of anaerobic fungi when challenged by different substrates indicates how the fungal CAZyme repertoire and fungal metabolism are adjusted in response to an altered environment. Often, waste streams containing biomass in industrial settings can vary in composition, potentially affecting bioreactor function through shifts in community composition and metabolic function [73,74]. Examining these changes using RNA-Seq reveals how variations in the composition of growth substrates impacts biomass breakdown and product generation. Differential regulation of CAZymes and associated enzymatic machinery was examined for *C. churrovis* co-cultivated with *M. bryantii* and was compared to *C. churrovis* fungal monocultures, both grown on Avicel®, reed canary grass, glucose, fructose, and xylan. A proportionally greater number of genes annotated as CAZymes and enzymatic machinery was upregulated in fungal-methanogen co-cultures relative to fungal monocultures than were downregulated on lignocellulose- and hemicellulose-rich substrates, reed canary grass and Avicel®. The opposite was true for co-cultures grown on substrates rich in soluble sugars, glucose, fructose, and xylan as shown in the Supplementary Figure 7.1.4A. The total number of genes upregulated or downregulated for individual CBM, GH, CE, PL, and GT families are shown in Supplementary Figure 7.1.4B-D.

However, the majority of the ten most highly upregulated genes in these categories in fungal-methanogen co-culture relative to fungal monoculture on all substrates were annotated as either CBM 18 family proteins or fungal dockerin domains, the majority of which were associated with genes of unknown function. Table 2.2 shows the top ten most highly

upregulated fungal genes according to log2fold change values annotated as CAZymes or associated enzymatic machinery in co-cultures of the anaerobic fungus *C. churrovis* and the methanogen *M. bryantii* relative to monocultures of *C. churrovis* grown on multiple substrates. The CBM family with the most abundant number of genes in the sequenced genome, CBM 18, was consistently the gene classification with the greatest log2fold change of any CAZyme or enzymatic machinery on all substrates in fungal-methanogen co-cultures relative to fungal monocultures. Furthermore, the same CBM 18 gene (*Caecomyces churrovis* protein ID 407913) had the greatest log2fold change in fungal-methanogen co-cultures relative to fungal monocultures on reed canary grass, glucose, and fructose substrates. CBM family 18 modules contain approximately 40 amino acid residues and include members with functions linked to modules with chitinase activity or which are lectins [75,76]. The modules may therefore either be attached to chitinase catalytic domains or in non-catalytic proteins in isolation or as multiple repeats. These carbohydrate-binding proteins possess diversity in ligand specificity and the ability to maintain enzymes in proximity of the substrate, increasing enzyme concentration and potentially leading to more rapid degradation of polysaccharides. These features make these proteins excellent candidates for use in biotechnological applications designed for biomass breakdown [77–80].

Avicel		
ID	Log2fold Change	CAZyme Annotation
607438	8.00	CBM 18
547795	5.96	CBM 18
629343	5.46	DOC
620648	5.14	GT 17
513365	4.96	CBM 87
548447	4.85	CBM 1
100311	4.71	CBM 18
528501	4.58	GH 78
430401	4.42	DOC/GH 30
207551	4.15	CBM 18

Reed Canary Grass		
ID	Log2fold Change	CAZyme Annotation
407913	5.94	CBM 18
607438	5.08	CBM 18
434710	4.87	DOC
547795	3.91	CBM 18
620648	3.55	GT 17
526368	3.37	CBM 18
574117	3.29	DOC
607594	2.94	DOC
136193	2.93	DOC
579030	2.83	CBM 18

Glucose		
ID	Log2fold Change	CAZyme Annotation
407913	9.08	CBM 18
596610	7.02	GH 73
555679	5.95	CBM 18
622576	5.87	CBM 18
590554	5.55	DOC
126623	5.39	CE 6/DOC
198053	4.81	CBM 18
498097	4.52	DOC
621851	4.45	GH 25
593248	4.40	DOC

Fructose		
ID	Log2fold Change	CAZyme Annotation
407913	7.72	CBM 18
403091	6.48	GT 71
198053	6.13	CBM 18
529683	5.74	DOC
436379	4.91	CBM 18
498097	4.52	DOC
401262	4.42	DOC
136193	4.19	DOC
620648	4.10	GT 17
607438	4.08	CBM 18

Xylan		
ID	Log2fold Change	CAZyme Annotation
198053	8.95	CBM 18
126623	7.72	CE 6/DOC
524258	6.25	CBM 18
407913	5.73	CBM 18
563945	5.29	CBM 18
531011	5.23	CBM 18
627323	5.06	CBM 18
513365	4.53	CBM 87
622031	4.11	CBM 18
527510	3.92	CBM 18

Table 2.2. Table of the top ten upregulated fungal genes annotated as CAZymes or associated enzymatic machinery in co-cultures of the anaerobic fungus *C. churrovis* and the methanogen *M. bryantii* relative to fungal monocultures of *C. churrovis* grown on multiple substrates. Co-cultures of the anaerobic fungus and the methanogen and fungal monocultures were grown on Avicel®, reed canary grass, glucose, fructose, and xylan. Differential expression of fungal genes in co-cultures relative to fungal monocultures was determined using DESEQ2. The ten genes with the highest log2fold change in expression in co-culture relative to fungal monoculture are shown in the table above for each substrate and organized into the following classifications: carbohydrate binding module family (CBM), dockerins (DOC), carbohydrate esterase family (CE), glycoside hydrolase family (GH), and glycosyltransferase family (GT). Dockerin-fused CAZymes are indicated by a forward slash between annotations. CBMs were highly upregulated, indicating that there may be an increase in enzymatic machinery that aids in anchoring CAZymes to substrates in co-culture, even when grown on soluble sugars.

The observation that CAZymes, fungal dockerins, and other biomass degrading machinery are upregulated in all co-cultures, even those grown on glucose is in agreement with previous studies conducted for fungal-methanogen co-cultures on reed canary grass and glucose at mid-log growth stage [49,50]. Since the majority of the top ten genes upregulated

on all substrates were annotated as either CBM 18 family proteins or fungal dockerin domains, this strongly suggests that co-culture with the methanogen *M. bryantii* results in the transcriptional upregulation of enzymatic machinery associated with biomass degradation. Although no transcriptional upregulation of scaffoldin-encoding genes was initially detected in this study, likely due to the more stringent log2fold change cutoff used to determine significant upregulation, Pre-ranked Gene Set Enrichment Analysis (GSEA) of the entire set of regulated genes revealed that upregulated scaffoldins are significantly enriched in co-cultures grown on Avicel® and reed canary grass [81,82]. These results agree with the finding by Swift et al. that transcription of fungal cellulosome components increases in co-culture [49]. Another possibility is that the production of CBM18 transcripts is not related to plant biomass breakdown but instead to interactions between the fungus and methanogen since differential expression is observed across all conditions, including growth on glucose. Many of the dockerin domains not attached to CAZymes contain a CotH kinase protein domain. Previous work showed that approximately 20% of DDPs identified in five previously sequenced anaerobic fungi belonged to spore coat protein CotH and were also present in bacterial cellulosomes [18]. These dockerin domain proteins belonging to spore coat protein CotH have been speculated to be involved in plant cell wall binding, although this remains to be experimentally validated [83].

The top ten most highly upregulated genes according to log2fold change annotated as CAZymes, CBMs, or fungal dockerins in co-cultures of *C. churrovis* with *M. bryantii* grown on reed canary grass were compared to those upregulated in co-cultures of the same methanogen, *M. bryantii*, with fungal strains *A. robustus* (previously published) and *N. californiae,* grown on the same substrate [49]. A plot of the proportion of genes containing

domains belonging to CAZyme gene families or associated enzymatic machinery upregulated in co-cultures of the three different fungal strains paired with the same non-native methanogen, *Methanobacterium bryantii* relative to fungal monocultures grown on a reed canary grass substrate is included in Figure 2.3. The number of genes regulated in CBM, GT, PL, CE, and GH families in the three fungal strains in co-culture versus fungal monoculture on reed canary grass substrate are shown in Supplementary Figure 7.1.5. The most highly upregulated gene for each strain was a CBM family 18 protein for both the *N. californiae* strain and the *C. churrovis* strain and a Carbohydrate Esterase (family 1) protein for the *A. robustus* strain. For each strain, at least three of the top ten genes were fungal dockerin domains, fused to CAZymes or genes of other function. A high proportion of upregulated genes for all three strains contained dockerin domains and a relatively high proportion of genes containing CBM family 1 or CBM family 18 domains were upregulated for multiple strains as well, as shown in Figure 2.3. This comparison suggests that co-cultivation with a methanogen likely encourages substrate channeling between synergistic enzymes for both rhizoid-forming fungal strains (*A. robustus* and *N. californiae*) and non-rhizoid-forming fungi (*C. churrovis*) [18,49]. Previously, it was suggested that a smaller proportion of CAZyme transcripts containing dockerin domains in the transcriptome of *C. churrovis* indicated a greater dependence on free enzymes compared to rhizoid-forming gut fungal genera [45]. Nevertheless, with comparative transcriptomic data, upregulation of these non-catalytic modules and CBMs is clearly observed when *C. churrovis* is cultured with *M. bryantii*. This could indicate that anaerobic fungi, regardless of their usual mode of biomass deconstruction, will respond to the presence of other microbes by increasing binding to fibrous substrates.

This would allow them more direct access to sugars released during biomass breakdown, which might otherwise be consumed by other microbes.

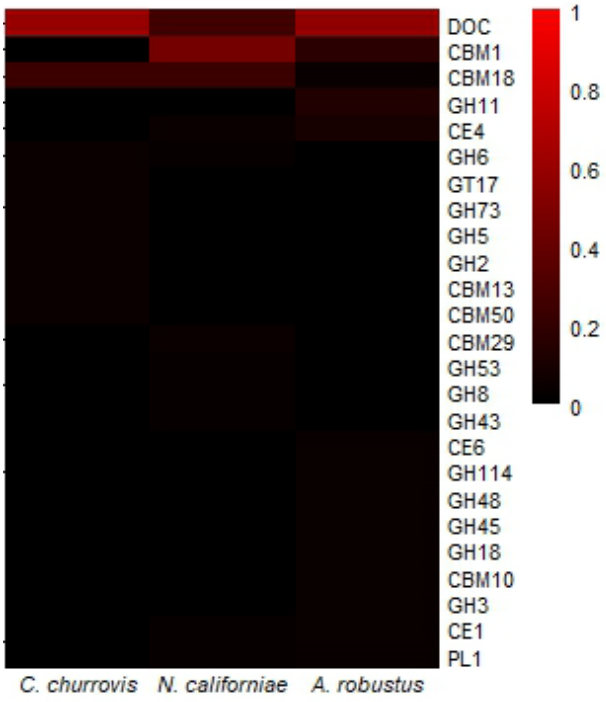


Figure 2.3. Plot of the proportion of genes containing domains belonging to CAZyme gene families or associated enzymatic machinery upregulated in co-cultures of three different fungal strains paired with the same non-native methanogen, *Methanobacterium bryantii* relative to fungal monocultures grown on a reed canary grass substrate. Three different strains of anaerobic fungi, *Anaeromyces robustus*, *Neocallimastix californiae*, and *Caecomyces churrovis* were used to form separate co-cultures with *M. bryantii* and grown on a reed canary grass substrate along with monocultures of each fungus on the same substrate. Differential expression of fungal genes in co-cultures relative to fungal monocultures was determined using DESEQ2. A heatmap of the proportion of genes containing domains belonging to CAZyme gene families or associated enzymatic machinery is shown above. Gene domains were organized into the following classifications: carbohydrate binding modules (CBM), dockerins (DOC), glycoside hydrolases (GH), glycosyltransferases (GT), polysaccharide lyases (PL), and carbohydrate esterases (CE).

2.2.4 Fungal co-culture with a methanogen may enhance PFL function and production of bottleneck enzymes in sugar pathways

Transcriptional regulation coupled with HPLC analysis was used to determine the impact of methanogen co-cultivation on fungal sugar utilization, genes potentially associated with hydrogenosome function, secondary metabolite production, and membrane protein regulation in stable, non-native fungal-methanogen co-cultures. Previous studies of fungal-methanogen co-cultures described increased sugar utilization in co-culture [54,84]. As such, we hypothesized that genes encoding enzymes involved in sugar catabolism would be upregulated in *C. churrovis* and *M. bryantii* co-cultures relative to fungal monocultures. While some enzymes within these pathways showed changes for each substrate, no co-culture condition resulted in uniform up or downregulation of all enzymes within a given sugar pathway, as shown in Supplementary Figure 7.1.6. The enzymes that were upregulated in fungal-methanogen co-culture relative to fungal monoculture on the same substrate may represent bottlenecks in these catabolic pathways. We suspected that sugar utilization in co-cultures could also be increased through upregulation of sugar transporters in the co-culture condition. We instead observe that in the presence of Avicel® and xylan, *M. bryantii* induces transcriptional upregulation of genes that appear to encode proteins homologous to prokaryotic Substrate Binding Proteins (SBPs), as well as Class C G-Protein Coupled Receptors (GPCRs) as seen in Supplementary Table 7.1.2 [85–87]. While the function of these protein domains and receptors remains unknown, we speculate that they may be involved in the increased binding of sugar polymers in the presence of the methanogen; or in establishing physical interactions between the methanogens and fungi [88].

A previous study showed that anaerobic fungal genomes encode a wide array of biosynthetic enzymes of natural products including secondary metabolites - small, bioactive molecules known to mediate a variety of interactions between microorganisms [89–92]. The majority of these genes were not significantly differentially expressed between co-culture and monoculture conditions on the various substrates in this study. However, two of these fungal genes were highly upregulated in co-culture (*p*-adjusted <0.01). The first is a non-ribosomal peptide synthetase (NRPS)-like gene (protein Id 604712), which was upregulated eight-fold during growth on fructose and on Avicel®. The second, a polyketide synthase (PKS; protein Id 402343) was four-fold upregulated in co-culture compared to monoculture during growth on xylan and reed canary grass, suggesting that some fungal secondary metabolites may mediate the interaction between *C. churrovis* and *M. bryantii*, depending on the specific substrate. Co-culture interaction may be most notable on Avicel® and xylan substrates, as both transporters and secondary metabolite biosynthesis genes were upregulated in co-culture for both of these substrates.

Based on previous studies noting an increase in metabolites produced by the ATP-generating fungal hydrogenosome during co-culture with methanogens, we hypothesized that genes associated with hydrogenosomal function would be upregulated in methanogen co-culture [50,54]. A list of genes associated with the fungal hydrogenosome of the *C. churrovis* strain was constructed based on homology with known hydrogenosome components, shown in Supplementary Table 7.1.3. FASTA sequences from known hydrogenosomal components identified in the fungal strain *Neocallimastix lanati* [44] were aligned to filtered model proteins of *C. churrovis* using the blastp alignment program in MycoCosm [62]. One or more genes within the *C. churrovis* genome aligned to all listed hydrogenosomal enzymes found in

N. lanati. Regulation of these genes in co-culture compared to monoculture was examined for each substrate. As shown in Supplementary Table 7.1.3, 21 genes were homologous to both pyruvate formate lyases (PFLs) that were identified in the *N. lanati* genome [44]. This enzyme reversibly converts pyruvate and CoA into acetyl-CoA and formate, which plays a central role in anaerobic glucose fermentation [93]. It has been shown that this enzyme is functional in hydrogenosomes of the anaerobic fungal species *Piromyces* sp. E2 and *Neocallimastix* sp. L2 [94]. The most notable upregulation of PFLs was observed in cultures grown on xylan and fructose, where 15 of the 21 PFL genes identified by homology were upregulated in co-cultures compared to monocultures grown on xylan and two genes identified by homology were upregulated in co-cultures compared to monocultures grown on fructose as shown in Supplementary Table 7.1.3. Five additional genes annotated as PFLs (or formate C acetyltransferases) according to Enzyme Commission (EC) number rather than homology to the *N. lanati* genome were upregulated on xylan and one additional gene was upregulated on fructose. One of these genes (Protein ID 428490) was upregulated in co-culture on all substrates examined except reed canary grass. A previous study examining transcriptional regulation of co-cultures of the native fungus-methanogen pairing *Pecoramyces* sp. F1 with the methanogen *Methanobrevibacter thaueri* versus monoculture of the fungus grown on glucose did not detect a difference in expression levels of PFL genes (although upregulation was detected at the protein level) [50].

Although we hypothesized that genes associated with the hydrogenosome would be transcriptionally upregulated in the co-culture relative to the fungal monocultures based on the metabolic data collected in previous work, transcriptional upregulation of genes associated with hydrogenosomal function is limited, with the exception of pyruvate formate lyases in co-

cultures grown on xylan and fructose. It is important to note that further studies are needed to confirm that this transcriptional upregulation of PFLs is associated specifically with the hydrogenosome, as PFLs function in both the cytosol and the hydrogenosome. However, as a complement to the transcriptional information regarding metabolic function in this study, end point metabolites present in the supernatant were measured using HPLC upon harvest of the co-cultures and monocultures (Figure 2.4). Increases in the amount of acetate produced in co-culture and the absence of significant amounts of ethanol and lactate indicate that some of these genes may potentially be associated with hydrogenosome function for cultures grown on fructose, since pyruvate can either be converted to lactate or ethanol by PFLs functioning in the cytosol or converted to acetate by PFLs functioning within the hydrogenosome. Ethanol was also absent in cultures grown on xylan, although higher levels of lactate were observed in co-culture in addition to higher levels of acetate, indicating that both cytosolic and hydrogenosomal PFLs may be upregulated in co-culture. GSEAPreranked analysis also indicated that upregulated genes were enriched in pathways associated with pyruvate metabolism and glycolysis for co-cultures grown on xylan, in agreement with the observed upregulation of PFLs [81,82].

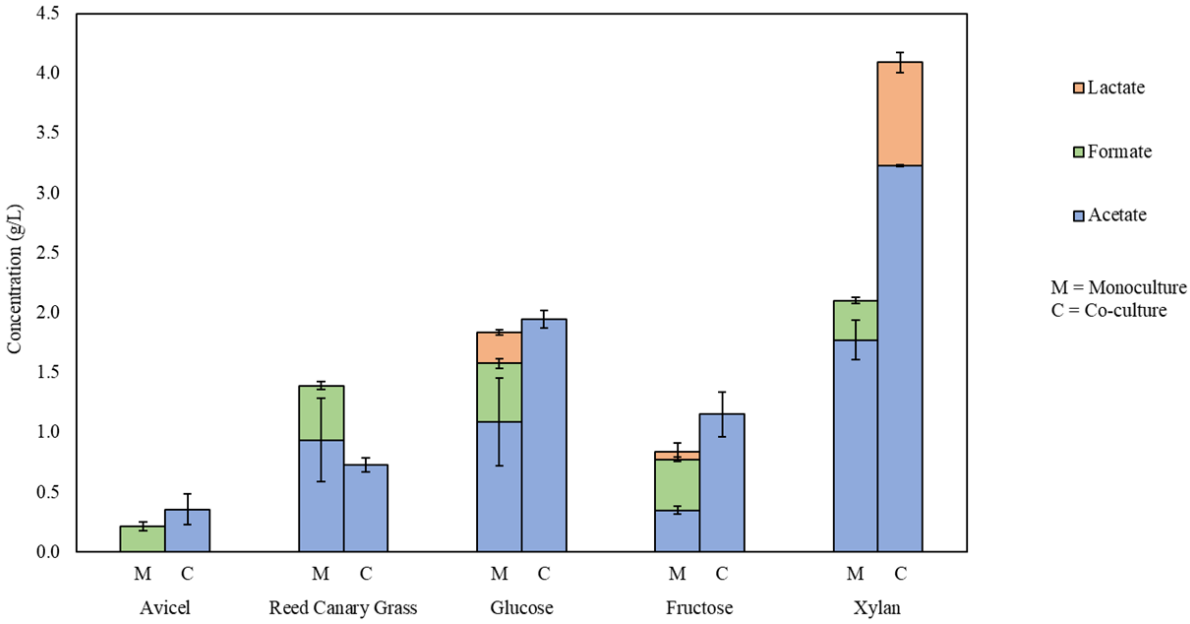


Figure 2.4. Accumulated metabolites for co-cultures of *C. churrovis* paired with *M. bryantii* versus monocultures of *C. churrovis* upon harvest. HPLC data is shown for co-culture and monoculture grown on each substrate. No formate was observed in co-culture on any substrate, suggesting that *M. bryantii* is capable of metabolizing formate. Trace amounts of ethanol were present in the cultures but fell below the 0.1 g/L limit of detection. This, in conjunction with increased levels of acetate in co-culture, indicates that some of the PFLs upregulated in co-cultures grown on xylan and fructose may be functioning within the hydrogensome.

While analysis of the end-point metabolites of *A. robustus* paired with *M. bryantii* in previous work did not indicate a statistically significant difference in the level of formate in co-culture versus monoculture, formate was absent in the *C. churrovis* and *M. bryantii* co-culture samples but present in fungal monocultures [49]. Earlier studies concluded that this type strain of *M. bryantii* (DSM 863 M.o.H.) was unable to produce methane from formate in pure culture [95,96]. The discovery of a formate transporter and several copies of formate dehydrogenase genes upon sequencing the methanogen's genome has suggested the possibility of growth on formate [72]. The observed upregulation of PFL genes and the absence of formate in co-cultures in the current study provides evidence that this strain of *M.*

bryantii can utilize formate under certain conditions. A similar phenomenon has been observed for co-cultivation of a formate-producing *Piromyces* fungal species and the natively associated methanogen *Methanobrevibacter thaueri*, a methanogen that has been shown incapable of growth on formate [58,97]. It is possible that cultivating these methanogens under the conditions required for co-culture with rumen anaerobic fungi stimulates formate utilization by inducing function of the formate transporter and formate dehydrogenase discovered upon sequencing the genome [72].

2.3 Conclusions

Here, we have sequenced the first high-quality genome of a non-rhizoidal fungus, *Caecomyces churrovis*, revealing an abundance of diverse CAZymes and the highest number of CBM family 18 domains among anaerobic fungi sequenced to date. We found that co-cultivation of the *C. churrovis* fungal strain with the non-native methanogen *M. bryantii* enhanced production of transcripts containing these chitin-binding CBM 18 domains across a variety of substrates. Upregulation of CBMs and dockerin domains in fungal-methanogen co-culture with the same non-native methanogen relative to fungal monoculture on a lignocellulose-rich substrate was also observed for two other previously sequenced fungal strains, *A. robustus* and *N. californiae*. We hypothesize that the function of CBMs belonging to family 18 may not be directly related to plant biomass breakdown but instead to interactions between the fungus and methanogen since upregulation of transcripts containing these domains is observed across multiple cultivation conditions, including both cellulose and lignocellulose-rich substrates as well as soluble sugars. Upregulation of genes associated with sugar pathways and the functioning of the hydrogenosome for *C. churrovis* and *M. bryantii* co-cultures relative to fungal monocultures of *C. churrovis* also suggests that co-culture with

a methanogen may enhance pyruvate formate lyase (PFL) function under certain cultivation conditions and production of key enzymes in sugar utilization pathways. Overall, these observations enhance our understanding of the mechanistic interactions between anaerobic fungi and associated methanogens, which aids in our ability to design synthetic biomass-degrading microbial consortia.

2.4 Materials and Methods

2.4.1 Growing and harvesting cultures for RNA extractions

Anaerobic serum bottles containing 80 mL of modified Medium C ("MC-") with 0.8 mL 100 × vitamin solution and 0.8 g reed canary grass were inoculated with cultures of *C. churrovis* and *M. bryantii*: 1.0 mL of *C. churrovis* or a combination of 1.0 mL of *C. churrovis* and 1.0 mL of *M. bryantii* (DSM No.-863, DSMZ) (routine cultures were cultivated as described previously by Swift et al.) [49]. The fungal and methanogen co-cultures and fungal monocultures were grown anaerobically at 39°C in Hungate tubes filled with 9.0 mL of autoclaved modified Medium C [98] ("MC-"), containing 1.25 g/L yeast extract, 5 g/L Bacto™ Casitone, and 7.5 vol% clarified rumen fluid, with either 0.1 g of milled reed canary grass, 0.1 g Avicel®, 0.1 g xylan, 0.5 mL of a 0.1 g/mL sterile filtered glucose stock solution, or 0.1 g/mL of a sterile filtered fructose stock solution as the growth substrate, and supplemented with vitamin solution post-autoclaving [99]. Pressure production was used as a proxy for fungal growth, as described previously [100]. Daily pressure measurements were taken using a probe pressure transducer to determine when the cultures reached the mid-log growth phase, based upon previous pressure growth curves measured to stationary phase growth. Upon reaching mid-log growth phase, cultures were harvested and stored for later

RNA extraction. After sampling the headspace gas of the culture to determine end-point methane and hydrogen concentrations for monocultures and co-cultures, a volume of 1.2 mL of the culture supernatant was pipetted off of the top of the culture and stored at -20ºC for later HPLC analysis. The remainder of the culture was transferred to a 15 mL Falcon® tube and spun down at 10,000 g and 4ºC for 6 minutes. The remaining supernatant was then decanted or pipetted off depending upon the integrity of the remaining cell pellet and replaced with 1 mL of RNA-later and mixed by pipetting. Samples were then stored at -80ºC until extraction.

2.4.2 Measuring hydrogen and methane production

End-point methane and hydrogen measurements for both monocultures and co-cultures were taken from the headspace of the culture tubes before harvesting the cultures. Daily measurements and sampling were performed to monitor the growth of the co-cultures and monocultures. First the pressure in each sample was measured using a pressure transducer [101], and the headspace composition was measured on a gas chromatograph (GC)-pulsed, discharge helium ionization detector (Thermo Fisher Scientific TRACE 1300) [102]. Finally, the headspace pressure of the sample was vented to return the headspace to atmospheric pressure. The total moles of headspace gas were calculated using the ideal gas law. Gas concentrations for H_2 and methane were calculated using an external standard calibration method. The gas concentration could then be multiplied by the number of moles present both before and after the pressure sampling in order to determine the moles of H_2 or methane produced. It was assumed that the amount of gas dissolved in the liquid media was negligible for these calculations.

2.4.3 HPLC analysis

Levels of volatile fatty acids present in the supernatant of both co-cultures and monocultures were measured using an Agilent1260 Infinity HPLC (Agilent)**.** Samples were prepared by acidifying to 5 mM using sulfuric acid and subsequently incubating at room temperature for 5 minutes. Samples were then centrifuged for 5 minutes at 21,000 g. The supernatant was syringe filtered into an HPLC vial (Eppendorf™ FA-45-24-11) using a 0.22 µm PVDF filter. Samples were analyzed on an Agilent 1260 Infinity high-performance liquid chromatography system (HPLC, Agilent, Santa Clara, CA) equipped with an auto-sampler unit (1260 ALS). Separation of formate, acetate, and lactate was achieved with a Bio-Rad Aminex® 87H Ion Exclusion Column for organic acids (Part No. 1250140, Bio-Rad, Hercules, CA) with a mobile phase of 5 mM sulfuric acid. In-house standards were prepared with MC- blank culture medium as a base and sodium formate (ACS Grade, Fisher Chemical S648500), sodium acetate (ACS Grade, Fisher Chemical S210500), and L-lactic acid sodium (99%, extra pure, Acros Organics 439220100) at VFA concentrations of 0.1 and 1 g/L.

2.4.4 Genome sequencing and annotation of anaerobic fungus Caecomyces churrovis

The *Caecomyces churrovis* fungal strain was isolated as described by Henske et al. [45]. Genomic DNA was isolated from cultures grown for 5-7 days on glucose to reduce the interference of plant material during cell lysis. DNA was extracted using the MoBio PowerPlant Pro kit. DNA was isolated from 5-10 cultures grown in 40 mL volumes and pooled together by collecting the DNA in the same silica column. This process was repeated until the total amount of DNA isolated was greater than 12 µg. The *C. churrovis* genome was sequenced using the PacBio sequencing platform. >10kb fragments were size selected using Blue Pippin Size Selection, then 10 ug of genomic DNA was sheared to >10kb fragments

using Covaris g-Tubes. The sheared DNA was treated with exonuclease to remove single-stranded ends and DNA damage repair mix followed by end repair and ligation of blunt adapters using SMRTbell Template Prep Kit 1.0 (Pacific Biosciences). The library was purified with AMPure PB beads and size selected with BluePippin (Sage Science) at >10 kb cutoff size. PacBio Sequencing primer was then annealed to the SMRTbell template library and sequencing polymerase was bound to them using Sequel Binding kit 2.0. The prepared SMRTbell template libraries were then sequenced on a Pacific Biosystems' Sequel sequencer using v3 sequencing primer, 1M v2 SMRT cells, and Version 2.0 sequencing chemistry with 6 hour & 10 hour movie run times. 6mA modifications were detected using the PacBio SMRT analysis platform (pb_basemods package; smrtanalysis version: smrtlink/8.0.0.80529). 6mA modifications were then filtered and methylated genes were identified following the methods described in Mondo et al., 2017 [69]. The assembly was completed with Falcon which generates better assemblies than competing methods likely due to an improvement in isolation of high molecular weight DNA and sequencing larger DNA fragments [22,103,104]. While annotating fungal genomes present a challenge due to the lack of anaerobic fungal gene content in existing databases, the genome was annotated using the JGI Annotation Pipeline, which employs a variety of gene modelers to discover genes [62]. In addition to homology-based modelers, ab-initio gene discovery tools and RNAseq based methods were used for annotation. Models were determined to be allelic if they were located in regions on smaller scaffolds that were > 95% identical at the nucleic acid level and > 50% of the smaller scaffold was covered by these regions. The CAZymes of the *C. churrovis* genome were detected and assigned to families by the curators of the CAZy database using the methods used for the daily

updates of the CAZy database [76,105]. Other fungal genomes included in comparisons were sequenced previously [18,43,44].

2.4.5 Extracting RNA from experimental samples

Samples were removed from storage at -80ºC and thawed on ice. After thawing, samples were spun down for 6 minutes at 4ºC and 10,000 g and RNA*later*™ was removed. Cells were lysed for the reed canary grass and Avicel® cultures using bead beating for 1 minute in 30 second intervals and cells were lysed for glucose, fructose, and xylan cultures using liquid nitrogen grinding. Total RNA was extracted using the RNeasy Mini kit (QIAGEN) following the protocol for "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi" including an on-column DNase digestion. An Agilent TapeStation was used to determine the quality of the sequenced RNA and Qubit High Sensitivity RNA Assay was used to determine concentrations.

2.4.6 RNA sequencing and data analysis

Stranded RNASeq library(s) were created and quantified by qPCR for both monoculture and co-culture samples. Stranded cDNA libraries were generated using the Illumina Truseq Stranded mRNA Library Prep kit. mRNA was purified from 1 ug of total RNA using magnetic beads containing poly-T oligos. mRNA was fragmented and reversed transcribed using random hexamers and SSII (Invitrogen) followed by second strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, adapter ligation, and 8 cycles of PCR. The prepared library was quantified using KAPA Biosystems' next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. For genome annotation, the quantified library was then prepared for sequencing on the Illumina HiSeq

sequencing platform utilizing a TruSeq paired-end cluster kit, v4. Sequencing of the flow cell was performed on the Illumina HiSeq 2500 sequencer using HiSeq TruSeq SBS sequencing kits, v4, following a 2x150 indexed run recipe. Reads filtered for artifacts and trimmed for quality were assembled into consensus sequences using Trinity v. 2.3.2 [106]. For differential gene expression analysis, sequencing of the libraries was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent kits, S4 flowcell, and following a 2x150 indexed run recipe. The filtered reads from each library were aligned to the *Caecomyces churrovis* genome using HISAT2 version 2.1.0 [107]. Strand-specific coverage was generated using deepTools v3.1 [108]. Raw gene counts were generated using featureCounts, with only primary hits assigned to the reverse strand were included in the raw gene counts [109]. Raw gene counts were used to evaluate the level of correlation between biological replicates using Pearson's correlation and determine which replicates would be used in the DGE analysis. DESeq2 (version 1.18.1) [110] was subsequently used to determine which genes were differentially expressed between pairs of conditions. The parameters used to call a gene DE between conditions were p-value < 0.05 and a log2fold change greater than 2. This log2fold change cutoff is more stringent than the typical cutoff used in previous studies to account for variation in undefined rumen fluid components across different batches of media. Raw gene counts, not normalized counts, were used for DGE analysis since DESeq2 uses its own internal normalization. Subsequent analysis was done using the filtered model gene catalog for *C. churrovis* provided for download on the MycoCosm website [62]. Pre-ranked Gene Set Enrichment Analysis (GSEA) of regulated genes in co-cultures relative to fungal monocultures for each substrate condition was conducted using 1,000 permutations and weighted enrichment statistics [81,82]. The TOPCONS web server was used to determine

consensus prediction of membrane protein topology for upregulated and downregulated gene sets and sequences were annotated using Pfam and the HMMER web server [85,111,112].

3 Co-cultivation of anaerobic fungi with *Clostridium acetobutylicum* bolsters butyrate and butanol production from cellulose and lignocellulose

Adapted from work that was accepted for publication in *Journal of Industrial Microbiology & Biotechnology* by Jennifer L. Brown, Matthew A. Perisin, Candice L. Swift, Marcus Benyamin, Sanchao Liu, Vasanth Singan, Yu Zhang, Emily Savage, Christa Pennacchio, Igor V. Grigoriev, and Michelle A. O'Malley, Co-cultivation of anaerobic fungi with *Clostridium acetobutylicum* bolsters butyrate and butanol production from cellulose and lignocellulose, Copyright 2022, with permission from Oxford University Press

3.1 Introduction

Synthetic microbial consortia are promising potential avenues for bio-based chemical production, including the production of biofuels from waste materials. The co-cultivation of microbes with complementary metabolic functions also eliminates the need for genetic manipulation of recalcitrant microbes and provides a division of labor between members to avoid metabolic burden [113,114]. Stable microbial consortia are found in natural environments ranging from soil microbiomes to the digestive tract of herbivores. Across these environments, consortium stability and function depend upon several factors, including functional complementation, redundancy between community members, and nutrient cross-feeding to relax metabolic burdens [47,115,116].

Microbial consortia drive lignocellulose breakdown in the digestive tract of herbivores [117,118], and provide an attractive avenue for lignocellulosic biofuel production. Recently anaerobic fungi were shown to be efficient degraders of lignocellulosic biomass, eliminating

the need for costly and energy-intensive pretreatment processes that produce inhibitory byproducts [18,60,119]. The anaerobic fungi also release excess sugars during lignocellulose breakdown [59] as well as other fermentation products such as acetate, lactate, H_2 and ethanol. In the herbivore rumen, these fungal metabolites are understood to support the growth of other gut microbes such as archaeal methanogens to release methane [25,120,121], but they can also be used to drive growth and bioproduction in microbes that are not native to the rumen microbiome [59,122–124]. While the anaerobic fungi lack reliable genetic tools [125], they can still be harnessed for bioproduction as members of synthetic consortia. These consortia combine the powerful carbohydrate active enzyme (CAZyme) production of anaerobic fungi with genetically tractable microbes that can cross-feed on fungal fermentation products to synthesize value-added chemicals and fuels from lignocellulose.

Here, we constructed and evaluated several synthetic microbial consortia that paired anaerobic fungi with the anaerobic bacterium *Clostridium acetobutylicum*. *C. acetobutylicum* is not native to the rumen microbiome, and cannot digest lignocellulose, but can produce acetone, butanol, and ethanol from fermentable sugars (via ABE fermentation) [126]. Two key phases occur during batch fermentation of *C. acetobutylicum* [127–132]. During exponential growth, *C. acetobutylicum* produces acetate and butyrate resulting in a lower culture pH (acidogenesis). These short chain fatty acids are then reassimilated during solventogenesis, corresponding to an increase in pH, production of acetone, n-butanol, and ethanol, and initiation of bacterial sporulation [133,134]. While microbially sourced biobutanol is not currently economically competitive with petrochemical synthesis, advances in genetic engineering of *C. acetobutylicum* and improvements to downstream processes have been shown to elevate n-butanol production in *C. acetobutylicum* [135,136].

Pairing *C. acetobutylicum* with an efficient lignocellulosic biomass degrader like an anaerobic fungus provides an economic advantage by enabling bio-based production of fuels from low-cost feedstocks [137]. For example, the excess sugars released by anaerobic fungi during biomass breakdown [59] could potentially support the growth of *C. acetobutylicum*, which can utilize both hexose and pentose sugars but has been shown to preferentially metabolize hexose sugars over pentose sugars [138]. Similar co-culture strategies have been previously used, where anaerobic fungus *Pecoramyces* sp. F1 and associated methanogens were able to pretreat and saccharify lignocellulosic biomass to support ethanol production by the bacterium *Z. mobilis* [124]. Moreover, a previous study combined *C. acetobutylicum* with undefined rumen fluid to hydrolyze pretreated agave and enhance hydrogen and butanol production [139]. Based on prior work and our current knowledge of anaerobic fungal metabolism [44,59], we hypothesized that metabolic cross-feeding of lactate and/or succinate between anaerobic fungi and *C. acetobutylicum* would enable synergistic growth . In turn, *C. acetobutylicum*'s uptake of the lactate produced by co-cultured anaerobic fungi would relieve production inhibition experienced by the fungi in monoculture.

Here, we construct and compare two co-culture strategies to anaerobically produce butyrate and butanol from plant-derived biomass. In the first strategy, we simultaneously co-cultured several strains of anaerobic fungi with bacterium *C. acetobutylicum* on lignocellulose and measured generated fermentation products over the course of nearly 30 days. In the second strategy, fungi were initially cultivated for 22 days to release fermentable sugars and metabolic byproducts, and *C. acetobutylicum* was added directly to fungal supernatant to facilitate butyrate/butanol production. There was at least 4.5 mM more average butyrate produced in the one-stage cultivation condition versus the two-stage condition, with as much

as 30 mM of butyrate being produced by *C. acetobutylicum* paired with the *N. californiae* strain after 30 days of fermentation. Alternatively, significantly more butanol was produced in all experimental conditions compared to *C. acetobutylicum* monoculture controls for long-term cultivation, with as much as 9 mM butanol being produced by *C. acetobutylicum* paired with the *N. californiae* strain in the two-stage experimental condition. While there remains significant room for optimization following these initial studies, we have shown that long-term cultivation of different strains of anaerobic fungi and *C. acetobutylicum* is a promising route for bio-butyrate and biobutanol production, and that lactate cross-feeding likely occurs between anaerobic fungi and *C. acetobutylicum*, which bolsters butyrate production by *C. acetobutylicum* in the co-cultivation condition.

3.2 Results and Discussion

Anaerobic fungi produce a diverse array of CAZymes capable of converting lignocellulosic biomass to soluble sugars that can support the growth of other microbes [59]. Here, we leveraged metabolic end products of fungal growth (e.g. fermentable sugars, lactate, acetate, and formate) to enable metabolic cross-feeding to enhance production of industrially-relevant biochemicals through synthetic co-culture. Based on previous work that showed assimilation of the fermentation product lactate by *C. acetobutylicum* [140–142], a microbe commonly used in ABE bioprocesses, we hypothesized that partnership between anaerobic fungi and this anaerobic bacterium would elevate bacterial butyrate or butanol production via metabolic cross-feeding of fungal-produced lactate (Figure 3.1) [143–145].

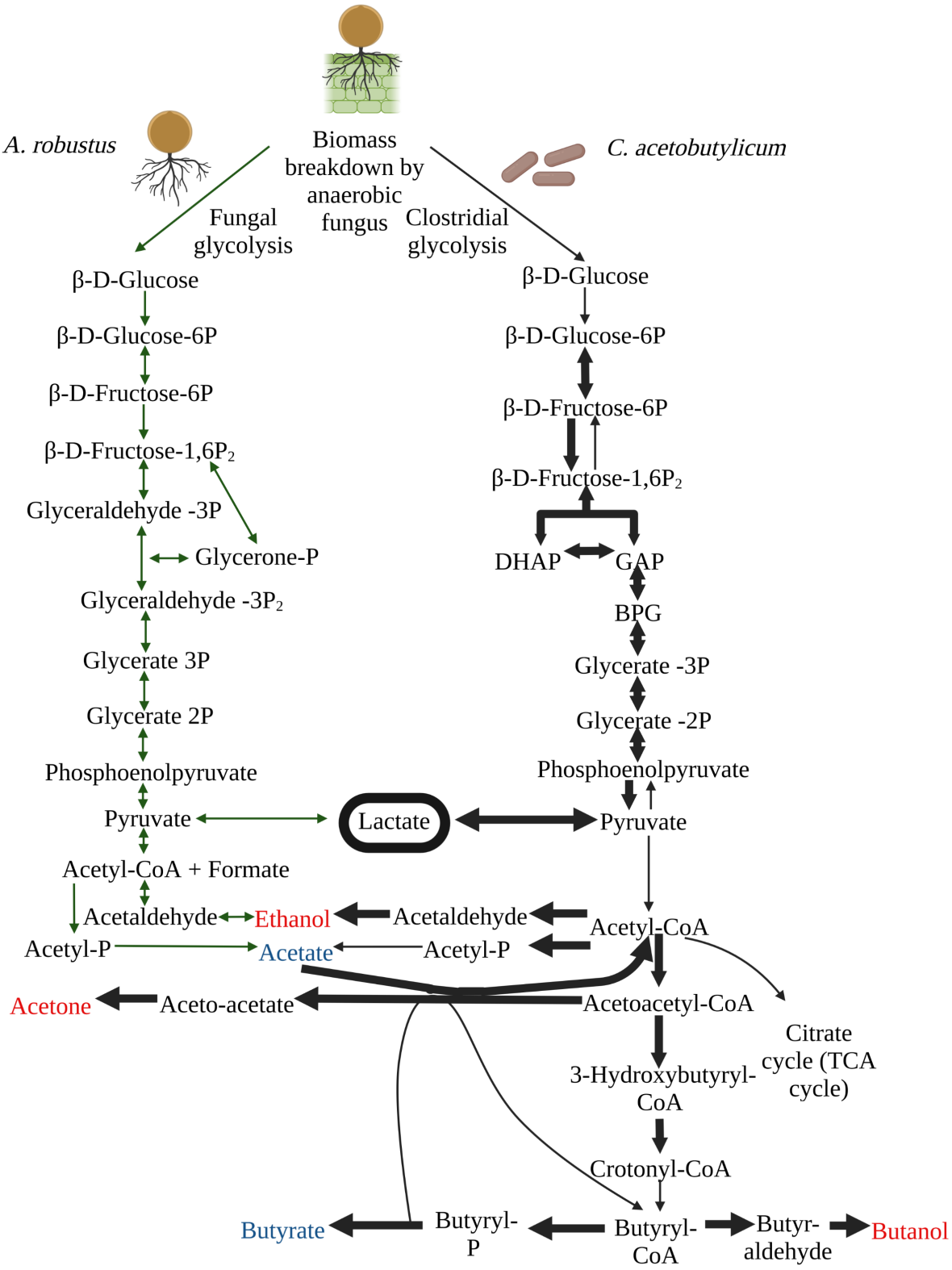
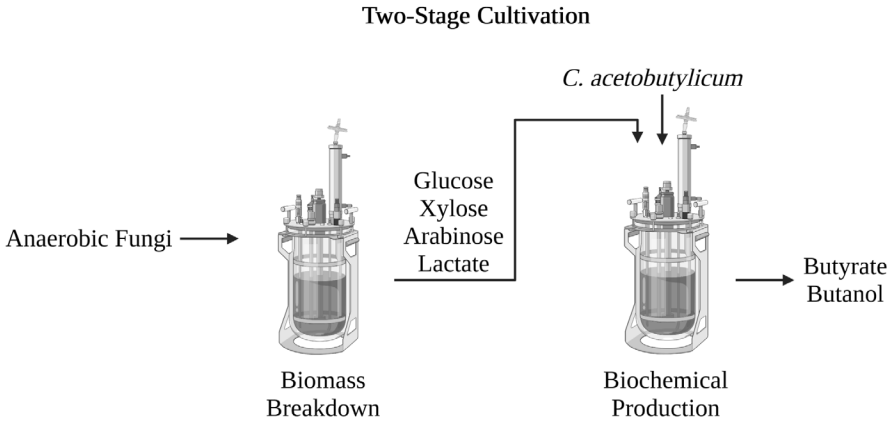

A. robustus
Biomass breakdown by anaerobic fungus
C. acetobutylicum
Fungal glycolysis
Clostridial glycolysis
β-D-Glucose
β-D-Glucose-6P
β-D-Fructose-6P
β-D-Fructose-1,6P2
Glyceraldehyde -3P
Glycerone-P
Glyceraldehyde -3P2
Glycerate 3P
Glycerate 2P
Phosphoenolpyruvate
Pyruvate
Lactate
Acetyl-CoA + Formate
Acetaldehyde
Ethanol
Acetyl-P
Acetate
Acetone
Aceto-acetate
DHAP
GAP
BPG
Glycerate -3P
Glycerate -2P
Acetyl-CoA
Acetoacetyl-CoA
3-Hydroxybutyryl-CoA
Crotonyl-CoA
Butyryl-CoA
Butyryl-P
Butyrate
Butyr-aldehyde
Butanol
Citrate cycle (TCA cycle)

Figure 3.1. Metabolic map of major fermentation products and potential for lactate cross-feeding in an anaerobic co-culture system composed of anaerobic fungi and anaerobic bacterium *C. acetobutylicum*. Metabolic map of major fermentation products and potential for lactate cross-feeding in an anaerobic co-culture system composed of anaerobic fungi and anaerobic bacterium *C. acetobutylicum*. The breakdown of plant biomass (lignocellulose or cellulose) is carried out by enzymes secreted by anaerobic fungi, enabling conversion of released glucose by the fungus and bacteria. Major metabolic steps in glucose utilization are shown for the anaerobic fungus *A. robustus* as well as the anaerobic bacterium *C. acetobutylicum*. Red text is used to denote primary products produced by *C. acetobutylicum* under solventogenesis conditions. Blue text denotes primary products produced by *C. acetobutylicum* under acidogenesis conditions. Lactate (circled) is produced by both *C. acetobutylicum* and anaerobic fungi and it is hypothesized that *C. acetobutylicum* can crossfeed lactate via a mechanism for lactate metabolism based on the lactate oxidation pathway in *Acetobacterium woodii*. This mechanism couples a flavin adenine dinucleotide (FAD)-dependent lactate dehydrogenase with an electron flavoprotein complex to convert a reduced ferredoxin, lactate, and two oxidized nicotinamide adenine dinucleotides (NAD) to an oxidized ferredoxin, pyruvate, and two reduced nicotinamide adenine dinucleotides (NADH) [146,147]. Bold arrows denote that the TPM count of at least one gene associated with the conversion is equal to or exceeds the median TPM count (491.85) for all genes expressed in the pathways shown via RNA-Seq. Annotations were obtained from Crown et al., 2010 and Dash et al., 2014; genes associated with lactate formation were obtained from KEGG [143–145]. Genes associated with lactate formation are also in agreement with i802 *C. acetobutylicum* model [145]. Image made using Biorender.

To test our hypothesis, we both co-cultivated anaerobic fungi with *C. acetobutylicum* and grew *C. acetobutylicum* in spent fungal supernatant in a defined medium (Figure 3.2). The supernatant of two-stage cultures grown in spent fungal supernatant was cultivated for 22 days before *C. acetobutylicum* inoculation and then sampled for fermentation products after 10 days of *C. acetobutylicum* growth (Figure 3.2A). One-stage, or simultaneously co-cultivated, culture supernatant was sampled after 29 days of growth (Figure 3.2B). We cultivated the fungus on a complex lignocellulosic substrate, reed canary grass, in order to better reflect cultivation conditions found in industrial bioprocesses and because anaerobic fungi are capable of growth on unpretreated lignocellulose. *C. acetobutylicum* was paired with

one of three different strains of anaerobic fungus, *Neocallimastix californiae, Anaeromyces robustus* or *Caecomyces churrovis* to test whether the metabolic co-cultivation strategy was generalizable across anaerobic fungal strains.

A



B

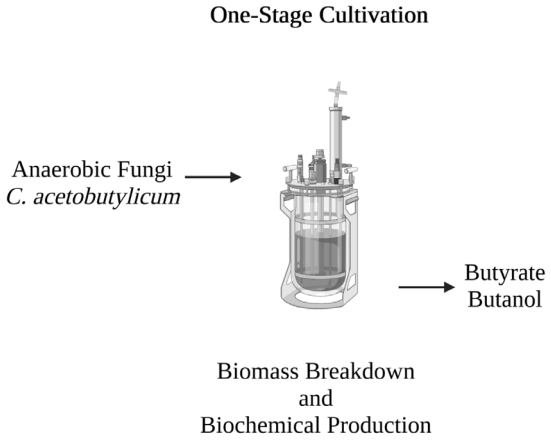


Figure 3.2. (A) Schematic of the two-stage anaerobic cultivation experiment to produce biobutanol and bio-butyrate from cellulose or lignocellulose. The anaerobic fungus (either *C. churrovis*, *N. californiae*, or *A. robustus*) was inoculated into a culture containing reed canary grass and allowed to grow for 22 days. After 22 days of fungal growth, anaerobic bacterium *C. acetobutylicum* was inoculated directly into fungal supernatant with the reed canary grass substrate still remaining in it and grown for 10 days. Analogous short-term experiments were also conducted, whereby anaerobic fungus *A. robustus* was inoculated into a culture containing filter paper and allowed to grow for 8 days to release fermentation products. After 8 days of fungal growth, anaerobic bacterium *C. acetobutylicum* was inoculated directly into sterile-filtered spent fungal cultures and grown for another 56 hours. (B) Schematic of a one-stage simultaneous anaerobic cultivation experiment to produce biobutanol and bio-butyrate from cellulose or lignocellulose. Anaerobic fungal strains *C. churrovis, A. robustus, or N. californiae* were inoculated into a culture containing reed canary grass as well as *C. acetobutylicum* to facilitate simultaneous metabolic cross-feeding and produce biobutanol and bio-butyrate over a period of 29 days. Analogous short-term experiments were also conducted, whereby anaerobic fungus *A. robustus* was inoculated into a culture vessel containing filter paper and allowed to grow for 24 hours prior to inoculation with *C. acetobutylicum* for simultaneous growth and release of fermentation products. Image made using Biorender.

3.2.1 Gas production provides evidence of established anaerobic co-cultures

Proliferation of all monocultures and co-cultures was non-invasively monitored by gas pressure production measurements as a proxy for growth, as shown in Figure 3.3A-D. In all cases, fermentation gas pressures are well beyond typical values seen for fungal monoculture growth [59], indicating that both anaerobic fungi and anaerobic bacteria actively grow in both one-stage and two-stage cultures. For the two-stage growth scheme, *C. acetobutylicum* was quickly able to establish in the culture without an appreciable lag phase and rapidly contributed to fermentation gas production long after cessation of fungal growth as evidenced by the long pressure plateau (Figure 3.3A-C). Nevertheless, one-stage fermentation with both *C. acetobutylicum* and the anaerobic fungal strains led to synergistic growth and greater pressure production relative to the two-stage fermentation for all strains tested. Notably, co-cultures that included fungal strains with extensive rhizoidal networks (*A. robustus* and *N. californiae*) [20] resulted in the highest accumulated gas pressures (Figures 3.3A-B) compared to those that contained the non-rhizoid forming fungus *C. churrovis* [45] (Figure 3.3C). While non-rhizoidal strains such as *C. churrovis* are well-suited for cultivation conditions that allow the concentrations of anaerobic fungi to be measured and subsequent flux calculations to be performed [148], the current study suggests a potential trade-off to using non-rhizoid-forming fungi to liberate sugar from lignocellulose, which ultimately limits its ability to generate nutrients for a co-cultured partner like *C. acetobutylicum*. Gas pressure readings also showed that addition of *C. acetobutylicum* to reed canary grass in the absence of fungi led to negligible growth, as this bacterium does not possess CAZymes to hydrolyze lignocellulose (Figure 3.3D).

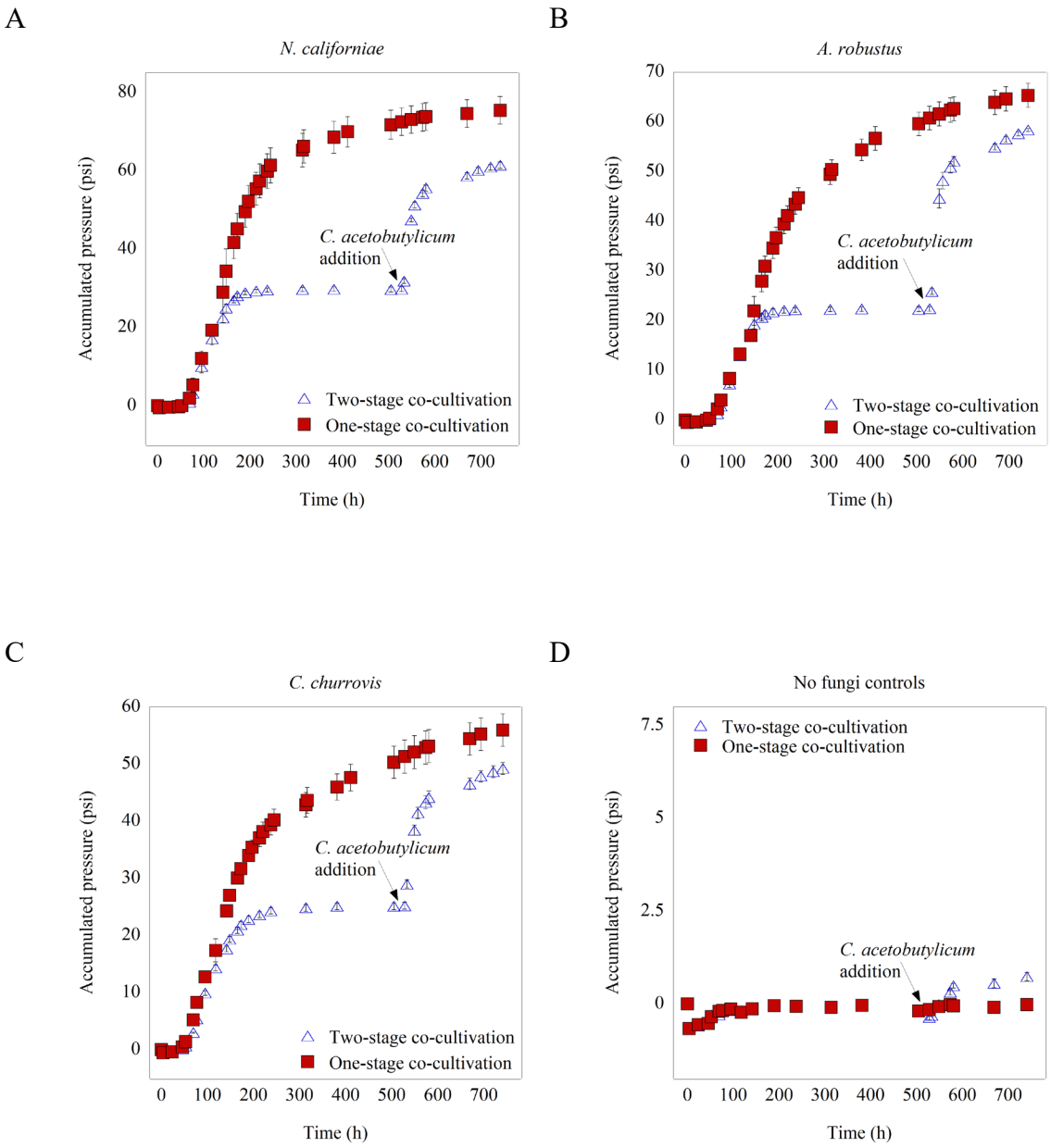


Figure 3.3. Cumulative pressure data for long-term experiments indicate increased pressure production in the one-stage co-cultivation condition. An arrow is used to indicate when *C. acetobutylicum* was added to cultures for all two-stage conditions. One-stage fermentation with both *C. acetobutylicum* and the indicated fungal strains led to synergistic growth and greater pressure production relative to the two-stage condition for each fungal strain tested. This provides further evidence, in addition to the HPLC data, that suggests that *C. acetobutylicum* cross-feeds lactate from the anaerobic fungi and benefits fungal growth. The mean value is plotted for each set of replicates and error bars indicate standard deviation.

3.2.2 Excess sugars released by anaerobic fungi support growth of C. acetobutylicum

HPLC analysis of spent monoculture and co-culture supernatant provided evidence that lignocellulose-derived sugars supported the growth of *C. acetobutylicum* in co-cultures (Figure 3.4). Rhizoidal fungi (*N. californiae* and *A. robustus*) led to the release of 7–11 mM of glucose and 8–11 mM xylose in all experimental conditions, similar to the observation in a previous study that excess sugars released by anaerobic fungi could support the growth of *Saccharomyces cerevisiae* [59]. In both two-stage and one-stage co-culture conditions, a drastic reduction of fermentable sugars was seen, which is evidence of their assimilation by *C. acetobutylicum*. Sucrose and arabinose were also released by all strains of anaerobic fungi as shown in Supplementary Figure 7.2.1A and B, but sucrose was not significantly utilized by *C. acetobutylicum* in any experimental condition. While arabinose was consumed by *C. acetobutylicum* in all experimental conditions, the concentration of arabinose released (5-6 mM) was significantly less than the amount of glucose and xylose released by *N. californiae* and *A. robustus*. It has been demonstrated previously that *C. acetobutylicum* consumes arabinose before xylose [149], however, *C. acetobutylicum* will produce more acetate than butyrate when metabolizing arabinose [149]. The greater increase in butyrate compared to acetate for our data supports the observation that a greater amount of glucose and xylose is consumed compared to arabinose. As compared to the rhizoid-forming fungi, the non-rhizoid forming *C. churrovis* produced a far lower concentration of fermentable sugars (1–2 mM glucose above autoclaved controls). The two-stage cultivation condition has as much or less glucose or xylose as the one-stage cultivation condition for each fungal strain pairing. This is in agreement with the previous observation that less pressure was produced in the two-stage cultivation condition than in the one-stage cultivation condition for all fungal strain pairings

as shown in Figure 3.3. More sugar would be required in the two-stage cultures if the growth of two-stage cultures eventually exceeded the growth of one-stage cultures. It is reasonable to presume that the fungi are dead or dormant in two-stage cultures, preventing *C. acetobutylicum* from accessing more sugar from the biomass substrate.

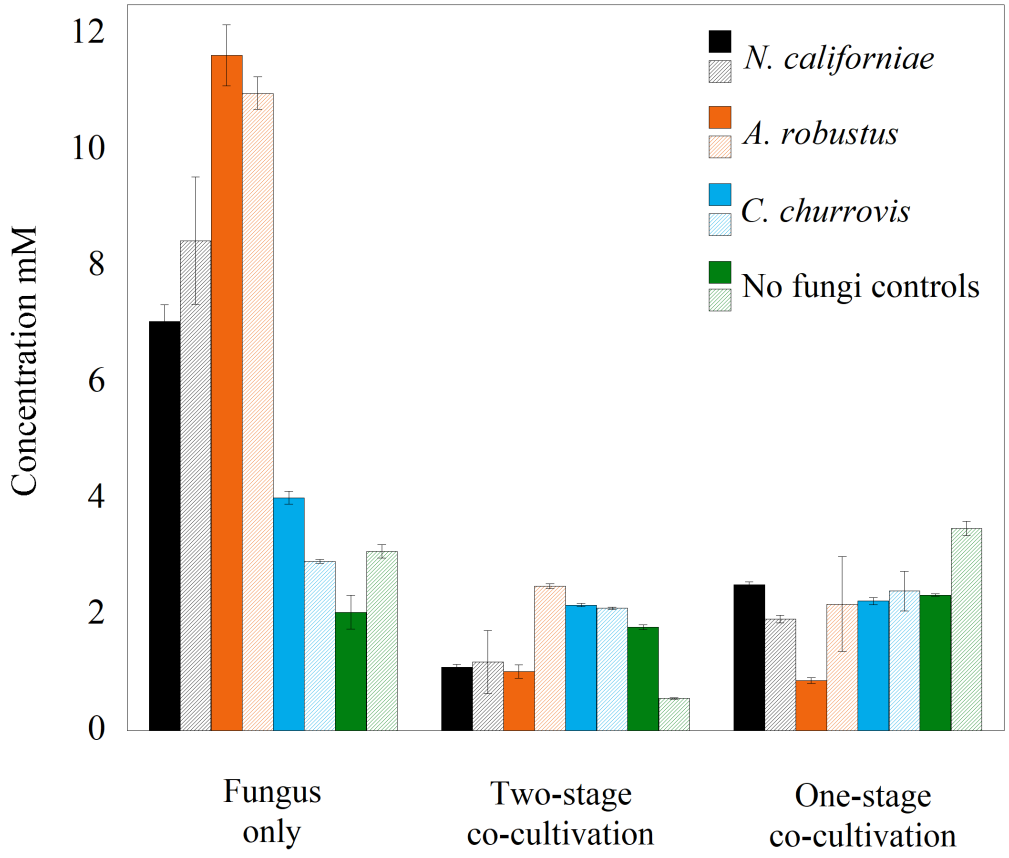


Figure 3.4. **Released fermentable sugars in fungal monocultures versus fungal-bacterial co-cultures grown in M2 media on reed canary grass.** Sugar concentrations were measured after 29 days of microbial growth for the one-stage co-cultivation condition or 10 days of *C. acetobutylicum* growth for the two-stage co-cultivation condition grown in spent fungal supernatant that the fungi had grown for 22 days previously. Solid fill indicates glucose measured and patterned fill indicates xylose measured. Colors correspond to a particular fungal strain used in the monoculture or co-culture as provided in the legend. Higher levels of glucose and xylose were released compared to sucrose and arabinose (graphs for sucrose and arabinose included in the supplement). With the exception of sucrose, sugars released by the fungi were significantly depleted in all experimental cultures containing *C. acetobutylicum*, indicating that sugars released by the fungus can sustain growth of *C. acetobutylicum*. The mean value is plotted for each set of replicates and error bars indicate standard deviation.

3.2.3 Co-cultivation results in increased butyrate production, lactate cross-feeding, and increased butanol production

In all co-culture conditions tested, significant amounts of both butanol and butyrate were produced from lignocellulose for all co-culture combinations tested (Figure 3.5). The amount of butyrate produced by *C. acetobutylicum* one-stage co-cultivation condition was higher than the amount of butyrate produced in the two-stage condition after almost 30 days across all fungal strains tested in the co-culture (Figure 3.5A). There was at least 4.5 mM more average butyrate produced in the one-stage cultivation condition versus the two-stage condition for all fungal strains in the study. Conversely, lower levels of lactate were detected in all experimental conditions compared to fungal controls (Figure 3.5B), providing evidence that *C. acetobutylicum* likely cross-fed lactate produced by the anaerobic fungi, which bolstered butyrate production (Figure 3.5A). The concentrations of acetate and ethanol were found to be slightly to moderately elevated in all co-culture conditions relative to monoculture controls (Supplementary Figure 7.2.2A and B). Additionally, no significant changes were found in the concentrations of formate in the co-cultures versus monocultures (Supplementary Figure 7.2.2C), which further supports that lactate assimilation by *C. acetobutylicum* was responsible for elevated butanol and butyrate production. It has been shown previously that clostridium species, *Clostridium beijerinckii*, *Clostridium propionicum*, and *Clostridium tyrobutyricum* were able to ferment lactate [142,150,151]. *C. acetobutylicum* cultures have been shown to metabolize lactate in corn steep liquor and in semi-defined medium with glucose and lactose [140,141]. *C. acetobutylicum* strain P262 has been shown to use lactate as an energy source in the presence of acetate as a co-substrate [142].

These cultures also reached the solventogenic growth phase of *C. acetobutylicum*, as demonstrated by the butanol production in all experimental conditions, shown in Figure 3.5C. Higher butanol levels were observed in the two-stage experimental condition compared to one-stage co-cultivation for co-cultures formed with the *A. robustus, N. californiae*, and *C. churrovis* strains. These results suggest that two-stage cultivation shifts *C. acetobutylicum* to the solventogenesis phase of growth, which is preferable for production of butanol under these conditions. Conversely, one-stage production under these conditions keeps within the acidogenic growth phase to promote production of more butyrate. This earlier shift to solventogenesis could also explain the lower pressure production in the two-stage condition as shown in Figure 3.3.

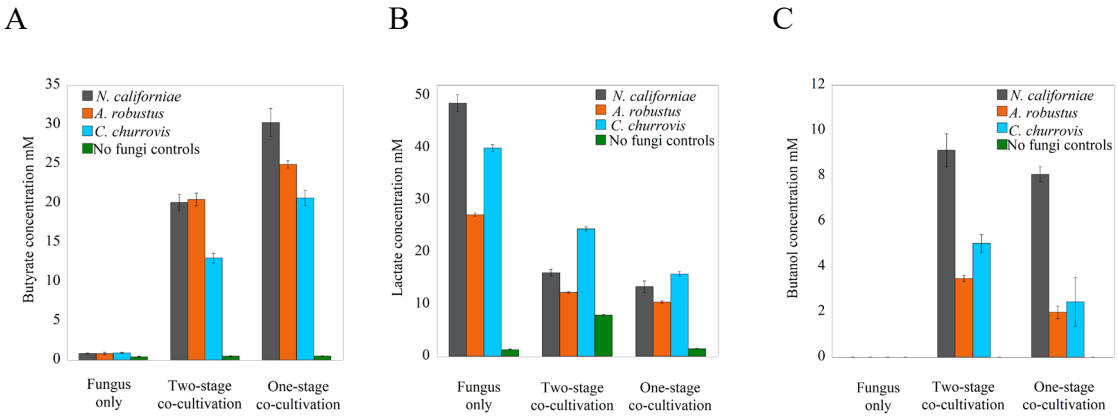
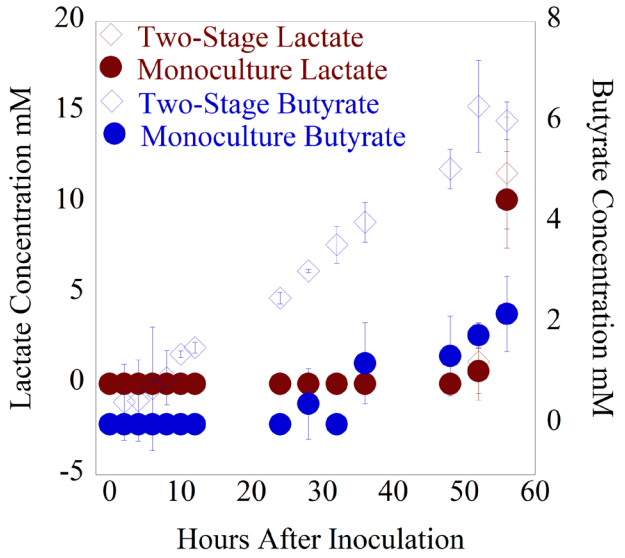


Figure 3.5. **Production of butyrate, lactate, and butanol in cultures grown in M2 on reed canary grass.** Concentrations were measured after 29 days of microbial growth for the one-stage co-cultivation condition or 10 days of *C. acetobutylicum* growth in the two-stage co-cultivation condition grown in spent fungal supernatant that the fungi had grown in for 22 days previously. Lactate cross-feeding occurs in both experimental conditions. Butanol was produced in the long-term cultivation condition, in contrast to the short-term cultivation condition. Butyrate levels were significantly increased for all experimental conditions relative to controls. The mean value is plotted for each set of replicates and error bars indicate standard deviation.

3.2.4 Short-term two-stage cultivation of anaerobic fungi and C. acetobutylicum *also bolsters butyrate production*

We also both co-cultivated the anaerobic fungus *Anaeromyces robustus* and *C. acetobutylicum* and grew *C. acetobutylicum* in spent *A. robustus* fungal supernatant in a defined medium short-term (see methods) to examine any metabolic and/or transcriptional response of *C. acetobutylicum* to fungal co-cultivation. While fungal metabolites and released sugars did not accumulate in an amount that exceeded the limit of detection for HPLC readings in the duration of the experiment (8 days of growth), a significant increase in butyrate production was observed in *C. acetobutylicum* cultures grown in spent fungal media versus *C. acetobutylicum* controls grown in defined media, as shown in Figure 3.6A. These cultures were kept in the anaerobic chamber in loosely capped culture vessels so gas production could not be measured. OD600 levels were measured upon harvest but were comparable for both conditions. At the final timepoint upon harvest at 56 hours, over 2.5 times more butyrate was measured in the cultures of *C. acetobutylicum* grown in spent fungal supernatant versus the *C. acetobutylicum* controls grown in defined media. Production of lactate, acetate, and ethanol did not significantly differ between experimental and control conditions as shown in Figure 3.6A and Supplementary Figure 7.2.3, although the amount of lactate measured in the culture at 55 hours after inoculation (when the cultures were harvested for RNA extraction) significantly increased in both experimental and control cultures while the amount of measured ethanol in the cultures dropped, indicating a metabolic switch to the conversion of pyruvate to lactate instead of acetyl-CoA.

A



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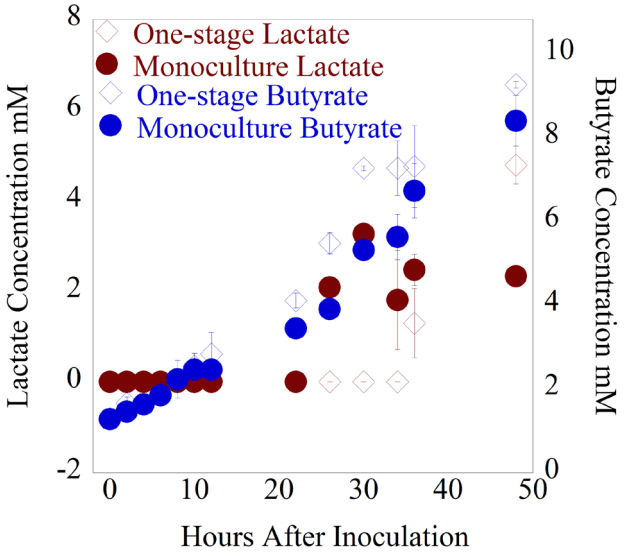


Figure 3.6. (A) Timecourse graph of lactate and butyrate production for *C. acetobutylicum* cultivated in anaerobic fungal supernatant (short-term co-cultivation) and *C. acetobutylicum* monoculture controls grown in media the fungi had not grown in previously. Sugar release was also measured but did not exceed the limit of detection (0.1 g/L). (B) Timecourse graphs (short-term co-cultivation) of lactate and butyrate production for *C. acetobutylicum* co-cultured with the anaerobic fungal strain *A. robustus* and *C. acetobutylicum* monoculture controls. Sugars released were also measured but did not exceed the limit of detection in any of the cultures (0.1 g/L). Significantly higher levels of lactate were detected in the cultures in which *C. acetobutylicum* was co-cultivated with actively growing *A. robustus*, even though fungal monoculture controls did not grow enough to produce fungal metabolite levels above the limit of detection. The mean value is plotted for each set of replicates and error bars indicate standard deviation.

For the two-stage co-culture with *A. robustus*, we transcriptionally verified the metabolic pathways of *C. acetobutylicum*, including core carbon metabolism genes (glycolysis, acetate, butyrate, butanol, and acetone fermentative pathways) as shown in Figure 3.1. Both *C. acetobutylicum* and *A. robustus* produce lactate and acetate. Ethanol is produced by both microbes when *C. acetobutylicum* enters the solventogenic phase of metabolism. Transcripts per million (TPM) counts indicated that genes associated with core carbon metabolism were actively expressed. The observed metabolic switch to the conversion of pyruvate to lactate instead of acetyl-CoA at 56 hours after inoculation (when the cultures were harvested for RNA extraction) was also reflected in TPM counts. TPM counts for genes associated with the conversion of pyruvate to lactate exceeded the median TPM count for all genes in core carbon metabolism pathways, while TPM counts for genes associated with the conversion of pyruvate to acetyl-CoA fell below the median TPM count (shown in Figure 3.1).

As mentioned previously, 2.5 times more butyrate was produced in cultures of *C. acetobutylicum* grown in spent fungal supernatant from *A. robustus* cultures than in cultures grown in media controls without fungal supernatant. Overall, the high expression of the gene (CA_P0035) that produces the enzyme acetaldehyde dehydrogenase (EC 1.2.1.10)/alcohol dehydrogenase AdhE (EC 1.1.1.1) is in agreement with the high levels of ethanol production relative to acetate. The lack of butanol would also indicate that this enzyme is associated with the conversion of acetyl-CoA to ethanol as opposed to the conversion of butyryl-CoA to butanol. Butyryl-CoA is metabolically downstream of acetyl-CoA, thus the shunting of acetyl-CoA to ethanol is a possible explanation for the limited butanol production [152].

3.2.5 RNA-Seq indicates that two-stage cultivation may decrease time required to reach solventogenesis and to relieve carbon catabolite repression

Differential gene expression analysis of *C. acetobutylicum* in the short-term two-stage cultivation condition with *A. robustus* hydrolysate versus the *C. acetobutylicum* monoculture condition revealed that 94 genes were upregulated in the two-stage condition and 64 genes were upregulated in the monoculture condition. Three of the top ten genes upregulated in the two-stage condition are associated with solventogenic pathways (Table 3.1). Genes CA_P0163 and CA_P0164 encode the alpha and beta subunits of butyrate-acetoacetate CoA-transferase (*ctfA* and *ctfB*) associated with the conversion of acetoacetyl-CoA to acetoacetate, indicating that metabolism in the two-stage condition is likely further along in solventogenesis compared to the monoculture. The alcohol dehydrogenase *adhE1* (CA_P0162), which catalyzes butanol production, was also upregulated in the two-stage condition, further supporting that *C. acetobutylicum* was further into solventogenesis compared to monoculture. It is well-established that these pSOL1 megaplasmid genes are expressed at the onset of solventogenesis [153,154] and were shown to be transcriptionally upregulated in a previous study at the onset of solvent formation [155]. In a study by Alsaker et al., other pSOL1 megaplasmid and chromosomal genes (CA_P0165, CA_C3299, and CA_C3298) were upregulated at the onset of solvent formation, and expression increased continuously throughout stationary phase with the exception of CA_C3299 [155]. In our study, CA_P0165 was also upregulated in the two-stage condition at a log2fold change of 1.63 (not reaching the cutoff for the top ten most highly upregulated genes). Since butanol was not detected in the cultures upon harvest and pSOL1 megaplasmid genes CA_C3299 and CA_C3298 were not yet upregulated, it is possible that the switch to solventogenesis was just beginning at the time

of harvest. Though the monoculture displayed reduced expression of the solvent genes mentioned above, CA_C0017 which encodes a seryl-tRNA synthetase was upregulated in the monoculture and has been shown previously to be upregulated during solventogenesis [152]. These results may indicate that *C. acetobutylicum* reaches the solventogenesis phase faster in the two-stage experimental culture condition compared to monoculture.

Table 3.1. **Top ten upregulated genes in *C. acetobutylicum* in the two-stage co-cultivation condition versus *C. acetobutylicum* monoculture.** Genes highlighted in yellow are associated with metabolic pathways involved in metabolite production for *C. acetobutylicum*. Genes highlighted in blue are associated with cellulose degradation, although *C. acetobutylicum* has not been shown to degrade cellulose.

Locus Tag	Gene Product Name	Log2fold Change
CAC3428	hydroxylamine reductase	3.91
CA_P0163	butyryl-CoA: acetoacetate CoA-transferase alpha subunit (EC 2.8.3.9)	2.28
CAC2750	hydroxylamine reductase	2.20
CAC3682	potassium-transporting ATPase subunit A	2.18
CAC3526	FMN-binding protein	2.15
CAC0161	ABC transporter (permease)	2.15
CAC0910	Putative cellulosomal scaffolding protein precursor, secreted; cellulose-binding and cohesin domain	2.11
CA_P0162	acetaldehyde dehydrogenase (EC 1.2.1.10)/alcohol dehydrogenase AdhE (EC 1.1.1.1)	2.09
CAC0911	cellulose 1,4-beta-cellobiosidase (EC 3.2.1.91)	2.05
CA_P0164	butyryl-CoA: acetoacetate CoA-transferase beta subunit (EC 2.8.3.9)	2.03

Of the genes upregulated in the monoculture condition, CA_C3037 which encodes the carbon catabolite repressor *ccpA* was particularly interesting. This finding corresponded to several predicted or experimentally confirmed [156] CcpA regulated genes that were upregulated in the two-stage condition, including genes encoding a potassium-transporting ATPase (CA_C3682), glucanase (CA_C2807), pentose utilization enzyme (CA_C1349), and pectate lyase (CA_C1968). These upregulated genes indicate that the two-stage condition resulted in relieved carbon catabolite repression at the time of sampling.

Although it does not degrade lignocellulose, the *C. acetobutylicum* genome does contain several cellulase-encoding genes and a complete cellulosome cluster of genes, and a low level of induction of cellulase activity has been shown to occur during growth on xylose [157]. Genes CA_C0910 and CA_C0911, both highlighted in blue in Table 3.1, are associated with cellulose-binding and cellulose degradation [158]. Notably, these genes have not been found to be regulated by CcpA [156]; however, CA_C0911 has been shown to have decreased protein abundance for *C. acetobutylicum* grown on glucose compared to xylose, thus indicating potential carbon catabolite mediated regulation [157]. In addition to the two genes associated with cellulose degradation found in the top ten upregulated genes list, 5 more are upregulated with a log2foldchange greater than 1, for a total of 7 out of the 12 genes associated with cellulose degradation [158] being upregulated in the two-stage cultivation condition, shown in Supplementary Table 7.2.1. One of the genes associated with cellulose-binding and cellulose degradation (*celF*, CA_C0911), a family 48 glycoside hydrolase enzyme with exoglucanase activity, is a conserved feature between cellulosomes produced by clostridial species and appears to contribute to cellulosome function [157].

3.2.6 Short-term one-stage cultivation results in increased lactate production

In our short-term study, butyrate production in the co-cultivation experimental condition did not significantly differ between the co-cultivation and monoculture control conditions (Figure 3.6B). However, twice the lactate was produced in the co-cultivation condition versus the *C. acetobutylicum* monoculture control (Figure 3.6B). This could be due to lactate production by the fungus, although lactate levels in fungal monoculture controls did not exceed the limit of detection during the experiment; therefore, it is also possible that the fungus was instead supporting increased lactate production by *C. acetobutylicum* through the

release of sugars from the cellulose substrate. Acetate and ethanol levels were slightly higher in the co-cultivation conditions, as shown in Supplementary Figure 7.2.4, again due to either metabolite production by the anaerobic fungus or due to increased clostridial growth due to the release of sugars. No butanol production was observed in either of the short-term cultivation conditions, indicating that the cultures did not enter the solventogenic growth phase in the duration of these experiments, making it unlikely that the observed increase in ethanol in the single stage co-cultivation condition was due to increased clostridial growth. The drop in ethanol and corresponding increase in lactate was not observed for the one-stage co-cultivation condition, but this could be due to a slightly earlier harvest time in an effort to obtain high-quality fungal RNA from the cultures. However, the lactate flux through the anaerobic fungi and *C. acetobutylicum* could be high despite the low steady-state concentration; resolving this flux would require transcriptomic sequencing of the mixed culture.

3.3 Conclusions

Higher levels of butyrate and butanol in long-term fungal and *C. acetobutylicum* cultures reveal that creating consortia that include these two microbes could be a promising future avenue of industrial bio-butyrate and biobutanol production from lignocellulosic feedstocks, specifically. Evidence is presented in this study that strongly suggests lactate cross-feeding between anaerobic fungi and *C. acetobutylicum*. This cross-feeding is likely a cause of the observed increase in butyrate production in the experimental co-cultivation conditions explored in this study. Additional experiments are needed to definitively pinpoint this metabolic exchange, particularly carbon tracing experiments since lactate is produced by both *C. acetobutylicum* and anaerobic fungi. RNA extraction methods also need to be developed

to examine the late-stage cultures and reliably sequence mixtures of prokaryotic and eukaryotic organisms in co-culture. Future studies could include co-cultivating anaerobic fungi and *C. acetobutylicum* to convert non-homogenous lignocellulose feedstock to produce tunable outputs. As demonstrated in this study, fungal strains vary in their capacity to release excess sugars from lignocellulosic biomass, so other fungal strains not used in this work could yield superior results in a similar co-culture. Combining anaerobic fungi with methanogens to potentially enhance lignocellulose degradation could increase the amount of excess sugars released to support the growth of *C. acetobutylicum* [124]. As demonstrated by a previous study that combined *C. acetobutylicum* with undefined rumen fluid to hydrolyze pretreated agave and enhance hydrogen and butanol production [139], varying experimental conditions such as temperature and pH could further enhance biobutanol production. It may be possible to tune products or induce early solventogenesis by supplementing the medium, which would keep the fermentation pH high and prolong fungal growth [159–161].

3.4 Materials and Methods

3.4.1 Routine microbial cultivation

The anaerobic gut fungal strains, *Neocallimastix californiae* [18,60], *Anaeromyces robustus* [18,60], and *Caecomyces churrovis* [45,123], were isolated as described previously. The fungal cultures were anaerobically cultivated at 39°C in Hungate tubes with 100% CO_2 headspace, 10 mL of Medium C (MC) or Minimal Media 2 (M2), and 0.5 g reed canary grass as the carbon source as described previously [98,99]. The reed canary grass was provided by the US Department of Agriculture, Agricultural Research Service, US Dairy Forage Research Center, and it was milled in a Model 4 Wiley Mill (Thomas Scientific) using a 4-mm screen

size (courtesy of P. J. Weimer). Growth of anaerobic fungi was monitored via a pressure transducer method used to measure the accumulation of fermentation gas in the headspace of the culture tubes [100].

Clostridium acetobutylicum ATCC 824 obtained from the American Type Culture Collection (ATCC) was anaerobically maintained as a spore suspension stock in potato glucose medium (PGM) [162] containing 150 g/L potato (shredded, boiled for 1 hour, and filtered through cheesecloth), 1% glucose, 30 mM $CaCO_3$, and 4 mM $(NH_4)_2SO_4$. *C. acetobutylicum* spore stock was revived by heating 1 mL at 80°C for 10 minutes on a heat block before adding heat-shocked spore stock to a 15 mL Falcon® tube containing 8 mL of clostridium growth media (CGM) [130] and 0.5 g/mL glucose solution. This culture was subsequently used to make a 1:10, 1:100, and 1:1000 dilution in 3 additional Falcon® tubes prepared with 8 mL CGM and 0.5 g/L glucose solution. The spore stock dilution cultures were grown at 39°C in an anaerobic chamber for approximately 24 hours until one of the dilutions reached an OD600 of 0.8-1, at which time it could be used to inoculate a seed culture for the experiments.

3.4.2 Short-term simultaneous (one-stage) co-cultivation

An *A. robustus* seed culture was grown by adding 2 mL of an *A. robustus* 10 mL culture grown for 3 days in a Hungate tube in Medium C [98] with reed canary grass as a substrate to an autoclaved PYREX 250 mL Delong Shaker Flask with Extra-Deep Baffles containing 0.8 g filter paper and 38 mL of anaerobic undefined Medium B at 39°C [163]. This culture and all other seed and experimental cultures were grown unshaken in a modified version of Medium B either defined (no yeast extract or Bacto Casitone added) or undefined

(yeast extract and Bacto Casitone added) with only 1g/L Na_2CO_3 in order to lower the pH of the media, making it suitable to cultivate both microbes used in the study [163]. The Whatman filter paper was cut into ~ 0.5 inch strips for all cultures. This culture was grown in undefined Medium B for 3 days at 39°C in an AS-580 gloveless anaerobic chamber (Anaerobe Systems, Morgan Hill, CA, USA) before being used to inoculate experimental cultures. Two mL of the *A. robustus* seed culture was used to inoculate four PYREX 250 mL Delong Shaker Flasks with Extra-Deep Baffles containing 0.8 g filter paper, 34 mL defined Medium B, and 2 mL 10 wt/vol% maltodextrin solution at 39°C to form the experimental co-cultures after the *A. robustus* has grown for 24 hours to establish the fungal population since *A. robustus* grows slower than *C. acetobutylicum*. At the same time, four additional *A. robustus* cultures were inoculated with 2 mL of *A. robustus* seed culture in autoclaved PYREX 250 mL Delong Shaker Flasks with Extra-Deep Baffles with 36 mL anaerobic MB, 2 mL maltodextrin, and 0.8 g filter paper at 39°C to serve as *A. robustus* monoculture controls grown in the anaerobic chamber at 39°C.

C. acetobutylicum spore stock was revived by heating 1 mL at 80°C for 10 minutes on a heat block before adding heat-shocked spore stock to a 15 mL Falcon® tube containing 8 mL of CGM and 0.5 g/mL glucose solution. This culture was subsequently used to make a 1:10, 1:100, and 1:1000 dilution in 3 additional Falcon® tubes prepared with 8 mL CGM and 0.5 g/L glucose solution. The spore stock dilution cultures were grown at 39°C in an anaerobic chamber for approximately 24 hours until one of the dilutions reached and OD600 of 0.8-1, at which time it could be used to inoculate a seed culture. The 1:1000 dilution reached OD600 of 1.386 at approximately 24 hours, therefore 2 mL of that culture was used to inoculate a *C. acetobutylicum* seed culture in an autoclaved yeast shaker flask filled with 36 mL anaerobic

undefined Medium B and 2 mL of 10 wt/vol% maltodextrin solution at 39°C. This *C. acetobutylicum* seed culture was grown for 24 hours at 39°C in an anaerobic chamber before being used to inoculate experimental cultures. After approximately 24 hours, the culture had reached an OD600 of 0.973. Four *C. acetobutylicum* monoculture controls were inoculated with two mL of the *C. acetobutylicum* seed culture in autoclaved yeast shaker flasks containing 36 mL defined Medium B and 2 mL 10 wt/vol% maltodextrin at 39°C. After the flasks inoculated with the 2 mL of the *A. robustus* seed culture had grown in the anaerobic chamber at 39°C for approximately 24 hours, 2 mL of the *C. acetobutylicum* seed culture was added to each flask to form the co-cultures.

All cultures were covered with parafilm until the *C. acetobutylicum* inoculum was added to prevent evaporation of the liquid. A sample of 250 µL of media from all four replicates of the *C. acetobutylicum* monocultures, *C. acetobutylicum* and *A. robustus* co-cultures, and *A. robustus* monocultures was taken before inoculation and placed in 1.5 mL Eppendorf™ tubes and stored at -80°C for later HPLC analysis. After inoculation, a 250 µL sample of each culture supernatant was taken at 2-hour intervals for a twelve-hour period and stored in 1.5 mL Eppendorf™ tubes at -80°C for later analysis. The culture was then allowed to grow without sampling for 10 hours overnight, then 250 µL samples for HPLC analysis were again collected and stored at -80°C for later HPLC analysis at 4-hour intervals for 12 hours, with a final reading taken two hours later. The cultures were then allowed to grow undisturbed for another twelve hours before collecting a final 250 µL HPLC sample from each culture before harvesting the cultures for RNA extraction. The OD600 measurement of each culture was taken before harvest and ranged from 0.07-0.119.

3.4.3 Short-term sequential (two-stage) co-cultivation

An *A. robustus* seed culture was grown by adding 1 mL of an *A. robustus* 10 mL culture grown in a Hungate tube in complex media with a reed canary grass substrate to an autoclaved yeast shaker flask with 0.8 g filter paper, 37 mL of anaerobic defined Medium B, and 2 mL of 10 wt/vol% sterile filtered maltodextrin solution at 39°C in an AS-580 gloveless anaerobic chamber (Anaerobe Systems, Morgan Hill, CA, USA). The culture grew undisturbed at 39°C in the anaerobic chamber for 3 days. After 3 days, the culture was checked for signs of growth. Bubbling was observed in the culture, indicating that it grew successfully. Four autoclaved yeast shaker flasks containing 0.8 g filter paper, 37 mL defined anaerobic Medium B, and 2 mL of 10 wt/vol% maltodextrin were inoculated with 1 mL of the *A. robustus* seed culture. The flasks were then placed in the 39°C incubator in the anaerobic chamber to grow for eight days. After eight days, the fungal supernatant was sterile filtered and 35 mL was transferred to four autoclaved yeast shaker flasks containing 0.8 g filter paper and inoculated with 1 mL of the *C. acetobutylicum* seed culture once the seed culture had reached an OD600 of 1.049; the preparation of the *C. acetobutylicum* seed culture is discussed below.

C. acetobutylicum spore stock was revived by heating 1 mL at 80°C for 10 minutes on a heat block before adding heat-shocked spore stock to a 15 mL Falcon® tube containing 8 mL of CGM and 0.5 g/mL glucose solution. This culture was subsequently used to make a 1:10, 1:100, and 1:1000 dilution in 3 additional Falcon® tubes prepared with 8 mL CGM and 0.5 g/L glucose solution. The spore stock dilution cultures were grown at 39°C in an anaerobic chamber for approximately 24 hours until one of the dilutions reached an OD600 of 0.8-1, at which time it could be used to inoculate a seed culture. One mL of the 1:100 dilution was used

to inoculate a yeast shaker flask containing 37 mL of anaerobic undefined Medium B and 2 mL of maltodextrin at 39°C. The seed culture was placed in the 39°C incubator in the anaerobic chamber to grow for approximately 36 hours, until the OD600 reached 0.8-1.0. When the seed culture reached an OD600 of 1.049, four autoclaved yeast shaker flasks containing 0.8 g filter paper, 37 mL anaerobic defined Medium B, and 2 mL 10 wt/vol% maltodextrin at 39°C were inoculated with 1 mL of the *C. acetobutylicum* seed culture to serve as *C. acetobutylicum* monoculture controls.

All cultures were covered with parafilm until the *C. acetobutylicum* inoculum was added to prevent evaporation of the liquid. A sample of 250 µL of media from all experimental replicates was taken before inoculation for later HPLC analysis. Six samples were taken at 2-hour intervals following inoculation the first day of growth. After a 12-hour undisturbed growth period overnight, samples were then taken every 4 hours the second day of growth for a 12-hour period. After another 12-hour undisturbed growth period the second night, samples were again collected every 4 hours for an 8-hour period on the third day of growth, at the end of which time the cultures were harvested, after a total of 56 hours of *C. acetobutylicum* growth. All HPLC samples (250 µL each) were immediately stored in 1.5 mL Eppendorf™ tubes at -80°C for later HPLC analysis. These cultures did not reach the OD600 range of the previous cultures, but the cultures were still harvested at this time so that they would still be comparable to the previous co-cultivation experiment's time frame (although the two-stage cultures were allowed to grow for a slightly longer period).

3.4.4 Long-term co-culture cultivation (both one-stage and two-stage)

Prior to *C. churrovis*, *N. californiae*, or *A. robustus* inoculation, an aliquot of *C. acetobutylicum* spore stock (500 µL) was heat shocked for 10 min at 80°C to revive spores. This aliquot was added to 5 mL MC with 0.5% glucose in a round-bottom 10 mL culture tube and incubated (without shaking) overnight at 39°C in an anaerobic chamber with 100% CO_2. For the one-stage co-cultivation condition, 50 µL of the *C. acetobutylicum* culture (OD600 = 0.6) was inoculated into a Hungate tube with 10 mL M2 and 0.5 g reed canary grass at the same time as fungal culture passaging and grown for 29 days without shaking. For the two-stage co-cultivation condition, fungi were grown for 22 days without shaking and then *C. acetobutylicum* was inoculated (50 µL of an OD600 = 0.64 overnight culture). The culture was then grown for 10 days without shaking after *C. acetobutylicum* inoculation, with the residual reed canary grass substrate. *C. acetobutylicum* was added to blank media as controls using the same methods described for the experimental cultures. Growth of all monocultures and co-cultures were monitored by gas pressure measurements. After each measurement, the gas pressure was vented to 0 psi [100].

3.4.5 Harvesting cultures for RNA extraction and sequencing of C. acetobutylicum *(short-term two-stage condition)*

Cultures of *C. acetobutylicum* were grown in the hydrolysate of anaerobic fungus *A. robustus* and harvested after 56 hours of growth as described above. The supernatant was decanted off the filter paper into 50 mL Falcon® tubes and the filter paper was discarded. The cell pellet was spun down at 10,000 g and 4°C for 20 minutes. Visible cell pellets formed for each culture, confirming growth despite low OD measurements. The supernatant was removed from the cell pellet using an automated pipette. A quantity of 500ul of RNA*later*™ was added

to each cell pellet before storing the samples at -80°C. The samples grown for the co-cultivation condition were also harvested for RNA extraction, but sequencing failed for those samples.

The cultures were thawed on ice for RNA extraction. The samples were initially spun down in the 50mL Falcon® tubes they had been frozen in at 4°C and 15,000 g for 20 minutes, but the cells did not form a sufficient pellet in the Falcon® tube, so the sample suspended in RNA-later was then transferred by pipetting to a 1.5 mL Eppendorf™ tube with a conical bottom and spun down in the microcentrifuge for 3 minutes at 20,000 RCF and 4°C. The RNA*later*™ was then removed by pipetting from each cell pellet before resuspending in 600 µL buffer RLT. The cell pellet and buffer RLT were then transferred by pipetting to a mortar filled with liquid nitrogen for grinding with a pestle to lyse the cells. After lysis, the samples were processed in the Qiacube using the RNeasy Mini protocol for animal cells with DNase digestion (eluted in 50 ul RNAse-free water). The samples were then stored at -80°C until sequenced.

For transcriptome sequencing, rRNA was removed from 100 ng of total RNA using Qiagen FastSelect 5S/16S/23S for bacterial rRNA depletion (and additional FastSelect plant and/or yeast rRNA depletion) (Qiagen) with RNA blocking oligo technology. The fragmented and rRNA-depleted RNA is reverse transcribed to create first strand cDNA using Illumina TruSeq Stranded mRNA Library prep kit (Illumina) followed by the second strand cDNA synthesis which incorporates dUTP to quench the second strand during amplification. The double stranded cDNA fragments are then A-tailed and ligated to JGI dual indexed Y-adapters, followed by an enrichment of the library by 10 cycles of PCR. The prepared libraries were then quantified using KAPA Illumina library quantification kit (Roche) and run on a

LightCycler 480 real-time PCR instrument (Roche). The quantified libraries were then multiplexed and the pool of libraries was then prepared for sequencing on the Illumina NovaSeq 6000 sequencing platform using NovaSeq XP v1 reagent kits (Illumina), S4 flow cell, following a 2x150 indexed run recipe. Raw fastq file reads were filtered and trimmed using the JGI QC pipeline resulting in the filtered fastq file. Using BBDuk, raw reads were evaluated for artifact sequence by kmer matching (kmer=25), allowing 1 mismatch and detected artifact was trimmed from the 3' end of the reads [164]. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed (minimum length 25 bases or 1/3 of the original read length - whichever is longer). Filtered reads from each library were aligned to the *C. acetobutylicum* ATCC 824 reference genome (IMG taxon ID 637000076.fna 1275968) using HISAT2 version 2.2.0 [107,128]. Strand-specific coverage was generated using deepTools v3.1 [108]. Raw gene counts were generated using featureCounts, with only primary hits assigned to the reverse strand were included in the raw gene counts [109]. Raw gene counts were used to evaluate the level of correlation between biological replicates using Pearson's correlation and determine which replicates would be used in the DGE analysis. One of the four replicates for each condition was removed from the DGE analysis because the Pearson correlation co-efficient fell below .85 for these outliers for at least one other sample in the group. DESeq2 (version 1.28.1) was subsequently used to determine which genes were differentially expressed between pairs of conditions. The parameters used to call a gene DE between conditions were an absolute $\log_2$fold change greater than 1 and a p-adjusted value less than 0.05 [110]. The average TPM value of either the experimental condition or of the control (or

both) for all differentially expressed genes was above a conservative TPM cutoff of 3, indicating biological relevance. Raw gene counts, not normalized counts, are used for DGE analysis, as DESeq2 uses its own internal normalization.

3.4.6 Detection of sugars and fatty acids by high-performance liquid chromatography (HPLC)

Levels of volatile fatty acids and sugars present in the supernatant of short-term experimental cultures were measured using an Agilent1260 Infinity HPLC (Agilent). Samples were prepared by acidifying to 5 mM using sulfuric acid and subsequently incubating at room temperature for 5 minutes. Samples were then centrifuged for 5 minutes at 21,000 g. The supernatant was syringe filtered into an HPLC vial (Eppendorf™ FA-45-24-11) using a 0.22 µm PVDF filter. Samples were analyzed on an Agilent 1260 Infinity high-performance liquid chromatography system (HPLC, Agilent, Santa Clara, CA) equipped with an auto-sampler unit (1260 ALS). Separation was achieved with a Bio-Rad Aminex® HPX-87H Ion Exclusion Column for organic acids (Part No. 1250140, Bio-Rad, Hercules, CA) set to 35°C and a flow rate of 0.6 mL/min with a mobile phase of 5 mM sulfuric acid and a 20 µL injection volume. In-house standards were prepared with blank culture medium as a base and sodium formate (ACS Grade, Fisher Chemical S648500), sodium acetate (ACS Grade, Fisher Chemical S210500), L-lactic acid sodium (99%, extra pure, Acros Organics 439220100), n-butyric acid (99%, Acros Organics, Cat. No. 108111000), D-(+)-glucose (Sigma-Aldrich Cat. No. G8270), D-(+)-xylose (Sigma-Aldrich Cat. No. X1500-500G), 1-butanol, 99.7%, Chromasolv Plus (Sigma-Aldrich Cat. No. 34867), and ethyl alcohol (molecular biology grade, Sigma-Aldrich Cat. No. E7023) at concentrations of 0.1 and 1 g/L to 2 g/L (dependent upon the upper concentration limit of the experimental samples).

Quantities of volatile fatty acids and sugars for long-term cultures were measured using an Agilent 1200 equipped with a refractive index detector. After 31 days, cultures were centrifuged at 3700 RPM for 10 min. The supernatant was filtered through a 0.2 μm sterile filter membrane and a 1 mL aliquot was stored at -20°C until acidification for analysis. Acidification process was the same as that described for the short-term cultures. Separation was achieved with a Bio-Rad Aminex® HPX-87H Ion Exclusion Column for organic acids (Part No. 1250140, Bio-Rad, Hercules, CA) set to 65°C and 0.6 mL/min and eluted with a mobile phase of 3.25 mM and 5mM sulfuric acid and a 20 μL injection volume. Quantification was based on an external calibration curve using pure known components as standards. Stock solutions of 1, 2.5, 5, 10, 25, 50, 100, 250, and 500 mM of pure known components were utilized. Calibration curves were generated by triplicate injections of each calibrator.

4 High-quality RNA extraction and regulation of carbohydrate active enzymes is correlated with stage of growth in anaerobic fungi

4.1 Introduction

Comparative transcriptome profiling is difficult to apply to non-model organisms since traditional nucleic acid extraction protocols and approaches do not often translate well to these systems [165]. This is particularly true when working with fungi that have extensive rhizoid or mycelial networks and chitin-rich cell walls [166], which require difficult lysis and extraction protocols to isolate sufficient quantities and quality of nucleic acids [167–169]. For example, anaerobic fungi are non-model organisms that serve as a valuable source of diverse carbohydrate active enzymes (CAZymes) with powerful biomass degrading capabilities [18,22,43–45] that are attractive for use in biotech applications to generate value-added products from low-cost waste materials [18,60,119]. Moreover, they also produce unique natural products [89,170] that likely enable their function, which could be harnessed as an emerging class of antimicrobials or as therapeutic compounds. Several research teams have worked to develop unique lysis and extraction approaches to overcome challenges associated with obtaining high quality genomic DNA from anaerobic fungi to tap into this biotechnology potential [60,61,171,172].

While advancements to overcome the challenges associated with extracting DNA and RNA from non-model microbes such as anaerobic fungi have been made, universally effective RNA extraction methods have not been well established for anaerobic fungi. For example, it is extremely challenging to extract similar quantities of high quality RNA in lag, exponential growth, and stationary phases that may prove relevant to deciphering the function of certain

fungal genes [173]. Most gut fungal RNA studies to date have collected RNA data from one timepoint in the mid-log growth phase and focused on differential regulation of CAZymes and/or the biosynthetic genes that encode natural products within anaerobic fungi, as altered by substrate or co-cultivation with other organisms [49,50,123]. The ability to collect RNA-Seq data for a full time course across all growth regimes provides valuable information regarding when the CAZyme and biosynthetic genes of interest are expressed. Determining how widely expression varies as a function of growth phase would also inform bioreactor design to maximize production of target products (e.g. enzymes or metabolites) produced by anaerobic fungi either in isolation or in co-culture.

To monitor gene expression, a reliable method to collect high-quality RNA for transcriptomic analysis is needed. This study investigates how time of harvest affects RNA quality, RNA concentration, and transcriptional regulation with a focus on biomass-degrading enzymes and other cellulosome components. We chose to examine both fungal-methanogen co-cultures and fungal monocultures since previous studies [49,50,123] demonstrated that transcription of CAZymes increases at a given timepoint in the fungal growth phase when co-cultivated with a methanogen. Fungal monocultures of *Anaeromyces robustus* and co-cultures of *A. robustus* and the methanogen *Methanobacterium bryantii* were cultivated on filter paper and harvested at 24-hour time points from the 2nd day of growth to the 7th day of growth post-inoculation for RNA extraction and subsequent RNA quality and quantity assessment. The optimal range for RNA harvest in anaerobic fungal monocultures and co-cultures was 2-5 days of growth post-inoculation. During this window of growth, overall fungal CAZyme regulation in anaerobic fungal co-cultures with methanogens versus fungal monocultures was dependent upon time of harvest. Genes encoding fungal cellulosome components were

upregulated in co-cultures of fungi and methanogens relative to fungal monocultures with variation in expression occurring at 24-hour intervals. These findings underscore that timing and phase of fungal growth are important factors to consider when designing experiments and deciphering transcriptomic regulation patterns.

4.2 Results and Discussion

4.2.1 High-quality RNA is harvested from anaerobic fungi and fungal-methanogen co-cultures 2-5 days post-inoculation under batch growth conditions

High quality RNA is the gold standard for transcriptomic studies since it remains unclear whether RNA degradation occurs uniformly across the transcriptome or at different rates [174]. Degradation that is not uniform could result in inaccurate expression levels for genes of interest that do not accurately reflect *in vivo* production levels [174]. Mechanical lysis through both bead beating and liquid nitrogen grinding has provided high-quality RNA for many previous transcriptional studies, effectively breaking through the chitin-rich rigid cell walls of anaerobic fungi to release nucleic acids [20]. To avoid heat generation associated with bead beating that leads to RNA degradation, liquid nitrogen was used to extract nucleic acids for this study.

Fungal monocultures of *A. robustus* and fungal-methanogen co-cultures of *A. robustus* and the methanogen *M. bryantii* were grown on the cellulosic substrate, Whatman filter paper. RNA was extracted from fungal monocultures and fungal-methanogen co-cultures harvested on days 2-7 post-inoculation into batch anaerobic culture using the liquid nitrogen grinding lysis method. While there is no universally accepted criterion to determine whether a given RNA sample is suitable for inclusion in a given study, quality metrics such as RNA Integrity

Number (RIN) [175] are often used to determine relative sample quality [174]. Samples from the anaerobic fungal monocultures as well as the fungal-methanogen co-cultures harvested on days 2-5 post-inoculation into anaerobic batch cultures were sequenced on an Illumina NovaSeq sequencer (co-culture samples from day 6 were also sequenced).

A plot of RNA concentrations and RINe (RNA Integrity Number Equivalent) scores for cultures harvested over 10 days of growth is shown in Figure 4.1A (monocultures) and Figure 4.1B (co-cultures). RNA degradation was most pronounced in cultures harvested on days 6 and 7, which marks the beginning of stationary phase based on accumulated pressure measurements, likely leading to the failure to sequence monoculture samples collected on day 6 and failure to sequence both monoculture and co-culture samples collected on day 7. While there is no generally accepted criterion for sample inclusion based on quality, samples with RINe scores as low as 3.95 have been included in previous published studies [176], and the average concentration of samples harvested on days 2-7 in this study exceeded that threshold. RIN and RINe values are both ranked on a scale from 1 to 10, with the highest value indicating no degradation; however, in contrast to RIN, RINe is a representation of the relative ratio of the signal in the fast zone to the 18s peak signal and provides a faster method of determining total RNA integrity [177]. RIN and RINe have been shown to be equivalent for an Agilent 2200 TapeStation system and the Agilent R6K ScreenTape (measuring RINe) and the Agilent 2100 Bioanalyzer (measuring RIN) [177]. The Joint Genome Institute (JGI) recommends an RNA Quality Number (RQN) above 6.0 for samples submitted for sequencing [178]. All sample groups (harvested on days 2-7) had an average RINe score that exceeded this threshold (Figure 4.1).

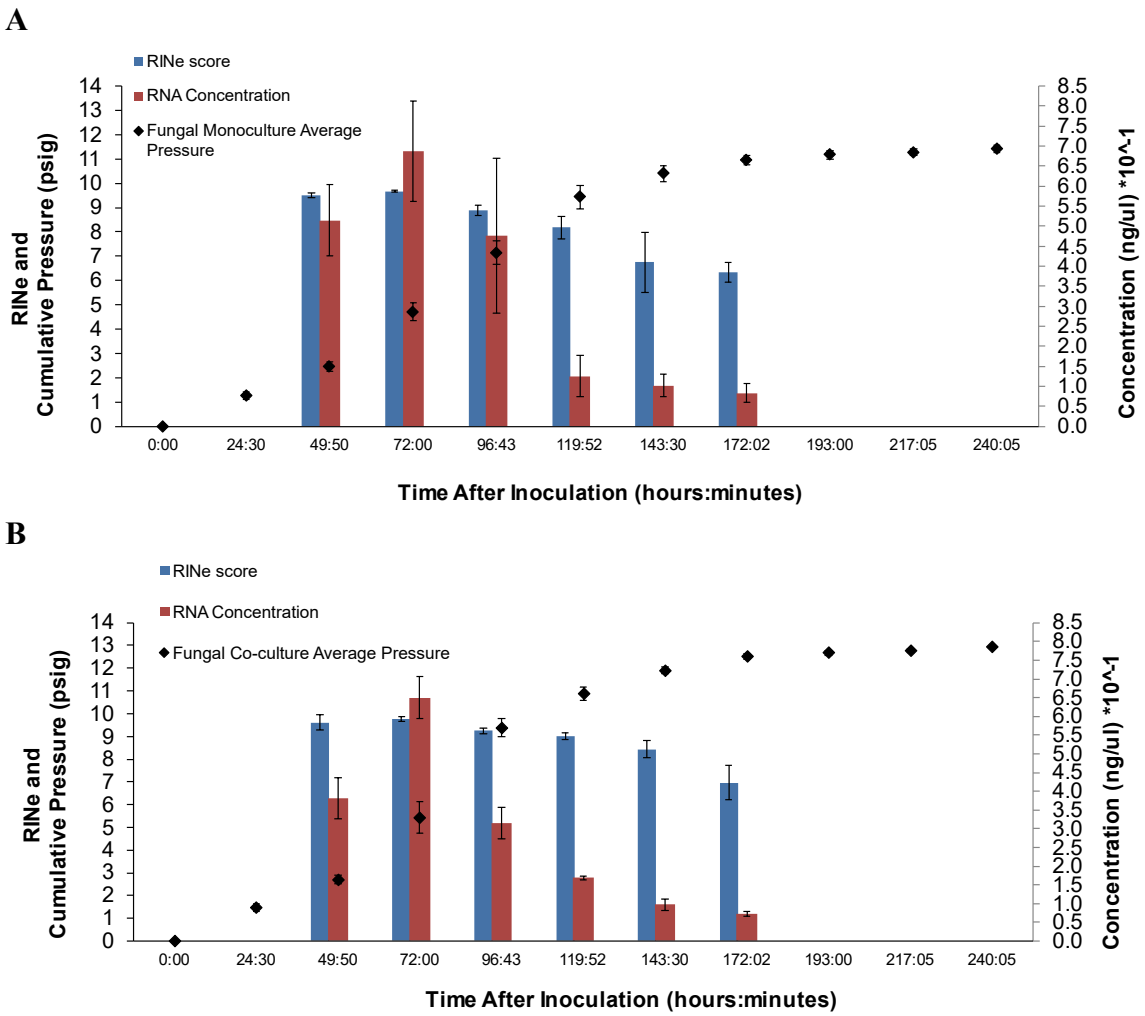


Figure 4.1. RNA concentrations and RINe scores for cultures harvested over 7 days of growth post-inoculation for fungal monocultures of *A. robustus* (A) and fungal-methanogen co-cultures of *A. robustus* and *M. bryantii* (B) both grown on a cellulose substrate (Whatman filter paper). RNA was extracted from cultures harvested on days 2-7 using a liquid nitrogen grinding lysis method. Samples were sequenced from both conditions on days 2-5 (co-culture samples from day 6 were also successfully sequenced). RNA degradation was more pronounced and RNA concentration decreased in cultures harvested on days 6 and 7, likely leading to the failure to sequence monoculture samples collected on day six and both monoculture and co-culture samples collected on day 7.

RNA concentrations for both fungal monocultures and fungal-methanogen co-cultures were above at least 30 ng/µL in cultures harvested on days 2-4 during exponential growth phase, then average concentrations dropped to half of that amount or less for cultures harvested on days 5-7 marking the end of the exponential growth phase and the beginning of

the stationary growth phase, which could have also contributed to the failure to sequence monoculture samples collected on day six and failure to sequence both monoculture and co-culture samples collected on day 7. The JGI recommends that eukaryotic RNA samples in the low input category have a concentration range of 10-1000 ng/ul [175]. The average concentration of fungal monoculture samples harvested on days 2-6 and the average concentration of fungal-methanogen co-cultures harvested on days 2-4 met this criterion (although the average concentration of co-cultures harvested on day 5 was extremely close – average concentration for these samples was 9.7). These findings indicate that the optimal range for RNA harvest from this anaerobic fungus ranges from days 2-5 post-inoculation encompassing the exponential growth phase, as based on both quantity and quality measures and whether the harvested samples could be sequenced. The fungus used in this study, *A. robustus*, is a polycentric fungus with multinucleate rhizomycelia [18,69]. This range could vary for other fungal strains or microbial pairings due to biological differences such as the presence or absence of rhizoidal structures, since nuclei are present in the rhizomycelium of polycentric fungi [20,179]. Therefore, future studies should be conducted to determine whether this range for capturing sufficient RNA quantity/quality is generalizable across anaerobic fungal genera.

4.2.2 Overall CAZyme regulation in anaerobic fungal co-cultures depends on time of harvest during exponential growth phase

Multiple previous studies [49,50,123] have found that fungal CAZymes are upregulated during co-cultivation with a methanogen under multiple growth conditions (such as media formulation, substrate, or using a particular fungal strain). However, these studies used only one, or at the most two, time points of RNA collection during the growth phase of

fungal monocultures and co-cultures, calling into question whether these findings would hold true throughout the entire duration of co-culture cultivation [49,50,123]. One previous study investigated transcriptional response in exponential gut fungal monocultures for six timepoints over a relatively short 28-hour time period when pulsed with glucose [60]. Recent work has determined how CAZymes are regulated at the mid-log growth phase and late growth phase of a gut fungal monoculture, as well as in co-culture with a methanogen grown on glucose [50], noting a change in CAZyme regulation between the growth phases. However, it remains unclear how differences in growth stage affect the outcome of CAZyme-focused transcriptional studies and to what extent the timeline of cultivation drives differences seen in these studies. Narrowing in on the optimal timeframe for maximal expression of CAZymes is also important to inform bioprocessing strategies that seek to use anaerobic fungi, since the prevalence of CAZymes within a bioreactor determines the efficiency with which a batch culture can degrade plant biomass substrates.

DESEQ2 was used to determine that 1002 unique genes were differentially expressed (419 upregulated and 583 downregulated) by the anaerobic fungus *A. robustus* over the 4 days (days 2-5 post-inoculation) examined in fungal-methanogen co-culture versus fungal monoculture. Days 2-4 post-inoculation fell within the exponential growth phase and day 5 post-inoculation marked the beginning of the stationary growth phase based on measurements of pressure accumulation in the headspace of the cultures, which serves as a proxy for growth in the absence of quantitative methods to measure fungal cells grown on an insoluble substrate [100]. While average accumulated pressure was slightly higher overall in the co-cultivation condition, co-cultivation did not appear to affect when the shift from exponential growth to stationary growth occurred relative to fungal monoculture. Out of the unique genes that were

differentially expressed, 200 of those genes encoded fungal CAZymes. GSEApreranked analysis of CAZyme regulation revealed that CAZymes were enriched in upregulated genes in co-culture versus monoculture for cultures harvested on day 3 and day 5 (significant at false discovery rate, FDR, of <25%), but not for cultures harvested on days 2 and 4. The day post-inoculation that cultures were harvested affects the total number of CAZymes regulated and whether or not more CAZyme genes were upregulated or downregulated when comparing co-cultures to monocultures. These findings reveal that the overall upregulation of fungal genes annotated as CAZymes in fungal-methanogen co-cultures relative to fungal monocultures observed in previous studies was likely strongly dependent upon time of harvest.

These cultures were grown on filter paper, a cellulose substrate, and therefore results could vary when cultures are grown on other substrates, such as glucose or lignocellulose. Previous work indicated that a common regulatory network for diverse CAZymes is upregulated for a variety of substrates, however, results also indicated that gene expression of specific enzyme types for similar reactions were differentially regulated as a function of growth substrate [60]. This would demonstrate a substrate-specific catabolic response also occurs in response to the presence of a particular growth substrate [60].

4.2.3 Cellulosome components are transcriptionally upregulated in batch co-culture of fungi and methanogens with variation in expression at 24-hour intervals

The breakdown of biomass by anaerobic fungi is aided by extracellular fungal cellulosomes that consist of a catalytic complex that includes dockerins, carbohydrate binding modules, and CAZymes grouped together for improved hydrolysis [18]. A previous study examining one timepoint for RNA harvest indicated that growth on insoluble substrates such

as filter paper, Avicel®, or reed canary grass induced expression of fungal cellulosomes for enhanced degradation in fungal monoculture relative to growth on glucose [60]. A second study found that co-culture of a non-rhizoidal fungal strain with a methanogen increased transcription of carbohydrate binding modules and dockerin domains in co-cultures grown on cellulose (Avicel®) [123]. We would therefore expect expression of cellulosome components and transcriptional upregulation of carbohydrate binding modules and dockerins in co-culture to enhance degradation capability for growth on the cellulosic filter paper substrate used in this study.

We found that regulation of fungal genes annotated as dockerins and carbohydrate binding modules as well as the CAZymes glycoside hydrolases, carbohydrate esterases, glycosyl transferases, and polysaccharide lyases in fungal-methanogen co-culture relative to fungal monoculture varied at each 24-hour timepoint of the exponential growth phase, indicating that the conclusion that co-cultivation with a methanogen upregulates expression of cellusome components reached by previous studies is dependent upon time of harvest as shown in Figure 4.2.

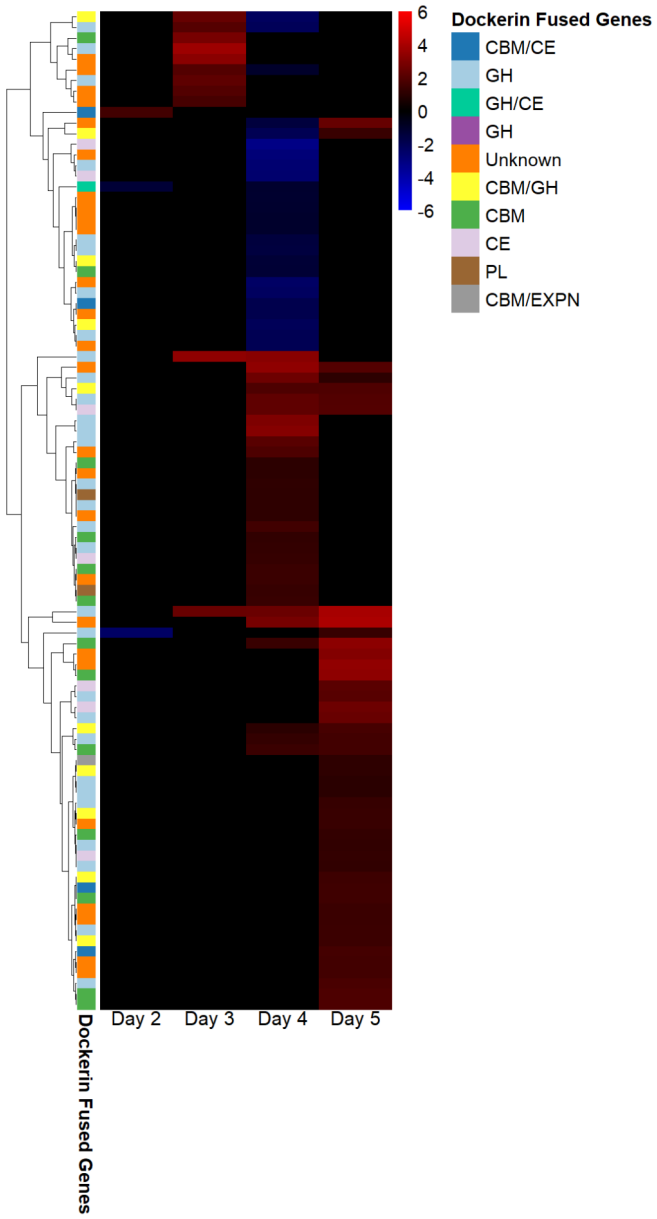


Figure 4.2. **Dockerin regulation in *A. robustus* and *M. bryantii* co-culture versus *A. robustus* monoculture indicates that transcriptional upregulation of these cellulosome components is dependent upon time of batch culture harvest post-inoculation.** Regulated genes annotated as containing dockerin domains were upregulated on days 3 and 5 – none were downregulated at these harvest timepoints, although downregulation of genes annotated as containing dockerin domains was observed for cultures harvested on days 2 and 4. The legend indicates which type of CAZyme the dockerin is fused to and whether a CBM is also present. "Unknown" indicates that the dockerin is fused to a gene of unknown function. Transcriptional upregulation of these cellulosome components in fungal-methanogen co-cultures relative to fungal monocultures is dependent upon time of harvest for batch cultures grown on a cellulose substrate, with regulation of largely unique dockerin-fused gene groups at harvest on a given day of growth post-inoculation.

These results suggest that co-culture with a methanogen bolsters the upregulation of cellulosome components observed previously in fungal monocultures grown on insoluble substrates, such as cellulose, relative to growth on soluble substrates at specific points in the growth phase. Only four genes annotated as CAZymes, CBMs, and/or containing dockerin domains were upregulated in the co-cultivation condition for cultures harvested on day 2. It is possible that this occurred because the fungus had not yet transcriptionally responded to the presence of the methanogen.

Many fungal glycosyl hydrolases (GHs) assist in breaking down the cellulosic and hemicellulosic components of plant biomass [180]. While several genes annotated as glycosyl hydrolases were downregulated on day 4 and two genes annotated as GHs were downregulated on day 2, most differentially expressed genes annotated as GHs were upregulated on days 3 and 5 as shown in Figure 4.3.

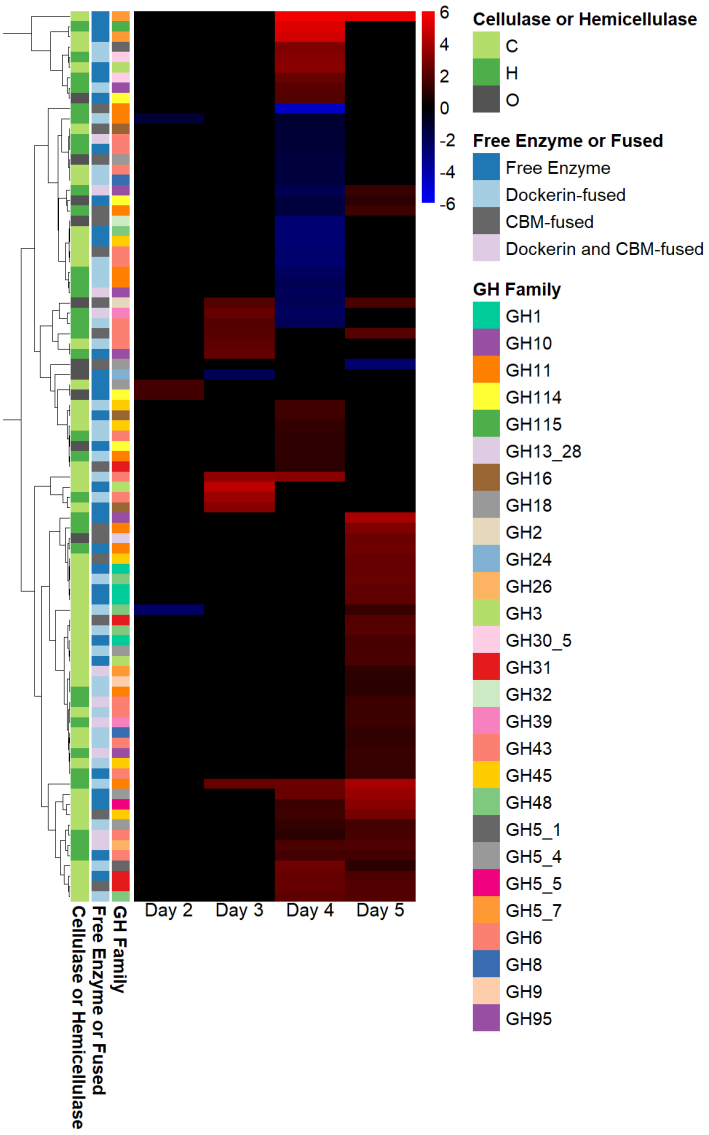


Figure 4.3. **Glycosyl hydrolase regulation in co-cultures of *A. robustus* and *M. bryantii* versus *A. robustus* monoculture grown on a cellulose substrate reveals sequential upregulation of hemicellulase and cellulase enzymes.** While several genes annotated as glycosyl hydrolases were downregulated on day 4 and two genes annotated as GHs were downregulated on day 2, Only one gene annotated as a GH was downregulated on days 3 and 5 – all other differentially expressed genes annotated as GHs were upregulated on days 3 and 5 in a regulation pattern similar to that observed for regulation of genes containing dockerin domains. The majority of genes annotated as GHs that were upregulated in fungal-methanogen co-culture on day 3 were hemicellulases and the majority of genes annotated as GHs that were upregulated in fungal-methanogen co-culture on days 4 and 5 were cellulases. This observation could be attributed to an adaptive upregulation of enzymes in co-culture to free the core of plant biomass before cellulase transcription increases. The legend indicates whether a given gene annotated as a GH is classified as a cellulase (C), a hemicellulase (H), or other (O) and whether the GH is a free enzyme or fused to a CBM and/or dockerin domain.

The majority of genes annotated as GHs that were upregulated in fungal-methanogen co-culture on day 3 were hemicellulases and the majority of genes annotated as GHs that were upregulated in fungal-methanogen co-culture on days 4 and 5 were cellulases. Since hemicellulases remove the hemicellulose in plant biomass to provide access to cellulose [181], this observed pattern of regulation could be due to an adaptive upregulation of enzymes in co-culture to free the core of plant biomass before transcription of genes encoding cellulases increases, even though cellulose was the only substrate present in this experiment [60].

The removal of hemicellulose from plant biomass to free cellulose is accompanied by pectin removal by polysaccharide lyases (PLs) and carbohydrate esterases (CEs). Regulation of genes annotated as CEs and PLs are shown in Supplementary Figures 7.3.1 and 7.3.2. While some genes annotated as CEs were downregulated on day 4 of growth post-inoculation, genes annotated as CEs and PLs were only upregulated in the co-cultivation condition on days 3 and 5 of growth post-inoculation. This demonstrates that co-cultivation with a methanogen increases transcription of genes associated with the pectin removal process of biomass breakdown in the presence of cellulose, even if pectin is absent. GTs were only downregulated (exclusively on day 4) and not regulated on any of the other days as shown in Supplementary Figure 7.3.2. These findings collectively indicate that CAZyme production may not be consistent over the entire exponential growth phase. The transcriptional regulation on days 3 and 5 would suggest that the previously observed patterns of cellulase and cellulosome component upregulation on insoluble substrates such as filter paper relative to growth on soluble substrates is enhanced by co-cultivation with a methanogen, as indicated by previous studies that look at only one or two specific time points.

In addition to CAZyme regulation, regulation of biosynthetic genes has been examined in transcriptional studies [89,170]. It is traditionally thought that secondary metabolite (SM) production occurs during the stationary growth phase of a microbe [182]. However, in agreement with previous work [89,170], in both *A. robustus* monoculture and *A. robustus* and *M. bryantii* co-culture, we observe upregulation of only a few putative biosynthetic genes in *A. robustus* at later days in the growth phase. Contrary to expectations, the ten most highly expressed predicted SM core genes are significantly upregulated at earlier growth (day 2) compared to successive days for both monoculture and co-culture conditions, as shown in Supplementary Table 7.3.1. These findings, in agreement with previous studies, suggest that anaerobic fungi may be unique in their tendency to transcriptionally upregulate biosynthetic genes early in the growth phase before other microbes typically make the metabolic shift to secondary metabolite production in the stationary growth phase.

In the synergistic relationship that exists between fungi and methanogens, the methanogens remove hydrogen produced by the fungi and convert it to methane, potentially resulting in increases in fungal production of acetate, formate, lactate, and ethanol over time. No significant differences were observed in formate, lactate, and ethanol levels between monocultures of *A. robustus* and co-cultures of *A. robustus* and *M. bryantii* during this experiment as shown in Supplementary Figures 7.3.3-7.3.5. Slightly higher acetate and glucose levels were present in co-cultures relative to monoculture on day 5 as shown in Supplementary Figure 7.3.6. A lack of statistically significant differences in fermentation products is in agreement with what was observed previously for a comparison of fermentation products produced in monocultures of *A. robustus* versus co-cultures of *A. robustus* and *M. bryantii* grown on a reed canary grass substrate, except lactate was detected in the co-cultures

but not in the monocultures on the third day of growth [49]. (Although there was a higher amount of acetate present in co-cultures on day 5 of this experiment, there was no statistically significant difference in acetate levels on day 3).

It is notable that the methanogen *M. bryantii* does not appear to utilize formate in this fungal-methanogen pairing grown on filter paper up through day 5 of growth. This contrasts with previous studies in which *M. bryantii* was observed to utilize formate produced by the fungus in a pairing of *M. bryantii* with *A. robustus* grown on filter paper for 10 days [48] and another study in which *M. bryantii* was observed to utilize formate produced by the fungus in a pairing of *M. bryantii* with *C. churrovis* grown on Avicel®, reed canary grass, glucose, fructose, and xylan [123]. This observation implies that the previous hypothesis that co-culture with rumen anaerobic fungi stimulates formate utilization by inducing the function of a formate transporter and formate dehydrogenase in the *M. bryantii* genome [37] may not occur until much later in the growth phase for certain strains or growth conditions.

4.3 Conclusions

Here, we have demonstrated the importance of designing transcriptional studies of anaerobic fungi that sample the entirety of the lag, exponential, and stationary growth phase and establish a method and time frame for the extraction of high-quality RNA from the anaerobic fungal strain *A. robustus* grown on a cellulose substrate. While there is value in determining gene expression for given conditions at a fixed point in time, we have shown for the anaerobic fungus *A. robustus* that the expression and upregulation of genes of biotechnological interest in a co-cultivation condition with the methanogen *M. bryantii*, such as CAZymes and biosynthetic genes, vary throughout the growth phase. These findings have

implications for bioreactor design or future studies to identify secondary metabolites, since this study has shown that timing could be crucial in harnessing the potential of anaerobic fungi and perhaps other anaerobic microorganisms in those applications.

4.4 Materials and Methods

4.4.1 Growing and harvesting cultures for RNA extractions

Anaerobic serum bottles (120 mL total volume) containing 80 mL of modified Medium C [98] ("MC-") with 0.8 mL 100 × vitamin solution [99] and 0.8 g reed canary grass were inoculated with cultures of the anaerobic fungus *A. robustus* [18,60] and the methanogen *M. bryantii*: 1.0 mL of *A. robustus* or a combination of 1.0 mL of *A. robustus* and 1.0 mL of *M. bryantii* (DSM No.-863, DSMZ) (routine cultures were cultivated as described previously by Swift et al.) [49]. The reed canary grass was provided by the US Department of Agriculture, Agricultural Research Service, US Dairy Forage Research Center, and was milled in a Model 4 Wiley Mill (Thomas Scientific) using a 4-mm screen size (courtesy of P. J. Weimer). The fungal and methanogen co-cultures and fungal monocultures were grown anaerobically at 39°C in Hungate tubes filled with 7.0 mL of autoclaved modified Medium C ("MC-") [98], containing 1.25 g/L yeast extract, 5 g/L Bacto™ Casitone, and 7.5 vol% clarified rumen fluid, with 0.08 g filter paper (Whatman GE Healthcare Life Sciences, Grade 3, 23 mm, 100 circles, CAT no. 1003-323, Lot No 16932763) as the growth substrate, supplemented with 0.1 mL vitamin solution post-autoclaving, and inoculated with 0.8 mL of the appropriate 80 mL inoculum culture at mid-log growth phase [99]. Pressure production was used as a proxy for fungal growth, as described previously [100]. Daily pressure measurements were taken using a probe pressure transducer [100]. Once methane was detectable in the co-cultures indicating

that a successful co-culture had formed (starting at 48 hours post-inoculation), 3 or 4 cultures were harvested at 24-hour intervals and stored for later RNA extraction. End-point methane measurements for co-cultures were taken from the headspace gas of the culture tubes before harvesting the cultures. First, the pressure in each sample was measured using a pressure transducer [101], and the headspace gas composition was measured on a gas chromatograph (GC)-pulsed, discharge helium ionization detector (Thermo Fisher Scientific TRACE 1300) [102]. Finally, the headspace pressure of the sample was vented to return the headspace to atmospheric pressure.

After sampling the headspace gas of the culture to determine methane was present in co-cultures, the cultures were opened in an anaerobic chamber and the colonized filter paper was transferred to a 15 mL Falcon® tube containing 1 mL of RNA-later using sterilized tweezers. The Falcon® tube was then removed from the anaerobic chamber and immediately stored at -80ºC until later extraction. A volume of 5 mL of the culture supernatant was transferred to an Eppendorf™ tube and stored at -20ºC for later HPLC analysis.

4.4.2 Extracting RNA from experimental samples

Samples were removed from storage at -80ºC and thawed on ice. After thawing, the cell pellets of *A. robustus* fungal monocultures or *A. robustus* and *M. bryantii* co-cultures stored in RNA*later*™ were spun down for 6 minutes at 4ºC and 10,000 g and RNA*later*™ was removed. Cells were lysed using liquid nitrogen grinding. Total RNA was extracted using the RNeasy Mini kit (QIAGEN) and a QIAcube following the RNeasy Mini protocol for animal cells with QIAshredder homogenization and the optional on-column DNase digestion. An

Agilent TapeStation was used to determine the quality of the sequenced RNA and Qubit High Sensitivity RNA Assay was used to determine concentrations.

4.4.3 RNA sequencing and data analysis

Stranded RNASeq libraries were created and quantified by qPCR for both monoculture and co-culture samples. For differential gene expression analysis, sequencing of the libraries was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent kits, S4 flowcell, and following a 2x150 indexed run recipe. The filtered reads from each library were aligned to the *Anaeromyces robustus* genome using HISAT2 version 2.1.0 [107]. Strand-specific coverage was generated using deepTools v3.1 [108]. Raw gene counts were generated using featureCounts, with only primary hits assigned to the reverse strand were included in the raw gene counts [109]. Raw gene counts were used to evaluate the level of correlation between biological replicates using Pearson's correlation and determine which replicates would be used in the DGE analysis. DESeq2 (version 1.18.1) [110] was subsequently used to determine which genes were differentially expressed between pairs of conditions. The parameters used to call a gene DE between conditions were p-value < 0.05 and a log2fold change greater than 1. Raw gene counts, not normalized counts, were used for DGE analysis since DESeq2 uses its own internal normalization. Subsequent analysis was done using the filtered model gene catalog for *A. robustus* provided for download on the MycoCosm website [62]. Pre-ranked Gene Set Enrichment Analysis (GSEA) of regulated genes in co-cultures relative to fungal monocultures for each substrate condition was conducted using 1,000 permutations and weighted enrichment statistics [81,82].

4.4.4 HPLC analysis

Levels of volatile fatty acids present in the supernatant of both co-cultures and monocultures were measured using an Agilent1260 Infinity HPLC (Agilent)**.** Samples were prepared by acidifying to 5 mM using sulfuric acid and subsequently incubating at room temperature for 5 minutes. Samples were then centrifuged for 5 minutes at 21,000 g. The supernatant was syringe filtered into an HPLC vial (Eppendorf™ FA-45-24-11) using a 0.22 µm PVDF filter. Samples were analyzed on an Agilent 1260 Infinity high-performance liquid chromatography system (HPLC, Agilent, Santa Clara, CA) equipped with an auto-sampler unit (1260 ALS). Separation of formate, acetate, glucose, ethanol, and lactate was achieved with a Bio-Rad Aminex® 87H Ion Exclusion Column for organic acids (Part No. 1250140, Bio-Rad, Hercules, CA) with a mobile phase of 5 mM sulfuric acid. In-house standards were prepared with MC- blank culture medium as a base and sodium formate (ACS Grade, Fisher Chemical S648500), sodium acetate (ACS Grade, Fisher Chemical S210500), and L-lactic acid sodium (99%, extra pure, Acros Organics 439220100), ethyl alcohol (molecular biology grade, Sigma-Aldrich Cat. No. E7023), and D-(+)-glucose (Sigma-Aldrich Cat. No. G8270) at VFA concentrations of 0.1 and 1 g/L.

5 Isolation of new fungal strains

5.1 Isolation and molecular classification of Piromyces *sp. E1M*

Piromyces sp. E1M was isolated from the feces of an Asian elephant at the Santa Barbara Zoo according to the method described by Henske et al., with the exception of using an anaerobic chamber for colony picking [45]. Feces from the Asian elephant were collected at the Santa Barbara Zoo and transported to the laboratory in a 50 mL Falcon® tube. The feces were diluted in 10 mL Medium C [98] with 0.1 mL chloramphenicol solution in a Hungate tube under anaerobic conditions with the lignocellulose substrate reed canary grass supplied as a carbon source. After subsequent growth indicated by pressure production and a clumped mat of lignocellulose substrate and fungus, 0.1 mL of the fungal culture supernatant was used to inoculate an anaerobic roll tube. The roll tube was allowed to grow for approximately 4 days until visible colonies formed. A single colony representing a clonal fungus was picked from the wall of the roll tube in an anaerobic chamber. The colony was placed in a Hungate tube containing 10 mL Medium C [98] with 0.1 mL chloramphenicol solution and reed canary grass supplied as a carbon source. The roll tube process was repeated at least three times to obtain pure cultures, alternating between single colony picking in roll tubes and growing liquid fungal cultures in the presence of chloramphenicol to prevent bacterial contamination. Microscopic analysis suggests that *P.* sp. E1M is monocentric and encysted zoospores germinate to form a rhizoidal system and a single oval or club-shaped zoosporangium approximately 6-9 μm wide (Figure 5.1A-C).

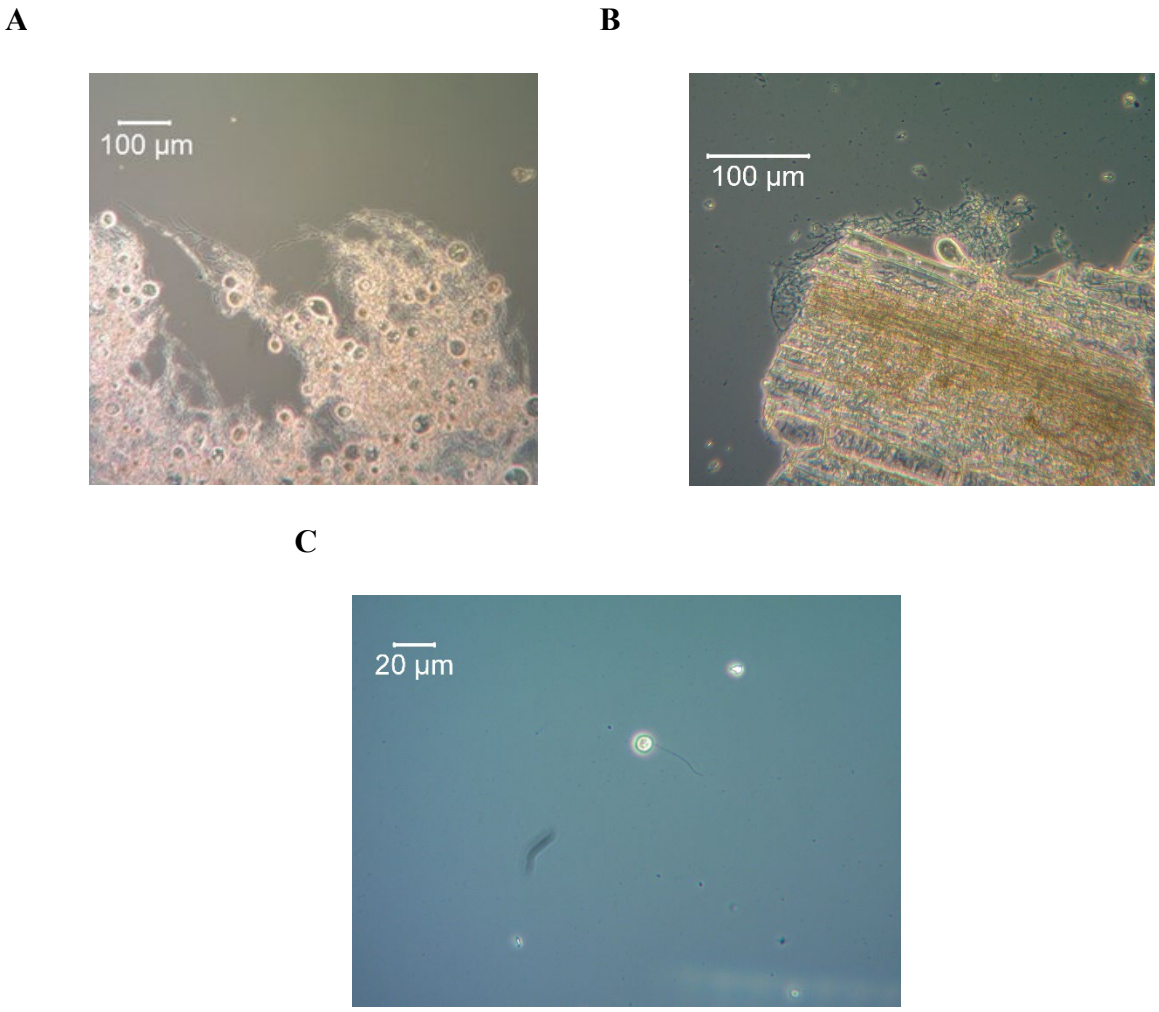
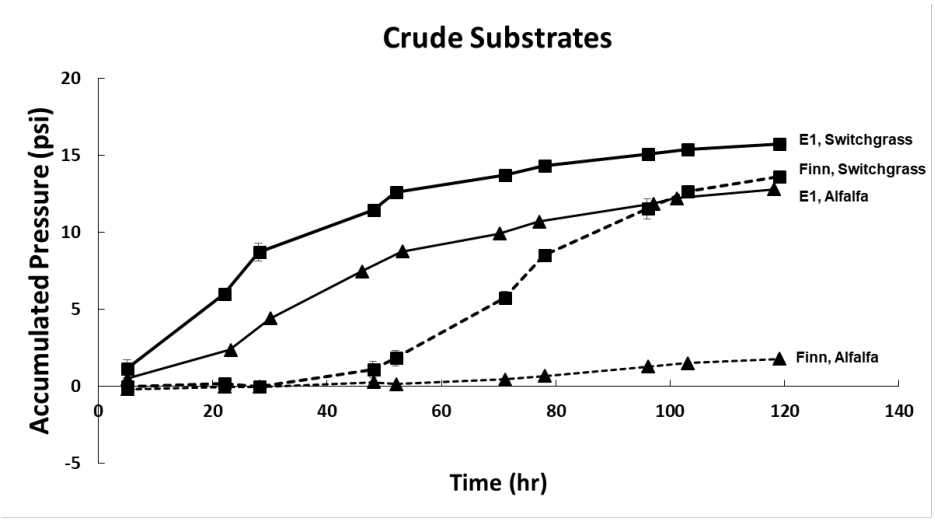


Figure 5.1. *Piromyces* sp. E1M microscopy images. The monocentric fungus germinates to form a club-shaped or oval zoosporangium between 6 and 9 µm wide. Cell mats form when cultivated on soluble sugars, such as cellobiose (A). Rhizoidal networks form to assist in breaking down biomass substrates, such as reed canary grass (B). Motile uniflagellate zoospores are released when sporangia burst as part of the reproductive life cycle, as shown for a culture grown on cellobiose (C).

Both the internal transcribed spacer (ITS1 and ITS2) regions of the ribosomal RNA of the fungus were amplified with primers JB206/JB205 and the large subunit 28S rRNA (LSU) region was amplified using primers NL1/NL4 due to various limitations in using the ITS1 region as a taxonomic marker (Supplementary Table 7.4.1) [22,183,184]. The ITS1 and LSU

regions were significantly different compared to other *Piromyces* strains with ITS1 or LSU sequences available in the NCBI BLAST database, suggesting that it represents a novel strain. Growth characterization on glucose, cellobiose, switchgrass, and alfalfa substrates indicated that the isolate favored crude over soluble substrates, exhibiting particularly rapid growth on switchgrass. (Figure 5.2A and B, data collected by Nikola Malinov).

A



B

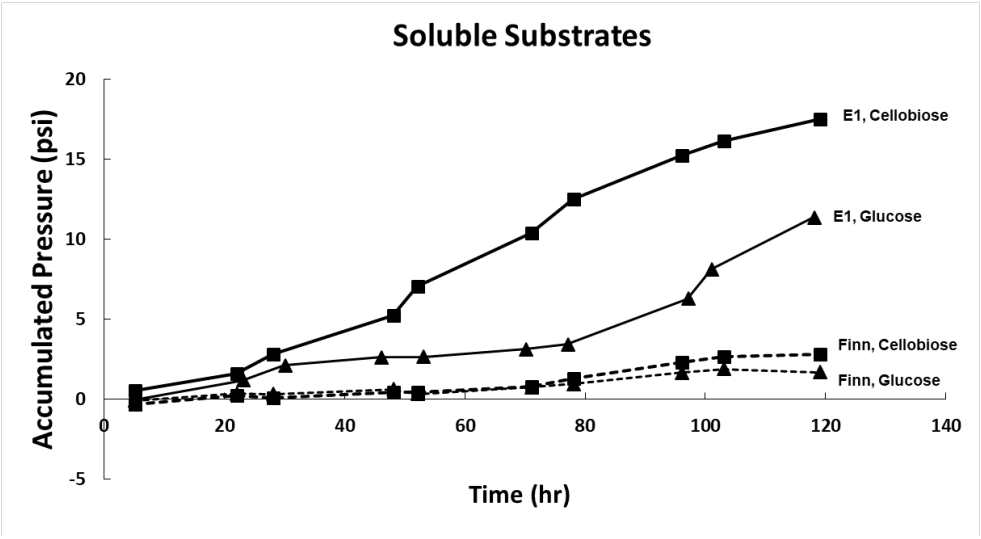


Figure 5.2. Growth curves determined by measuring gas production by the fungus *P.* sp. E1M on crude substrates, switch grass and alfalfa, (A) and soluble substrates, cellobiose and glucose (B). Growth was measured in the form of accumulated pressure in sealed cultures over time. Growth data indicated that the isolate favored crude over soluble substrates. Growth of the new isolate was compared to the growth of a previously isolated strain, *Piromyces finnis* [18,60], on the same substrates. Data collected by Nikola Malinov.

The fungal cultures were anaerobically cultivated at 39°C in Hungate tubes with 100% CO_2 headspace, 10 mL of Medium C (MC) [98], and 0.5 g of crude substrates or 0.5 mL of a 0.1 g/mL sterile filtered sugar stock solution as the carbon source as described previously [98].

Growth of anaerobic fungi was monitored via a pressure transducer method used to measure the accumulation of fermentation gas in the headspace of the culture tubes [100].

P. sp. E1M produced more pressure on each substrate tested compared to growth of the previously isolated *Piromyces* strain, *Piromyces finnis* (isolated from a horse) [18,60], over the course of 120 hours of growth post-inoculation. It is uncertain whether these differences are observed due to the *P. finnis* strain being cultivated continuously for several years after isolation or whether there are genetic differences between the two strains that result in more efficient substrate utilization by the new isolate *P*. sp. E1M.

Future genome sequencing and transcriptional studies will assist in clarifying the cause of this observed difference in growth based on pressure production. RNA was collected from cultures of the fungus grown on glucose, cellobiose, Whatman filter paper, and switchgrass at 39°C in Hungate tubes with 100% CO_2 headspace, 10 mL of Medium C (MC) [98], and 0.5 g of crude substrates or 0.5 mL of a 0.1 g/mL sterile filtered sugar stock solution as the carbon source as described previously [98]. The samples were harvested at mid-log growth phase and stored in 1 mL of RNA*later*™ at -80ºC until extraction. Cell pellets were thawed at room temperature, spun down, and the RNA*later*™ was removed from the samples before lysis. Cell pellets were lysed using liquid nitrogen grinding and RNA was extracted using the RNeasy Mini kit (QIAGEN) and a QIAcube that followed the RNeasy Mini protocol for animal cells with QIAshredder homogenization and the optional on-column DNase digestion. An Agilent TapeStation was used to determine the quality of the sequenced RNA and the Qubit High Sensitivity RNA Assay was used to determine concentrations. RNA was stored at -80ºC until sequencing.

5.2 Isolation and molecular classification of N. constans

The anaerobic fungus *Neocallimastix constans* was isolated from a consortium of fungi, methanogens, and antibiotic resistant bacteria. The consortium was originally enriched from the feces of a San Clemente Island goat at the Santa Barbara Zoo through extended cultivation on an alfalfa substrate and regular antibiotic treatment with penicillin and streptomycin [47]. Prior to roll tube isolation, 1 mL of the consortium culture was inoculated into 9 mL Medium C [98] in a Hungate tube with 0.1 mL chloramphenicol solution under anaerobic conditions with the lignocellulose substrate reed canary grass supplied as a carbon source. The consortium was cultivated in this way with inoculation into a fresh Hungate tube every 3-4 days of growth until methane could no longer be detected using gas chromatography and the culture did not appear cloudy, indicating that the methanogens and bacteria were no longer present due to treatment with chloramphenicol. The fungus was then isolated from this culture according to the method described by Henske et al., with the exception of using an anaerobic chamber for colony picking [45]. After subsequent growth indicated by pressure production and a clumped mat of lignocellulose and fungus, 0.1 mL of the fungal culture supernatant was used to inoculate an anaerobic roll tube. The roll tube was allowed to grow for approximately 4 days until visible colonies formed. A single colony representing a clonal fungus was picked from the wall of the roll tube in an anaerobic chamber. The colony was placed in a Hungate tube containing 10 mL Medium C [98] with 0.1 mL chloramphenicol solution and reed canary grass supplied as a carbon source. The roll tube process was repeated at least three times to obtain pure cultures of the fungus shown in Figure 5.3, alternating between single colony picking in roll tubes and growing liquid fungal cultures in the presence of chloramphenicol to prevent bacterial contamination.

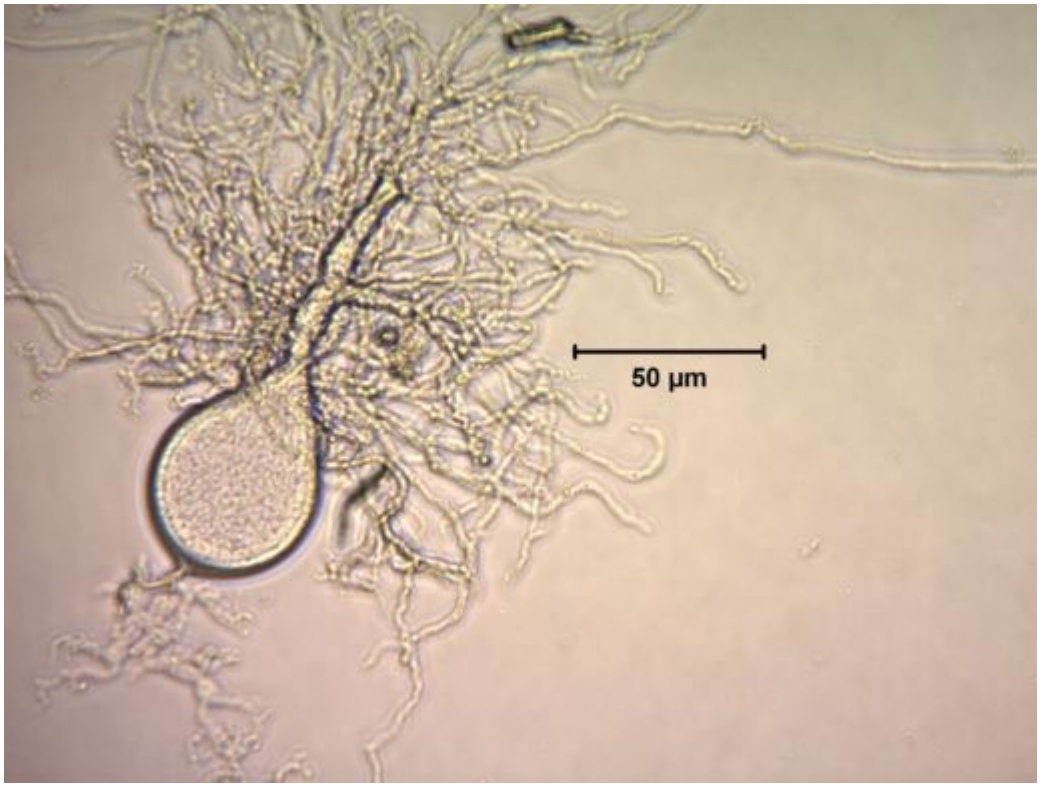


Figure 5.3. *N. constans* microscopy image. The anaerobic fungal strain *N. constans*, isolated from an enriched consortium obtained from the San Clemente Island goat at the Santa Barbara Zoo, forms a rhizoidal network to assist in the breakdown of plant biomass. Photo taken from Mycocosm, a fungal genomics resource provided by the Joint Genome Institute. (https://mycocosm.jgi.doe.gov/Neocon1/Neocon1.home.html)
Photo credit: Tejas Navaratna.

It has proven difficult to form consortia of anaerobic fungi and methanogens from individual isolates that can be cultivated long-term [48]. Enrichments of natural consortia selected through extended cultivation on a given substrate with antibiotic treatments tend to result in highly stable consortia, such as the one from which *N. constans* was isolated [47,48]. Due to the long-term cultivation of the enrichment consortium of anaerobic fungi, methanogens, and antibiotic resistant bacteria this fungal strain was obtained from, it is possible that this fungus has unique genetic or morphological characteristics that make it well-suited for co-cultivation with its companion methanogen. Genomic DNA from this

anaerobic fungal strain has therefore been sequenced using PacBio long-read sequencing. Complementary RNA collection and sequencing has been completed for genome annotation using the JGI annotation pipeline [62]. Future analysis will be completed to determine what features make this strain of anaerobic fungus unique and well-suited to co-cultivation with its companion methanogen. Initial genome assembly statistics from the Joint Genome Institute are included in Table 5.1 below.

Genome Assembly Statistics	
Genome Assembly size (Mbp)	187.12
Sequencing read coverage depth	61.03x
Number of contigs	558
Number of scaffolds	558
Scaffold N50	83
Scaffold L50	0.70

Table 5.1. Genome assembly statistics for *N. constans*. Sequencing read coverage depth is the number of times a given nucleotide has been read during sequencing. Contigs are overlapping DNA sequences used to reconstruct the original DNA sequence of a genomic region. Scaffolds are a portion of the genome composed of contigs and gaps between them. Scaffold N50 is the length at which scaffolds of that length or longer include half the bases of the assembly. Scaffold L50 is the number of scaffolds that are longer than or equal to the N50 length.

6 Overall Conclusions

6.1. Perspectives

This work has demonstrated that several strains of anaerobic fungi can be paired with non-native microbes possessing complementary metabolism to either potentially enhance biomass degrading capability (based on transcriptional data) or produce value-added chemicals. Although tools are still lacking to genetically modify anaerobic fungi, these fungi can be paired with other genetically tractable microbes to expand their applicability in industrial processes. Harnessing the biomass-degrading potential of anaerobic fungi could make bioprocesses more economically viable due to the fungi's ability to breakdown biomass waste into sugars that can support the growth of other microorganisms, providing a low-cost carbon substrate. The capability to pair microbes based on metabolic ties alone instead of native association is an important aspect of engineering consortia to carry out a desired process, since it provides greater flexibility in choosing potential consortia partners without the limit of native association.

6.2 Future Directions

Proteomics studies will need to be conducted to demonstrate that proteins encoding cellulosome components are produced from the upregulated transcripts in the studies discussed herein. It is possible that the upregulated transcripts may not be translated to proteins and therefore the transcriptional upregulation observed may not necessarily result in increased biomass degradation when fungi are paired with methanogens as suggested by transcriptional data. While some progress has been made developing techniques for dual transcriptomics studies of anaerobic fungi and bacteria [170], sequencing of these mixed

cultures still often fails, and further refinement of these sequencing techniques is needed in order to understand the transcriptional response of both microbes in the co-culture. Based on the findings in Chapter 4, future transcriptional studies should be designed to sample the entirety of the lag, exponential growth, and stationary phase of anaerobic fungi grown in batch cultures since upregulation of genes of biotechnological interest in a co-cultivation condition versus a monoculture condition could vary throughout different growth phases. The findings in Chapter 4 also hold implications for bioreactor design and the production of useful products from these microbial communities in a batch cultivation condition, demonstrating that timing must be taken into account when developing these processes. Methods to successfully extract sufficient quantities of high-quality RNA for all of these growth phases, including those that extend beyond those covered in this work, will need to be developed.

7 Appendices

7.1 Appendix A: Supplementary figures for chapter two

Supplementary Table 7.1.1. The number of proteins identified as cellulases, hemicellulases, and other accessory enzymes for six sequenced anaerobic fungi annotated from genome sequencing (see methods).

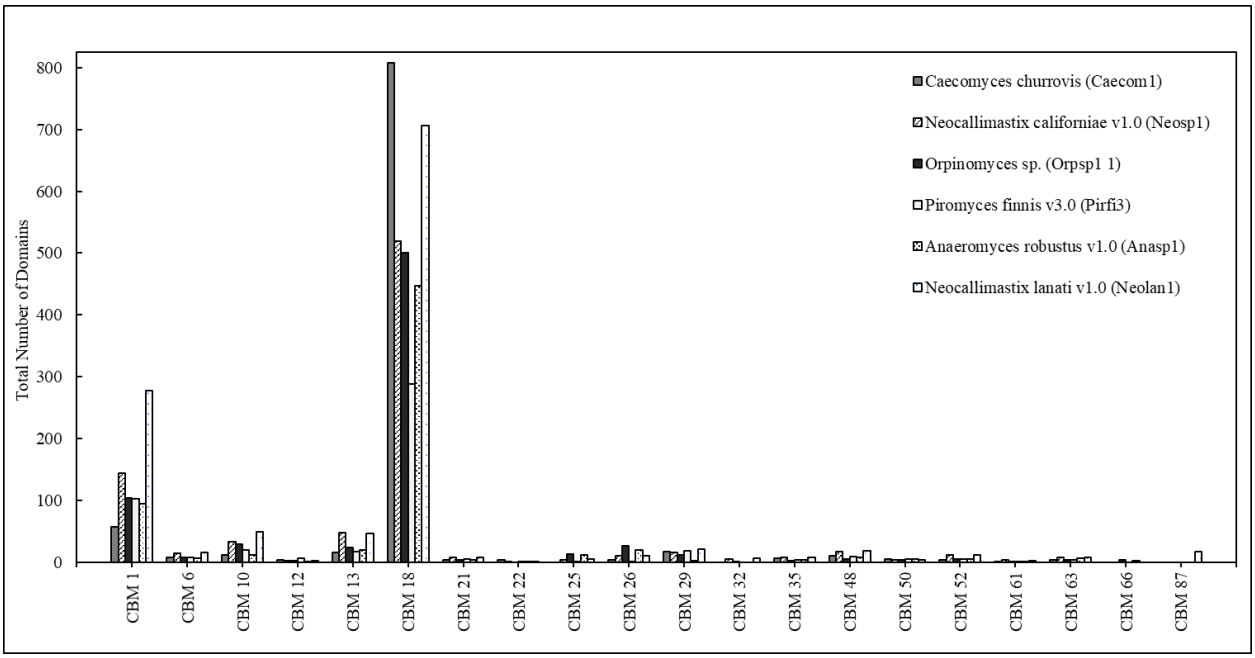
	Number of protein IDs					
Hemicellulases	*C. churrovis*	*N. californiae*	*A. robustus*	*P. finnis*	*N. lanati*	*O.* sp. C1A
GH11	33	24	29	36	90	45
GH43	25	43	18	14	48	31
GH10	12	58	15	21	59	32
GH39	7	9	5	2	8	3
GH30	4	4	2	1	4	3
Accessory enzymes						
Carbohydrate esterase (SGNH hydrolase domains)	51	68	39	23	79	39
Pectin Lyase	53	127	44	49	142	72
Polysaccharide deacetylase	43	93	49	44	96	48
Rhamnogalacturonate lyase	2	9	3	2	12	1
Pectinesterase	1	15	5	5	16	8
Glycosyl Hydrolase 88	0	2	0	1	2	0
Cellulases						
GH9	10	14	9	12	14	13
GH6	18	27	12	21	89	49
GH45	20	28	14	15	28	16
GH48	7	21	7	13	23	14
GH1	7	16	7	10	19	10
GH5	23	65	26	26	70	47
GH3	10	53	15	15	58	18
GH16	8	19	11	6	19	6
GH8	1	2	2	1	2	1
GH31	3	10	7	2	11	19
Total	338	706	319	319	889	475

Supplementary Table 7.1.2. Co-cultivation of *C. churrovis* with *M. bryantii* induces transcriptional upregulation of genes that appear to encode proteins homologous to prokaryotic Substrate Binding Proteins (SBPs), as well as Class C G-Protein Coupled Receptors (GPCRs). Among regulated transcripts are sequences encoding G-protein coupled receptors (7tm_3, PF00003) and sequences encoding putative Substrate Binding Proteins (SBP_Bac_1, PF01547; SBP_Bac_3, PF00497; and SBP_Bac_8, PF13416). The table lists transcriptional regulation of genes encoding proteins that have at least one predicted transmembrane segment. Approximately half of all sequences contain at least one Pfam. Conditions indicate the substrate on which cultures were grown, followed by whether *C. churrovis* transcripts were upregulated or downregulated in the co-culture condition relative to fungal monocultures.

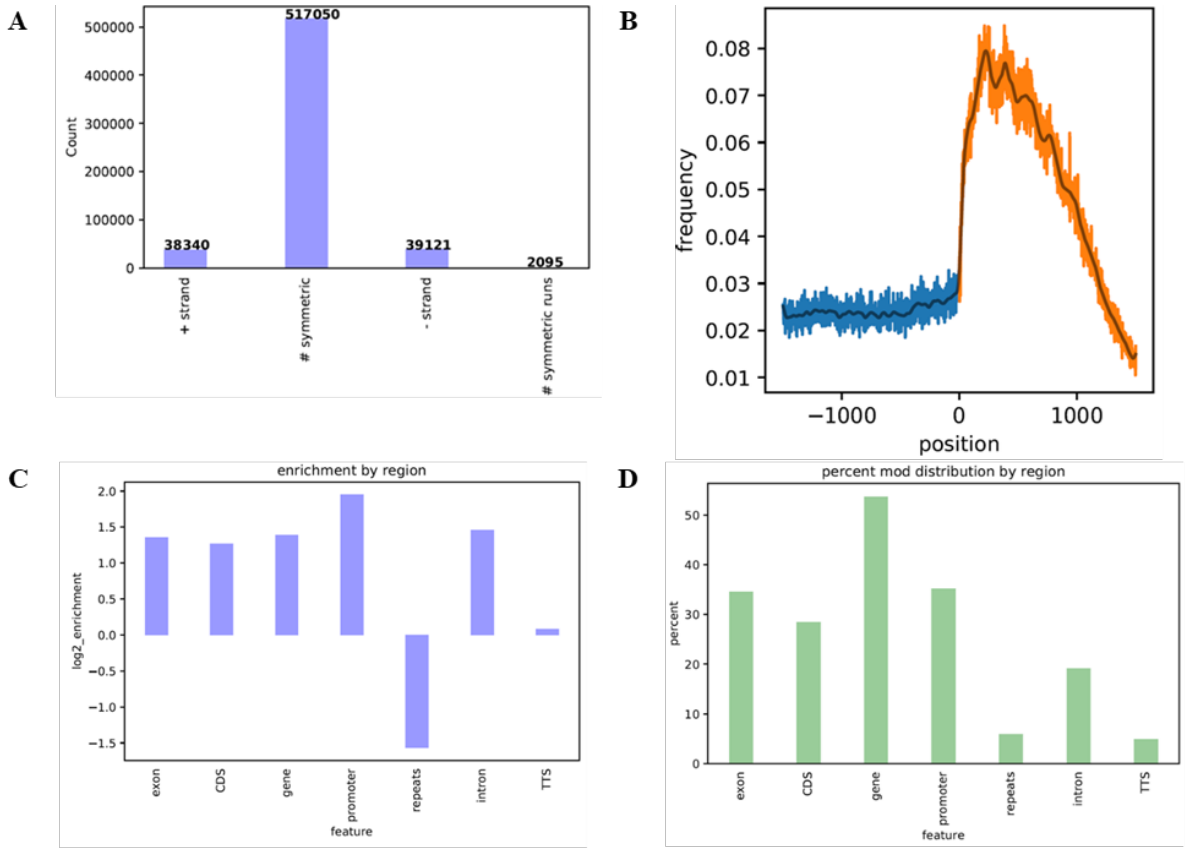
Condition	Number of transcripts affected	Transcripts with at least 1 Pfam hit	GPCR	SBP
Glucose Upregulated	149	77	1	0
Glucose Downregulated	203	123	5	21
Fructose Upregulated	87	48	1	0
Fructose Downregulated	66	33	1	5
Avicel Upregulated	321	183	21	41
Avicel Downregulated	22	15	0	1
Xylan Upregulated	175	101	15	8
Xylan Downregulated	278	164	1	2
Reed Canary Grass Upregulated	44	17	0	0
Reed Canary Grass Downregulated	18	13	0	0

Supplementary Table 7.1.3. One or more genes within the *C. churrovis* genome aligned to all listed hydrogenosomal enzymes for *N. lanati*, with a %identity cutoff of 70.0 and a %subject coverage cutoff of 80.0, with the exception of the complex 2 subunit D, which only had a %subject coverage of 59.0 (%identity was 79.6). A=Avicel®, R=reed canary grass, G=glucose, F=fructose, X=xylan

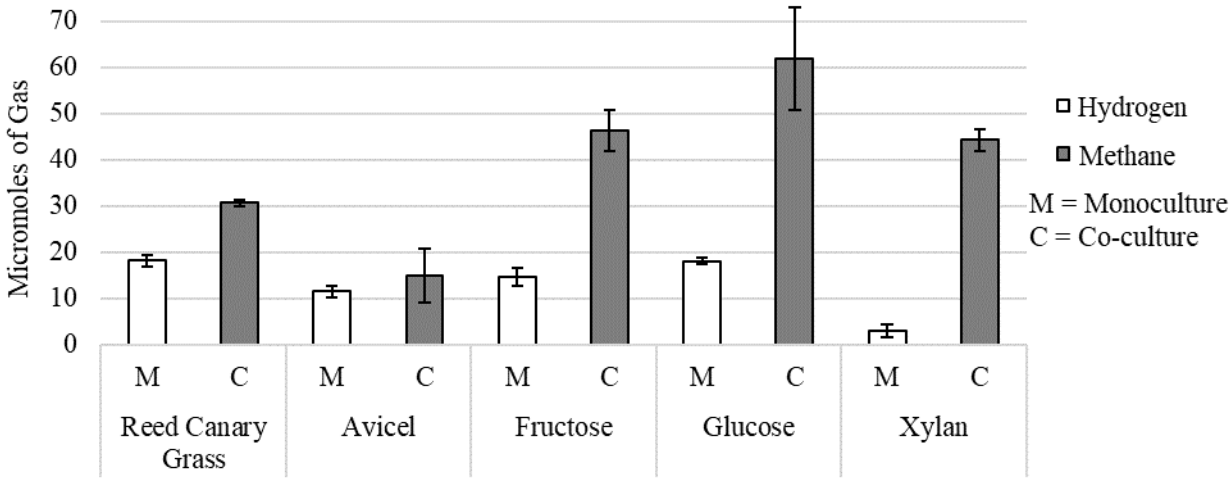
***C. churrovis* Protein ID**	**Enzyme**	**Upregulated or Downregulated (Substrate)**
428490	PFL1/PFL2	Upregulated (X, A, G, F)
11340	PFL1/PFL2	Upregulated (X) Downregulated (A)
193710	PFL1/PFL2	Upregulated (X) Downregulated (R)
193705	PFL1/PFL2	Upregulated (X) Downregulated (R)
420504	PFL1/PFL2	
107174	PFL1/PFL2	Upregulated (X, G)
417119	PFL1/PFL2	
621094	PFL1/PFL2	Upregulated (X)
431187	PFL1/PFL2	Upregulated (X)
214975	PFL1/PFL2	Upregulated (X, F)
621093	PFL1/PFL2	Upregulated (X)
277171	PFL1/PFL2	Upregulated (X)
622192	PFL1/PFL2	Upregulated (X) Downregulated (G)
277169	PFL1/PFL2	Upregulated (X)
418039	PFL1/PFL2	Upregulated (X)
416923	PFL1/PFL2	Upregulated (X)
537129	PFL1/PFL2	Upregulated (X)
635526	PFL1/PFL2	
413357	PFL1/PFL2	
572185	PFL1/PFL2	
462330	PFL1/PFL2	
454624	Ac:SucCoA trans.	
454624	Ac:SucCoA trans.	
453440	SucCoA syn. Sub. A	
519176	SucCoA syn. Sub. B	
526974	SucCoA syn. Sub. B	
243125	Hydrogenase 1	Downregulated (R, G)
456067	Hydrogenase 2	
557447	Complex 1: nuoF	Downregulated (G)
454874	Complex1: nuoE	Downregulated (X, G)
452336	Complex 2: sub. A	
416671	Complex 2: sub. B	
544208	Complex 2: sub. C	
417861	Complex 2: sub. D	
549900	Fumarase	
487810	ATP syn.: sub. Alpha	
443140	ATP syn: sub. Beta	
459763	ATP syn: sub. Delta	
523564	ATP syn.: sub. Gamma	Upregulated (G)



Supplementary Figure 7.1.1. *Caecomyces churrovis* has the highest number of CBM family 18 domains among sequenced anaerobic fungi to date.

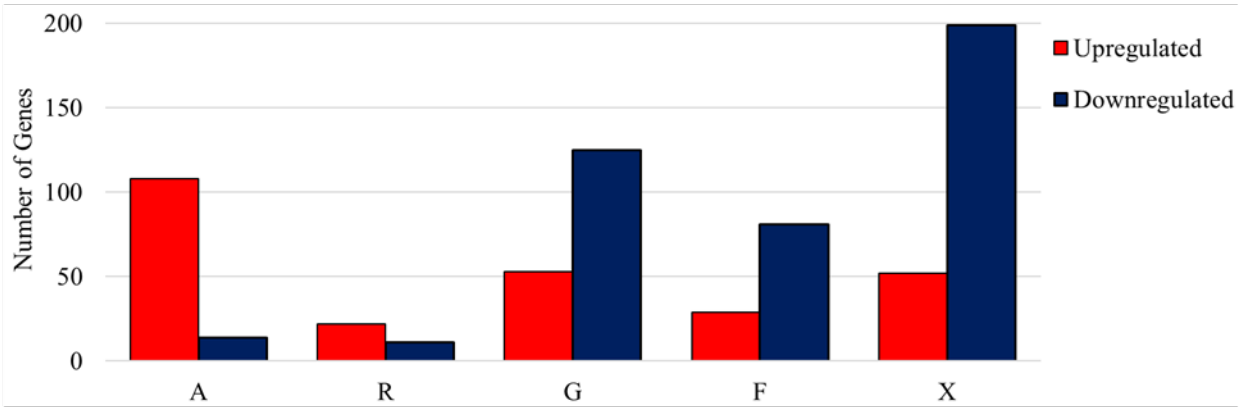


Supplementary Figure 7.1.2. 6mA modifications occur symmetrically at ApT dinucleotides and are concentrated in methylated adenine clusters (MACs) surrounding the transcriptional start sites of expressed genes. This finding agrees with previous work examining 6mA modification in 16 other fungal genomes, including other early-diverging fungi. As shown in Figure 7.1.2A, 92.2% of modifications were symmetric within AT context and 83.32% of all modifications were symmetric. In addition, 89.67% of modifications were in AT context. Figure 7.1.2B shows the frequency (# 6mA observed / # available sites) per position ±1500 bp surrounding transcriptional start sites in *C. churrovis*. A slight wave in modification frequency is observed following the start of the 5' utr, not seen before in other early-diverging fungi, but the presence of modifications at the start of genes is in agreement with previous studies of fungal 6mA modifications.[25] Figure 7.1.2C shows the log2fold enrichment of 6mA modifications by region. Log2_enrichment refers to the enrichment of 6mA at a given feature relative to the expected abundance of 6mA genome-wide, normalized by GC content. Figure 7.1.2D shows the percent of total 6mA modifications that are found within each region. Note that many of these regions overlap, such as introns and genes. Regions are defined as follows: exons – non-intron genic space, CDS – coding sequence only, gene – entirety of genic space, promoter – ±500bp surrounding transcriptional start sites, repeats – repetitive sequences identified using RepeatScout and RepeatMasker, intron – introns within genic regions, TTS – ±250bp surrounding transcription termination sites.



Supplementary Figure 7.1.3. End-point methane and hydrogen measurements for monocultures of *C. churrovis* and co-cultures of *C. churrovis* and *M. bryantii*. Gas chromatography was used to determine the concentration of methane and hydrogen in the headspace gas of co-cultures and monocultures on each substrate upon harvest for RNA extraction. No significant amount of hydrogen was detected in the co-cultures, and no methane was detected in the monocultures. Significantly higher amounts of methane were produced on soluble substrates.

A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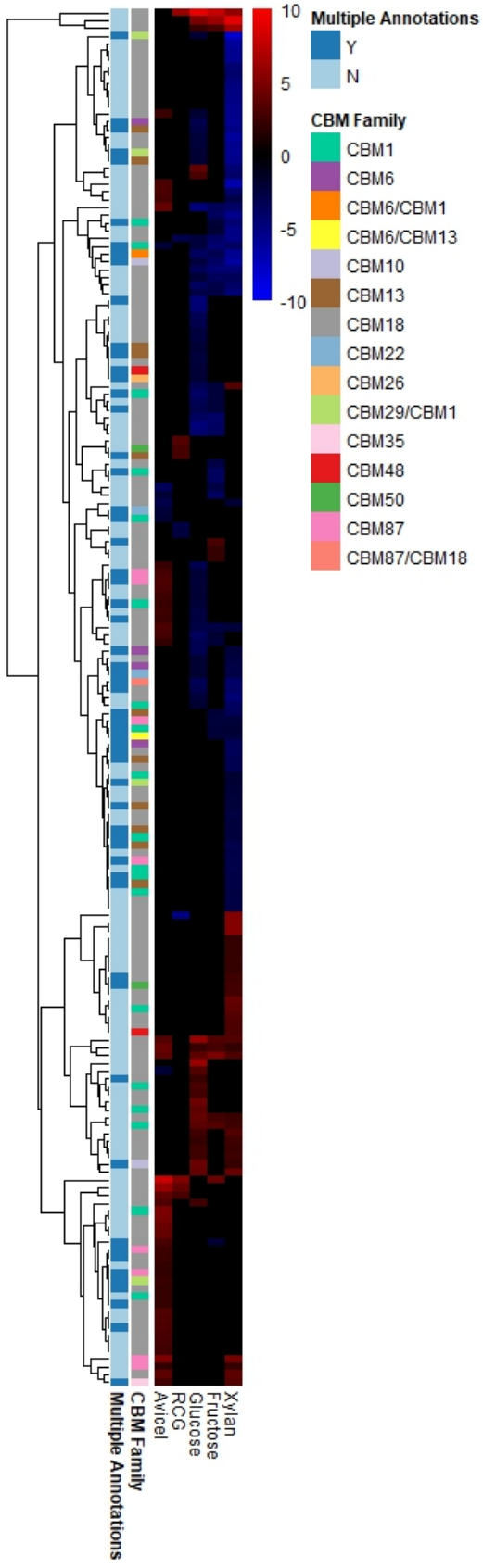
Upregulated
Downregulated

B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CBM26
CBM29/CBM1
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Fructose
Glucose
RCG
Avicel
CBM Family
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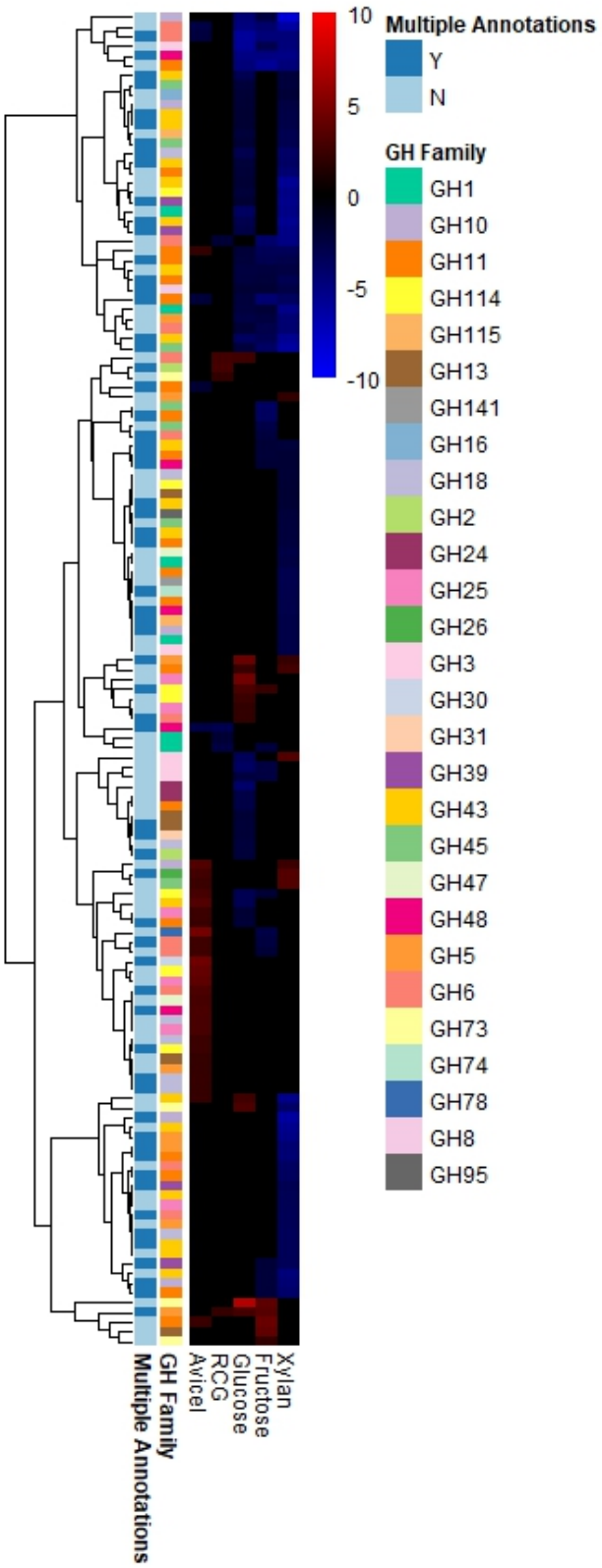
C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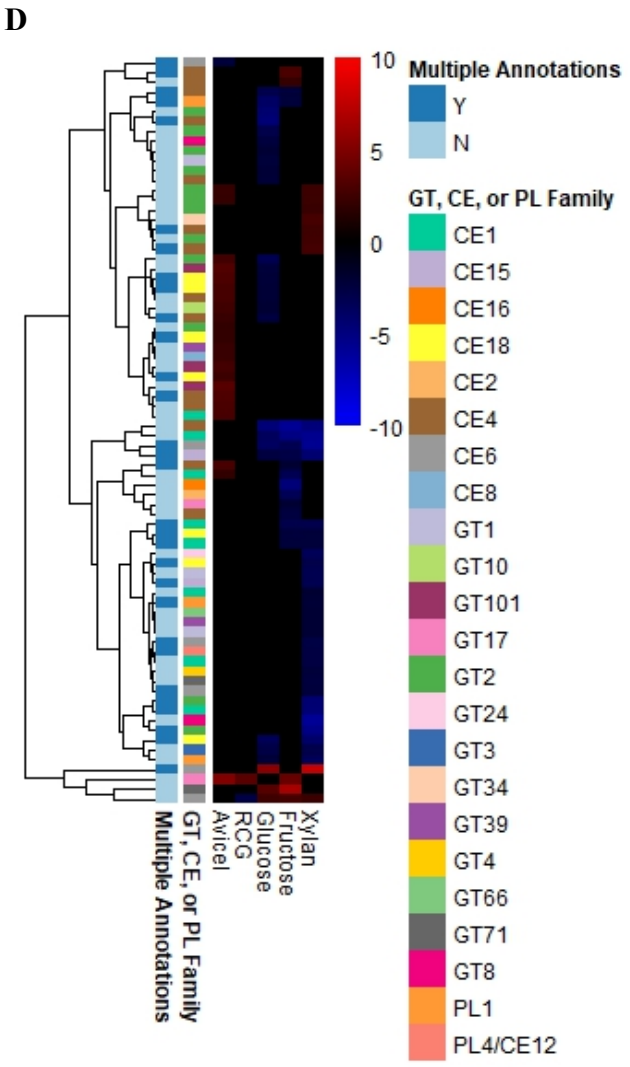
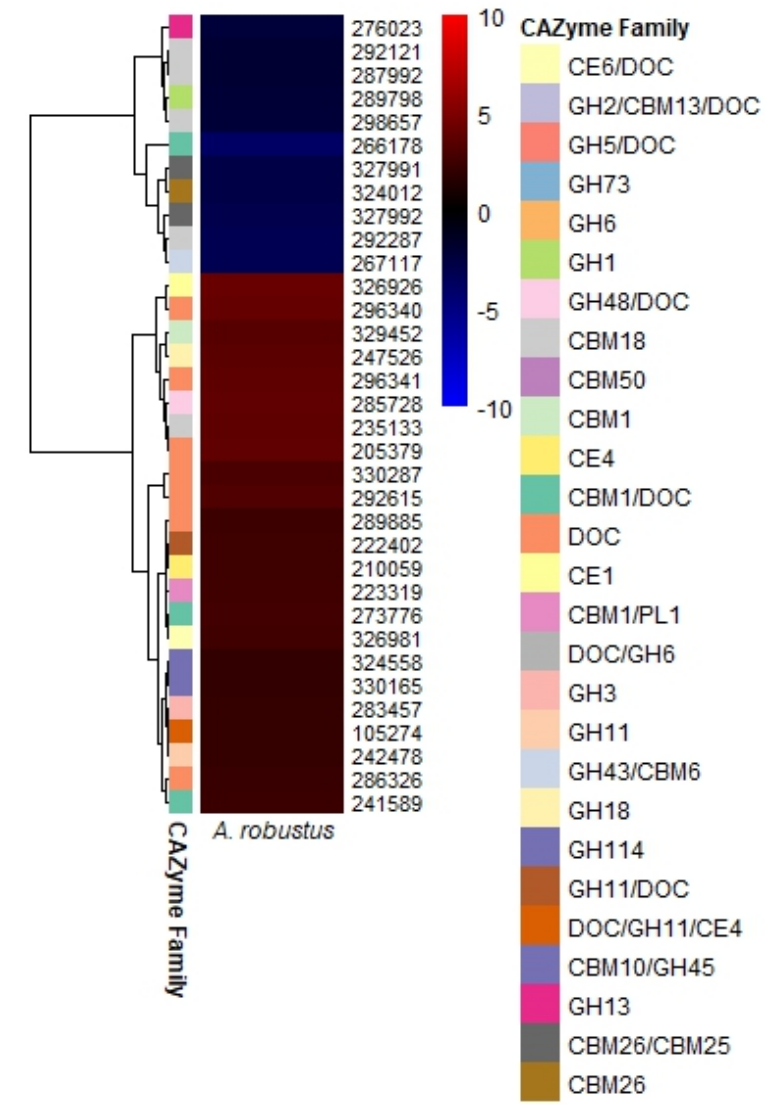


Supplementary Figure 7.1.4. The total number of genes annotated as CAZymes upregulated or downregulated overall (Figure 7.1.4A) and for carbohydrate binding module (CBM) (Figure 7.1.4B), glycoside hydrolase (GH) (Figure 7.1.4C), carbohydrate esterase (CE), polysaccharide lyase (PL), and glycosyltransferase (GT) families (Figure 7.1.4D) for fungal-methanogen co-cultures of *C. churrovis* paired with *M. bryantii* relative to fungal monocultures of *C. churrovis* on a range of substrates. A=Avicel®, R or RCG=reed canary grass, G=glucose, F=fructose, X=xylan, Y=yes, N=no.

A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292615
289885
222402
210059
223319
273776
326981
324558
330165
283457
105274
242478
286326
241589
A. robustus
CAZyme Family
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-10
CAZyme Family
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GH48/DOC
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GH13
CBM26/CBM25
CBM26

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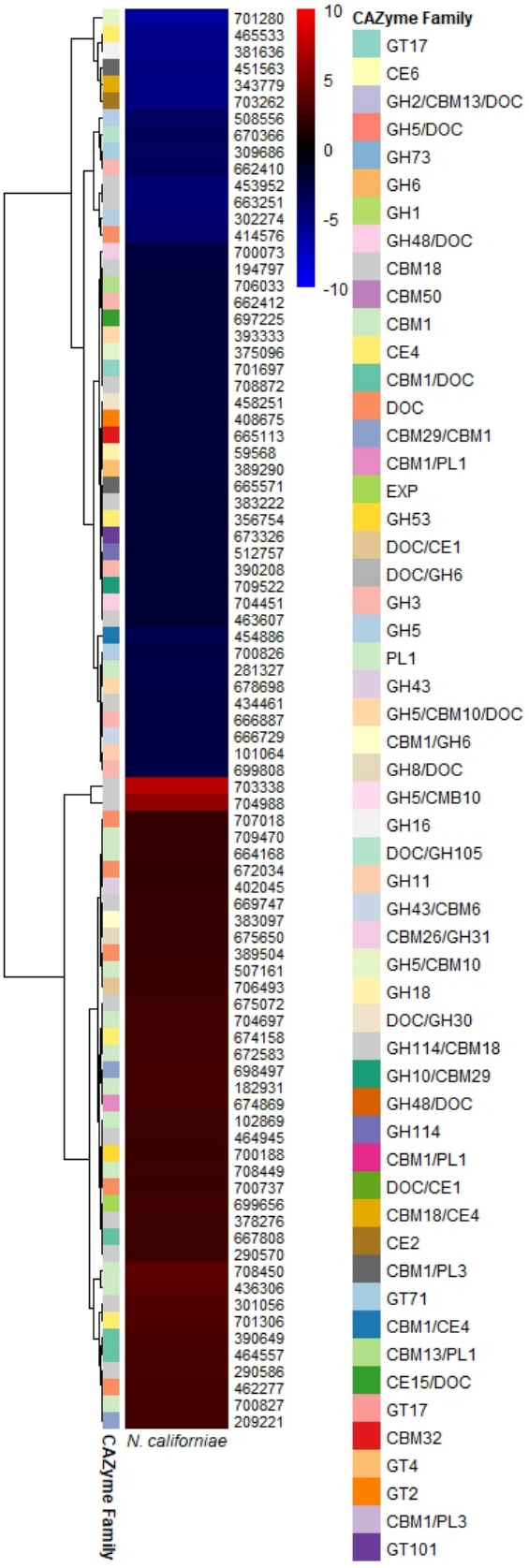
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700827
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CAZyme Family
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GH6
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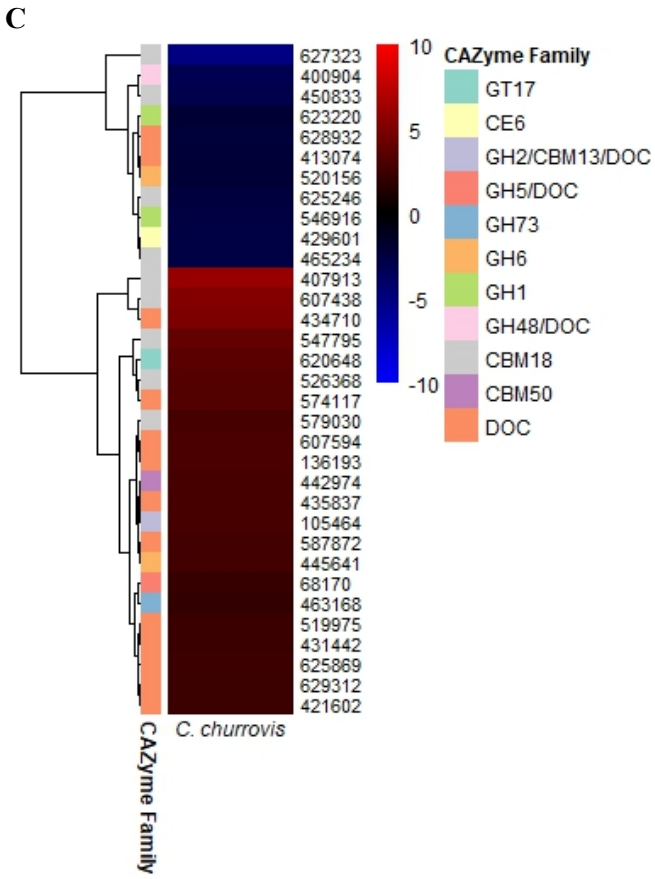
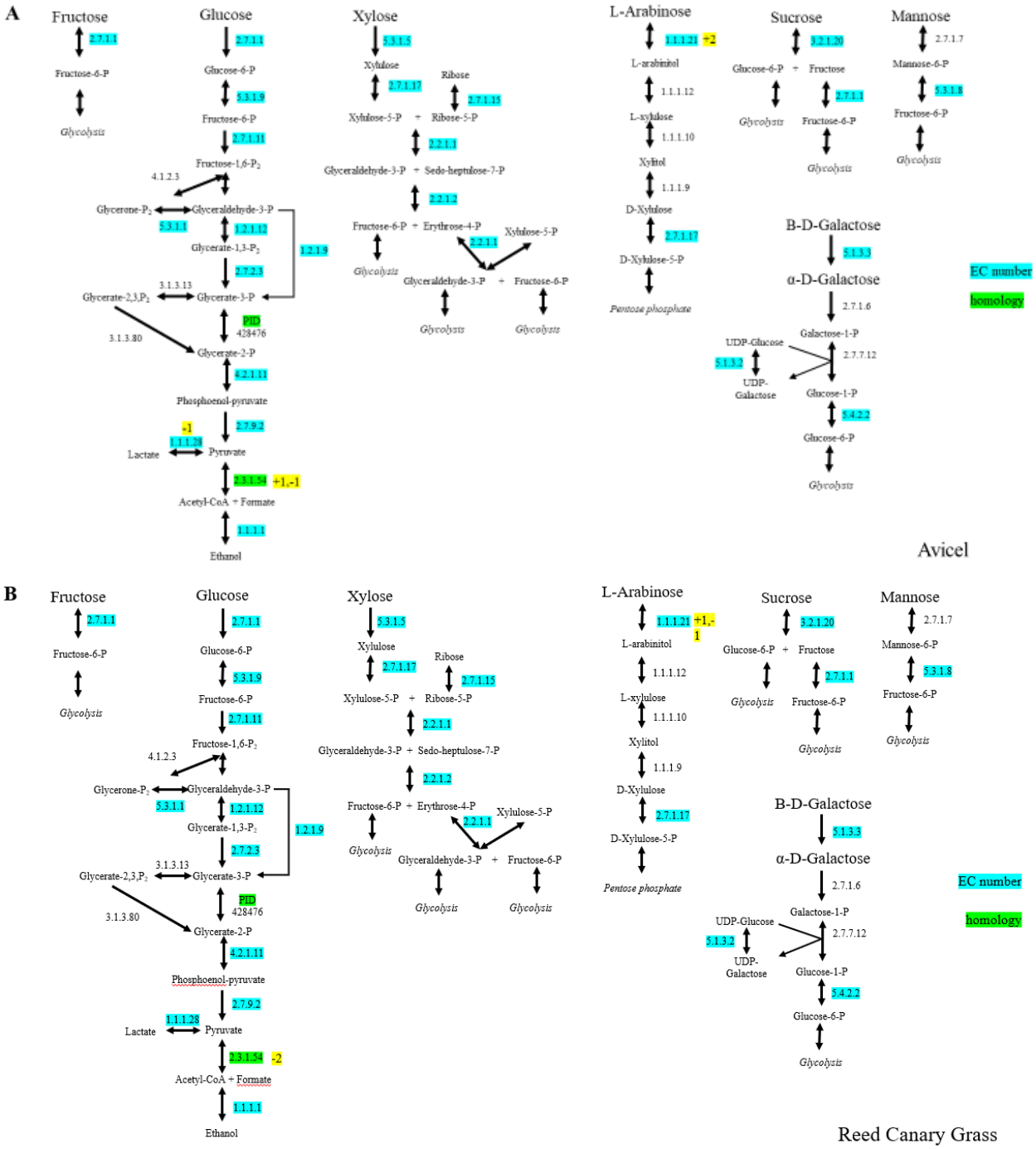


Supplementary Figure 7.1.5. Regulated genes annotated as dockerins, CBM, GT, PL, CE, and GH families in the three fungal strains *A. robustus* (A), *N. californiae* (B), and *C. churrovis* (C) in fungal-methanogen co-culture versus fungal monoculture on a reed canary grass substrate. CBM 1 and CBM 18 families were significantly regulated (significant regulation defined as number of genes regulated > or =5). The CBM 1 family was highly upregulated for the *N. californiae* strain. GH 3 and GH 5 were significantly downregulated in the *N. californiae* strain. GH=glycoside hydrolase, GT=glycosyltransferase, PL=polysaccharide lyase, CBM=carbohydrate binding module, CE=carbohydrate esterase, DOC=dockerin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Xylose
L-Arabinose
Sucrose
Mannose
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Xylulose
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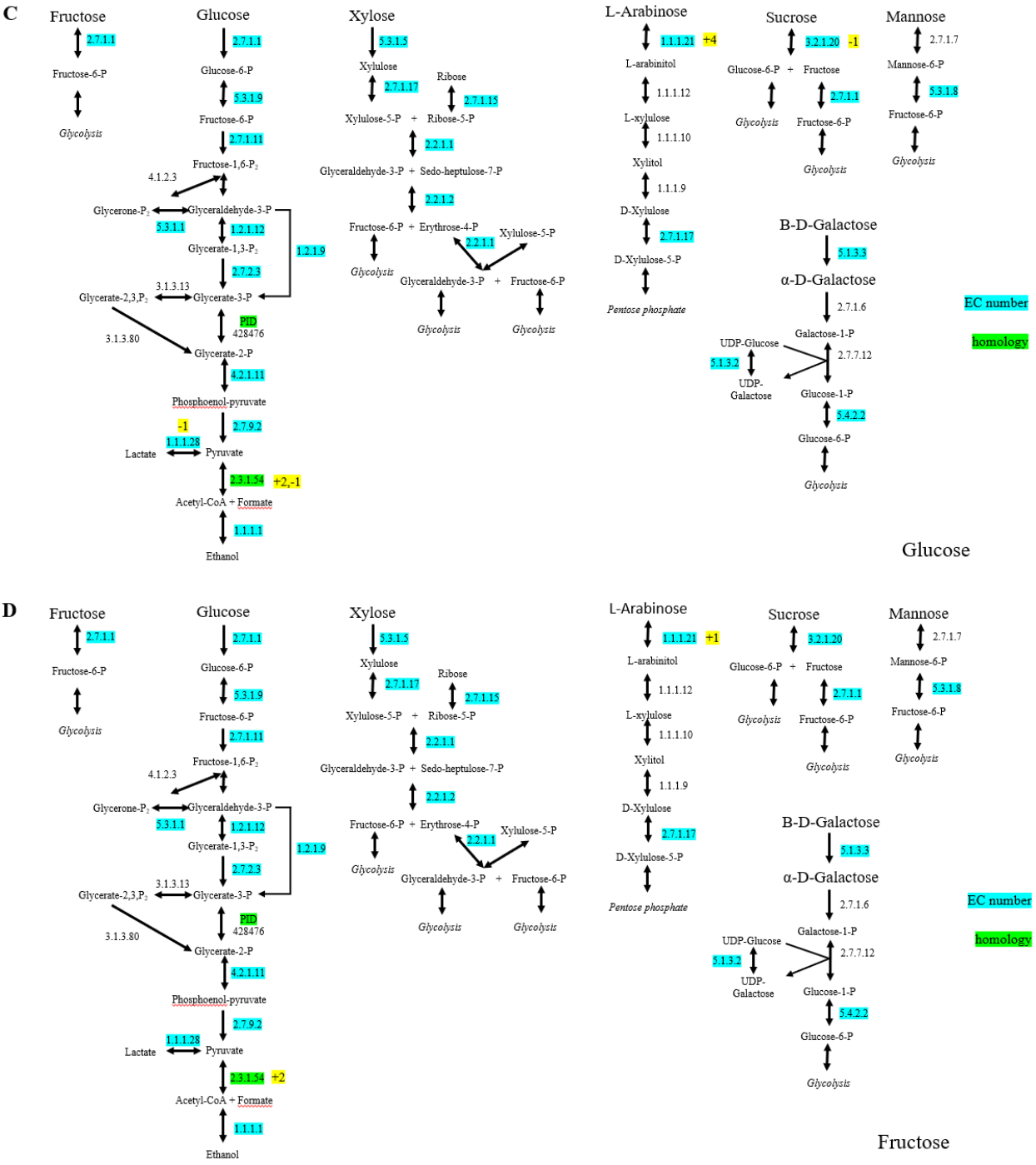
Glucose-6-P + Fructose
Mannose-6-P
Glycolysis
Fructose-6-P
Xylulose-5-P + Ribose-5-P
L-xylulose
Fructose-6-P
Fructose-1,6-P2
Glyceraldehyde-3-P + Sedo-heptulose-7-P
Xylitol
Glycerone-P2
Glyceraldehyde-3-P
Fructose-6-P + Erythrose-4-P
D-Xylulose
B-D-Galactose
Glycerate-1,3-P2
Glyceraldehyde-3-P + Fructose-6-P
D-Xylulose-5-P
α-D-Galactose
Glycerate-2,3,P2
Glycerate-3-P
Pentose phosphate
UDP-Glucose
Galactose-1-P
PID 428476
Glycerate-2-P
UDP-Galactose
Glucose-1-P
Phosphoenol-pyruvate
Lactate
Pyruvate
Acetyl-CoA + Formate
Ethanol
EC number
homology
Avicel
B
Reed Canary Grass


C
Fructose
Glucose
Xylose
L-Arabinose
Sucrose
Mannose
Fructose-6-P
Glycolysis
Glucose-6-P
Fructose-6-P
Fructose-1,6-P2
Glycerone-P2
Glyceraldehyde-3-P
Glycerate-1,3-P2
Glycerate-2,3,P2
Glycerate-3-P
PID 428476
Glycerate-2-P
Phosphoenol-pyruvate
Lactate
Pyruvate
Acetyl-CoA + Formate
Ethanol
Xylulose
Ribose
Xylulose-5-P + Ribose-5-P
Glyceraldehyde-3-P + Sedo-heptulose-7-P
Fructose-6-P + Erythrose-4-P
Xylulose-5-P
Glyceraldehyde-3-P + Fructose-6-P
L-arabinitol
L-xylulose
Xylitol
D-Xylulose
D-Xylulose-5-P
Pentose phosphate
Glucose-6-P + Fructose
B-D-Galactose
α-D-Galactose
Galactose-1-P
UDP-Glucose
UDP-Galactose
Glucose-1-P
Mannose-6-P
EC number
homology
Glucose
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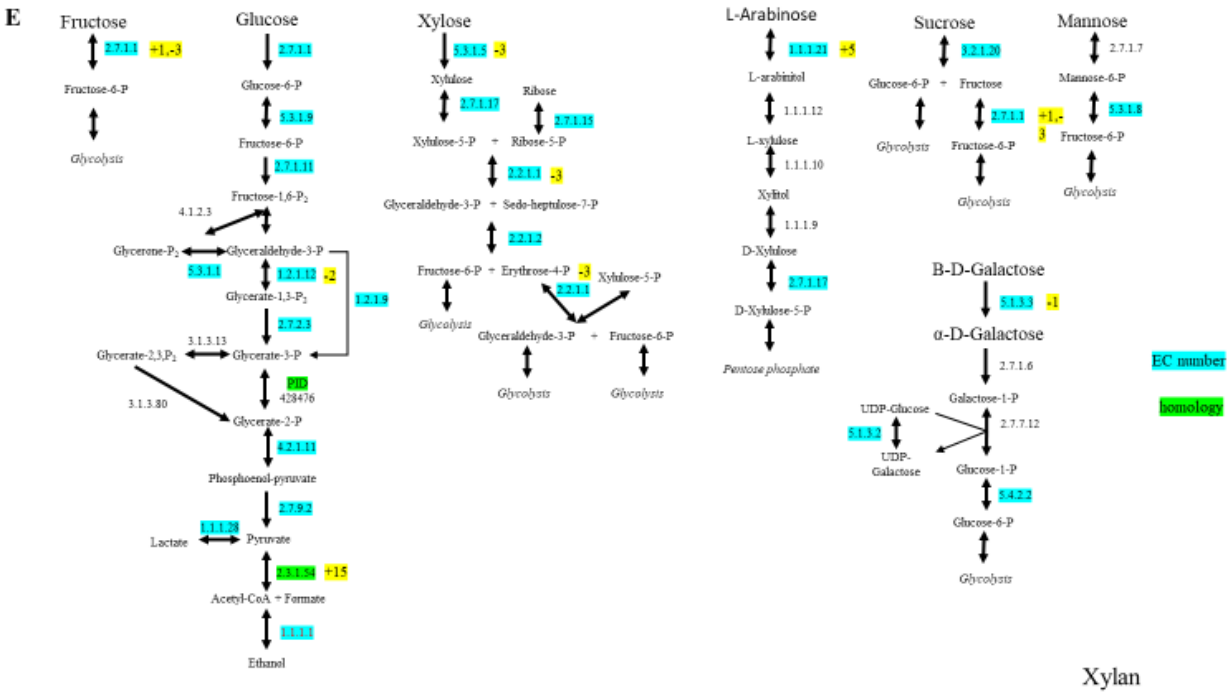
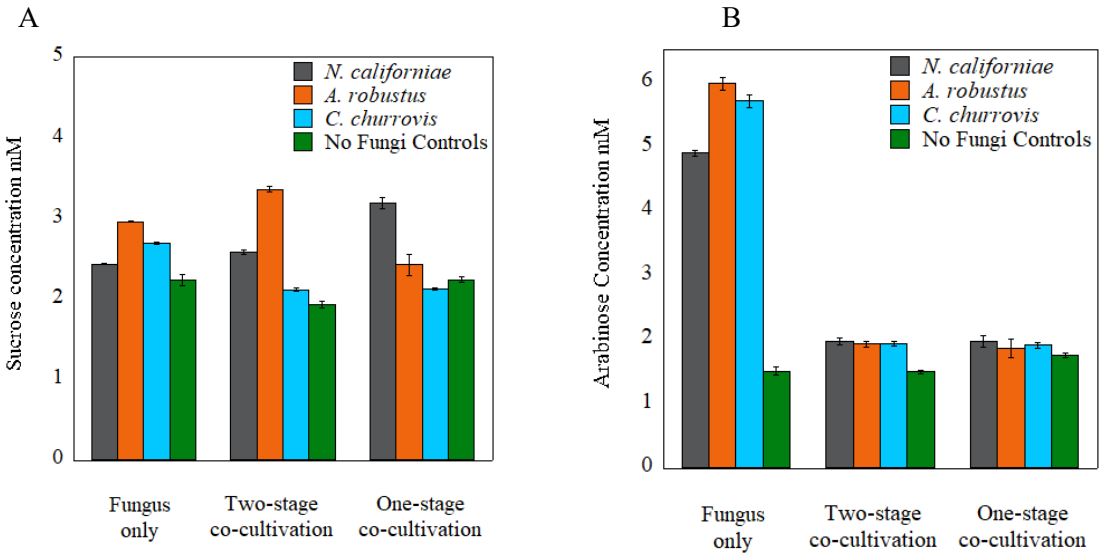
L-Arabinose
Sucrose
Mannose

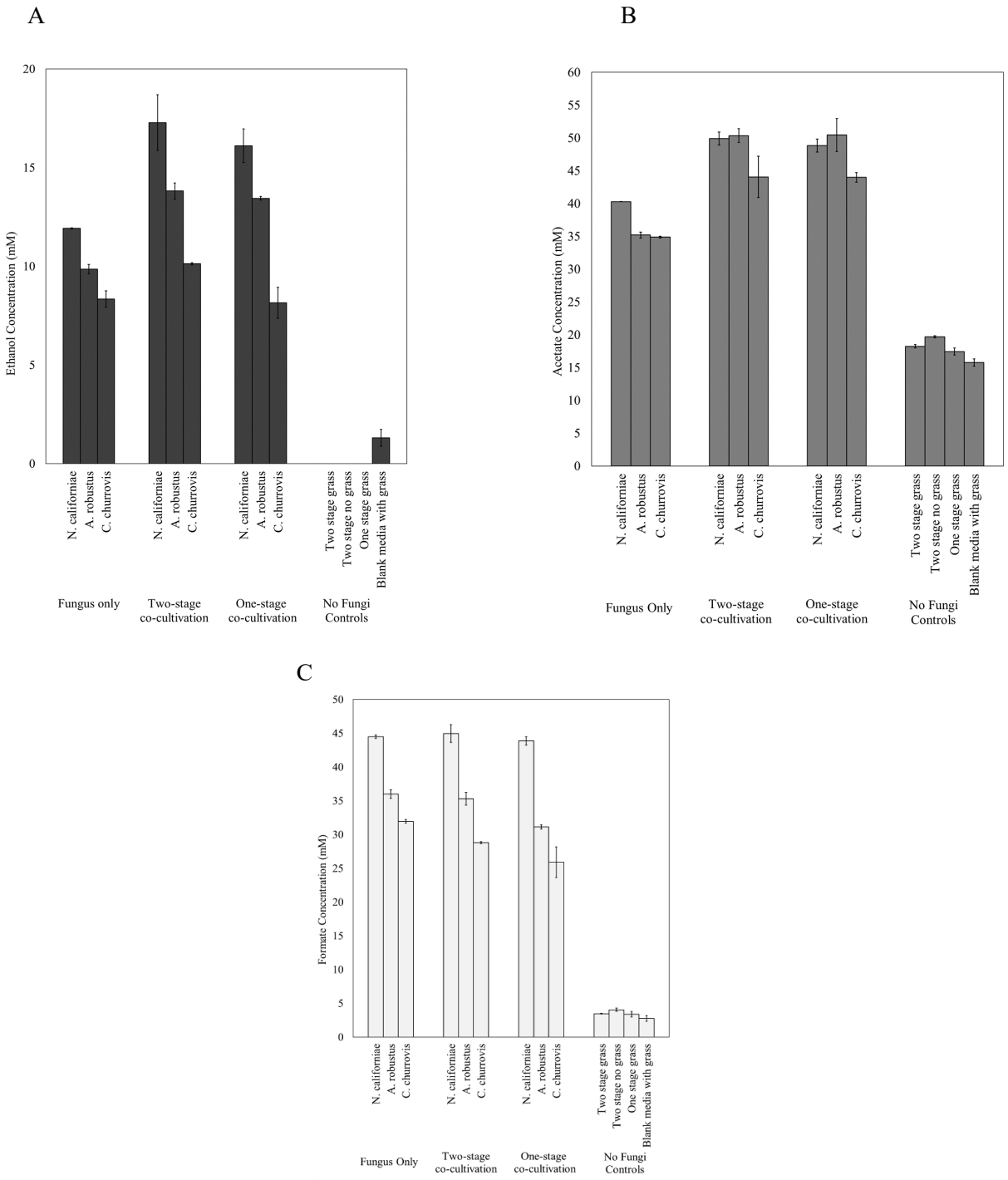


Supplementary Figure 7.1.6. Transcriptional regulation of genes within sugar pathways for co-culture versus monocultures of *C. churrovis* and *C. churrovis* paired with *M. bryantii*. The number of genes upregulated (+) or downregulated (-) that are annotated as each enzyme are highlighted in yellow. Enzymes were either identified by Enzyme Commission (EC) number (highlighted in blue) or homology (highlighted in green). Cultures grown on all substrates with the exception of reed canary grass had at least one gene annotated as a pyruvate formate lyase (PFL) upregulated. Results indicated that co-culture with a methanogen may enhance PFL function in cultures grown on fructose and xylan. PFLs were identified through homology to PFLs identified as hydrogenosome components in the *N. lanati* genome. Select enzymes in sugar pathways were upregulated in co-culture for some substrates, indicating enhanced production of bottleneck enzymes in sugar pathways. This figure depicts regulation in the cultures grown on Avicel® (A) reed canary grass (B), glucose (C), fructose (D), and xylan (E) substrates.

7.2 Appendix B: Supplementary figures for chapter three

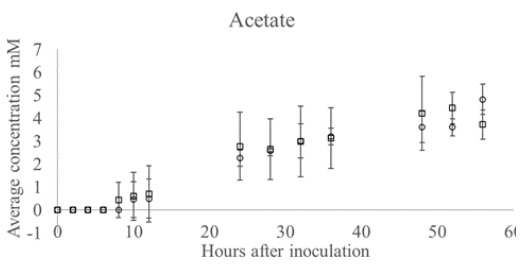


Supplementary Figure 7.2.1. Sucrose and arabinose sugars in cultures grown on M2 in reed canary grass long-term. With the exception of sucrose, sugars released by the fungi were significantly depleted in all experimental cultures containing *C. acetobutylicum*, indicating that sugars released by the fungus can sustain growth of *C. acetobutylicum*.

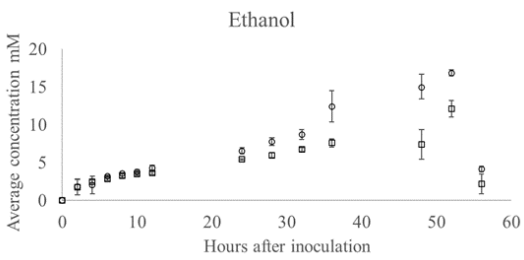


Supplementary Figure 7.2.2. Production of ethanol, acetate, and formate in cultures grown in M2 with reed canary grass after 29 days of microbial growth for the one-stage co-cultivation condition or 10 days of *C. acetobutylicum* growth in the two-stage conditions grown in spent fungal supernatant that the fungi had grown for 22 days previously. Levels of these metabolites did not vary significantly between the two-stage and one-stage cultivation conditions for any fungal strain used in the study.

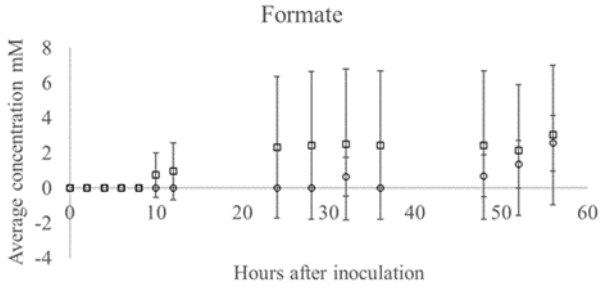
A



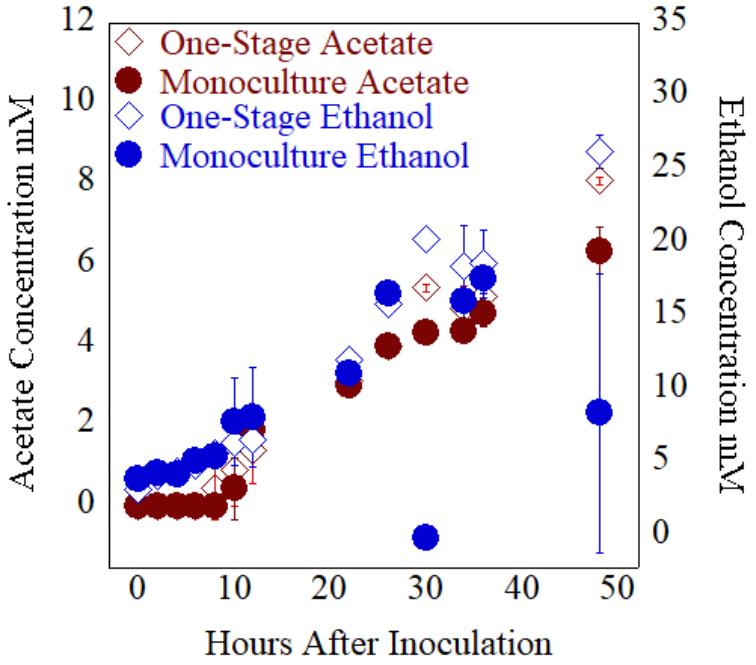
B



C



Supplementary Figure 7.2.3. Timecourse graphs of HPLC readings of acetate, ethanol, and formate production for *C. acetobutylicum* cultivated in anaerobic fungal supernatant (two-stage cultivation condition) and *C. acetobutylicum* controls grown in media the fungi had not grown in previously.

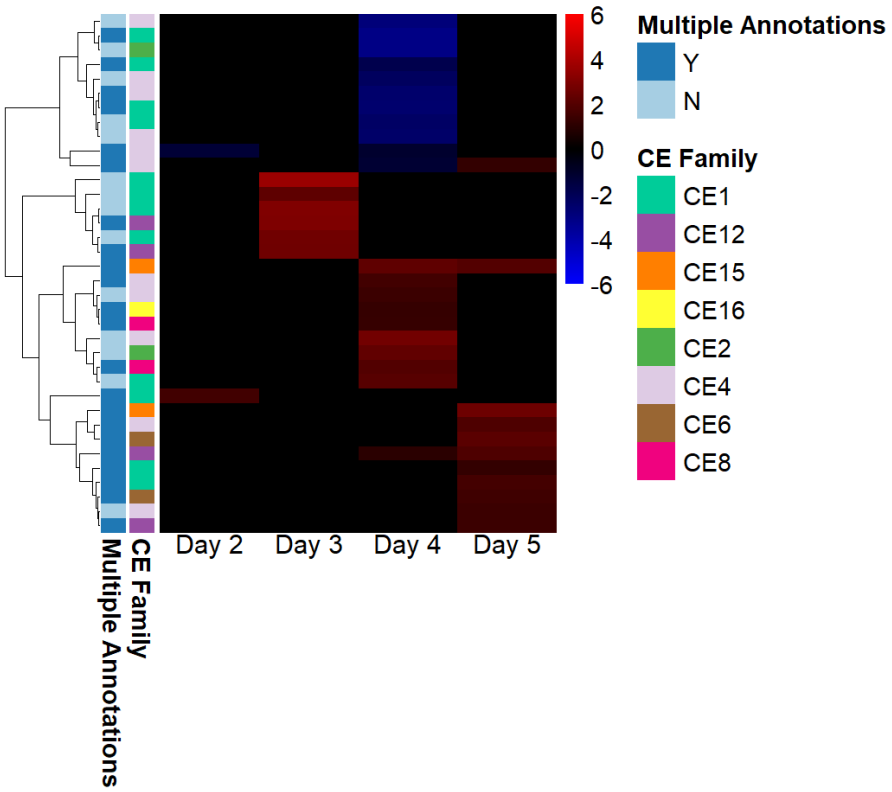


Supplementary Figure 7.2.4. Timecourse graph of HPLC readings of acetate and ethanol production for *C. acetobutylicum* co-cultured with the anaerobic fungal strain *A. robustus* (one-stage cultivation condition) and *C. acetobutylicum* monoculture controls.

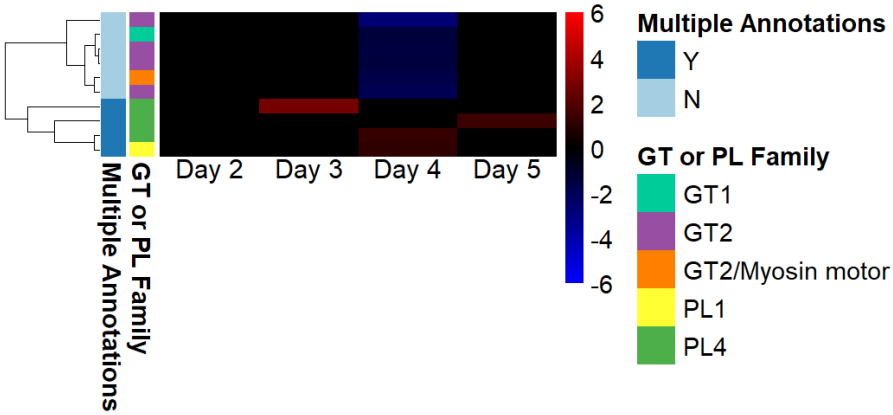
Supplementary Table 7.2.1. Upregulated *C. acetobutylicum* genes associated with cellulose degradation in the two-stage cultivation condition versus *C. acetobutylicum* monoculture. Differential gene expression analysis indicated that 7 out of the 12 genes associated with cellulose degradation were upregulated in the two-stage cultivation condition compared to *C. acetobutylicum* controls. None of the 12 genes associated with cellulose degradation were downregulated.

Locus Tag	Gene Product Name	Log2fold Change
CAC0911	cellulose 1,4-beta-cellobiosidase (EC 3.2.1.91)	2.05
CAC0912	Possible non-processive endoglucanase family 5, secreted; CelA homolog secreted; dockerin domain	1.93
CAC0913	endoglucanase Cel9G	1.77
CAC0916	endoglucanase (EC:3.2.1.4)	1.14
CAC0561	non-processive endocellulase	1.81
CAC0915	Endoglucanase A precursor (endo-1,4-beta-glucanase) (cellulase A), secreted; dockerin domain	1.19
CAC0910	Probably cellulosomal scaffolding protein precursor, secreted; cellulose-binding and cohesin domain	2.11

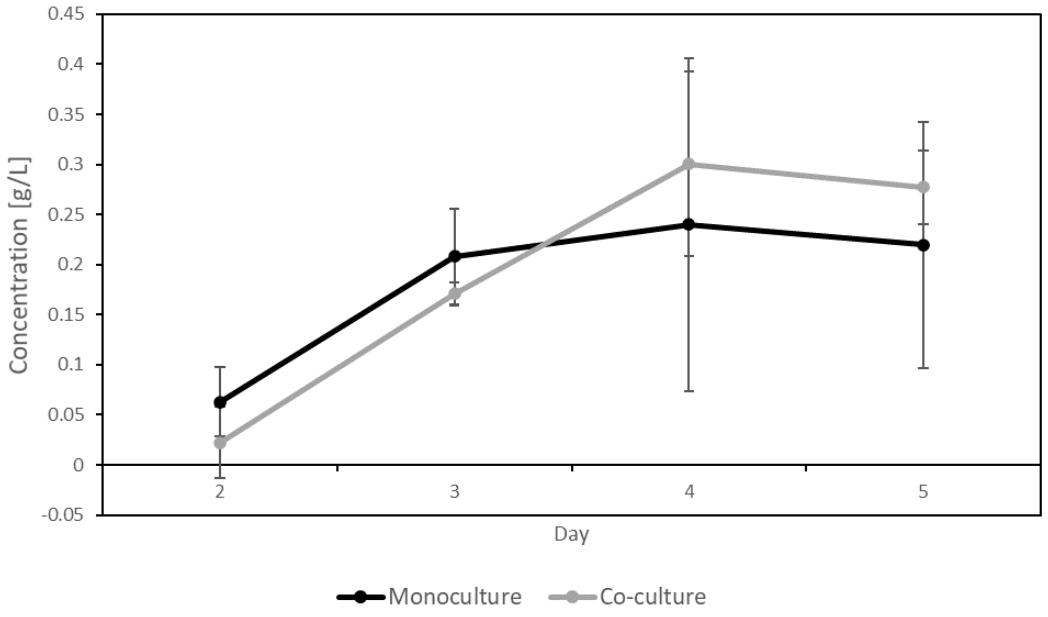
7.3 Appendix C: Supplementary figures for chapter four



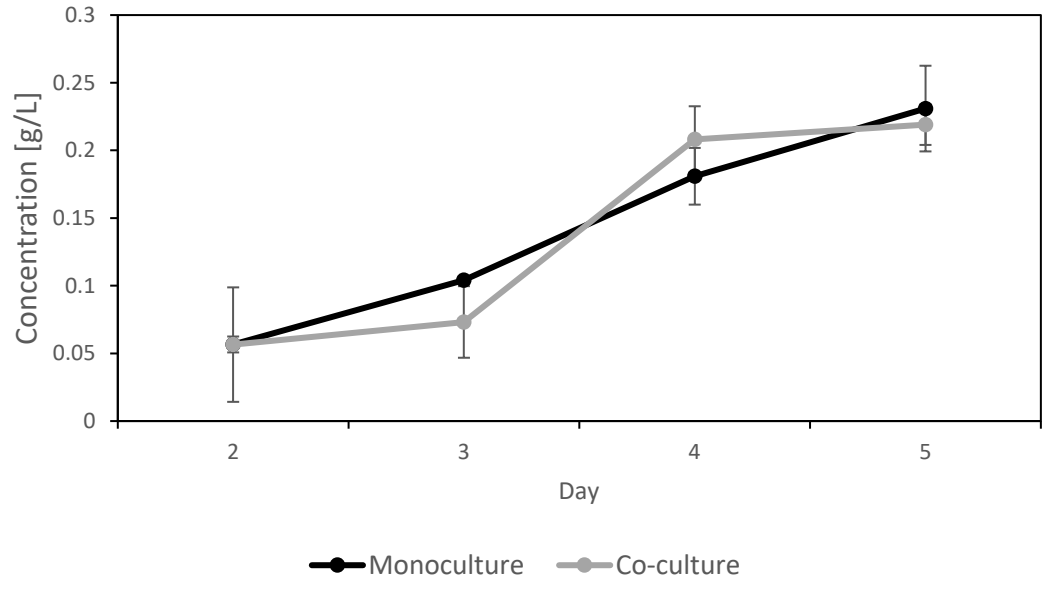
Supplementary Figure 7.3.1. CE regulation in *A. robustus* and *M. bryantii* fungal-methanogen co-culture versus *A. robustus* fungal monoculture. Regulated genes annotated as CEs were only upregulated (none downregulated) on days 3 and 5.



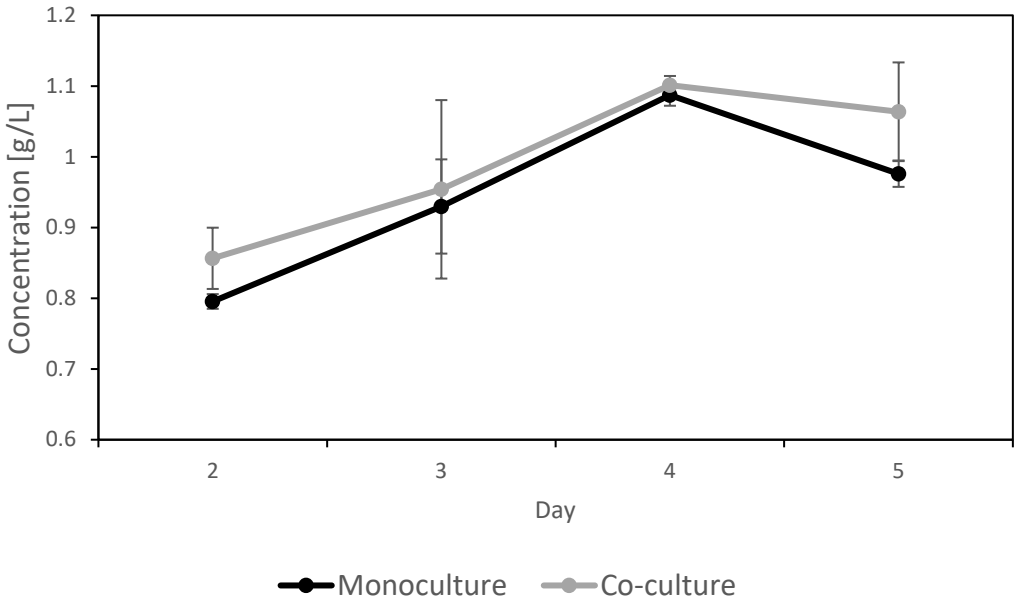
Supplementary Figure 7.3.2. GT regulation in *A. robustus* and *M. bryantii* fungal-methanogen co-culture versus *A. robustus* fungal monoculture. GTs were only downregulated on any of the days and PLs were only upregulated on any of the days.



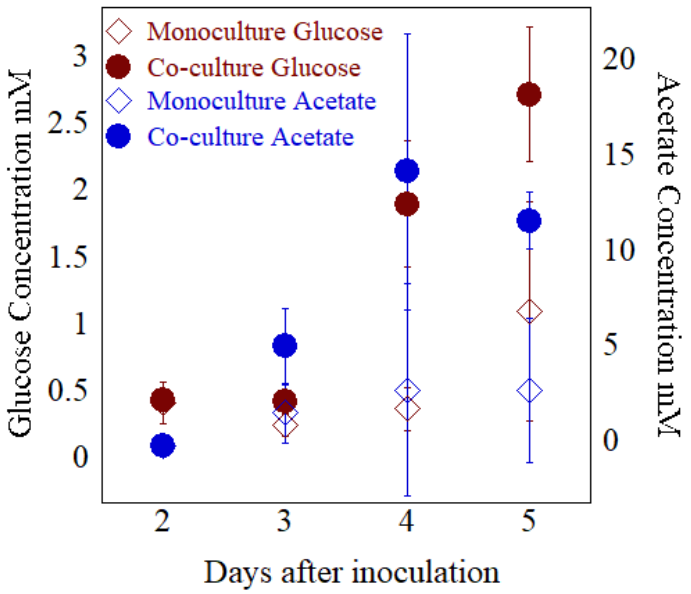
Supplementary Figure 7.3.3. Formate concentration over time for monocultures of *A. robustus* and co-cultures of *A. robustus* and *M. bryantii* grown on a cellulose substrate.



Supplementary Figure 7.3.4. Lactate concentration over time of *A. robustus* monocultures and *A. robustus* and *M. bryantii* co-cultures grown on a cellulose substrate.



Supplementary Figure 7.3.5. Ethanol concentration over time of *A. robustus* monocultures and *A. robustus* and *M. bryantii* co-cultures grown on a cellulose substrate.



Supplementary Figure 7.3.6. Co-cultivation of *A. robustus* with a methanogen increased acetate production and glucose release when grown on a cellulose substrate. Higher levels of glucose and acetate were present in *A. robustus* and *M. bryantii* co-cultures compared to *A. robustus* monocultures after 5 days of growth on a cellulose substrate (Whatman filter paper).

ProteinID	Predicted SM Type	Monoculture Log2FC	Monoculture p-value	Co-culture Log2FC	Co-culture p-value
245209	PKS	2.3	2.90E-15	0.7	0.04
204091	PKS	4.7	1.10E-33	4	1.80E-24
298303	PKS-Like	4	5.80E-31	3.9	4.40E-26
289077	PKS	3.4	2.10E-48	3.7	6.70E-51
330657	NRPS	1.5	1.70E-11	1.7	1.60E-12
271916	NRPS	0.9	3.50E-04	0.8	0.001
248107	PKS	2	6.20E-22	1.9	1.30E-10
212224	PKS	1.1	7.80E-04	1.3	1.60E-05
328517	PKS-Like	4.3	4.60E-41	4	1.40E-27

Supplementary Table 7.3.1. Nine predicted *A. robustus* secondary metabolite (SM) core genes are significantly upregulated at early growth compared to late growth. Early harvest is defined as samples harvested after two days of growth, and late harvest as samples harvested after five days of growth in monoculture and six days in co-culture. Classes of secondary metabolites upregulated: PKS=polyketide synthase, NRPS=nonribosomal peptide synthase. Log2FC=log2-fold change.

7.4 Appendix D: Supplementary figures for chapter five

Supplementary Table 7.4.1. The *Piromyces* sp. E1M ITS1 (E1_1_206-JB206_H12.ab1) and LSU (E1_1-LSU_E03.ab1) sequences.

>E1_1_206-JB206_H12.ab1

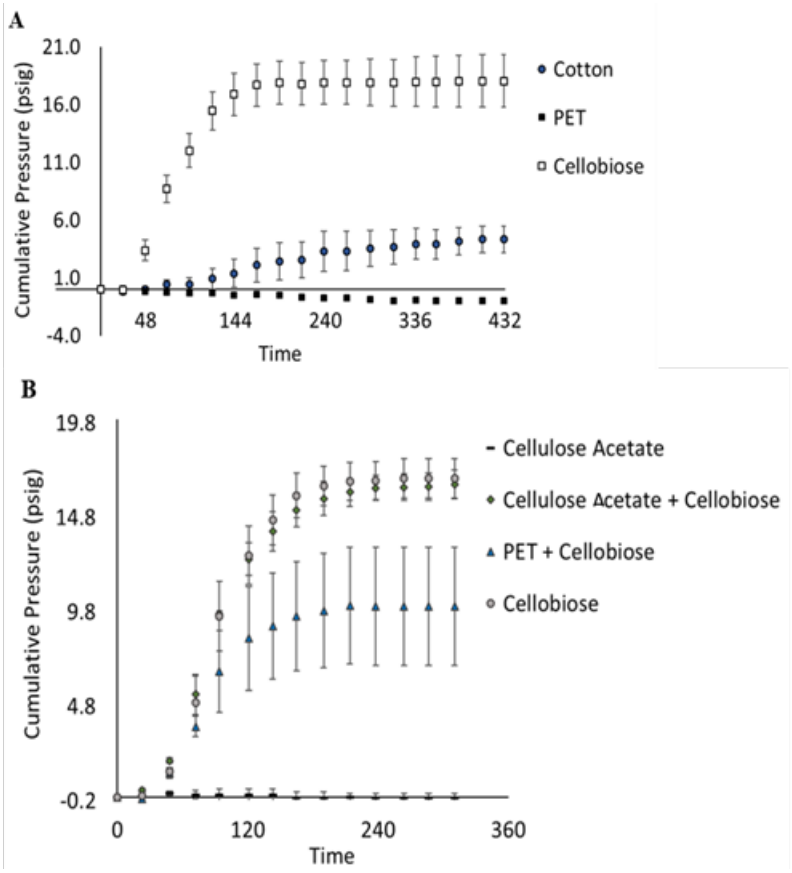
GNNNNNNNNNNNNNNNNCTGANNGNGANCNAGNNNTTNNGTTTAAATT
NNNNCCTTNTNANGTTTGGATTTTCTAATCAAAAATTAAAATNNGTTTAGGTT
ATTCCTTTTTACGGGAAAATTTTATCCAATTTTATTTTAAAATTAGAAAAATTC
AAATTTTAAAAAAATTTAAAGGGGAACAACCTGGATCGGTGTAAAACACTCA
TAACCATAAAAAACAATGGTTTTTATGAAAATATTTTACTGATACCCAAACAG
ACATACTTTTTTAGTAAACTAAAAAAGTGCAATATGCGTTCGAAGAATCCATG
AATCACGTATTCCGCAATTCACACCACTTATCGCATTTTGCTGCGTTCCTCATC
GNTGCNAGAACCAAGAGATCCATTGGCAAAAGTTGGTTTTATATTATAAAAA
TAATTTTTANACCAANGNANACCAAAAGTTTAATTTTAAAAAAAGGGTCTTTT
TNAAAAATTACNGCCCCCCCCCNGNNAATCTTTTGTCATTAAAANNNCCANTT
NAACAGGNAAANANNTTNAAAANTTTTAAAAAAAAAATTNTTNCNANCNAAC
NAATTCACNNNGGGNAGGATTATNANCCGCCNANNNAAAAAACGTNNACCA
TTTNTNNNNGNTCCNNCCGNAGGTNCNCCNACGGAANCNTTGTTNNNNNNNT
NNNNNCNNAAAAA

>E1_1-LSU_E03.ab1

NNNNNNNNNNNNANTNCGGNNAGTGAAGCGGGAAGAGCTCAAATTTGAAA
TCTTCAAGGTTCTACCTTGACGAATTGTAGTTTAAAGAAGTGTTTTCTGTTGAA
GTTTTGGTAAAAGTTCTTTGGAATAGGACATCTTAGAGGGTGAGAATCCCGTA
TTTGACCATTATTTTCAGCTGTGTGATACACTTTCAAATAGTCGGATTGTTTGG
GAATGCAGTCCAAAATGGGTGGTAAATTTCATCTAAAGCTAAATATTGGCGA
GAGACCGATAGCGAACAAGTACCGTGAGGGAAAGATGAAAAGAACTTTGAA
AAGAGAGTTAAACAGTACGTGAAATTGTCAAAAGGGAAGCGTTTGACACCAG
TGTTGTTTTCTCGAAAATCAATTAAAAANAGTTGGATTTTGTGTTGTATTGACC
TTAACAGCTTGCTTCACTCTTTTCCTGCTTTTTTTAATGCACTTTTCGTTTAACA
AGTCAACATCAGTTTCTTTTGTTGTAAAAGGGTCATTGGAAGGTGGCTTTCTCT
TCGGAGAAAGTGTTTATAGCCTTTGATCTCTGCAATCGGAGAGACTGANGTCT
GCAGCGTANATCCCTTCGGGGTNAGATACATTTGTGCTAAGCTACAGCTTTTC
ATANACAACTTGTTGACTATGTTTAGTCNTGTGACTTACCCGCTTATGCTATNT
NNNCTANGCNTNANGGATGCTNACAAANTGGNNTTNNANNNACCCNNCTTNG
AAANNACGGAACNNANNNNN

7.5 Appendix E: Anaerobic fungi and potential for microplastic degradation

We also conducted studies to investigate whether anaerobic fungi are capable of degrading microplastics present in the OFMSW. The capability of the fungi to degrade polyethylene terephthalate (PET) and cellulose acetate, two common environmental pollutants originating from textiles and cigarette filters, respectively, was tested relative to cotton (cellulose) microfiber degradation. Pressure accumulation resulting from H_2 production by the fungi was used to create growth curves on each substrate, as shown in Supplementary Figure 7.5.1.



Supplementary Figure 7.5.1. Pressure growth curves for the fungus *N. californiae* (A) and *A. robustus* (B) indicate that anaerobic fungi are not able to grow on PET microplastic as a sole carbon source, but that the fungi can grow in the presence of the microfibers when provided a soluble sugar carbon source, such as cellobiose.

The fungal samples containing PET microfibers produced less pressure than controls with no carbon source available, indicating that the PET microfibers may inhibit fungal growth, and those samples containing cellulose acetate only produced as much pressure as the controls with no carbon source. Since the fungi could not survive on plastic as a sole carbon source, additional co-digestion experiments were performed with the plastic microfibers and soluble sugars to determine whether the fungi would be able to grow in the presence of the fibers when other carbon sources were available. While the PET fibers still seemed to exhibit a mildly inhibitory effect on fungal growth, the fungi were able to grow on soluble sugars in the presence of the fibers. These results reveal the potential to design consortia with other plastic-degrading microbes if the fungal enzymes do not degrade the microplastics.

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