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Integration of *Clostridium thermocellum* Consolidated Bioprocessing With Thermochemical Pretreatments for Fuel Ethanol Production From Switchgrass

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

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by

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Chapters 1-6 of this dissertation will be submitted or are currently in Peer Review for publication in various scientific journals. Specifically, Chapters 3 and 5 have been submitted and are in Peer Review for publication in *Biotechnology for Biofuels*.

Dedication

This dissertation is dedicated to my parents, Dr. (Mrs.) Shobha D. Kothari and Dr. Dushyant C. Kothari, for their love, support, understanding, and encouragement.

ABSTRACT OF THE DISSERTATION

Integration of *Clostridium thermocellum* Consolidated Bioprocessing With Thermochemical Pretreatments for Fuel Ethanol Production From Switchgrass

by

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Dr. Charles E. Wyman, Chairperson

There is an urgent need to replace petroleum-based transportation fuels with renewable and sustainable fuels to reduce the deteriorating impact of greenhouse gas emissions on climate change. Biofuels would not only provide a sustainable energy source but also help countries reduce their dependence on imported petroleum. Ethanol made from corn starch and cane sugar is presently the largest biotechnology-based product and commands a large share of the alternative fuels market. However, it is important to move towards making ethanol from lignocellulosic biomass that, unlike corn starch and cane sugar, does not have an important alternative use as food. However, biological conversion of this plentiful material suffers from high enzyme costs that stymie competitiveness. *Clostridium thermocellum* is a multifunctional ethanol producer capable of enzyme production, enzymatic saccharification, and fermentation that is fundamental to the consolidated bioprocessing (CBP) approach of ethanol production

from lignocellulosic biomass. CBP eliminates the supplementation of expensive enzymes that are required in the traditional approach of ethanol production. However, the recalcitrance of lignocellulosic biomass is still a hindrance to effective ethanol production. *C. thermocellum* is unable to achieve complete biomass digestion and sugar release without pretreatment of lignocellulosic biomass. This work focuses on extensive process development for effective integration of CBP with four different thermochemical pretreatments of switchgrass. First, cellulose loading for *C. thermocellum* flask fermentations was optimized to understand the impacts of substrate structural features on digestion by *C. thermocellum* under non-inhibitory conditions. Next, the impact of various cellulose properties including, but not limited to, crystallinity, surface area, pore size, and degree of polymerization, was studied on *C. thermocellum* digestion of model cellulosic substrates compared to fungal enzymatic hydrolysis. With an extensive understanding of *C. thermocellum* fermentations on model substrates the interdependency of switchgrass structural features with thermochemical and biological digestion was studied. Process configurations to achieve complete cellulose solubilization and total sugar release from switchgrass were finally defined. The comprehensive nature of integration of four different thermochemical and two different biological approaches in this work is unparalleled and could provide a platform to systematically develop cost effective ethanol production from lignocellulosic biomass in the future.

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Chapter 1. Introduction

The contents of this chapter will be used for publication in a scientific journal in part or in full

1.1. The need for alternative transportation fuels

Energy consumption in the United States (US) is dominated by coal, petroleum, and natural gas that contributed about 16%, 36%, and 29%, respectively, accounting for 81% of the total 97.7 quadrillion Btu energy consumption in 2015 according to the US Energy Information Administration (US EIA) [1]. Electricity generation and transportation accounted for the majority of US energy consumption, about 39% and 29%, respectively, in 2015, according to the US EIA. The use of fossil resources promotes unidirectional flow of carbon from underneath the earth's surface to the atmosphere in the form of carbon dioxide [2]. The result is increasing concentrations of greenhouse gases that are causing global climate change. Further, emissions from burning of fossil fuels also include volatile organic compounds, carbon monoxide, nitrogen dioxide, ground level ozone, and particulate matter that have detrimental health impacts [3]. Exposure to traffic related air pollutants, such as, ozone, particulate matter, sulfur dioxide, and nitrogen dioxide, have been known to exacerbate asthma, especially in children, and have other serious detrimental impacts on human health [3]. The economic value of human health impacts from fossil fuels use for electricity alone has been estimated to be $361.7-886.5 billion annually (2.5-6.0% of the nation domestic gross product) [4]. A number of policies including, Air Pollution Control Act 1955 that provided funds for federal research in air pollution, Clean Air Act 1963 that was the first legislation on air pollution control, and Air Quality Act 1967 that for the first time conducted extensive air pollution monitoring and enforced proceedings on areas affected by interstate air pollution transport, have been enacted to control air pollution [5].

Amendments to the Clean Air Act extended federal legal authority in 1990. Further Energy Policy Act 2005 and US Energy and Independence Act 2007 have also been enacted at the Federal level over the past 13 years to control the deteriorating impact of fossil fuels use. At the state level, policies such as the California Global Warming Solutions Act 2006 or Assembly Bill 32 (AB32) have been enacted [6]. Specifically, AB32 requires a reduction of California greenhouse gas emissions to 1990 levels by 2020. This is a reduction of about 15% below emissions expected if no changes are made to the current energy scenario. Further long term goals of 40% reduction in emissions by 2030 and 80% reduction by 2050 compared to the 1990 levels have been set in place.

The transportation sector consumed about 75% of the total petroleum used in the US in 2015 according to the US EIA and contributed to 27% of total US greenhouse gas emissions in 2015 according to the US Environmental Protection Agency (US EPA) [1, 7]. Thus, the transportation system presents one of the greatest needs for reduction in carbon dioxide, particulate matter, and emissions, especially in California. As one of the measures, in 2015, the US EPA issued a new limit on ground level ozone, the biggest source of which is ground transportation, of 70 parts per billion (ppb) down from the 75 ppb adopted in 2008 [8]. These levels are expected to have the worst impact on California because 19 counties within the state will not meet this standard until at least the year 2037, 12 years longer than any county outside the state, as projected by the EPA. Even though natural gas and coal reserves are more abundant than petroleum, the extensive use of petroleum for transportation shows that liquid fuels are particularly valued for this

sector because of their high energy density, convenience in refueling, and high power. This is confirmed by the near total dependence (about 92% in 2015 according to the US EIA) of the US transportation sector on petroleum [1]. Thus, it is of utmost importance to reduce petroleum use in the transportation sector and replace it with renewable and sustainable fuels to achieve lower and better standards for all pollutants. Further, with the largest reserves of oil located in politically unstable regions, petroleum supplies are vulnerable to politically motivated disruptions, and heavy dependence of transportation on petroleum makes it particularly vulnerable to such disturbances [1, 9]. Along those lines, the US Energy and Independence Act of 2007 was passed that requires the domestic production of 36 billion gallons of biofuels by 2022 [10]. To support this act, a federal program, the Renewable Fuel Standard, was started that enforced a minimum volume of renewable fuels to be sold by blending increasing amounts of such fuel in with traditional petroleum based fuels by 2022. Along those lines, the Low Carbon Fuel Standard was adopted in California in 2009 which requires the carbon intensity of transportation fuels to be reduced by a minimum of 10% in 2020. This is expected to achieve 16 million metric tons of greenhouse gas emission reduction. Thus, sustainable liquid fuels are needed to meet human energy needs in the transportation sector while reducing greenhouse gas emissions, pollutants, and the heavy dependence on volatile sources of petroleum

1.2. Cellulosic ethanol to replace petroleum

Lignocellulosic biomass, including fast growing grasses and trees and abundant agricultural residues, is the only known sustainable resource that is low enough in cost and available in sufficient quantity to support production of liquid transportation fuels at a scale that can significantly reduce petroleum dependence. Production of such lignocellulosic fuels can substantially reduce greenhouse gas emissions and contribute to solving a mounting global environmental threat. In particular, on the order of 1.4 billion tons of lignocellulosic biomass has been projected to be available each year in the US at a cost of less than $60/ton, about the same price as petroleum at $20/barrel. This low cost, abundant resource could displace about 100 billion gallons of gasoline annually compared to the approximately 125 billion gallons consumed [11]. Although ethanol currently made from corn starch in the US and cane sugar in Brazil is the largest biomass-based product and commands a large share of the renewable fuels market, both compete with food, rely on resources that are insufficient to substantially reduce petroleum dependence, are expensive, and create environmental concerns. In addition, greenhouse gas reductions from corn ethanol production are much lower than offered by production and use of lignocellulosic fuels according to the US EPA. The use of cellulosic ethanol produced from switchgrass, a lignocellulosic energy crop, is projected to achieve a 115-125% reduction in the mean greenhouse gas emissions compared to gasoline sold or distributed as transportation fuel in 2005 as opposed to 30% for sugarcane ethanol and 10-30% for corn ethanol with a 30 years' Time Horizon (time period for which biofuel production is projected to occur) [12]. Further, the combined cost of climate change and

health effects due to greenhouse gases and fine particulate matter ($PM_{2.5}$) for each billion ethanol equivalent gallons of fuel produced and combusted were estimated in 2008 to be $123-208 for cellulosic ethanol depending on the lignocellulosic feedstock of choice compared to $472-952 million depending on the choice of bio-refinery heat source for corn ethanol and $469 million for gasoline [12]. Thus, it is urgent to transition to making ethanol from lignocellulosic biomass that along with having multiple other advantages also does not compete with food, unlike corn starch and cane sugar. In addition, ethanol has favorable fuel properties including high octane and high heat of vaporization that support more efficient use than gasoline [13], and ethanol can be upgraded further to drop-in fuels and hydrocarbons, such as jet fuel, gasoline, and diesel [14]. The US Energy Independence and Security Act encourages the shift to lignocellulosic ethanol by mandating production of 21 billion gallons of this fuel out of the total target volume of 36 billion gallons of biofuels by 2022. [10] It is, thus, not only important to use lignocellulosic ethanol as a sustainable energy source but also to develop technologies to produce it and make it available at low cost in large amounts in the near future.

1.3. Fuel ethanol from lignocellulosic biomass

Lignocellulosic biomass includes agricultural residues such as corn stover and sugarcane bagasse, forestry residues, municipal solid waste, herbaceous crops such as switchgrass and miscanthus, and woody crops such as poplar and pine [15]. The research presented here focuses on conversion of switchgrass as an ideal energy feedstock for the production of ethanol. Switchgrass is a perennial C4 grass native to North America with a

total cell wall fraction of about 80% of the plant dry weight. It is highly productive, with about 36.7 Mg/ha being achieved in field trials on marginal lands with low agricultural input and use of conventional equipment and established harvesting processes. Biofuels made from switchgrass could potentially contain 500% or more energy than needed for its cultivation. At the same time, switchgrass is tolerant to diseases and insects and can improve soil quality as well as carbon sequestration due to its extensive root system that increases carbon storage in the soil [11, 16].

For biological conversion routes, the large amount of carbohydrate polymers (>65% wt., dry basis) in lignocellulosic materials such as switchgrass can be deconstructed into sugars for fermentation to ethanol. The state of the art technology in industry for ethanol production from lignocellulosic biomass is based on the primary steps of size reduction, pretreatment, enzyme production, enzymatic hydrolysis, fermentation, and product recovery, as represented in Figure 1.1 [17, 18]. This process makes use of cellulolytic enzymes produced and purified from *Trichoderma reesei* coupled with non-cellulolytic microorganism, such as, *Saccharomyces cerevisiae* for fermentation [19-21]. The high processing costs of these operations especially that of production and purification of enzymes from *T. reesei*, are of immediate concern [22]. In order for ethanol to compete with petroleum in the fuel market, the cost of ethanol production should ideally be able to compete with the low cost of extracting petroleum from under the earth. A cost competitive approach to deconstruct biomass and increase polysaccharides accessibility is, therefore, a necessity. In turn a substantial research effort

is required to reduce the amount / eliminate the use of enzymes required in the process to reduce the overall cost of operations [22]. Along those lines, a consolidated bioprocessing (CBP) approach eliminates the expensive enzyme production step and others by using a single organism that combines enzyme production, biomass saccharification, and carbohydrate fermentation all in one organism as shown in Figure 1.1. *Clostridium thermocellum,* in particular, is a very promising CBP organism [23].

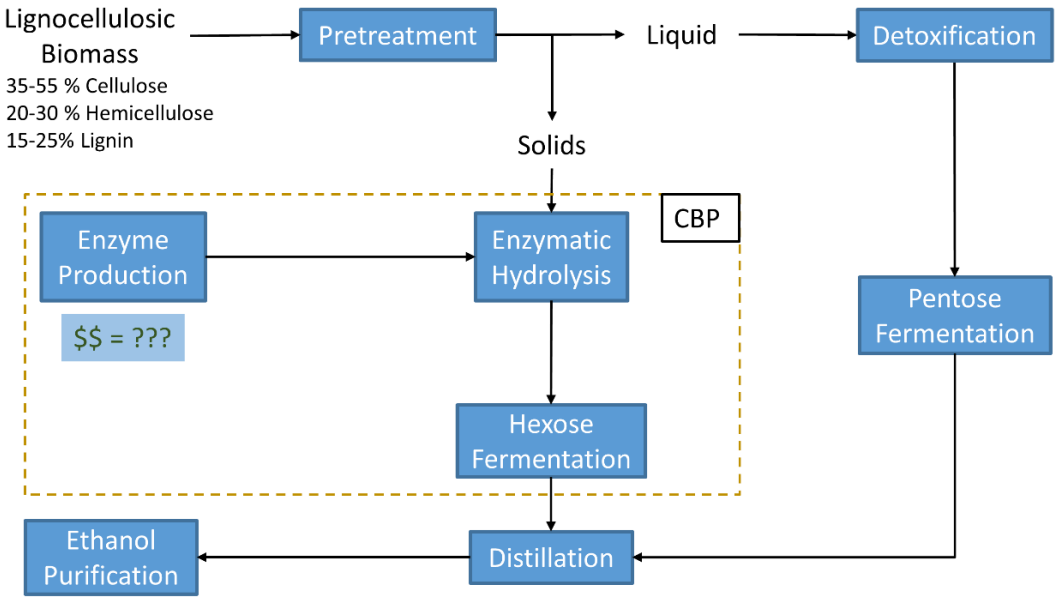

Figure 1.1. Representative process configuration for ethanol production from lignocellulosic biomass

However, the biggest barrier for enhanced production of fuel ethanol from lignocellulosic biomass is still the recalcitrance of biomass [18, 24-27]. Lignocellulosic biomass is structurally complex, with much of the long cellulose chains held together as crystalline fibers by hydrogen bonds. These long cellulose fibers are in turn glued

together by hemicellulose, a polymer of several sugars, and lignin, a phenolic polymer. In addition to supporting plants, this matrix protects them from attack by microorganisms and insects [26, 28-30]. The natural recalcitrance of the plant biomass leading to the physical unavailability of carbohydrates affects effective biomass deconstruction and carbohydrates digestion to fermentable sugars. Cellulose accessibility affected by the physical availability of cellulose for further enzymatic saccharification is termed as cellulose macro-accessibility [31]. The presence of lignin and hemicellulose in the lignocellulosic matrix influences the physical availability of cellulose and thus its macro-accessibility. Therefore, biomass augmentation such as by pretreatment or co-treatment, appear to be necessary to enhance biological deconstruction by fungal enzymes or *C. thermocellum* and other CBP organisms [25, 32, 33]. A variety of pretreatment technologies can prepare lignocellulosic biomass for high sugar yields by achieving distinctive changes in the solids compositional and structural characteristics [34-38]. Dilute acid and hydrothermal pretreatments are leading technologies to reduce biomass recalcitrance by removing some lignin and most of the hemicellulose. Dilute alkali pretreatment, on the other hand, removes some hemicellulose and significant amounts of lignin [17]. By comparison, co-solvent enhanced lignocellulosic fractionation (CELF), a novel fractionation technology that our UCR team recently developed, employs tetrahydrofuran (THF) in a miscible solution with water to effectively remove and recover most of the hemicellulose and lignin from lignocellulosic biomass with high yields and thereby dramatically reduce the recalcitrance of the solids produced compared to prior methods [39]. However, once cellulose from lignocellulosic biomass is made

macro-accessible through biomass augmentation processes, the properties of cellulose itself can affect enzyme adsorption and thus enzymatic saccharification of cellulose. The availability of binding sites on cellulose for enzyme adsorption is termed as cellulose micro-accessibility [31]. Cellulose properties, such as, water retention value (WRV), specific surface area, crystallinity, degree of polymerization, surface charge, and other physical characteristics are expected to influence cellulose micro-accessibility [31, 40-42]. The first step in enzymatic saccharification is the swelling of the substrate leading to an increase in surface area and therefore, an increase in the availability of enzyme binding sites on cellulose [43]. WRV and Simon's staining techniques are widely used to measure the swelling ability of the biomass and cellulose surface area, respectively [44-51]. The second step during hydrolysis is the adsorption of enzymes on the binding sites of the substrate. Cellulose crystallinity, pore size distribution, surface charge, and overall physical characteristics of the substrate influence enzyme adsorption [52-55]. Finally, the molecular weight and degree of polymerization of cellulose is expected to affect overall substrate digestion [31, 42, 56-58].

1.4. Goals and objectives

Considering all the sustainable sources of energy currently studied, it can be inferred that cellulosic ethanol is the most promising in the near future to power transportation. However, to truly begin displacing petroleum with ethanol, it is important, first and foremost, to cost-effectively produce it in large quantities from cellulosic biomass. *C. thermocellum* based consolidated bioprocessing is a promising bioprocess for

the production of ethanol from lignocellulosic biomass. However, *C. thermocellum* alone cannot effectively digest biomass and therefore, in this dissertation research, we have aimed to integrate *C. thermocellum* fermentation with the use of different thermochemical pretreatment techniques to achieve high biomass deconstruction. Keeping that in mind, the main goals of this work were to:

- Define process configurations to maximize sugar release from pretreatments coupled with *Clostridium thermocellum* consolidated bioprocessing, and
- Understand mechanisms underlying chemical and biological deconstruction of lignocellulosic biomass by combined pretreatment and biological conversion.

The specific objectives to achieve these goals were to understand the:

a) Impact of substrate loading on *C. thermocellum* cellulolytic and metabolic performance
b) Impact of cellulose properties on *C. thermocellum* compared to fungal enzymatic saccharification
c) Impact of switchgrass thermochemical pretreatments on *C. thermocellum* consolidated bioprocessing and fungal enzymatic hydrolysis
d) Interdependence of switchgrass structural features with thermochemical and biological digestion

1.5. Dissertation organization and research approach

In this dissertation, Chapter 2 begins with an in-depth literature review of *C. thermocellum* based CBP for ethanol production. The chapter starts with a history of *C. thermocellum* and the discovery of its complex, cell surface attached, multi-functional cellulase system called the cellulosome. Substrate regulated proteins involved in cellulose binding and the cellulosomal enzymes involved in the breakdown of the complex lignocellulosic substrate are described. A description of *C. thermocellum*'s metabolic pathways and research efforts to genetically modify these metabolic pathways to improve ethanol yield, titer, and tolerance of the organism are extensively reviewed. Finally, an analysis of research performed on *C. thermocellum* digestion of lignocellulosic biomass is provided to show the lack of extensive research on the integration of thermochemical pretreatment with *C. thermocellum* fermentation of real world lignocellulosic biomass, specifically for ethanol production.

Chapters 3 and 4 focus on *C. thermocellum* fermentations of model cellulosic substrates. In order to understand the impact of lignocellulosic biomass compositional and structural features on *C. thermocellum* fermentations and vice versa, it was first necessary to understand fermentations on model cellulosic substrates. Cellulose in lignocellulosic biomass, unlike model cellulosic substrates, is physically less accessible and thus differentiating substrate effects from organism effects on *C. thermocellum* fermentation of lignocellulosic biomass would be indiscernible without an understanding of fermentation itself. In Chapter 3, Avicel® PH-101, a model cellulosic substrate, was

used to address underlying metabolic features affected only by substrate loading independent of the physical availability of cellulose influenced by the presence of hemicellulose and lignin in lignocellulosic biomass. The best substrate loading for *C. thermocellum* flask fermentations with no bacterial inhibition was defined in this chapter. Using this substrate loading, Chapter 4 reports the impact of varying cellulose properties, including its crystallinity, degree of polymerization, surface are, pore size and micro-accessibility, on *C. thermocellum* compared to fungal enzymatic hydrolysis of model substrates. Model cellulosic substrates including Avicel® PH-101, α-cellulose, cellulose acetate, Sigmacell Type 50, cotton linter, and Whatman™ 1 filter paper were chosen to represent a wide spectrum in cellulose properties. This chapter further helped in understanding the process of biological digestion and revealed vital mechanistic information to guide the research toward understanding the interdependence of lignocellulosic substrate properties and substrate digestion.

Chapters 5 and 6, finally, integrate biomass thermochemical pretreatments and *C. thermocellum* CBP compared to fungal enzymatic hydrolysis for maximum sugar release from switchgrass. Four different thermochemical pretreatments, hydrothermal, dilute acid, dilute alkali, and CELF, were employed to produce solids with dramatically different physical and compositional characteristics. These pretreatments are known for their ability to solubilize xylan (hemicellulose) and lignin from lignocellulosic biomass to different extents producing solids with different compositions of cellulose, hemicellulose, and lignin [40, 60]. The comprehensive nature of this work with the use of four different

thermochemical pretreatment methods and two different biological digestion approaches is unparalleled. A major contribution of this research was to understand how removing hemicellulose and lignin separately and together by different pretreatment strategies affected deconstruction of switchgrass by *C. thermocellum* compared to fungal enzymes as described in Chapter 5. Deconstruction of the biomass was typically measured as sugar release, specifically glucan conversion / solubilization / yield for CBP or fungal enzymatic hydrolysis (EH). Another performance parameter followed was metabolites production, including ethanol, acetic acid, and lactic acid, by *C. thermocellum* during CBP. Further, an important aspect of this research was to characterize biomass before and after pretreatment and after biological conversion of the pretreated biomass to sugars to understand causes for differences in performance among the various pretreatments and biological systems as presented in Chapter 6. Through various collaborations, advanced characterization techniques, including solid state nuclear magnetic resonance (NMR), 2D heteronuclear single quantum coherence (HSQC) NMR, scanning electron microscopy, gel permeation chromatography, X-ray diffraction were applied to gain these insights. Relationships between total sugar release from switchgrass and parameters that contribute to overall biomass recalcitrance, such as syringyl (S) lignin to guaiacyl (G) lignin monolignol ratio, p-hydroxyphenyl (H) monolignol content, lignin interunit linkages, hydroxycinnamates content, cellulose crystallinity and degree of polymerization, cellulose accessibility measured via Simons' staining, and substrate water retention provided vital perspectives on biomass deconstruction. These techniques were applied at UCR and through relationships with leaders in the field at the University of Georgia,

Athens (UGA); the University of Tennessee, Knoxville (UTK); the National Renewable Energy Laboratory (NREL), and Oak Ridge National Laboratory (ORNL).

To conclude, Chapter 7 underlines the importance of the findings of this dissertation research and provides future directions for this field of research. Overall, this work has aided in understanding how changes in structural and microscopic biomass features by pretreatment can account for enhanced biological digestion performance. This knowledge of thermochemical and biological deconstruction of biomass helped us define promising process configurations for maximum fermentable sugar release and ethanol yields from switchgrass using *C. thermocellum* CBP. At the same time this work has also helped us identify important levers on which to focus future research to improve the overall process of fuel ethanol production. Thus, this work is expected to provide a strong platform for promising research directions for others in the field to pursue, specifically for the integration of pretreatments with consolidated bioprocessing.

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Chapter 2. A review of *Clostridium thermocellum* as a biocatalyst for ethanol production from lignocellulosic biomass

The contents of this chapter will be used for publication in a scientific journal in part or in full

2.1. Abstract

Clostridium thermocellum is found in guts of rumen, soil, hot springs, etc. in a complex, natural co-culture system depending on cross-feeding for vitamins and on other organisms as electron sinks. *C. thermocellum*'s cellulosome is a multi-enzyme, multi-functional complex for effective breakdown of cellulose, hemicellulose, and other components of lignocellulosic biomass. The organism prefers to metabolize cellobiose and cellodextrins more than glucose, saving ATP required for transportation of hexoses and providing an advantage in a complex, natural environment with other organisms that consume glucose for metabolic activity. *C. thermocellum* is a prime candidate for a native cellulolytic strategy of CBP, and its metabolism is being genetically altered to improve ethanol production and tolerance. Even though *C. thermocellum* possesses an effective cellulolytic system and is being provided with an improved metabolic system for ethanol production, biomass augmentation techniques, such as pretreatment and cotreatment, are required to aid *C. thermocellum* digestion of real world lignocellulosic biomass. However, the organism has mostly been studied with ideal substrates and not extensively with augmented real world biomass. Comprehensive and systematic *C. thermocellum* fermentation development and optimization in conjunction with biomass augmentation technologies for maximum sugar release and ethanol production is crucial for successful industrial use of *C. thermocellum* CBP for ethanol production. This review revisits the isolation of *C. thermocellum* in the first half of the 1900s and the discovery of its cellulosome in the 1980s, describes its metabolism and successful genetic alterations of

its metabolic pathways, and discusses *C. thermocellum* fermentation of lignocellulosic biomass while pointing out the need for future process optimization.

2.2. Introduction

2.2.1. The need for feedstock-independent processes:

Ethanol production process optimization heavily depends on understanding the impact of substrate, substrate properties, cellulose properties, and substrate pretreatments on further biological operations. There are various types of biomass, such as, agricultural crops, woody crops, herbaceous crops, municipal waste, and others that vary significantly in their bulk composition of cellulose, hemicellulose, and lignin [1]. Often the type of lignocellulosic biomass used for ethanol production would depend on the location of the production plant and its proximity to the feedstock considering high biomass transportation costs [2, 3]. Further, each biomass type has multiple species and natural variants that could change the bulk composition and properties of the feedstock [4-6]. Also, growth conditions, fertilizers, amount of water used, salinity, and harvesting time and conditions, among others are all expected to affect biomass composition [7-9]. In light of numerous substrate related variations that could be encountered in the process of ethanol production from lignocellulosic biomass, it is necessary to move towards feedstock-independent processes. Thus, understanding the impact of feedstock properties and composition on further biological digestion for each type of biocatalyst used is of utmost importance. Uniquely, *C. thermocellum* is able to adjust its cellulosomal composition and activity based on the substrate supporting growth [10]. *C. thermocellum*

as a biocatalyst for ethanol production is a significant step toward a feedstock-independent process. This review focuses on *C. thermocellum*, its history, the discovery of its cellulosome, the organism's metabolism, and successful genetic modifications of the organism towards understanding industrial utilization of this unique biocatalyst for the production of ethanol (and other metabolites) from lignocellulosic biomass.

2.2.2. Consolidated bioprocessing for ethanol production

Consolidated bioprocessing (CBP) is a bioprocess that directly converts lignocellulosic biomass into ethanol with the use of microorganisms that can produce their own enzyme consortium to hydrolyze polysaccharide chains in biomass into simple sugars and also ferment the sugars to produce ethanol and other desired products. It is a combination of enzyme production, biomass saccharification, and carbohydrate fermentation [11-18]. Because CBP eliminates the expensive enzyme production step and is inherently simpler than the conventional approach, the process has a high cost saving potential. However, there is no microorganism known to us that can deconstruct biomass efficiently as well as ferment the sugars into useful products in economically desired amounts under industrially harsh conditions [14, 18, 19]. Inherent part of the carbon cycle, cellulolytic microorganisms that are present in the soil, aquatic environments, and guts of animals, degrade the cellulose in plant biomass to other forms of carbon, returning it back to the earth [19-22]. *Clostridium thermocellum* and *Caldicellulosiruptor bescii,* in particular, are very promising cellulolytic microorganisms that can be used for CBP [11, 13, 16, 18]. A lot of research has been carried out in the direction of native cellulolytic

strategy, an approach in which the fermenting abilities of promising cellulolytic microorganisms are genetically improved for high product yields and titers and has been shown to be successful [19]. Another approach is the recombinant cellulolytic strategy which aims at conferring cellulolytic ability to an organism that naturally produces the desired metabolite in high yields and titer. Yeast has been the most common target organism for the recombinant cellulolytic strategy [12, 17]. However, processing lignocellulosic biomass at higher solids loading is expected to be the limiting step in CBP compared to the production of ethanol at high titers. Further, conferring an organism with cellulolytic activity is expected to be more challenging than improving an organism's metabolic activity. Therefore, the native cellulolytic strategy is considered more promising for ethanol production from lignocellulosic biomass [19].

2.3. History of *C. thermocellum*

2.3.1. *C. thermocellum* ATCC 27405

C. thermocellum is a gram positive, thermophilic, anaerobic, bacteria that was first identified and thought to have been isolated in 1926 [23]. However, this culture lost its ability to ferment cellulose when grown on glucose and multiple subsequent attempts to isolate *C. thermocellum* had failed [24]. Mcbee et al. were able to isolate *C. thermocellum* with demonstrable purity using techniques developed by Hungate decades later in 1948 [25, 26]. This strain is available today as ATCC 27405. Since then extensive studies on the cellulolytic and metabolic activities of *C. thermocellum* have been reported for the production of ethanol and other metabolites. AS-39 is a mutant strain, which

showed higher cellulase activities, was isolated from *C. thermocellum* ATCC 27405 by Shinmyo et al. A complex GS medium containing yeast extract was developed based on the CM-3 cellobiose medium by Garcia-Martinez et al. in 1980 for *C. thermocellum* ATCC 27405 growth, which was used to study enzyme production by the organism [27]. Xylan degrading ability of *C. thermocellum* was also reported in this study. Johnson et al. developed a modified GS-2 medium reported in 1981 containing sodium citrate dehydrate to prevent precipitation of salts in the original GS medium [28]. The same group developed a minimal defined medium (MJ) for *C. thermocellum* ATCC 27405 growth in 1981 and reported the organism's requirement of four vitamins specifically: biotin, pyridoxamine, B12, and p-aminobenzoic acid. *C. thermocellum* exists in nature in close association with other microbes to meet its need for vitamins through interspecies cross-feeding [29]. Ng et al. in 1981 used the GS medium to study ethanol production by *C. thermocellum* in a pure culture and a co-culture with *Clostridium thermohydrosulfuricum* [30]. The combination of *C. thermocellum*'s effective cellulolytic activity with *C. thermohydrosulfuricum*'s efficient metabolic activity led to higher cellulolytic and metabolic yields (100 mM ethanol and 10 mM acetic acid) for the co-culture on 1% Solka-Floc compared to a *C. thermocellum* monoculture (28 mM ethanol and 24 mM acetic acid). Further, *C. thermocellum* was also able to break down xylan from aspen wood (untreated and SO_2 treated) and steam-exploded poplar wood to xylose and xylobiose which *C. thermohydrofulfuricum* was able to metabolize. Johnson et al., in 1982, further found that Avicel solubilization increased in the presence of calcium ions and dithiothreitol to similar rates of cellulases found in *T. ressei* [31]. Brener et al. in

1983 looked at the effect of cellobiose concentration on the metabolic profile of *C. thermocellum* AS-39 and suggested that a 0.8% cellobiose loading was optimal for ethanol production and ethanol to acetate ratio during flask fermentations [32]. UV mutagenesis produced a mutant strain that was ethanol tolerant from the parent *C. thermocellum* ATCC 27405 as shown by Girard et al [33]. The mutant strain, proposed to have had an alteration of membrane properties affecting permeation of the inhibitor, fermented 63 g of cellulose into 14.5 g of ethanol (grown on 50-70 g/L MN300 cellulose). A simultaneous decrease in hydrogen production was also observed compared to ATCC 27405 strain. Lynd et al. further reported the processibility of *C. thermocellum* ATCC 27405 fermentations of cellulose and pretreated mixed hardwood in batch and continuous cultures for ethanol production using the GBG medium in the late 1980s [34, 35]. Further, Hogsett reported the development of a new medium, Medium for Thermophilic Clostridia (MTC), based on the GBG medium developed by Lynd et al. and the MJ medium developed by Johnson et al [28, 34-36]. MTC is extensively used for *C. thermocellum* growth and fermentations today and has been modified into a low carbon medium as well for the organism [37].

2.3.2. *C. thermocellum* LQ8, JW20, and others

Simultaneously, *C. thermocellum* LQ8 strain obtained from L. Y. Quinn's laboratory from the Department of Bacteriology at the Iowa State University, was studied in a co-culture with *Methanobacterium thermoautotrophicum* for the production of methane from cellulosic substrates in 1971 [38]. In this work, Weimer et al. reported that a

methanogen, such as *M. thermoautotrophicum,* acted as an electron sink for *C. thermocellum* in the co-culture allowing for more hydrogen production by *C. thermocellum* which was then converted to methane by the methanogen. In the absence of the methanogen, however, *C. thermocellum* produced acetic acid, ethanol, hydrogen, and carbon dioxide. Ng et al., in 1977, reported a high cellulase yield for *C. thermocellum* LQ8 grown on native cellulose, α-cellulose, and cellulose with low degree of polymerization as opposed to when grown on carboxymethycellulose or other carbohydrate sources and no cellulase production when grown on cellobiose [39]. Obtained from a contaminated culture of LQ8 in 1981, *C. thermocellum* LQRI was shown to be able to grow on and ferment glucose and produce carboxymethycellulase when grown on cellobiose or glucose [30, 40]. *C. thermocellum* JW20 was isolated from Louisiana cotton bale in 1979 by Wiegel et al., whereas, *C. thermocellum* JW 1 was isolated from river mud in Georgia in the early 1980s [41-43]. Wiegel et al. proposed that *C. thermocellum* sporulates when the pH dropped below 6.4 while remaining attached to the substrate (2% Avicel or Solka-Floc). Thus, *C. thermocellum* took advantage of being attached to the substrate when the conditions were not suitable for growth and this concept was utilized for strain purification. [43]. Today, multiple newer *C. thermocellum* strains, DSM 2360, BC1, NB2, 5g, T2, and others have also been discovered from various sources such as biogas plants, cow dung, and bio-composts [44].

2.4. *C. thermocellum* cellulosome

2.4.1. The discovery of cellulosome

The early work on ATCC 27405 (also known as DSM 1237), LQ8 (also known as DSM 1313), and JW20 (also known as DSM 4150) *C. thermocellum* strains by Johnson et al., Garcia-Martinez et al., Weimer et al., Ng et al., and Wiegel et al. paved the way for future identification of enzymes important in the cellulosome. Another wild type strain *C. thermocellum* YS was isolated at the General Electric CRD Center (Schenectady, N. Y.) from soil samples from hot springs in Yellowstone National Park [45] in 1983. Bayer et al. and Lamed et al extensively studied *C. thermocellum* YS to purify its carbohydrate binding factor (CBF) [46, 47]. Bayer et al. in 1983 further also isolated an adherence-defective mutant, *C. thermocellum* AD2, following an enrichment procedure, [45]. Comparison of AD2 and the wild type YS strains of *C. thermocellum* indicated a single entity that was responsible for the adherence of *C. thermocellum* cells on cellulose. *C. thermocellum* was shown to have two CBFs, a cell surface attached CBF and a cell free extracellular CBF. However, the cell associated CBF was actually recognized to be a multi-functional organelle, serendipitously leading to the discovery of "cellulosome", which showed antigenic, cellulose-binding, and multiple cellulolytic activities, as shown by Lamed et al. [47]. The antigenic and cellulolytic activities were reported to be physically separated on the cellulosome. The organization of various cellulases and other subunits in the cellulosome was expected to aid in effective delivery of enzymes to the substrate. Coughlan et al. in 1985 described the cellulolytic enzyme complex of *C. thermocellum* as very large and this complex was resolved into two major complexes

[48]. They reported that *C. thermocellum* JW20 produced a larger enzyme complex first during the early stages of growth that attached to cellulose which then yielded a smaller enzyme complex through disaggregation that is available in the culture liquid. This smaller complex may then reattach to cellulose. Lamed et al. described the interaction of cellulosome and cellulose as very economical which would be crucial for the survival of *C. thermocellum* (and other cellulose degrading anaerobes exhibiting a cellulosome) in nature [49]. Such high premetabolic efficiency is expected to compensate for the low ATP generation per hexose (compared to an aerobic organism) during metabolism.

2.4.2. Cellulosomal structure and binding:

Bayer et al. showed the presence of protuberances, cellulosomes, on the *C. thermocellum* YS cell surface using a new staining technique for scanning electron microscope. The mutant AD2 strain was shown to not have these protuberances and is therefore, an adherence-defective strain [50]. Protuberances were described as being able to protract a "contact corridor" to cellulose attaching onto the substrate and degrading it mostly to cellobiose and some glucose. The cells would then desorb leaving behind some adsorbed cellulosome structures that are then expected to continue to break down the substrate. The contact corridors were not observed when the *C. thermocellum* YS strain was grown on cellobiose but the protuberances were present. On the other hand, the adherence defective AD2 strain showed no signs of protuberances on its cell surface and only released extracellular cellulosomes [51]. Cellulosomes were reported to be very stable and treatments including, guanidince hydrochloride, urea, nonionic detergents, and

pH and ionic strength extremes, did not break the cellulosome into its subunits [51]. Further, purified cellulosomes showed lower adherence suggesting the importance of the attachment of cellulosome on the cell surface [45]. An antigenic S1 subunit with no cellulolytic function was also discovered and was proposed to have an organizational or cell surface attachment function [45, 51]. The S1 subunit was renamed "scaffoldin" which is now known to be the core of a cellulosome as shown in Figure 2.1. The noncatalytic scaffoldin of *C. thermocellum*, as reported in 1994 by Bayer et al., has a single carbohydrate binding domain (CBD), nine different cohesins that directly interact with the other catalytic subunits of the cellulosome, and a terminal dockerin that was proposed to help in cellular attachment [52]. The cohesins are connected to each other through linkers that are rich in proline and threonine residues. The gene encoding the scaffoldin of *C. thermocellum* was then sequenced and labelled cipA and the protein encoded by that gene, known as the cellulosome integrating protein, was labelled CipA by Gerngross et al. in 1993 [53]. The CipA dockerin was found to specifically bind to a new type of cohesin domain found on three proteins on *C. thermocellum* cell surface, SdbA, ORF2p, and OlpB in 1995 [54]. Thus, these three proteins were proposed to play an important role in the anchoring of CipA and therefore, the cellulosome to the cell surface as represented in Figure 2.1. OlpB has four cohesins, ORF2p has two, and SdbA has a single cohesion that binds to the dockerin on CipA. Dror et al. in 2003, showed that CipA, OlpB, and ORF2p, are regulated by growth rate at the transcriptional level [55]. During high growth conditions (exponential phase) the ORF2p component had 4 to 6-fold transcript level in comparison to the other anchoring proteins. Once the growth rate

slowed, expression of both the olpB and orf2 genes increased. Interestingly, the adherent deficient *C. thermocellum* mutant AD2 develops adherence after growing on cellulose with a longer lag phase compared to the wild type YS strain. A mutation in orf2 gene in the AD2 strain would explain the lag phase and development of adherence later in growth when olpB gene expression occurs at lower growth rates [55].

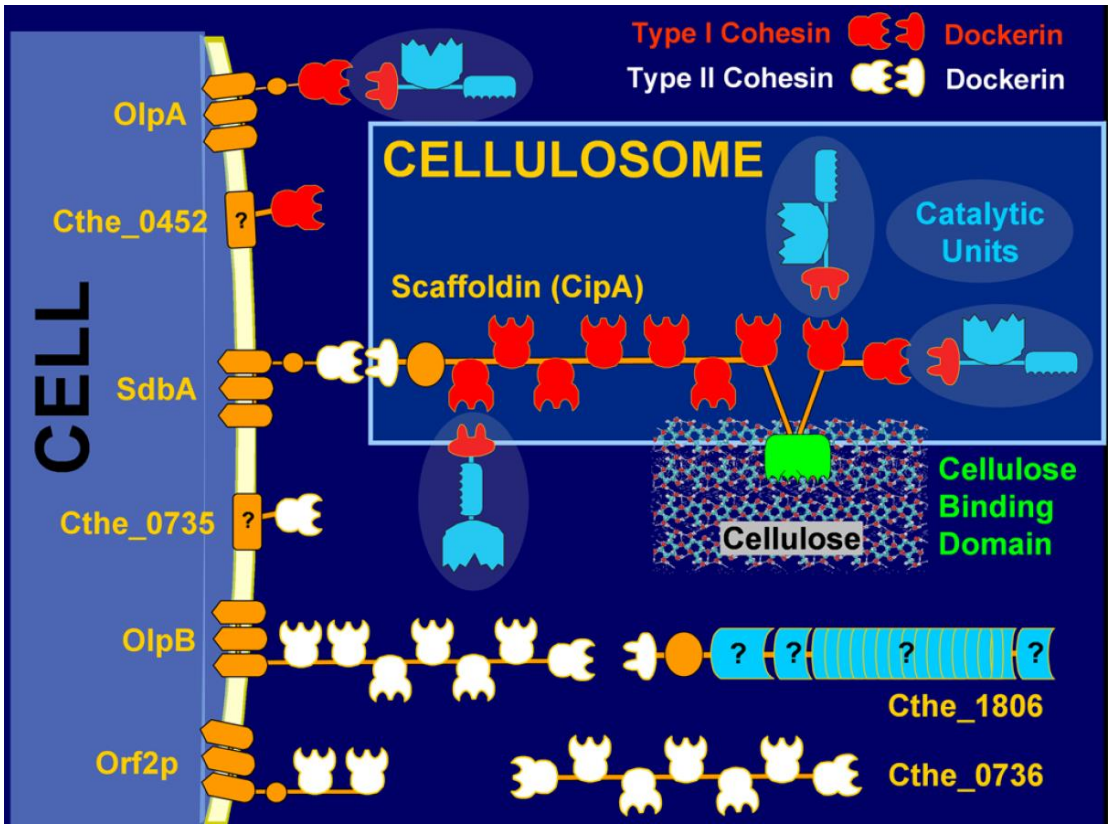

Figure 2.1. *C. thermocellum* cellulosome [10]*

*Reprinted as is from *Public Library of Science One*, 4(4), Raman B, Pan C, Hurst GB, Rodriguez Jr M, Mckeown CK, Lankford PK, Samatova NF, Mielenz JR, Impact of pretreated switchgrass and biomass carbohydrates on Clostridium thermocellum ATCC 27405 cellulosome composition: A quantitative proteomic analysis, Copyright (2009), Public Library of Science (Open Access: https://creativecommons.org/licenses/by/4.0/)

2.4.3. Cellulosomal enzymes

Cellulosomes have also been known to regulate enzymes on the cellulosome complex based on substrate and growth conditions [10, 55-58]. Dror et al. showed that the regulation of CelS protein, an exoglucanase, depended on the growth rate [58] and Mishra et. al. showed a similar growth dependence of endoglucanase celA, celC celD, celF gene regulation [59]. None of the endoglucanases were shown to be expressed during initial growth and the expression only increased late in the exponential phase or after cessation of growth. The expression of celA, celD, and celF genes was shown to occur during late exponential growth phase; whereas, the celC and celD were expressed in the stationary phase [59]. In contrast, the exoglucanase celS expression was shown to decrease in early stationary phase. The difference in regulation patterns of endo- and exoglucanases indicates an overall strong regulation of different cellulolytic enzymes on the cellulosome [58]. Thus, the organism overall utilizes a combined strategy to regulate both the catalytic components of the cellulosome and the proteins required for attachment of the cellulosome to the cell surface based on the substrate and growth conditions the organism encounters [55]. Overall, the cellulosome consists of a non-catalytic CipA scaffoldin unit comprised of cohesion units connected by linkers that bind to catalytic units, a carbohydrate binding domain / module, and a dockerin that binds to cell surface protein cohesins. The cellulosome cohesin – catalytic unit dockerin interaction was termed Type I to distinguish from the Type II cell surface protein cohesin – cellulosome dockerin interaction [55]. The *C. thermocellum* ATCC 27405 cellulosome consists of over 70 different single enzymes assembled on CipA out of which 24 are known to be

cellulases and their regulation is substrate and growth dependent [10, 60]. In fact, *C. thermocellum* has redundant cellulases as well and there is a debate on why the organism expresses such a vast number of different cellulases [10, 60-62]. CelS, CelK, CbhA, and CelO are the four exoglucanases on *C. thermocellum*'s cellulosome that belong to the GH48, GH9, and GH5 families, respectively, with CelS being the most abundant and CelO the least. GH9 exoglucanases attack celluloses from the non-reducing end and their expression was higher when *C. thermocellum* was grown on pretreated switchgrass, whereas, CelS and CelO attack from reducing end of cellulose. *C. thermocellum* endoglucanases are 9, 1, and 12 proteins from the GH5, GH8, and GH9 families, respectively with CelA being the most abundant. Xylanases have been shown to be expressed independent of growth [51, 63, 64] and XynA, XynC, XynZ, and XghA were expressed highly on cellobiose and cellulose [10]. *C. thermocellum* also has enzymes to degrade pectin from lignocellulosic biomass suggesting that the enzymes in *C. thermocellum* are designed to break down a plant biomass completely and not just cellulose and hemicellulose [10]. Specifically, PL1A, PL1B, and PL9 pectate lyases in the *C. thermocellum* cellulosome were found to be endo pectate lyases that require calcium ions for high activity [65].

2.5. *C. thermocellum* metabolism:

2.5.1. Sugar uptake

Zhang et al. went as far as calling *C. thermocellum* a "cellulose-using specialist" because the organism's ability to utilize a narrow range of substrates limited mostly to

cellodextrins and cellobiose and only to a certain extent glucose [66]. In fact, *C. thermocellum* prefers to utilize cellobiose instead of glucose when provided with both substrates. Adenosine-binding cassette system in the organism allows for effective cellodextrins transport and by assimilating cellodextrins *C. thermocellum* is able reduce the otherwise high ATP requirement for substrate transport [66]. This further gives *C. thermocellum* an advantage over other organisms in the environment that require glucose for metabolism [67]. Cellobiose phosphorylase catalyzes the reversible phosphate-dependent phosphorolysis of β-1,4-glycosidic bonds. The α-D-glucose 1-phosphate formed in this manner is then available for the Embden-Meyerhof glycolytic (EMP) pathway [68]. Similarly, cellodextrin phosphorylase is responsible for the phosphorolysis of β-1,4-oligoglucans [69]. *C. thermocellum* also has intracellular β-glucosidases for the hydrolytic cleavage of cellobiose to glucose but phosphorolysis is preferred due to ATP generation [69]. A metabolic consequence of cellodextrin and cellobiose phophorylases in *C. thermocellum* would be the accumulation of glucose since glucose utilization is the rate-limiting step especially at high substrate loadings [70]. Glucose accumulation has been associated with a drop in pH of the fermentation broth due to the formation of organic acids [71]. Further, glucose accumulation during *C. thermocellum* fermentation is also possible due to low cell associated β-glucosidase activity [70-75]. However, there is no phosphorolytic cleavage benefit for *C. thermocellum* during growth on glucose and the transport of glucose is more energetically demanding per hexose compared to cellobiose or cellodextrins [66]. The amount of ATP saved by the transport of cellodextrins opposed to glucose transport, which is only a fraction of total ATP

generated by an aerobic organism but very valuable to an anaerobic organism, would then be available for microbial growth. However, *C. thermocellum* has been shown to utilize both glucose and cellobiose in continuous cultures as opposed to its preference for the disaccharide in batch cultures [76].

2.5.2. Understanding *C. thermocellum* metabolic profile and pathways

Lamed et al., Ng et al., and Brener et al. extensively studied the metabolic profile of *C. thermocellum* AS-39 and LQRI strains on cellobiose and cellulose in the early 1980s to show that ethanol, hydrogen, carbon dioxide, lactate, and acetate were the organism's major fermentation products [30, 32, 77]. The AS-39 strain produced 230, 110, 12, 325, 121 μmoles compared to LQRI strain which produced 157, 125, 24, 346, 286 μmoles of ethanol, acetic acid, lactic acid, carbon dioxide, and hydrogen respectively after 18 hours in 10 ml of cellobiose complex (CC) medium with 8 g/L cellobiose [77]. Minor contribution of succinic acid, butyric acid, and formic acid were also been reported [32]. Ng et al. showed improvement in ethanol yields for a co-culture of *C. thermocellum* with *C. thermohydrosulfuricum* compared to *C. thermocellum* monoculture which was attributed to the ability of *C. thermocellum* to hydrolyze cellulose and hemicellulose and utilization of mono and di-saccharides by *C. thermohydrosulfuricum.* In fact, ethanol production jumped from 31.2 g/L and 30.8 g/L from MN300 (0.8%) and Solka-Floc (1.0%) cellulose, respectively, by *C. thermocellum* monoculture to 88.9 g/L and 98.7 g/L in the coculture in 10 ml GS medium. Ethanol to acetate ratio jumped from 1.39 to 21.1 (mol/mol) on MN300 for the co-culture compared to the monoculture. Berberich et al.

showed a higher ethanol to acetate ratio on cellobiose in the presence of compressed solvents during *C. thermocellum* fermentation with whole cells with a simultaneous decrease in lactic acid production [78]. Similarly, Bothun et al. studied *C. thermocellum* growth and metabolism on cellobiose in a continuous culture which allowed them to understand the effect of hydrostatic pressure on the metabolic profile of the organism independent of substrate level, product level, cell age, etc. that are time dependent in batch fermentation [79]. Both studies showed that an increase in hydrostatic pressure during continuous fermentation led to an increase in carbon flow toward product formation and also specifically led to an increase in ethanol production in contrast to unpressurized and pressurized batch *C. thermocellum* fermentations [78, 79].

Metabolic profile and performance of an organism is expected to reveal the metabolic pathways that were used by the organism to produce the observed metabolites. Along those lines, a number of researchers studied the impact of hydrogen production and the presence or absence of hydrogen in the culture on ethanol production by *C. thermocellum*. Learning from the cocktail of microbes in guts of rumen, Weimer et al. showed a decrease in ethanol production with a co-culture of *C. thermocellum* and *M. thermoautotrophicum* in comparison to a *C. thermocellum* monoculture along with an increase in hydrogen and acetate production because of a continuous hydrogen removal by the methanogen in the co-culture [38]. Saccharolytic organisms in guts of rumen produce hydrogen to satisfy their electron flows since the gas is then utilized by methanogens as a substrate for methane production [80]. Further, Lamed et al. have

shown a higher ethanol production during unstirred batch *C. thermocellum* fermentations possibly due to accumulation of hydrogen in the fermentation broth [81]. Freier et al. corroborated that the concentration of hydrogen produced by *C. thermocellum* JW20 in the fermentation broth was reduced by agitation leading to a decrease in ethanol production [41]. An increased ethanol production was also observed in the presence of externally added hydrogen (50-70 g/L MN300 cellulose). Similarly, Buthen et al. reported a 100 fold increase in ethanol to acetate ratios when continuous *C. thermocellum* cultures were pressurized with hydrogen compared to unpressurized batch fermentation [79]. Lamed et al. further verified a hydrogen feedback mechanism in the organism's metabolic pathway by showing that exogenous hydrogen addition of 1 atm compared to 1 atm nitrogen addition to maintain anaerobic conditions led to an increase in ethanol/acetate ratio for *C. thermocellum* AS-39 from 1.2 to 2.0 (20 h in 10 ml CC medium). However hydrogen addition had no impact on *C. thermocellum* LQRI [77]. These studies speculated that the presence of dissolved hydrogen would affect the regulation of reduced and oxidized electron carriers in the organism's metabolic pathway [79, 81, 82]. Higher hydrogen production in the LQRI strain was related to the absence of electron flow from reduced ferredoxin or NADPH (nicotinamide adenine dinucleotide phosphate) to lactate or ethanol and the higher hydrogenase activity [77]. Formation of acetic acid is more energetically favored by *C. thermocellum* because of ATP generation leading to production of hydrogen in the process [82]. In a pure culture, as opposed to a gut of rumen, saccharolytic organisms have to produce their own electron sinks leading to the formation of NADH (reduced nicotinamide adenine dinucleotide) and thus ethanol

and lactate [80, 82]. Thus, in the presence of excess hydrogen in the system the organism is forced to shift its electron flow from reduced ferredoxin to NAD+ (oxidized nicotinamide adenine dinucleotide) leading to the formation of NADH which is then available for ethanol production. Lamed et al. further reported that both the LQRI and AS-39 strains followed the EMP pathway using a radioactive tracer study utilizing ^{14}C glucose [77]. The strains were also reported to have catabolic activities of fructose-1,6-biphosphate activated lactate dehydrogenase, coenzyme A acetylating pyruvate and acetaldehyde dehydrogenase, hydrogenase, ethanol dehydrogenase, and acetate kinase. Excess substrate availability would lead to an increase in fructose-1,6-biphosphate (FBP). Accumulation of FBP, caused by high substrate loadings and comparatively low product formation rate, triggers lactate production in *C. thermocellum* as reported for both DSM 1313 and ATCC 27405 strains [83, 84]. Further, LQRI and AS-39 strains were also shown to contain a ferredoxin linked pyruvate dehydrogenase and pyridine nucleotide oxidoreductase involved in electron transfer for pyruvate conversion to fermentation products [77]. Alcohol dehydrogenase activity was also shown to be NAD dependent and inhibited by both NAD and ethanol. Overall, the differences in the two strains were attributed to the metabolic control of electron flow variation which was shown to be greatly influenced by pyridine nucleotide oxidoreductase.

2.5.3. *C. thermocellum* metabolic pathway

Zhou et al. corrected that *C. thermocellum*, in fact, converts both glucose and cellodextrins to pyruvate through a modified Embden-Meyerhof-Parnas glycolytic

pathway as opposed to the traditional pathway, shown in Figure 2.2 [85]. *C. thermocellum* has been shown to not have a pyruvate kinase required for the conversion of phosphophenylpyruvate (PEP) to pyruvate, the last step in the EMP pathway

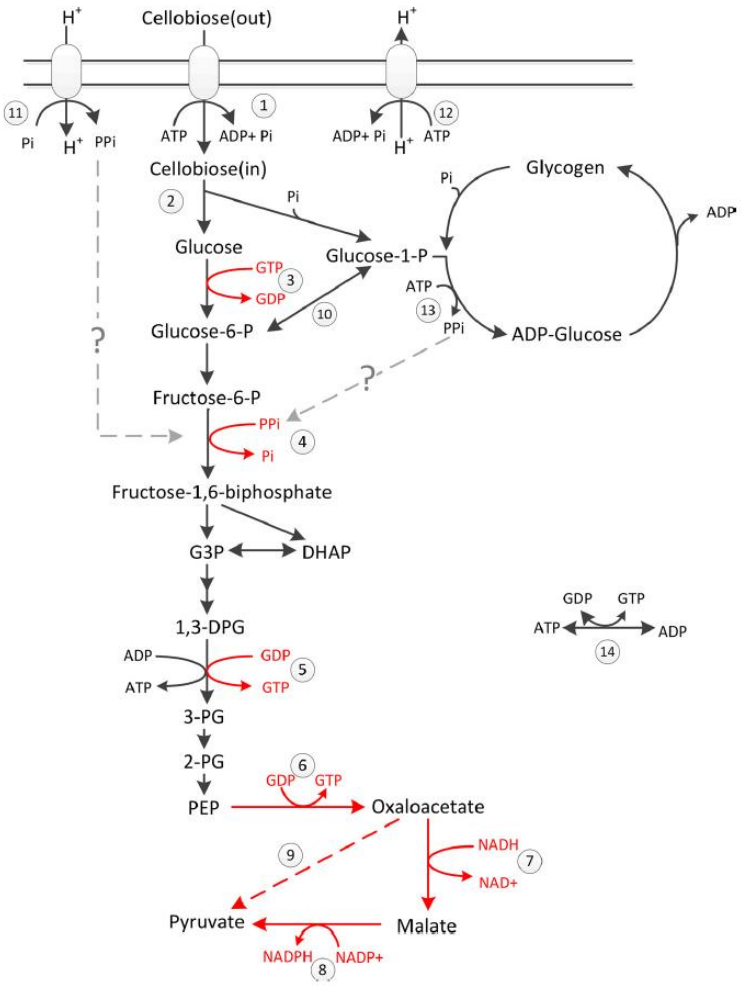

Figure 2.2. Atypical glycolytic pathway in *C. thermocellum* (Modified EMP pathway) [85]*

*Reprinted as is from *Applied and Environmental Microbiology*, 79(9), Zhou J, Olson DG, Argyros DA, Deng Y, van Gulik WM, van Dijken JP, Lynd LR, Atypical glycolysis in *Clostridium thermocellum*, 3000-3008, Copyright (2013), with permission from American Society for Microbiology

Deng et al. have shown that *C. thermocellum* converts PEP to pyruvate with a malate shunt that involves the conversion of PEP to oxaloacetate (OAA) by phosphoenolpyruvate carboxykinase, OAA is converted to malate by malate dehydrogenase, and malate to pyruvate by malic enzyme. Overall, this also leads to NADH to NADPH transhydrogenation [86]. As shown in Figure 2.3, downstream of the EMP pathway, Lo et al. further described the metabolic pathway for the formation of acetic acid, ethanol, lactic acid, and hydrogen as follows [87]: pyruvate is converted to acetyl-CoA in two ways, one of which is through pyruvate-ferredoxin oxidoreductase (PFOR) leading to carbon dioxide formation and electron transfer to reduced form of ferredoxin. Pyruvate is also converted to acetyl-CoA through pyruvate-formate lyase (PFL) leading to the formation of formic acid. Reduced ferredoxin can be reoxidized by hydrogen or NADH formation. Acetyl-CoA is converted to acetate by phosphotransacetylase (PTA) and acetate kinase (ACK). Acetyl-CoA is also converted to ethanol through aldehyde dehydrogenases and alcohol dehydrogenase converting NADH to NAD^+. Furthermore, Lactic acid production is considered overflow metabolism in *C. thermocellum* caused by the accumulation of fructose-1,6-biphosphate [37, 84]. NADH is utilized for lactate dehydrogenase activity required for the conversion of pyruvate to lactic acid.

2.5.4. Additional metabolites

Additionally, pyroglutamate, pyruvate, xylitol, fumarate, malate have been shown in the fermentation broth of *C. thermocellum* DSM 1313 grown on 100 g/L Avicel. 1,2,3-

butanetriol, 2-methyl-1-butanol (2M1B), 3-methyl-1-butanol (3M1B), 2,3-butanediol, isobutanol, 1-propanol, and amino acids were also detected in trace amounts during the high Avicel loading fermentation [29]. The formation of 1-propanol and 2M1B as a branch from threonine/isoleucine biosynthesis pathway and 3M1B from the leucine pathway, also present in yeast, had not been previously reported in *C. thermocellum*.

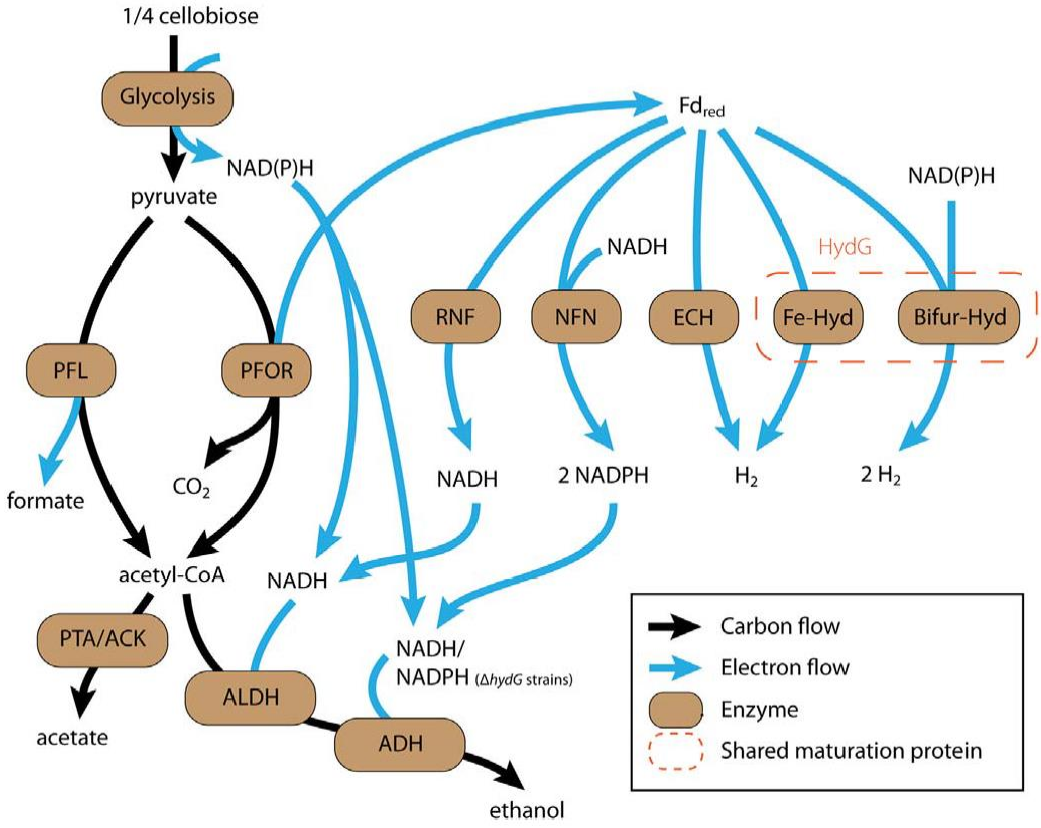

Figure 2.3: *C. thermocellum* metabolic carbon and electron flow [87]*

*Reprinted from *Metabolic Engineering*, 39, Lo J, Olson DG, Murphy SJ-L, Tian L, Hon S, Lanahan A, Guss AM, Lynd LR, Engineering electron metabolism to increase ethanol production in *Clostridium thermocellum*, 71-79, Copyright (2017), with permission from Elsevier

Isobutanol and 2,3-butanediol as products were also reported for *C. thermocellum* for the first time. The presence of citric acid cycle intermediates, fumarate and malate, signified a metabolic imbalance at the high substrate loading and pyruvate accumulation showed that the rate of catabolism exceeded pyruvate consumption at high substrate loading [29]. *C. thermocellum*'s vast metabolic profile including the production amino acids was attributed to cultivation under conditions different from those the organism would encounter in the nature, including the presence of other organisms in its environment [29]. Further, *C. thermocellum* has also been shown to be able to convert carbon dioxide to formate even though the organism lacks formate dehydrogenase. A reversed pyruvate:ferredoxin oxidoreductase was responsible for the formation of acetyl-CoA from carbon dioxide which can then be converted to formate by PFL [88].

2.6.*C. thermocellum* genetic modifications:

2.6.1. Ethanol tolerance and lactate deletion

Maintenance of membrane fluidity is important for an organism, especially at high temperatures. A highly fluid membrane, i.e. a membrane with high degree of molecular motion in the lipid bilayer, may make the membrane leaky, whereas, a membrane with low fluidity may prevent the transport of essential nutrient components. *C. thermocellum* has been proposed to respond to ethanol by producing a more fluid membrane in order to adapt to ethanol, which in turn affects physiology of the cell [89]. In contrast, ethanol tolerant strains have been shown to have fatty acid alteration in their membrane to increase rigidity that counteracts the fluidizing effect of ethanol [90]. The

cell membrane proteins of ATCC 27405 ethanol adapted (EA) strain has been shown to differ from wild type strain with a down regulation of carbohydrate transport and metabolism and an upregulation of proteins involved in chemotaxis and signal transduction [91]. Brown et al. sequenced the genome of the EA strain to show two single nucleotide polymorphisms in the alcohol dehydrogenase (adhE) genes and the transfer of this gene to the DSM 1313 strain led to ethanol tolerance in the mutant [92]. A decrease in NADH dependent alcohol dehydrogenase activity (ADH) was observed with an increase in NADPH ADH. Further, there was also a decrease in ethanol formation simultaneous with an increase in lactic acid production. In order to prevent from carbon and electron flow toward lactate production Biswas et al. deleted lactate dehydrogenase (ldh) from the DSM 1313 strain, which is a genetically more tractable strain than ATCC 27405 [93]. Successful deletion of the lactate pathway confirmed that production of lactate was a consequence of overflow metabolism in *C. thermocellum*. However, the fructose-1,6-biphosphate bottleneck, that usually leads to the direction of the carbon and electron flow to lactate, was hypothesized to not have been corrected and thus the flux to acetyl-CoA and thus acetate and ethanol was expected to be slow [93].

2.6.2. Acetate and formate deletion

Tripathi et al. deleted the pyrf gene in *C. thermocellum* DSM 1313 leading to uracil auxotrophy in the mutant strain so that a toxic uracil analog, 5-fluoroorotic acid, could be used for selection [94]. Using this technique they successfully deleted the phosphotransacetylase gene required for the production of acetate from acetyl-CoA. The

removal of the acetate pathway affected the pool of reduced ferredoxin in the organism since acetate formation is required when reduced ferredoxin is used for hydrogen production leading to growth defects in the organism that would need significant adaptation [94]. Cellular toxicity in the presence of purine antimetabolites (8-azahypoxanthine) due to phosphoribosyl transferase (Hpt), which is actually meant for reassimilation of purines such as hypoxanthine, xanthine, and guanine for DNA and RNA synthesis, in *C. thermocellum* was exploited as a selection marker by Argyros et al. They deleted hpt, followed by deletion of ldh and pta genes to eliminate both lactate and acetate production in *C. thermocellum* that led to a 54% theoretical maximum ethanol yield from Avicel [84]. Deletion of hpt as a selection marker has been used extensively since and was used in all reports reviewed here henceforth. Rydzak et al. deleted the genes encoding pyruvate:formate lyase (pflB) and PFL-activating enzyme (pflA) to eliminate formate production in *C. thermocellum* and also observed a 50% decrease in acetic acid production albeit with reduced growth rate on cellobiose. The reduced growth of the mutant strain was alleviated up to 80% of the parent strain growth rate with the supplementation of 5 mM formate [95]. Further, an increase in carbon dioxide was determined for the mutant strain with pfl deletion since naturally there was more cabron flux to acetyl-CoA through PFOR leading to carbon dioxide formation.

2.6.3. Manipulating electron flow and hydrogen production

Eliminating electron flux to hydrogen should increase availability of electrons for NADH production required for aldehyde and alcohol dehydrogenase activities for ethanol

production. *C. thermocellum* has three [FeFe] hydrogenases and one ferredoxin-dependent [NiFe] hydrogenase. [FeFe] hydrogenases use a single system for active site assembly: HydF maturase is the scaffold on which Fe active sites are assembled, HydE produces the ligands to bridge the two Fe molecules, and HydG cleaves tyrosine to produce –CN and –CO ligands on the active site [96]. Biswas et al. eliminated the hydrogenase maturase gene hydG and ech hydrogenase (encoding the [NiFe] hydrogenase) gene, leading to functional elimination of all four hydrogenases with no detectable hydrogen production and 64% theoretical maximum ethanol yield from cellobiose [96]. A decrease in acetic acid was also observed since acetic acid and hydrogen production are coupled for maintenance of redox balance [96]. Papanek et al further deleted metabolic pathways for all traditional fermentation products, acetate, lactate, formate, and hydrogen to increase ethanol yields from *C. thermocellum* to eliminate any major carbon or electron flux to by-product formation. This mutant strain showed similar yields to the hydrogenase deleted mutant developed by Biswas et al. with a further increase in ethanol yield to 75% theoretical maximum only with adaptations of the strain [97, 98]. This pointed out to the importance of hydrogenase deletion by Biswas et al. and that of directing electron flow toward more ethanol production. Lo et al. took advantage of this electron flow redirection in *C. thermocellum* to improve ethanol yields even further. The NADH-dependent reduced ferredoxin: $NADP^+$ oxidoreductase (NfnAB) and ion-translocating reduced ferredoxin: NAD^+ oxidoreductase (Rnf) complexes are known to be responsible for electron transfer between ferredoxin and nicotinamide co-factors in *C. thermocellum*. While NfnAB did not play a major role in

metabolism, Rnf deletion decreased ethanol production and Rnf overexpression along with hydG gene deletion led to a 66% theoretical maximum ethanol yield by *C. thermocellum* from Avicel (30% increase in ethanol production). The deletion of hydG was required to increase the reduced ferredoxin pool driving the reaction forward toward NADH production through Rnf overexpression.

2.7.*C. thermocellum* fermentation of lignocellulosic biomass:

2.7.1. Early work on lignocellulose fermentation by *C. thermocellum*

Lynd et al. were one of the first few to look at the integration of lignocellulosics pretreatment and *C. thermocellum* enzymatic hydrolysis / fermentation. They reported hydrolysis of dilute acid pretreated mixed hardwood using cell free broth recovered from *C. thermocellum* fermentations to show that 1% sulfuric acid pretreatments with 9 seconds residence at 220°C (10% w/v loading of substrate) allowed the *C. thermocellum* enzymes to achieve about 98% glucan solubilization [34]. Enzymatic hydrolysis was performed with 33% by volume and higher strength broth concentrations to show that higher broths strengths were required for digestion of pretreated mixed hardwood (90% birch and 10% maple from Wilner Wood Products, Norway ME) compared to Avicel. Interestingly, Lynd et al. also pointed out the high substrate affinity of *C. thermocellum* enzymes on pretreated wood compared to Avicel in this work. However, the use of cell free broths might have demanded the use of high pretreatment temperature and sulfuric acid concentration considering the importance of substrate microbe synergy for *C. thermocellum* [99]. Lynd et al. in a separate work also looked at batch and continuous

cultures of *C. thermocellum* on dilute acid pretreated mixed hardwood to determine the processibility of *C. thermocellum* fermentation for ethanol production from lignocellulosics. In this work, they showed that *C. thermocellum* growth on pretreated wood had a longer lag phase than Avicel with higher cellulase activities measured on wood [35]. A 69% substrate conversion was observed with an 8 g/L pretreated wood concentration with a dilution rate of 0.0833/h but the conversion decreased at higher substrate concentrations. Adsorption of *C. thermocellum* cell free cellulases on cellulose from pretreated hardwood (1% sulfuric acid and 9 seconds residence time) was shown to be very high by Bernardez et al [100]. However, the lignin from the pretreated material was also shown to adsorb enzymes significantly, higher than enzyme adsorption on Avicel, and therefore was expected to interfere with enzymatic hydrolysis. Further, they were also able to comment on the cellulosomal action of binding onto substrate, which involves only a single non-catalytic binding unit that allows for multiple catalytic units to adhere onto the substrate, as opposed to cellulase components from *Trichoderma reesei* that competitively bind onto the substrate [100].

Saddler et al. showed similar ethanol production by *C. thermocellum* from steam exploded Aspen wood compared to fermentation on Solka-Floc. Aspen was steam exploded using a high pressure gun with a 250 cc capacity and exposed to saturated steam at 560 psi for 20 seconds before extraction with water for 2 hours at room temperature (labelled wood fraction 1 or WF1). WF1 was further extracted with 0.4% NaOH, washed thoroughly, and mildly acidified with dilute H_2SO_4 to produce WF2.

WF2 was then treated with sodium chlorite (2% of biomass weight) to produce WF3. *C. thermocellum* monoculture was shown to produce 1.5 g/L ethanol from 1% Solka-Floc in a modified medium. Comparatively, *C. thermocellum* was shown to readily degrade 1% WF3 fraction of pretreated Aspen wood with glucose yields equivalent to fermentations on 1% Solka-Floc, which was readily utilized by *Zymomonas anaerobia* in a sequential culture to produce 1.6 g/L ethanol. Hörmeyer et al. showed that hydrothermal and methanol organosolv pretreatments at a temperature of 230ºC were required for efficient degradation of poplar wood and wheat straw by *C. thermocellum* in a pH regulated laboratory fermenter [101]. Donnison et al. looked at a number of extremely thermophilic microorganisms compared to *C. thermocellum* fermentation on various lignocellulosic substrates including SO_2 treated steam exploded *Pinus radiata*, acetone washed steam exploded *Eucalyptus Regnans*, deproteinized Lucerne fiber (alfalfa), and purified milled wood pulp [102]. Wood pulp was the most digested by all organisms in this study followed by Avicel, and all lignocellulosics were the least digested. Further, no weight loss in milled *P. radiata* chips (60 mesh) or unbleached coarse wood pulp (from hot kraft and thermomechanical processes) was detected after 7 days of incubation with *C. thermocellum*. Similarly, mild pulping in the laboratory (1 h in 0.4% NaOH, 1 h in 1M H_2SO_4, overnight in 2% sodium sulfite, followed by washing and filtering) did not help enhance *C. thermocellum* digestion of the substrate. A 3% weight loss was observed with steam explosion of *P. radiata* (200ºC for 10 min), whereas the weight loss increased to 21% with steam explosion of SO_2 treated *P. radiata* (soaked in 2.55% w/v SO_2 followed by steam explosion at 215ºC for 3 min and extensive water washing). Bleached *P.*

radiata wood pulp milled through a 40 mesh showed the highest weight loss of 75%. Highest ethanol to acetate ratio on a lignocellulosic substrate of 2.31 was obtained by *C. thermocellum* on alfalfa with other organisms showing an ethanol to acetate ratio below 0.25 on the same substrate [102].

2.7.2 Understanding and improving cellulosomal and metabolic performance

The earlier works focused on the integration of lignocellulosic biomass pretreatment with *C. thermocellum* fermentations for production of ethanol. In contrast, some of the more recent studies involving C. *thermocellum* and lignocellulosics have focused more on genetic modification of plant biomass, genetic modifications of *C. thermocellum* to improve ethanol yields and tolerance, and understanding and discovering cellulosomal enzymes and their activities on biomass. For example, Wei et al. using RNA-seq showed that 1211 genes in *C. thermocellum* grown on pretreated Yellow Poplar (YP; 0.21 wt% H_2SO_4 at 200ºC for 4 min) were up-regulated whereas 314 genes were down regulated (more than 2-fold compared to *C. thermocellum* grown on cellobiose) with a broad spectrum of classification. Specifically, some cellulosomal genes, cellodextrin transporter genes, and NADPH hydrogenase and alcohol dehydrogenase genes were up-regulated and proposed as useful candidates for future studies. Further, 30% and 39% ethanol and hydrogen production respectively by *C. thermocellum* ATCC 27405 was shown on pretreated YP [61]. Wilson et al., similarly, showed that growth and transcriptomic profiles of *C. thermocellum* ATCC 27405 were influenced by compositional differences in dilute acid pretreated Alamo switchgrass and

Populus (*Populus trichocarpa* and *Populus deltoids* F1 hybrid) (0.05 g sulfuric acid /g dry biomass at 190ºC with 1 min residence time in flow-through mode and 25 wt% total solids) [103]. Xu et al. showed that *C. thermocellum* JYT01 (derivative of *C. thermocellum* LQRI) cellulosome was tolerant of up to 5 mM furfural, 50 mM p-hydroxybenzoic acid and 1 mM catechol, which are known pretreatment-derived inhibitors, during hydrolysis of Avicel [104]. They also showed that the cellulolytic activity of the cellulosome on Avicel was promoted by formate, acetate, and lactate. Papanek et al. looked at fermentation of *C. thermocellum* AG553 (lactate, acetate, formate, and hydrogen production eliminated) fermentations on dilute acid pretreated poplar and switchgrass to validate the improvement in ethanol production observed on Avicel [97]. *C. thermocellum* AG553 produced 23.8 mM ethanol on pretreated poplar with a 62.6% theoretical maximum ethanol yield compared to 24.9 mM ethanol production and 65.5% theoretical maximum ethanol yield from Avicel. The corresponding wild type showed 13.4 mM and 13.0 mM ethanol production in pretreated poplar and Avicel, respectively. About 53% and 26% theoretical maximum ethanol yield was observed on pretreated switchgrass by the mutant and wild types strains, respectively [97].

2.7.3. Impact of lignocellulosic biomass, its natural variance, and genetic modifications

Dumitrache et al. showed that *C. thermocellum* ATCC27405 fermentation ethanol yield was 2.9 times higher on *Populus* genotype with high syringyl to guaiacyl (S/G) ratio when compared to a *Populus* genotype with a low S/G ratio [105]. Cellulose

accessibility and the lignin molecular weight were positively correlated with a high S/G ratio. It is though that high syringyl content is linked to longer lignin chains that has a lower interference with enzymatic activity. This research indicated *C. thermocellum* sensitivity to the composition of biomass, especially lignin, and therefore the ultimate success rate of conversion into ethanol [105]. Similarly, Yee et al. showed improved ethanol production by and alleviated substrate inhibition of *C. thermocellum* M1570 on dilute acid pretreated transgenic switchgrass with downregulation of caffeic acid O-methyl transferase (COMT) gene in the lignin pathway (COMT switchgrass) showing low lignin content and S/G ratio compared to wild type switchgrass [106]. Originally, Yee et al. and Fu et al. had showed inhibition of wild type *C. thermocellum* ATCC 27405 grown on dilute acid pretreated COMT switchgrass compared to wild type switchgrass that was alleviated with mild hot water extraction of the COMT switchgrass [107, 108]. Dilute acid pretreatment was performed in a hastelloy steel tubular reactor with a 2.5 g dry biomass per tube and 9 mL 0.5% sulfuric acid solution per gram of dry biomass loading that was heated in a sand bath set at 180ºC for 7.5 min. Washed biomass was soaked in water overnight and transferred to the sand bath set at 80ºC for 10 min and washed again to remove inhibitory compounds. Further, Yee et al. showed that *C. thermocellum* more readily fermented dilute acid and hot water pretreated COMT switchgrass than *Caldicellulosiruptor* species with latter organisms showing low levels of unconsumed sugars in the broth suggesting a negative impact of dilute acid pretreatment on both hydrolysis and fermentation by these organisms [107]. Thomas et al. varied hydrothermal pretreatment conditions to maximize sugar release from pretreatment of

poplar (BESC standard) and *C. thermocellum* fermentations combined and found 200ºC and 22.7 min to be the best pretreatment conditions. They further hydrothermally pretreated BESC standard poplar and other natural variants of poplar (SKWE 24-2 and BESC 876) with lower recalcitrance at the determined best conditions [109]. These low recalcitrant poplar natural variants have mutations in their 5-enolpyruvylshikimate-2-phosphate (EPSP) synthase gene that affects lignin biosynthesis and were shown to have loosely held cell wall structures, high water retention value, and high S/G ratio which were all positively correlated with glucan solubilization in the pretreated solids. *C. thermocellum* was also shown to more effectively digest glucan and reduce lignin molecular weight compared to that by fungal enzymes [109].

2.7.4. Impact of lignocellulosic biomass pretreatment

In a separate study, Thomas et al. have also shown complete polysaccharide solubilization by *C. thermocellum* when grown on a co-solvent enhanced lignocellulosic fractionation (CELF) pretreated poplar and corn stover, higher than that on dilute acid pretreated poplar and corn stover. The CELF-CBP combination was proposed to be a feedstock-independent process for ethanol production [110]. Shao et al. performed AFEX pretreatments on corn stover to better understand the microbial conversion of pretreated cellulosic biomass using *C. thermocellum* when compared to fungal cellulases for ethanol production [111]. *Saccharomyces cerevisiae* D5A was used in SSF experiments with 0.75 g AFEX corn stover or 0.22 g Avicel in 125 bottles with 41 mL deionized water, 2.5 mL 1 M citrate buffer at pH 4.5 and 0.15 mL corn steep liquor. A mixture of Spezyme cp and

β-galactosidase was used for hydrolysis. *C. thermocellum* fermentation were performed on 0.75 g AFEX corn stover or 0.22 g Avicel in 125 bottles with MTC medium. *C. thermocellum* and SSF with 10 FPU/g glucan enzyme loading showed equal rates and extents of solubilization on AFEX pretreated corn stover but SSF was shown to be more sensitive to cellulase loading than *C. thermocellum* fermentations to inoculum size in terms of solubilization extent. Further, particle size reduction was shown to affect *C. thermocellum* glucan solubilization more than hemicellulose removal, whereas, both seemed to have a significant impact on SSF [111]. Co-treatment, milling during *C. thermocellum* fermentation of lignocellulosics, as an alternative to thermochemical pretreatments was studied by Balch et al. to show that *C. thermocellum* was robust enough to survive ball milling, whereas yeast was not [112]. Total carbohydrate solubilization (TCS) was shown to be 0.88 for *C. thermocellum* co-treatment of switchgrass, higher than *C. thermocellum* fermentations of hydrothermal pretreatment of the biomass (200ºC for 15 min) with a 0.81 TCS and that of untreated switchgrass with a TCS of 0.45. Lignin recovered after co-treatment was also shown to be closer to its native structure compared to lignin recovered after *C. thermocellum* fermentations of hydrothermally pretreated switchgrass [112].

2.7.5. Alternative biomass pretreatment strategies for alternative products

Alternative pretreatment technologies for the production of metabolites other than ethanol by *C. thermocellum* have also been extensively studied. Liu and Cheng used a microwave assisted pretreatment (MAP) of corn stover in the presence of acid to improve

hydrogen production from thermophilic fermentations. MAP was proposed to have a high heating efficiency along with being operationally easy and therefore advantageous over thermal acid pretreatment (TAP) [113]. For acid based MAP, 10 g ground corn stover was added to a 120 mL solution containing varying amounts of H_2SO_4 (0.05, 0.1, 0.2, 0.3 or 0.4 N H_2SO_4). These mixtures were irradiated at 700 W for 5, 15, 30, 45, 60 or 90 min using a modified household microwave. In comparison, TAP experiments were conducted by boiling the corn stover-acid solution mixture for the same time durations. For hydrogen fermentation, seed was collected from a 5 L anaerobic digester with a working volume of 4 L which was started with a seed sludge from a local winery and operated in a semi-continuous mode for 6 months. Fermentation was performed in a 500 mL bottle with 48 mL of pretreated corn stover and 75 mL of seed inoculum, 147 mL of distilled water, and 30 mL of nutrient solution. Here, MAP was shown to have a clear advantage over TAP with the final hydrogen production being 182.2 mL with MAP pretreatment of corn stover at 0.3 N H2SO4 for 45 min, which was equivalent to 1.53 mol H_2/mol glucose. In contrast, the highest hydrogen production with TAP was with 0.2 N H2SO4 for 90 min with 139.8 mL hydrogen production [113]. A number of groups have further focused on microwave assisted pretreatments of lignocellulosics for production of cellulolytic enzymes, ethanol production, biogas production and hydrogen production. [114-118]. Li et al. improved upon the work performed previously on MAP to develop a dynamic microwave alkali pretreatment (DMAP) method. In comparison to MAP, DMAP has additional advantages such as large capacity, continuous and automated operation, high lignocellulosis hydrolysis efficiency, and a short time duration

[119]. Cornstalk was exposed to 2 L of NaOH with varying alkali loadings (0.02, 0.04, 0.08, 0.12, 0.16 g NaOH / g of cornstalk) and the suspension was exposed to irradiation at 15 second on/off intervals while stirring. A co-culture of *Clostridium thermocellum* DSM 7072 and *Clostridium thermosaccharolyticum* DSM 869 was used for batch fermentation in 125 mL bottles with a 10% by volume inoculum and 10 g/L of treated cornstalk equivalent added from the supernatant collected during DMAP. *C. thermosaccharolyticum* was further added after 24 hours [119]. The co-culturing method with *C. thermocellum* and *C. thermosaccharolyticum* for hydrogen production was developed separately [120]. The hydrogen yield was 105.61 mL/g of cornstalk with 45 min of pretreatment with DMAP, a liquid solid ratio of 50:1 mL/g, and a flow rate of 60 mL/s with 0.12 NaOH/g cornstalk. Compared to untreated cornstalk, the DMAP cornstalk had a 54.8% higher hydrogen production compared to MAP specifically with a significant increase in hemicellulose and cellulose degradation by the co-culture [119] . Similarly, a co-culture of *C. thermocellum* and butanol-producing *C. saccharoperbutylacetonicum* was studied on alkali (1% wt/vol NaOH) treated rice straw to show high butanol production (5.5 g/L) from 40 g/L pretreated biomass [121]. When cellulases (cellulase cocktail from Aspergillus Niger and β-glucosidase derived from almond) were added to the co-culture, further butanol production was observed, indicating the need for modification of organisms to increase enzyme production and/or activity for increased product formation. It was also found that cellulosomal exoglucanases were important for effective butanol production from delignified rice

straw, which was further improved by β-glucosidases that decrease cellobiose concentrations in the culture alleviating exoglucanase inhibition [121].

2.8. Inferences and future directions:

The effective cellulosomal cellulolytic activity of *C. thermocellum* is promising for sugar release from lignocellulosic biomass for the generation of a wide range of products. The multi-functional *C. thermocellum* cellulosome effectively adapts its enzymatic activities on the substrate of choice and efficiently degrades the substrate and produces metabolites. The *C. thermocellum* cellulolytic activity has further been assisted by various biomass augmentation techniques, including but not limited to thermochemical pretreatments, co-treatment, and microwave assisted pretreatments. *C. thermocellum* prefers to metabolize cellobiose and cellodextrins over glucose reducing the amount of ATP required for transportation per hexose, thus making more ATP available for other cellular functions. In its natural environment *C. thermocellum* is known to exist in combination with a number of other organisms obtaining vitamins through interspecies cross-feeding and using other organisms like methanogens as electron sinks. *C. thermocellum* wild type produces multiple useful metabolites, such as, ethanol, acetic acid, lactic acid, formic acid, and hydrogen and thus requires strategic fermentation process development or a genetically improved metabolism to produce the metabolite of choice with high yields and titers. *C. thermocellum* has been shown to produce high amounts of ethanol when pressurized with hydrogen making electrons available for ethanol production. In laboratory conditions, as opposed to natural

environments, under high substrate loadings *C. thermocellum* shows overflow metabolism producing amino acids and other metabolites not typically observed. A tractable genetic system for *C. thermocellum* has been developed for the DSM 1313 strain with the successful elimination of multiple pathways including formate, lactate, acetate, and hydrogen production. Electron transfer in *C. thermocellum*'s metabolism is considered especially important for manipulating hydrogen and ethanol production by the organism. Ethanol tolerance in the organism has been associated with changes in the cell membrane alleviating the fluidizing effect of ethanol.

The extensive reports on *C. thermocellum* have aided in making native cellulolytic strategy CBP a promising process for the production of ethanol from lignocellulosic biomass. However, in order for the industry to utilize *C. thermocellum* CBP, a directed, strategic, and an extensive process development effort is necessary. Even though *C. thermocellum* has an effective cellulolytic system, the organism by itself is not able to achieve effective solubilization of all of the polysaccharides from lignocellulosic biomass. Even though biomass augmentation techniques have been shown to aid *C. thermocellum* is digesting lignocellulosics, a comprehensive study on the integration of such augmentation techniques with *C. thermocellum* CBP is lacking. Most reports of *C. thermocellum* fermentations on lignocellulosics have focused on either understanding the cellulolytic / metabolic systems of the organism, testing genetically modified substrates, or understanding the mechanism of biomass breakdown by the organism. However, in order for the scientific knowledge on *C. thermocellum* to be

useful in an industrial process an engineering approach to development and optimization of the biochemical process in essential. Biomass augmentation should be optimized in combination with *C. thermocellum* fermentations for maximizing sugar release from lignocellulosics. Further, a substantial effort towards such optimizations with genetically modified *C. thermocellum* strains that are able to produce and tolerate ethanol (or other product of choice) at high concentrations is essential

2.9. References

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Chapter 3. Impact of cellulose loading on *Clostridium thermocellum* cellulolytic and metabolic performance

In Review at *Biotechnology for Biofuels*

Ninad Kothari, Maher Zaher, Priyanka Singh, Charles M. Cai, Rajeev Kumar, and Charles E. Wyman

3.1. Abstract

Clostridium thermocellum hydrolyzes polysaccharides from lignocellulosic biomass using its multi-functional cellulosomes and ferments the sugars released into ethanol and other metabolites in a single consolidated operation without the need to add expensive enzymes. However, accumulation of these metabolites at high substrate loadings can inhibit organism performance and obfuscate understanding how biomass substrate features impact the ability of *C. thermocellum* to deconstruct and utilize lignocellulosics. Thus, flask fermentations using *C. thermocellum* were performed on a cellulosic substrate at loadings ranging from 0.1-5 wt% glucan to determine the effect of substrate loading on metabolites production and glucan solubilization. An increase in ethanol and acetic acid production by *C. thermocellum* was observed from 0.1-0.5 wt% glucan loading of Avicel® PH-101. However, *C. thermocellum* significantly increased lactic acid production at 1wt% glucan Avicel loadings while the production of ethanol and acetic acid ceased. The drop in *C. thermocellum* metabolites yield, accounting for ethanol, lactic acid, and acetic acid, from 62% of glucan for 0.1-0.5 wt% glucan loadings of Avicel to about 40% at 1 wt% glucan loading further revealed inhibition of metabolic performance. Substantial inhibition of *C. thermocellum* by acetic acid and mild inhibition by ethanol at concentrations typically observed during fermentations coupled with a drop in pH contributed to the organism's poor metabolic performance at high substrate loadings. However, the observation that glucose continued to accumulate for fermentations of up to 5wt% glucan Avicel showed that enzymatic activity was maintained even after metabolites production had ceased. Because non-inhibitory conditions are needed to

understand the impact of substrate properties and substrate pretreatment technologies on *C. thermocellum* fermentations, glucan substrate loadings of 0.5 wt% or less should be employed in flask fermentations of *C. thermocellum* DSM 1313 strain. Furthermore, performing such studies in active pH controlled environments can further minimize masking effects of biological constraints from substrate effects. Strong inhibition by acetic acid compared to ethanol points to the desirability to genetically modify *C. thermocellum* to reduce or eliminate by-products formation and allow for higher substrate loadings and product titers in addition to high ethanol yields.

3.2. Introduction

Consolidated bioprocessing (CBP) is a simple bioprocess that employs *Clostridium thermocellum* and other organisms to produce multi-enzyme cellulase cocktails that digest complex lignocellulosic biomass and to ferment sugars released into useful metabolites [1-5]. The combination of enzyme production, enzymatic hydrolysis, and sugar fermentation in one step makes CBP a promising platform for ethanol production from biomass. Due to the recalcitrance of biomass to breakdown, cellulose is difficult to access and deconstruct by enzymes, thus warranting the need for biomass pretreatment to realize sufficient glucan conversion [6-10]. Because high substrate loadings are needed to produce high ethanol titers [11-13], determining the impact of substrate loading on *C. thermocellum* digestion performance is vital to guide process development. Furthermore, accumulation of metabolites at high substrate loadings can inhibit organism performance and obfuscate understanding how biomass substrate

features impact the ability of *C. thermocellum* to deconstruct and utilize lignocellulosics. Thus, it is important to understand the primary effect of substrate loading on *C. thermocellum* fermentations using model cellulosic substrates. Model substrates address underlying metabolic features affected only by substrate loading independent of the presence of hemicellulose and lignin that influence cellulose macro-accessibility in lignocellulosic biomass [8]. Avicel PH-101 was thus chosen as a model cellulosic substrate for this study.

C. thermocellum was first identified in 1926 [14, 15], isolated in the late 1940s [16, 17], and its unique cellulosome was first discovered in the 1980s [18]. Although its cellulolytic abilities have been studied extensively since, a comprehensive evaluation of the impact of substrate type and loading on *C. thermocellum* has not been done. Metabolites production by *C. thermocellum* DSM 1313 reported over the past decades has ranged from 0.7-1.32 g/L of ethanol, 0.75 – 2.74 g/L acetic acid, and 0.05 - 2.49 g/L lactic acid on Avicel and cellobiose at loadings of 5 and 20 g/L [14]. The initial cellulose loading has been shown to influence the metabolic flux of *C. thermocellum* ATCC 27405 grown on 1 and 5 g/L loadings of α-cellulose [19]. This report suggested that accumulation of organic acids and increased concentrations of dissolved product gases at high substrate loadings shifts the carbon flow from acetic acid to ethanol production. Further, effects of varying cellobiose loadings have been reported to influence hydrogen production by *C. thermocellum* ATCC 27405 [20]. Similarly, a change in end products ratio, accumulation, and yields have been shown with *C. thermocellum* AS-39 grown on

cellobiose loadings ranging from 0.2-5.0%, with 0.8% proposed to be an optimum for ethanol production and ethanol-acetate ratio [21]. *C. thermocellum* DSM 1313 exhibits overflow metabolism when grown on Avicel cellulose at 100 g/L loading achieving 11 g/L acetic acid and 13 g/L ethanol production in a bioreactor [12]. High product levels typically not observed in natural environments may trigger such metabolic changes in the organism. Furthermore, the wild type DSM 1313 strain has further been genetically modified to tolerate high ethanol concentrations observed with high initial cellulose concentrations including 20-100 g/L Avicel, 63 g/L MN300 cellulose, and 80 g/L Solka-Floc [12, 22, 23]. Similar effects of high substrate loadings on metabolites formation have been shown for other promising CBP organisms *Caldicellulosiruptor bescii* (200 g/L Avicel) and *Clostridium phytofermentans* (4 wt% AFEX pretreated corn stover) [11, 13].

Here we report for the first time in detail the influence of increasing initial loadings of Avicel PH-101, a model cellulosic substrate, on *C. thermocellum* DSM 1313 performance in flask fermentations. Results for only one substrate type are reported in this work since similar results were observed on Sigmacell Cellulose Type 50 and Whatman® 1 filter paper. Further, the effects of poor mixing at high substrate loadings, metabolites formation, pH change, and glucose formation on the organism's cellulolytic and metabolic activity were determined to understand the role these factors have on *C. thermocellum* performance at high substrate loadings. We also identified factors that potentially affect the metabolic profile of the organism at high substrate loadings.

3.3. Results and discussions

3.3.1. Impact of cellulose loading on *C. thermocellum* metabolites production

C. thermocellum fermentations were performed on Avicel® PH-101 at 0.1, 0.25, 0.5, 1, 2, and 5 wt% glucan with a 50 g working mass in 125 mL serum bottles with MOPS buffer (3-(*N*-morpholino)propanesulfonic acid (pK_a 7.20)) used to passively control pH at 7.0. To investigate the effect of substrate loading on metabolite production by *C. thermocellum*, the concentrations of acetic acid, ethanol, and lactic acid, the major metabolites produced by *C. thermocellum*, were measured after 7 days of fermentation, as reported in Figure 3.1.

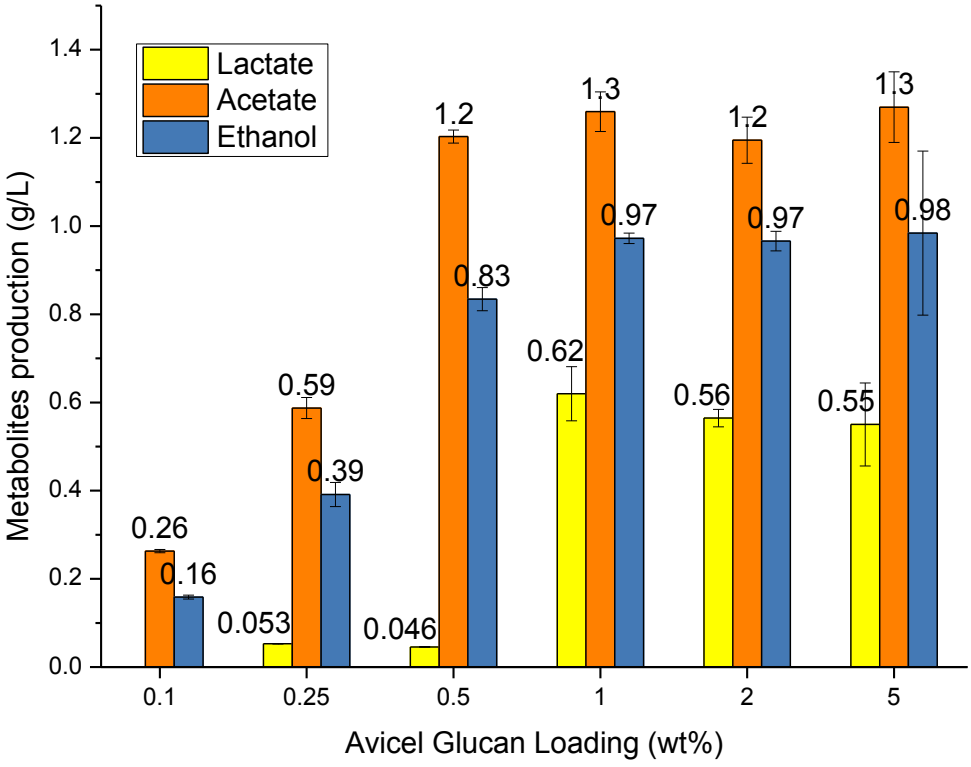

Figure 3.1. Metabolite concentrations produced by *Clostridium thermocellum* after 7 days of fermentation on 0.1-5 wt% glucan loadings of Avicel

The overall concentration of metabolites increased with substrate loading from 0.1 to 1 wt% glucan. These results show that *C. thermocellum* preferred to produce acetic acid and ethanol compared to the negligible amount of lactic acid production at low substrate loading of 0.1 wt% glucan. *C. thermocellum* prefers to produce acetic acid due to the generation of ATP also leading to the formation of hydrogen through electron flow from reduced ferredoxin [24]. Electron flow also leads to the formation of NADH (reduced nicotinamide adenine dinucleotide), which is increased in the presence of excess hydrogen during fermentation [24-27]. NADH is then available for aldehyde and alcohol dehydrogenase activities required for ethanol production from acetyl co-enzyme A (acetyl-CoA). The organism produced ~1.2 g/L acetic acid, ~0.8 g/L ethanol, and much lower lactic acid concentrations at 0.5 wt% glucan loading, as reported elsewhere for the *C. thermocellum* DSM 1313 grown on Avicel [14, 28]. However, *C. thermocellum* showed signs of stress at substrate loadings of 1 wt% and higher. A ~0.5 g/L increase in lactic acid concentration produced by *C. thermocellum* on 1 wt% glucan substrate loading compared to that on 0.5 wt% glucan substrate loading with no change in acetic acid and ethanol concentrations indicated that stressful conditions were encountered by the organism at and above 1 wt% glucan loadings of Avicel. Lactic acid production in *C. thermocellum* is considered overflow metabolism and not essential for carbon and electron flow [12, 29]. Fructose-1,6-biphosphate accumulation is considered a bottleneck in the formation of acetyl-CoA and thus ethanol and acetic acid production during *C. thermocellum* metabolism that leads to the formation of lactic acid instead [12, 22, 30]. Because the metabolic profile of the organism did not change significantly at substrate

loadings of 1-5 wt% glucan, metabolite formation is suggested to have ceased after a ceiling concentration of each metabolite was reached. Note that higher ethanol concentrations have been reported for this strain at 100 g/L substrate loadings in a bioreactor under highly controlled environments [12]. Even though the ethanol / acetate ratio (E/A) did not change significantly, E/A was highest for fermentations of 1-2 wt% glucan loading of Avicel because *C. thermocellum* was less tolerant to acetic acid than ethanol (Figure 3.2). A similar range of E/A ratios have been reported in a continuous culture of *C. thermocellum* ATCC 27405 on Avicel and for *C. thermocellum* LQRI fermentations with a 1% Solka-Floc and 0.8% MN 300 cellulose loadings [28, 31]. However, unlike the results of this work, E/A is also reported to vary significantly for *C. thermocellum* ATCC 27405 and its mutant strain AS-39 with varying loadings of cellobiose and α-cellulose [19-21].

3.3.2. Impact of product inhibition and pH change on *C. thermocellum* performance

Ineffective mixing, inhibition by high concentration of metabolites produced by the organism, and/or pH change to non-optimum conditions for the organism could impede *C. thermocellum* performance at higher substrate loadings. To determine if leveling off in metabolite concentrations produced by *C. thermocellum* was caused by mixing limitations at high substrate loadings, cellulose acetate was added over a range of loadings along with a fixed loading of Avicel prior to fermentation. *C. thermocellum* was unable to metabolize cellulose acetate and showed negligible metabolites production on

this substrate (Figure 3.3). Excess inert cellulose acetate would thus limit effective mixing without directly influencing metabolite production from Avicel.

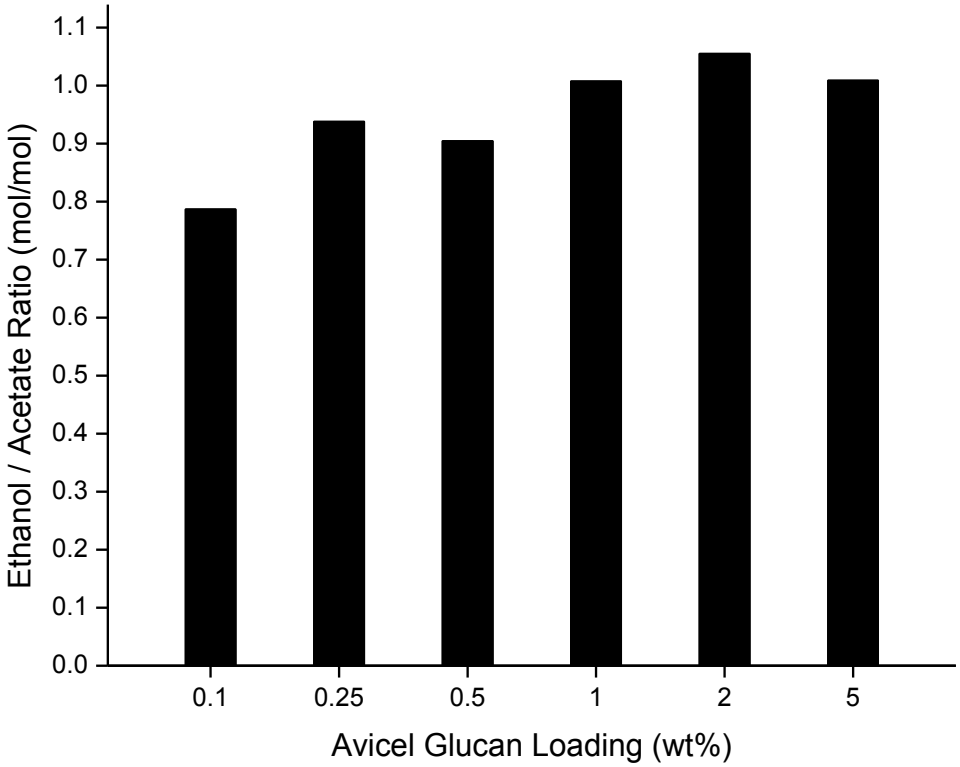

Figure 3.2. Ethanol / acetate ratio (mol/mol) produced by *Clostridium thermocellum* after 7 days of fermentation on 0.1-5 wt% glucan loadings of Avicel

Figure 3.4 shows that *C. thermocellum* produced similar amounts and concentrations of metabolites from 0.5 wt% Avicel after 7 days irrespective of cellulose acetate loadings from 0.1 wt% to 5 wt% added at the beginning of fermentation. In this regard, acetic acid production by *C. thermocellum* on Avicel mixed with cellulose acetate was calculated by subtracting the small amount of acetic acid produced by the organism on just cellulose acetate in control fermentations at the same loadings from the concentrations measured with the mixed solids. This correction may have contributed to

an apparent slightly lower acetic acid production by *C. thermocellum* from Avicel in the presence of cellulose acetate compared to when no cellulose acetate was added. Nevertheless, because *C. thermocellum* was not substantially affected at high substrate concentrations, mixing limitations appeared to not account for the leveling off in metabolite concentrations produced by the organism. This outcome suggests that inhibition of *C. thermocellum* fermentations at high substrate loadings was most likely caused by the buildup of metabolites at these loadings and/or a change in pH.

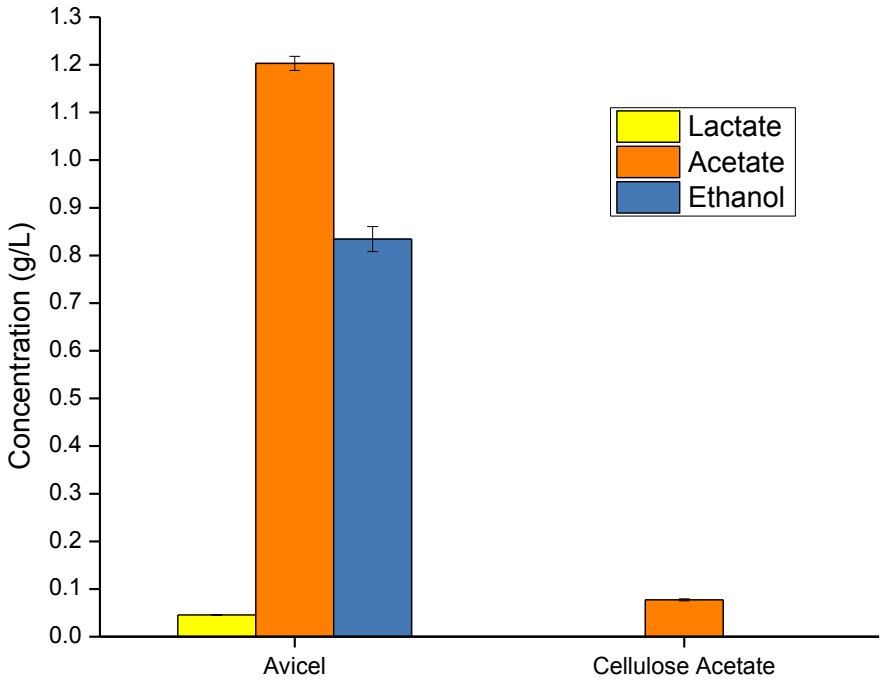

Figure 3.3. Metabolite production by *C. thermocellum* on 0.5 wt% glucan loading of Avicel and cellulose acetate after 7 days of fermentation

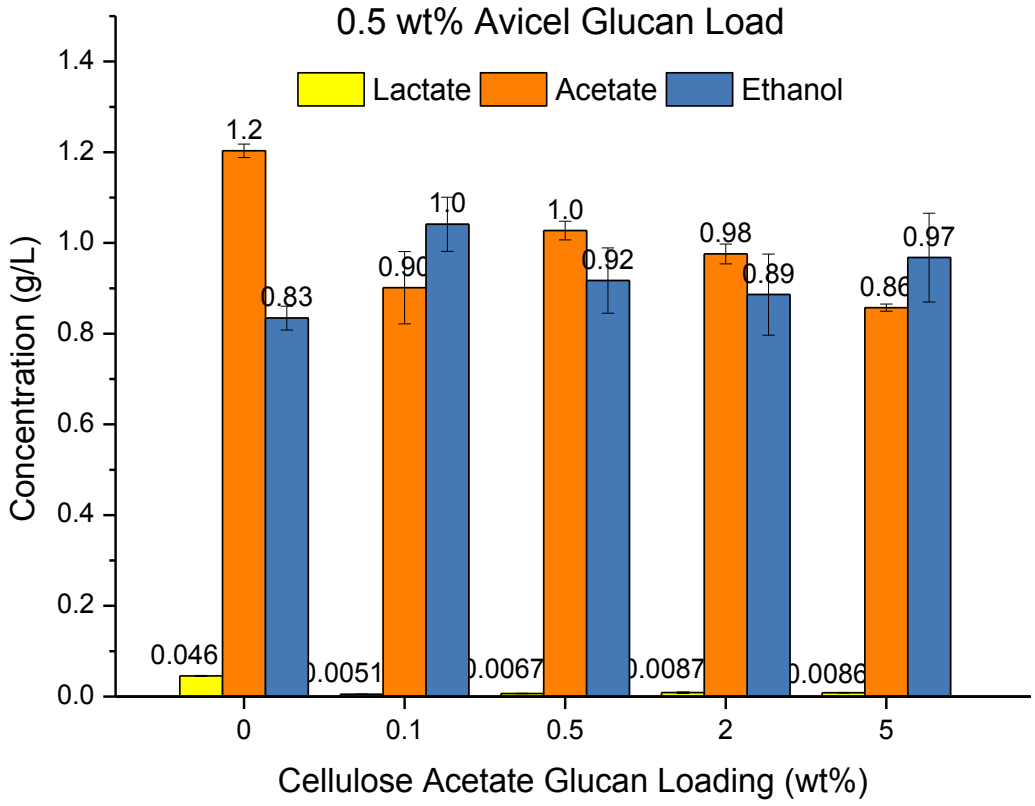

Figure 3.4. Metabolite production by *Clostridium thermocellum* after 7 days of fermentation on Avicel at 0.5 wt% glucan loading with increasing loading of inert cellulose acetate (0-5 wt%) to induce mixing limitation

Acetic acid and other carboxylic acids are known to act as uncouplers of the membrane pH gradient and thus affect biosynthesis and transport of metabolites causing inhibition [32]. To determine the extent of acetic acid inhibition of *C. thermocellum*, increasing concentrations of acetic acid were loaded along with a constant 0.5 wt% glucan Avicel loading for fermentations as shown in Figure 3.5. 0-1.5 g/L initial acetic acid loadings were employed since acetic acid production stopped at about 1.3 g/L during typical flask fermentations; therefore, minimal metabolite production was expected at and above 1.3 g/L initial acetic acid loading. Although the production of metabolites dropped with increasing concentrations of initial acetic acid, it did not cease even during

fermentations starting with 1.5 g/L acetic acid. In fact, *C. thermocellum* produced 0.5 g/L acetic acid after 7 days of fermentation even after starting with 1.5 g/L of that acid added exogenously. Thus, even though *C. thermocellum* was significantly inhibited at and above 1 g/L of acetic acid, its tolerance of at least up to 2 g/L of the acid suggested that acetic acid was not the only inhibitor in the system, with ethanol possibly inhibiting *C. thermocellum* as well.

Because ethanol has been shown to inhibit energy metabolism and sugar utilization by *C. thermocellum* and has also been reported to fluidize the organism's cell membrane [33-36], *C. thermocellum* fermentations were performed at a constant 0.5 wt% Avicel loading with increasing exogenous ethanol concentrations added before fermentation to determine the extent of ethanol inhibition of *C. thermocellum* reported in Figure 3.6. Originally, only low ethanol concentrations from 0-1 g/L were chosen since the maximum ethanol concentrations observed during typical flask fermentations reported in this study was between 0.8-1 g/L. Even though a drop in *C. thermocellum* ethanol production was observed at these low initial ethanol loadings, the extent of inhibition was low compared to acetic acid inhibition. Low ethanol inhibition with a lag phase, the length of which depended on initial exogenous ethanol concentration, has been reported for *C. thermocellum* grown on cellobiose [21].

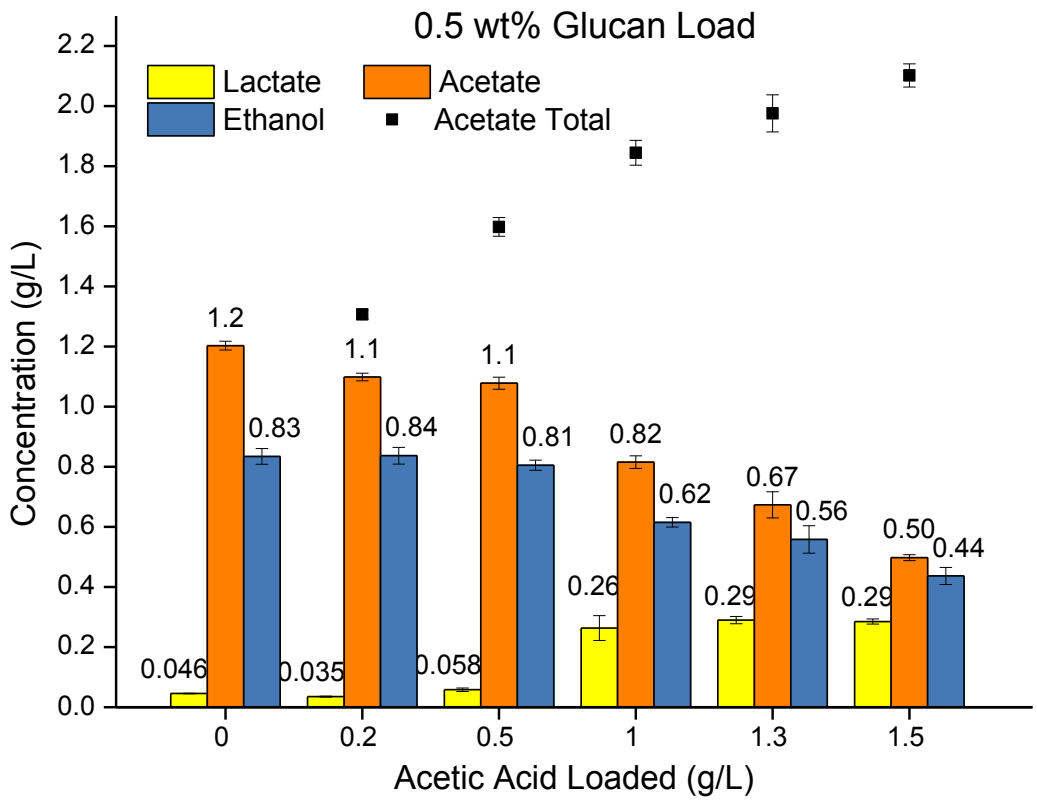

Figure 3.5. Metabolite production by *Clostridium thermocellum* after 7 days of fermentation on Avicel at 0.5 wt% glucan loading with increasing concentrations of acetic acid (0-1.5 g/L) added exogenously before inoculation

Thus, fermentations were next performed at higher initial ethanol loadings to determine ethanol tolerance of *C. thermocellum*. For up to 7 g/L of initial ethanol, there was no drop in acetic acid production and only slightly lower ethanol production compared to metabolite production by the organism when no ethanol was added before fermentation. However, both acetic acid and ethanol production decreased at initial ethanol loadings above 7 g/L, whereas lactic acid production increased substantially. Similarly, substantial inhibition of *C. phytofermentans* has been reported at ethanol concentrations above 20 g/L that reduced glucan and xylan conversion from AFEX pretreated corn stover [13]. Overall, even though *C. thermocellum* showed considerable

acetic acid inhibition during fermentations at all substrate loadings applied in this work, the organism was only mildly inhibited by ethanol.

However, additive inhibition of *C. thermocellum* by both ethanol and acetic acid is also likely. Therefore, we looked at metabolite production by *C. thermocellum* with exogenous addition of both ethanol and acetic acid before inoculation as reported in Figure 3.7. Concentration of acetic acid added exogenously was chosen to be 1.3 g/L, which was the maximum acetic acid concentration observed during typical flask fermentations reported in this study and was above 1 g/L at which *C. thermocellum* inhibition of acetic acid was expected. The concentration of exogenous acetic acid was kept constant at 1.3 g/L in all flasks, whereas, ethanol addition was varied at 0.8 g/L and 17 g/L. Exogenous ethanol concentration of 0.8 g/L was chosen because the same ethanol concentration was produced during *C. thermocellum* fermentations (without exogenous product additions) on 0.5 wt% glucan Avicel. Further, 17 g/L of exogenous ethanol addition was chosen since this concentration was significantly higher than 7 g/L, which was expected to be inhibitory to *C. thermocellum.* There was no significant change in the metabolic profile of the organism in the presence of exogenous ethanol and acetic acid compared to fermentations with an exogenous presence of only acetic acid. Thus, exogenous presence of low and high ethanol concentrations did not aggravate *C. thermocellum* acetic acid inhibition and additive inhibition of ethanol and acetic acid was not observed.

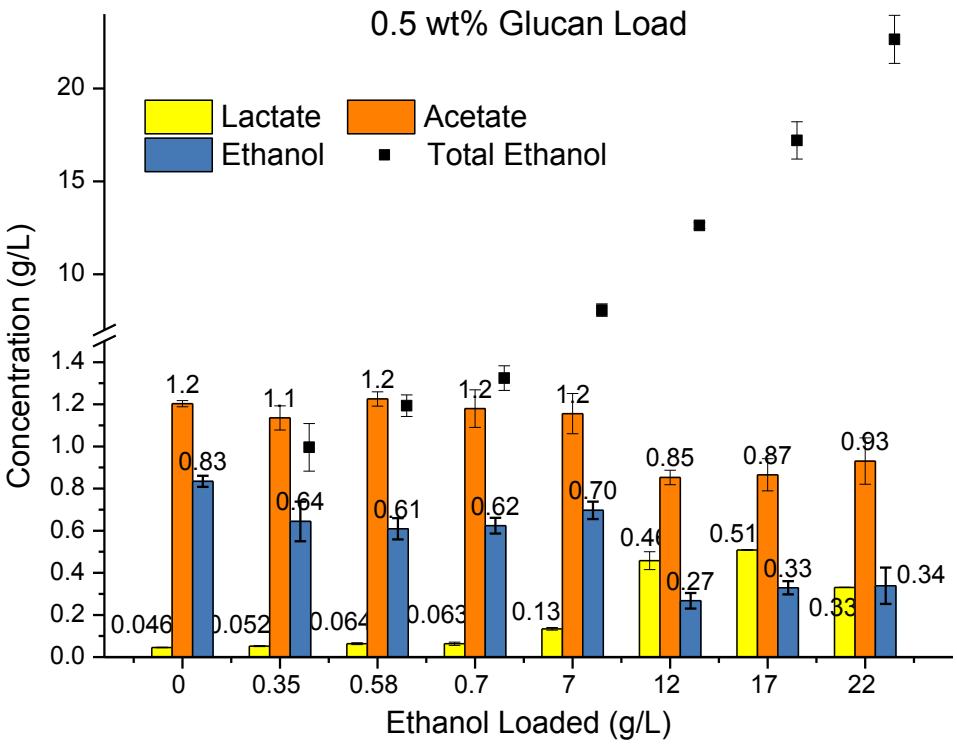

Figure 3.6. Metabolite production by *Clostridium thermocellum* after 7 days of fermentation on Avicel at 0.5 wt% glucan loading with increasing concentrations of ethanol (0-22 g/L) added exogenously before inoculation

Even in the presence of a buffer, a drop in measured pH to about 6.0 from neutral due to the production of acetic acid, increased production of lactic acid, and the presence of other organic acids during fermentation was observed. This drop in pH could have contributed to the ceasing of product formation. Therefore, a pH controlled bioreactor *C. thermocellum* fermentation was performed with a 2.0 wt% glucan loading of Avicel and a 3.5 L working volume for which a time profile of metabolite production by the organism is reported in Figure 3.8. A 2.0 wt% glucan Avicel loading was chosen for the bioreactor fermentation since *C. thermocellum* was inhibited at this substrate loading in flask fermentation.

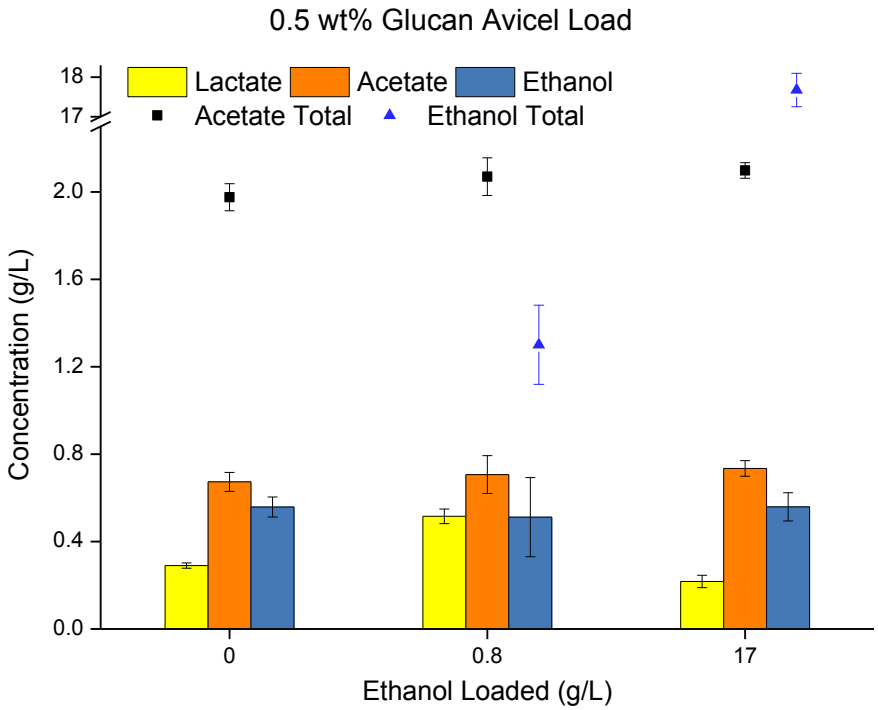

Figure 3.7. Metabolite production by *Clostridium thermocellum* after 7 days of fermentation on Avicel at 0.5 wt% glucan loading with increasing concentrations of ethanol (0-17 g/L) and 1.3 g/L acetic acid added exogenously before inoculation

There was no production of metabolites during the first 10 hours of fermentation and the long lag phase was expected because a 0.3% by volume inoculum was used as opposed to a 2% by volume inoculum size used during flask fermentations. Interestingly, ~3.5 g/L production of ethanol and acetic acid each observed after 4 days of fermentation was much higher than 1.3 g/L acetic acid and 0.9 g/L ethanol production typically observed after 7 days of flask *C. thermocellum* fermentations. A change in pH during *C. thermocellum* fermentations due to the formation of organic acids was determined to be substantially inhibitory to the organism. Interestingly, *C. thermocellum*, as a spore forming organism, is known to be robust and has been reported to sporulate due to low

pH without detaching from the substrate so that the organism can then continue fermentation after ideal conditions resume [37].

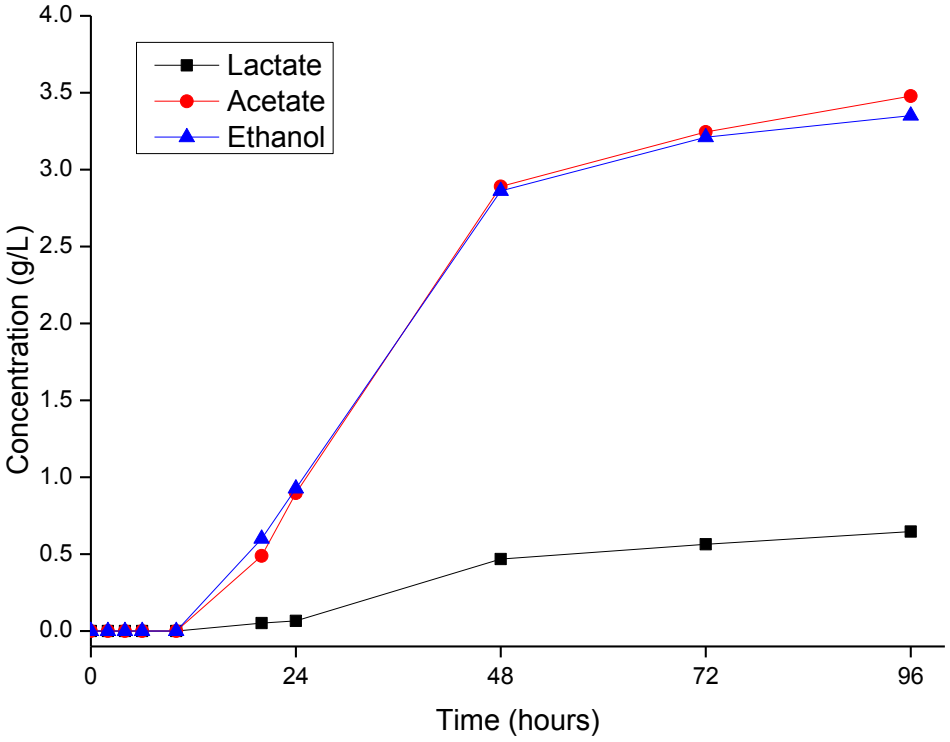

Figure 3.8. Metabolite production time profile during *Clostridium thermocellum* fermentation in a 5 L bioreactor with a 3.5 L working volume, a 2.0 wt% glucan Avicel loading, and active pH control at 7.0 (±0.2) using 2N KOH

3.3.3. Impact of substrate loadings on *C. thermocellum* metabolic *vs.* cellulolytic performance

Inhibitory factors could either affect the organism's metabolic or cellulolytic performance. The solubilization of more sugars and production of more metabolites by *Clostridium phytofermentans* supplemented with external fungal enzymes on 3% glucan loadings of AFEX pretreated corn stover suggested that the cellulolytic abilities of the

organism were inhibited before its metabolic capabilities [13]. This inhibition of *C. phytofermentans* was further attributed to high product concentrations. The inhibition by acetic acid or pH change reported in this work could have a similar effect on *C. thermocellum*. Thus, as shown in Figure 3.9, glucan breakdown was tracked during *C. thermocellum* flask fermentations in order to determine the effect of stressful conditions, induced by increasing cellulose loading, on cellulolytic and metabolic performance of *C. thermocellum*. Undigested glucan was assumed to be the same as total solids left undigested after fermentation since Avicel is known to be highly pure (>97% glucan) [38] and the cell mass was assumed to be negligible compared to the amount of undigested solids. The stoichiometric amount of glucan required for production of each metabolite by *C. thermocellum* is shown in Figure 3.10. Figure 3.9 reveals that the glucan required for production of metabolites by *C. thermocellum* dropped at substrate loadings of >0.5 wt% glucan. About 65% of the initial glucan loaded was attributable to production of metabolites at 0.1-0.5 wt% glucan loadings but dropped to only ~8% with fermentations performed at 5 wt% initial glucan loading. While the rest of the glucan is expected to have been used for cell growth, maintenance, enzyme production, and production of other metabolites [12, 39], a major portion of the glucan surprisingly accumulated as glucose, especially during fermentations performed with 1 and 2 wt% glucan loadings. Glucose accumulation of 9 g/L was observed during fermentations on 2 and 5 wt% glucan Avicel loading. This result indicates that the organism metabolism was inhibited by product formation and pH change long before the organism's cellulolytic abilities were impacted. The sharp increase in glucose accumulation after 0.5 wt% glucan

substrate loading along with the drop in metabolite yield without any change in overall glucan solubilization further supported this conclusion. Finally, the substantial drop in solubilization coupled with low glucose accumulation and low metabolite yield only observed for fermentations of 5 wt% glucan Avicel suggested inhibition of both cellulolytic and metabolic abilities of the organism at this substrate loading.

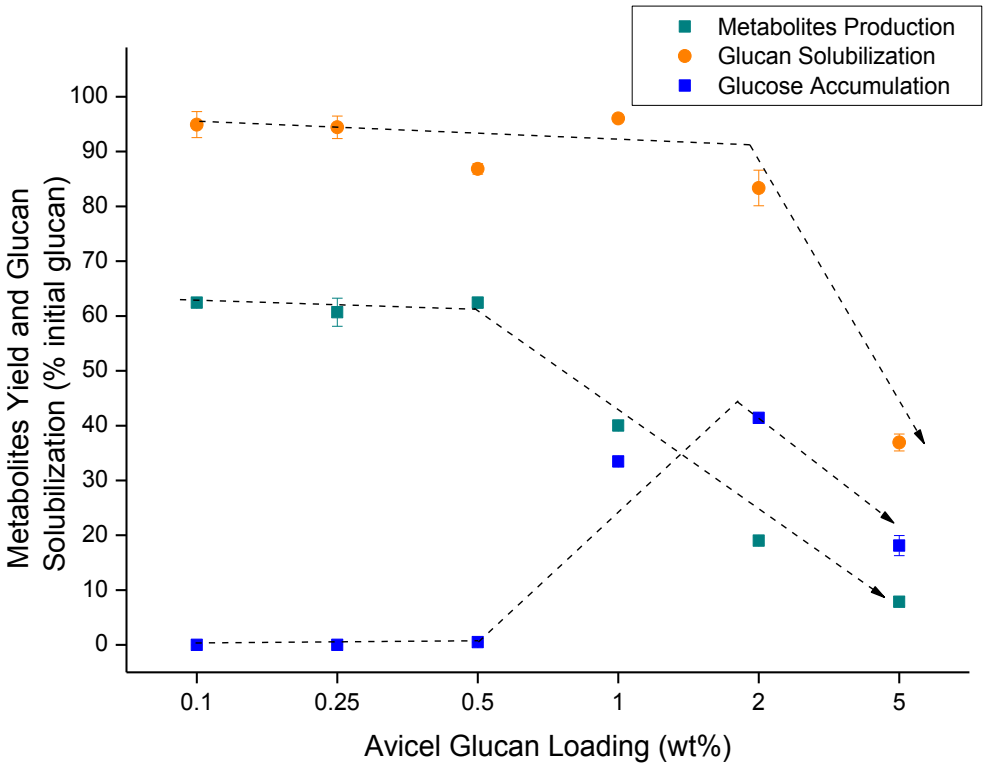

Figure 3.9. Tracking glucan after 7 days of *Clostridium thermocellum* consolidated bioprocessing on Avicel at 0.1-5 wt% glucan loadings

The glucose accumulation of 9 g/L by *C. thermocellum* grown on 2 and 5 wt% glucan Avicel loading is rather surprising because *C. thermocellum* prefers consumption of cellodextrins when grown on cellulose and has further been shown to prefer cellobiose

over glucose when presented with both substrates [40]. In fact, *C. thermocellum* produces intracellular phosphorylases to cleave both cellodextrins and cellobiose that are more beneficial from a bioenergetics perspective for ATP generation [40-44]. Similar glucose accumulation results reported for *C. thermocellum* cultures grown on both cellobiose and cellulose have been attributed to a pH drop in fermentation broth [45]. The pH dropped to 6.0 from neutral after 7 days during a number of flask fermentations reported in this study. In contrast, minimal sugar accumulation has been reported during *C. phytofermentans* fermentations on 2-4% AFEX pretreated corn stover [13].

The original hypothesis that *C. thermocellum* had cell membrane associated phosphorylase for the breakdown and uptake of cellobiose and cellodextrins could explain the accumulation of glucose [45]. However, it was later shown that the phosphorylase activity was, in fact, intracellular, and low extracellular β-glucosidase activities could be the reason for glucose production [44-49]. This mechanism coupled with high availability of cellodextrins that the organism prefers over glucose may explain the accumulation of glucose in the culture during high substrate loading fermentations [31]. However, the low availability of cellodextrins resulting from low substrate loadings of 0.1-0.5 wt% glucan may force *C. thermocellum* to utilize glucose at substrate limiting conditions during fermentations and thus showing negligible glucose accumulation.

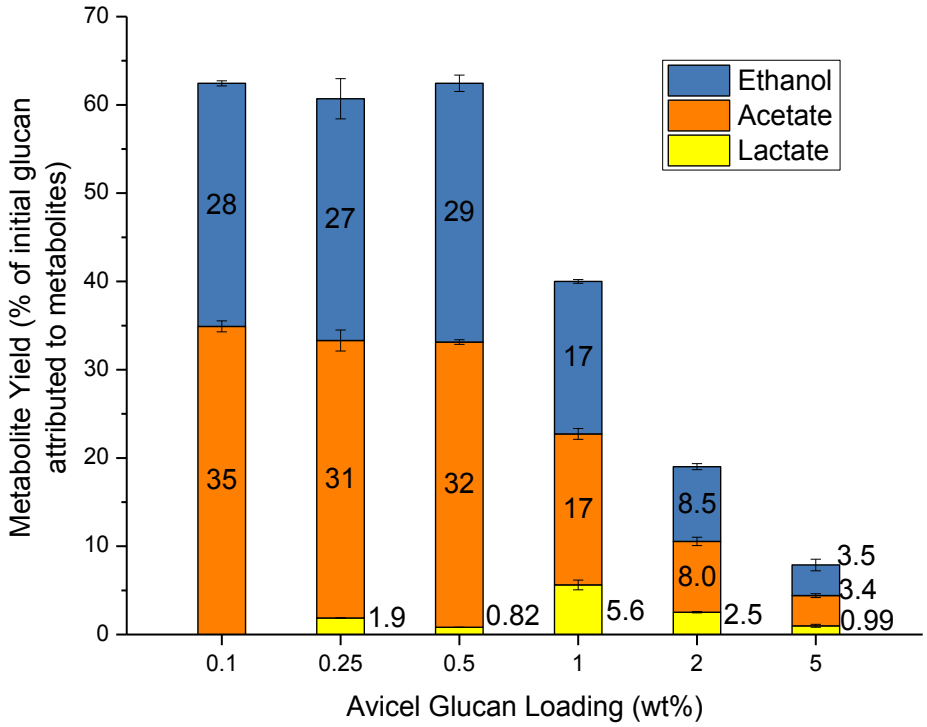

Figure 3.10 *Clostridium thermocellum* metabolite yields after 7 days of fermentation on 0.1-5 wt% glucan loadings of Avicel

3.4. Conclusions:

Clostridium thermocellum consolidated bioprocessing, a simple and efficient bioprocess, is promising for production of low cost fuel ethanol from lignocellulosic biomass. High lignocellulosic biomass substrate loadings are required for the production of ethanol at industrially viable titers [12, 13]. However, owing to the complexity of lignocellulosic biomass, it is vital to first understand the effect of high substrate loadings of model substrates on digestion and metabolite production by *C. thermocellum*. Therefore, this work reports the effect of varying 0.1 to 5 wt% glucan loadings of Avicel® PH-101 on *C. thermocellum* fermentations in order to determine the effect of substrate loading on glucan solubilization and metabolite production. Glucan

solubilization of about >90% was achieved for fermentations at all substrate loadings except the highest substrate loading of 5 wt% glucan, for which only about 37% of the glucan was solubilized by *C. thermocellum*. However, glucan conversion to metabolites dropped at substrate loadings of ≥1 wt% glucan with a simultaneous increase in glucose accumulation. This result suggested a drop in or cessation of metabolism by the organism even though substrate solubilization continued. A further drop in solubilization for 5 wt% glucan loading fermentations suggested ineffective enzymatic activity along with inhibited metabolic activity of the organism. The unexpectedly high glucose accumulation of 9 g/L observed for fermentation of 2 and 5 wt% glucan substrate loadings could result from the low β-glucosidase activity reported for *C. thermocellum*. Glucose accumulation has also been reported to result with a drop in pH during *C. thermocellum* fermentations owing to the formation of carboxylic acids as also observed in this work. High acetic acid inhibition and low ethanol inhibition of *C. thermocellum* along with a pH drop due to production of carboxylic acids contributed to cessation of metabolite production and eventual inhibition of enzymatic activity. *C. thermocellum* tolerated a total of about 2 g/L acetic acid and 22 g/L ethanol including the addition of exogenous acetic acid and ethanol, respectively. However, a maximum of only about 1.3 g/L acetic acid and 1.0 g/L ethanol were typically observed during fermentations on various substrates without the addition of exogenous products. This result suggested that along with product inhibition, the drop in pH contributed substantially to *C. thermocellum* inhibition. The negative impact of pH change on *C. thermocellum* metabolite production was corroborated by production of up to ~3.5 g/L acetic acid and

~3.5 g/L ethanol by *C. thermocellum* in a pH controlled bioreactor. The understanding of the effect of substrate loading on *C. thermocellum* fermentation is essential to differentiating its ability to deconstruct biomass from limitations in organism performance due to other factors. Additional studies are needed to determine the impact of solid loadings on pretreated lignocellulosic deconstruction. Further, a complete carbon and substrate mass balance for the fermentation along with a comprehensive determination of the metabolic profile of the organism, including the formation of hydrogen and carbon dioxide gases, will be helpful in understanding the organism's metabolic performance [12]. The strategy of manipulating metabolic performance of the organism by the addition of inhibitors or by-products to improve product yield is essential in development of *C. thermocellum* fermentation for ethanol production [50]. For example, exogenous addition of hydrogen has been shown to improve ethanol production by *C. thermocellum* [24-27]. Finally, genetic modification of *C. thermocellum* to overcome product and by-product inhibitions at high substrate loadings including elimination of by-product formation is also needed to achieve commercially viable high ethanol yields and titers.

3.5. Materials and methods:

3.5.1. Substrates:

Avicel® PH-101 (11365, Lot No. BCBN7864V), and cellulose acetate (419028, Lot No. MKBJ2957V) are commercially available (Sigma-Aldrich®, St. Louis, MO) and were used as is during fermentations. Avicel is known to be of high purity and was assumed to have 100% glucan for calculations. Cellulose acetate had a glucan content of 60.34%. Ethanol (E1028), acetic acid (A38-212), lactic acid (L6661) used as standards for HPLC analysis were obtained from Spectrum® Chemical Mfg. Corp. (Gardena, CA), Fisher Scientific™ (Fair Lawn, NJ), Sigma-Aldrich® (St. Louis, MO), respectively. Glucose (G8270), also used as an HPLC standard, was obtained from Sigma-Aldrich® (St. Louis, MO).

3.5.2. Bacterial Strain

Wild type *Clostridium thermocellum* DSM 1313 was kindly provided by Professor Lee R. Lynd at Dartmouth College, Hanover, NH. Stock culture was prepared in a 500 mL working volume with a 2% by volume inoculum in Media for Thermophilic Clostridia (MTC) without trace minerals (see Table 3.1) with a 5 g/L glucan Avicel loading. The concentrations of media components are shown in Table 3.1. The stock culture was transferred into 5 mL serum vials (Wheaton, Millville NJ) 8.5 hours after inoculation, stored at -80°C, and used as inoculum for seed cultures to be used in experiments.

Table 3.1. **Media for Thermophilic Clostridia (MTC) for *C. thermocellum* CBP**			
Solution	Component	Reactor concentration	Stock concentration
A	MOPS (buffer)	10 g/L	100 g/L
B	Citric acid potassium salt [$C_6H_5K_3O_7.H_2O$]	2 g/L	50 g/L
	Citric acid monohydrate	1.25 g/L	31.25 g/L
	Na_2SO_4	1 g/L	25 g/L
	KH_2PO_4	1 g/L	25 g/L
	$NaHCO_3$	2.5 g/L	62.5 g/L
C	NH_4Cl	1.5 g/L	75 g/L
D	$MgCl_2.6H_2O$	1 g/L	50 g/L
	$CaCl_2.2H_2O$	0.2 g/L	10 g/L
	$FeCl_2.4H_2O$	0.2 g/L	5 g/L
	L-cysteine hydrochloride monohydrate	1 g/L	50 g/L
E	Pyridoxamine dihydrochloride	0.02 g/L	1 g/L
	P-aminobenzoic acid	0.004 g/L	0.2 g/L
	D-biotin	0.002 g/L	0.1 g/L
	Vitamin B12	0.002 g/L	0.1 g/L

MOPS: 3-(*N*-morpholino)propanesulfonic acid (pK_a 7.20)

3.5.3. *Clostridium thermocellum* consolidated bioprocessing:

A 2% by volume inoculum of the stock cultures was used to prepare the seed cultures grown with a 5 g/L glucan loading of Avicel® PH-101 (Sigma Aldrich, St. Louis, MO) in a 50 mL working volume for 8-9 hours in MTC without trace minerals. The pellet nitrogen content and metabolites production by *C. thermocellum* grown in this manner in a working volume of 200 mL is shown in Figure 3.11. Pellet nitrogen content was analyzed according to methods published in literature [12, 51]. The seed culture was stored overnight in a refrigerator for about the same time for each experiment before inoculation the next day. All media solutions were purged and then sterilized separately in the autoclave except the vitamins solution which was passed through polyethersulfone (PES) syringe filters with 0.2 μm pores and 28 mm diameter (Corning® Life Sciences, Tewksbury MA) for sterilization. The separate media solutions were injected into nitrogen purged and autoclaved fermentations bottles with the appropriate amount of substrate and water added prior to purging. 45 seconds cycles of nitrogen at 14 psi and vacuum were performed for 27-30 minutes to purge all bottles and the autoclave was run at 121°C for 35 min for sterilization. Fermentations were carried out in a Multitron Orbital Shaker (Infors HT, Laurel MD) set at 60°C and 180 rpm after injecting a 2% v/v inoculum from the seed culture. The bottles were opened after 7 days of fermentation and liquid samples were collected for analysis in the HPLC to measure metabolites and glucose concentration. These samples were first filtered through 28 mm diameter polyethersulfone (PES) syringe filters with 0.2 μm pores (Corning® Life Sciences, Tewksbury, MA) and collected in 1.5 mL Simport® microcentrifuge tubes (Spectrum®

Chemical Manufacturing Corporation, New Brunswick, NJ). These were then centrifuged at 15,000 rpm for 10 min, the supernatant was collected and analyzed in the HPLC. All solid residues from the bottles were collected, rinsed thoroughly, and dried in a 70°C oven overnight to determine solids/glucan solubilization.

A 5 L Sartorius Biostat® A-plus (Sartorium Stedim, Bohemia, NY) bioreactor was used with a 3.5 L working volume for pH controlled *C. thermocellum* fermentations. Hamilton® (Reno, NV) probes were used to measure dissolved oxygen and pH of the fermentation broth and the bioreactor was maintained at 60°C with a heating jacket. The contents of the reactor were constantly mixed with a stirring speed of 300 rpm. A 2.0 wt% glucan loading of Avicel was used for the fermentation with a 0.3% by volume inoculum. The Avicel suspension in water was autoclaved for sterilization. A sparger was used to bubble nitrogen through the contents of the reactor to maintain anaerobic conditions before inoculation and for control of dissolved oxygen at 0% during fermentation. Sterilized MTC components and the inoculum were added separately while the bioreactor was being purged with nitrogen. Nitrogen gas and gases produced during fermentation were allowed to pass through a filter and the filter outlet was attached to a tube immersed in water. 2N potassium hydroxide was used for active pH control of the fermentation broth.

3.5.4. Analytical Procedures:

Waters® Alliance e2695 HPLC system (Waters Co., Milford MA) equipped with a Waters 2414 refractive index detector and a Bio-Rad Aminex HPX-87H column was used for analysis of liquid samples. The 5 mM sulfuric acid was used as the eluent at 0.6 mL/min. Empower™ 2 software package was used to integrate the HPLC chromatograms.

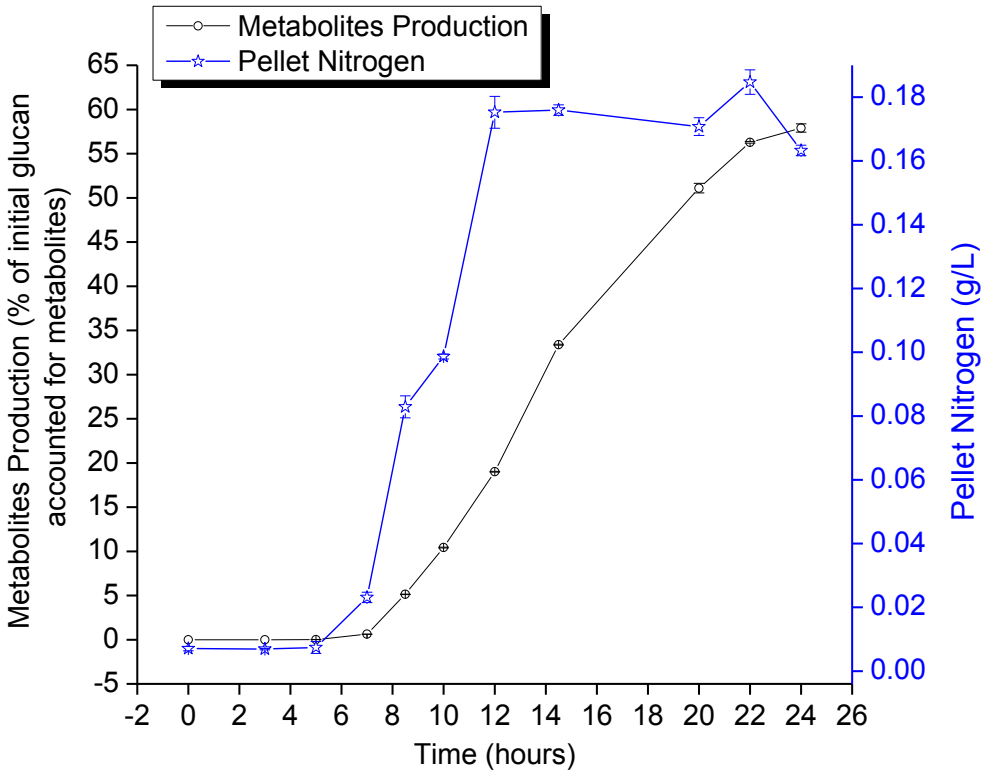

Figure 3.11 *C. thermocellum* growth profile in terms production of metabolites (ethanol + acetic acid + lactic acid) and pellet nitrogen content (g/L) as a proxy for cell growth for 24 hours under growth conditions in a bottle with 200 mL working volume and 5 g/L glucan Avicel® PH-101 loading without active pH control in MOPS buffer using a 2% (v/v) inoculum size

3.5.5. Calculations:

All experiments were performed in triplicates (except the pH controlled bioreactor fermentation which was performed once) and error bars on plots represent the standard deviation in the replicates. Anhydrous correction factor (= 0.9) was used to convert glucose concentrations measured in the HPLC to glucan in order to represent all yields in terms of the polymeric form of the sugar. Metabolite production was represented as the glucan required to produce each metabolite calculated through stoichiometry of the glucose to metabolite reactions. Glucose was converted to glucan using the anhydrous correction factor. Solids solubilization was calculated as the percentage loss of solids after fermentations. Avicel® PH-101 is of high purity and was assumed to be 100% cellulose/glucan which allowed for the use of solids solubilization and glucan solubilization interchangeably. All yields are based on initial glucan loaded before fermentation.

1. CBP % solids (glucan) solubilization =

$$\frac{(Mass\ of\ Dry\ Solids\ before\ CBP\ (g) - Mass\ of\ Dry\ Solids\ After\ CBP\ (g))}{Mass\ of\ Dry\ Solids\ before\ CBP\ (g)} \times 100$$

2. CBP metabolites production (g metabolites starting with 100 g glucan) =

$$\frac{(Metabolites\ Concentration\ from\ HPLC\ (g/L) \times Working\ Volume\ (L))}{Mass\ of\ Initial\ Glucan\ Loaded\ for\ CBP\ (g)} \times 100$$

3. CBP metabolites yield (% of initial glucan accounted for metabolites)

$$\frac{CBP\ Metabolites\ Production\ (g\ Metabolite\ Starting\ with\ 100\ Glucan) \times Anhydrous\ Factor}{Stoichometric\ Factor \times Molar\ Mass\ Ratio}$$

Anhydrous Factor for glucose = 0.9

4. Stoichiometric Factor (for a balanced glucose to metabolite reaction) =

$$\frac{Stoichiometric\ Coefficient\ of\ Metabolite}{Stiochiometric\ Coefficient\ of\ Glucose}$$

5. Molar Mass Ratio =

$$\frac{Molar\ Mass\ of\ Metabolite}{Molar\ Mass\ of\ Glucose}$$

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Chapter 4. Impact of cellulose properties on *Clostridium thermocellum* and fungal enzymatic saccharification*

The contents of this chapter will be used for publication in a scientific journal in part or in full

*This chapter was completed in collaboration with the University of California Riverside[1], National Renewable Energy Laboratoy[2], University of Tennessee Knoxville[3], and Oak Ridge National Laboratory[4]. *C. thermocellum* fermentations and fungal enzymatic hydrolysis experimentation and experimental design, planning, and management of project was done by Ninad Kothari[1]. Simons' staining, water retention value, scanning electron microscopy, and gel permeation chromatography were performed by Samarthya Bhagia[3], Chang Geun Yoo[3,4], and John R. Dunlap[3]. Solid state nuclear magnetic resonance was performed by Yunqiao Pu[3,4], and X-ray diffraction was performed by Ashutosh Mittal[2]

4.1. Abstract

The recalcitrance of lignocellulosic biomass hinders effective deconstruction of its complex cell wall structure to make cellulose available for enzymatic saccharification and fermentation to fuel ethanol. Further, once cellulose is physically available for biological solubilization, the properties of cellulose itself affect its digestion. Here, we study the impact of distinct properties of five model cellulosic substrates on substrate solubilization using two biological approaches. Commercially available cellulosic substrates, Avicel® PH-101, Sigmacell Cellulose Type 50, cotton linter, Whatman™ 1 filter paper (milled), and α-cellulose were chosen to represent a variety in cellulose properties. Cellulose crystallinity and crystallite sizes using X-ray diffraction and solid state nuclear magnetic resonance, surface area measured as total dye adsorption and pore size distribution measured as orange/blue dye adsorption ratio via Simon's staining method, water retention value (WRV), cellulose degree of polymerization (DP) using gel permeation chromatography, and scanning electron microscopy were performed to determine characteristic differences in the materials. *C. thermocellum* was overall unaffected by varying cellulose properties and showed similar solids solubilization and metabolite production on different materials. However, fungal enzymes showed large differences in solubilization performance of various substrates with highest glucan yield observed on filter paper followed by Avicel, Sigmacell and α-cellulose, and cotton linter. Fungal enzymatic digestion is driven by effective enzyme adsorption on cellulose influenced by cellulose surface area, pore size, and crystallinity. Further, the impact of substrate WRV on fungal enzymatic hydrolysis was confirmed by the difference in the

extent of digestion of dried Avicel that had lower WRV compared to Avicel. Fungal enzymes are proposed to be able to digest cellulose easily after effective enzyme adsorption on the substrate since cellulose DP did not impact the extent of digestion. *C. thermocellum* digestion of lignocellulosic biomass is driven mostly by physical availability of cellulose in the lignocellulosic matrix and is largely unaffected by cellulose properties once cellulose is made macro-accessible. In contrast, fungal enzymes require cellulose to be physically accessible as well as have properties amenable to digestion. Cellulose surface area, pore size distribution, and crystallinity, influencing enzyme adsorption, substantially impacted fungal enzymatic digestion.

4.2. Introduction

Lignocellulosic biomass is structurally complex with much of the long cellulose chains held together as crystalline fibers by hydrogen bonds which are in turn glued together by hemicellulose, a polymer of several sugars, and lignin, a phenolic polymer [1-3]. This complex architecture contributes to biomass recalcitrance to sugar release, which presents the primary barrier to competitive conversion of this low cost resource to transportation fuels. Furthermore, recalcitrance changes with plant type, further complicating biomass use [4]. Biomass augmentation is essential through either physical/chemical pretreatment or cotreatment of biomass to aid further solubilization by biological systems [5-9]. Traditionally used in ethanol production, enzymatic hydrolysis of cellulose is a heterogeneous reaction in which enzymes derived from *Trichoderma reesei* are used in solution to breakdown insoluble cellulosic substrates [10-13].

However, the high production cost and dosage of enzymes required to achieve industrially relevant sugar yields make them economically uncompetitive [14]. Consolidated bioprocessing, in contrast, is a simple and effective bioprocess that combines enzyme production, enzymatic hydrolysis, and fermentation by using organisms such as *Clostridium thermocellum* to perform all three operations in one step [15-19]. *C. thermocellum* produces a complex, multi-enzyme, multi-functional cellulosome that enhances biomass solubilization compared to the use of fungal enzymes [20, 21].

Accessibility of cellulose to solubilization can be divided into two types: macro- and micro-accessibility [22]. Macro-accessibility refers to the physical availability of cellulose influenced by the presence of lignin, hemicellulose, and other physical barriers in lignocellulosic biomass. Pretreatment of biomass may increase this physical access of cellulose to cellulolytic enzymes by deconstructing the complex plant cell wall structure. However, once the enzymes have gained physical access to cellulose the properties of cellulose itself, such as crystallinity, degree of polymerization, etc. may affect its hydrolysis by enzymes [11, 22-25]. These cellulose properties influence availability of cellulase binding sites in the polysaccharide, thus affecting cellulose micro-accessibility to enzymes [22]. Even though a number of studies have shown that solubilization by fungal enzymes is affected by cellulose micro-accessibility, these studies fail to systematically study the influence of a variety of cellulose properties on fungal enzymatic digestion and there is no consensus on the extent to which the cellulose properties affect

enzymatic digestion. Further, the effect of cellulose micro-accessibility on substrate solubilization and metabolite production by *C. thermocellum* has not been reported previously.

Cellulose biosynthesis influences the properties of cellulose that in turn affects cellulose micro-accessibility and digestibility of the substrate. Cellulose is a polymer of glucose linked via β-(1,4) glycosidic bonds that form at or outside the plasma membrane of plant cells [26]. Cellulose microfibrils containing multiple chains are packed together to form fibrils, which in turn are packed together to form a cellulose fiber. The presence of other plant cell wall polymers during the synthesis of microfibrils by the cellulose synthase complexes in higher plants leads to a variation in the number of chains present in the microfibril [27-29]. These cellulose chains are so tightly packed together that even water molecules would be unable to penetrate leading to ordered structures that are highly recalcitrant to hydrolysis [10]. Overall, accessible surface area, specific surface area and pore size of the substrate are expected to affect its hydrolysis [4, 11, 30]. Amorphogenesis, characterized by dispersion or swelling of cellulose leading to a decrease in compactness of the cellulose structure and/or cellulose crystallinity, has been proposed to occur in the initial stages of hydrolysis [10]. This further increases the available surface area for enzyme adsorption, thus, increasing cellulose micro-accessibility.

Early in evolutionary history, cellulose biosynthesis centered only on polymerization leading to the formation of the more stable cellulose II, which has a lower degree of polymerization (DP) than what is today known as native cellulose or cellulose I [31]. However, cell elongation and growth would be limited with the lower DP of cellulose II. Therefore, the evolutionary selection process led to the advent of chain ordering and ultimately the formation of microfibrils and native cellulose I with higher DP and increased functionality of the cell wall in the overall growth of the plants [11, 22, 23, 31]. Cellulose DP is known to affect digestion by fungal enzymes with lower DP of the substrate preferred for greater solubilization [32]. Cellulose II and III allomorphs are more susceptible to digestion by fungal enzymes than the native cellulose I [24, 33]. Each cellulose microfibril consists of ordered crystalline and disordered amorphous regions that coexist in a cross section rather than alternating along the axis of the microfibril [22, 27]. Crystallization during cellulose biosynthesis occurs while the cellulose units are arranged into a microfibril by a protein in the cellulose synthase complex and therefore directly related to hydrogen bond formation [26]. Cellulose hydrolysis by fungal enzymes has been shown to be negatively affected by substrates with high cellulose crystallinity [13, 34, 35]. Further, moisture uptake by cellulose is expected to increase with decrease in crystallinity [22] and therefore, both cellulose water retention value (WRV) and crystallinity are useful indicators of cellulose micro-accessibility [4, 13, 22]. Overall, however, there is still a debate on whether cellulose crystallinity and DP are important parameters to consider during cellulose digestion [11, 23, 25].

Here, we report the digestion performance of fungal enzymes compared to *C. thermocellum* on five model cellulosic substrates, Avicel® PH-101 (Avicel), Sigmacell Cellulose Type 50 (Sigmacell), Whatman™ 1 filter paper (filter paper; milled through 40 mesh), cotton linter, and α-cellulose. Model substrates were chosen for this study to avoid the negative effect of limited cellulose macro-accessibility, observed in lignocellulosic biomass, on cellulose digestion. These substrates were chosen to represent a wide range of commercially available cellulosic substrates. A sixth substrate was prepared by soaking Avicel in water at 30% solids loading and then drying the suspension overnight in a 105°C oven. An understanding of the differences in digestion performance of the two biological systems on these substrates is expected to help identify critical cellulose properties that influence cellulose digestion.

4.3. Results and discussion:

4.3.1. Substrate characterization

A suite of analytical techniques was applied to Avicel, Sigmacell, filter paper, cotton linter, and α-cellulose to determine cellulose crystallinity index (CrI) and crystallite size, cellulose number average and weight average degree of polymerization (DPn and DPw), cellulose surface area measured as total dye adsorption and pore size distribution measured as orange to blue dye adsorption ratio via Simons' staining, and cellulose water retention value (WRV). Scanning electron microscope (SEM) images were also taken to determine structural differences between the different cellulosic substrates. Glucan solubilization by both fungal enzymes and *C. thermocellum* was then

measured on these substrates to understand the effect of different characteristics of each cellulosic substrate on biological digestion.

Water retention value (WRV) is measured as water retained by a substrate after centrifugation under normal conditions and is a measurement of mass of water associated with the biomass itself and between biomass particles [36, 37]. WRV could indicate the surface area of cellulose since water would form more hydrogen bonds with more accessible hydroxyl groups on cellulose. This ability of the biomass to retain water has been directly correlated to its digestibility by enzymes [37-39]. Further, an increase in interaction between biomass and water has also been reported with increased biomass digestibility [37].

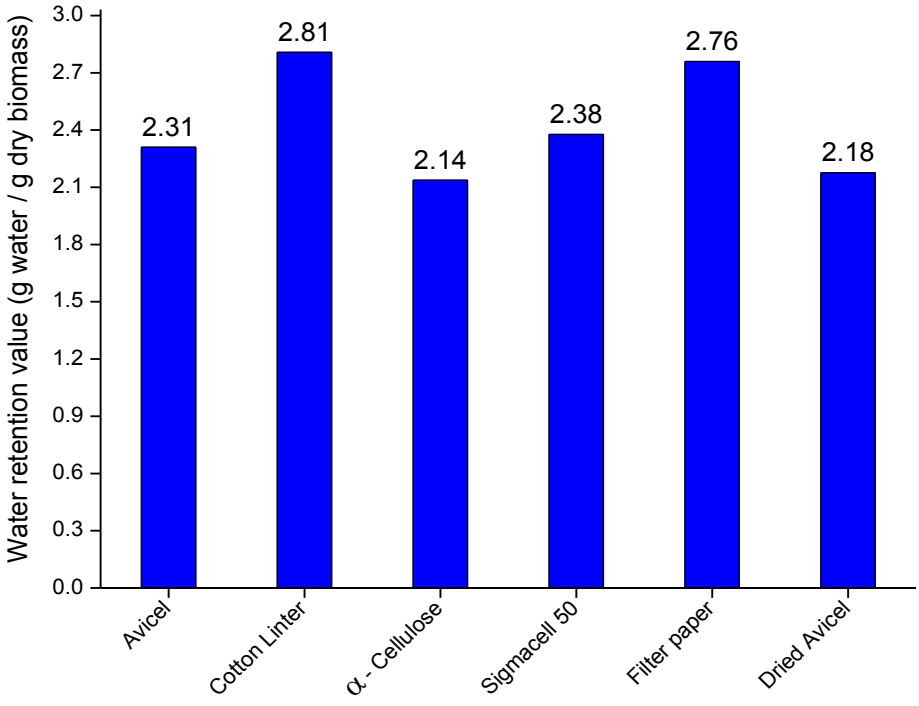

Figure 4.1. Water retention values for Avicel, Sigmacell 50, cotton linter, filter paper, and α-cellulose

Here, cotton linter and filter paper showed the highest WRV as reported in Figure 4.1, indicating that these materials have higher swellability and potentially higher cellulose surface area compared to other materials, which is expected to aid enzyme adsorption and further enzymatic digestion. The elongated and fibrous nature of cotton linter and filter paper particles seen in SEM images in Figure 4.2 could contribute to the high water retaining ability of these materials. Particles of α-cellulose showed a similar elongated shape but were less fibrous which could explain the lower WRV of this material compared to filter paper and cotton linter. Dried Avicel, which was prepared by rapidly drying a 30 wt% solids suspension of Avicel in a 105ºC oven, showed lower WRV compared to that for Avicel. The rapid drying of the material causes pore collapse and case hardening, possibly irreversible, leading to a lower ability of the material to adsorb or retain water [40, 41]. A direct comparison of fungal enzymes and *C. thermocellum* digestion performance on Avicel and dried Avicel will indicate the significance of substrate WRV in cellulose digestion [37].

Simon's staining method has been used to determine substrate specific surface area indicative of its accessibility and can be used to predict cellulose solubilization performance of enzymes on different substrates [4, 30, 42]. Dyes used in the Simons' staining method adsorb only to cellulose, as opposed to other polymers in the plant cell wall structure, and have a similar size profile to cellulases [43]. Specifically, the high molecular weight fraction of Direct Orange 15 has a high affinity to cellulose and binds to pores of size 5-36 nm [44].

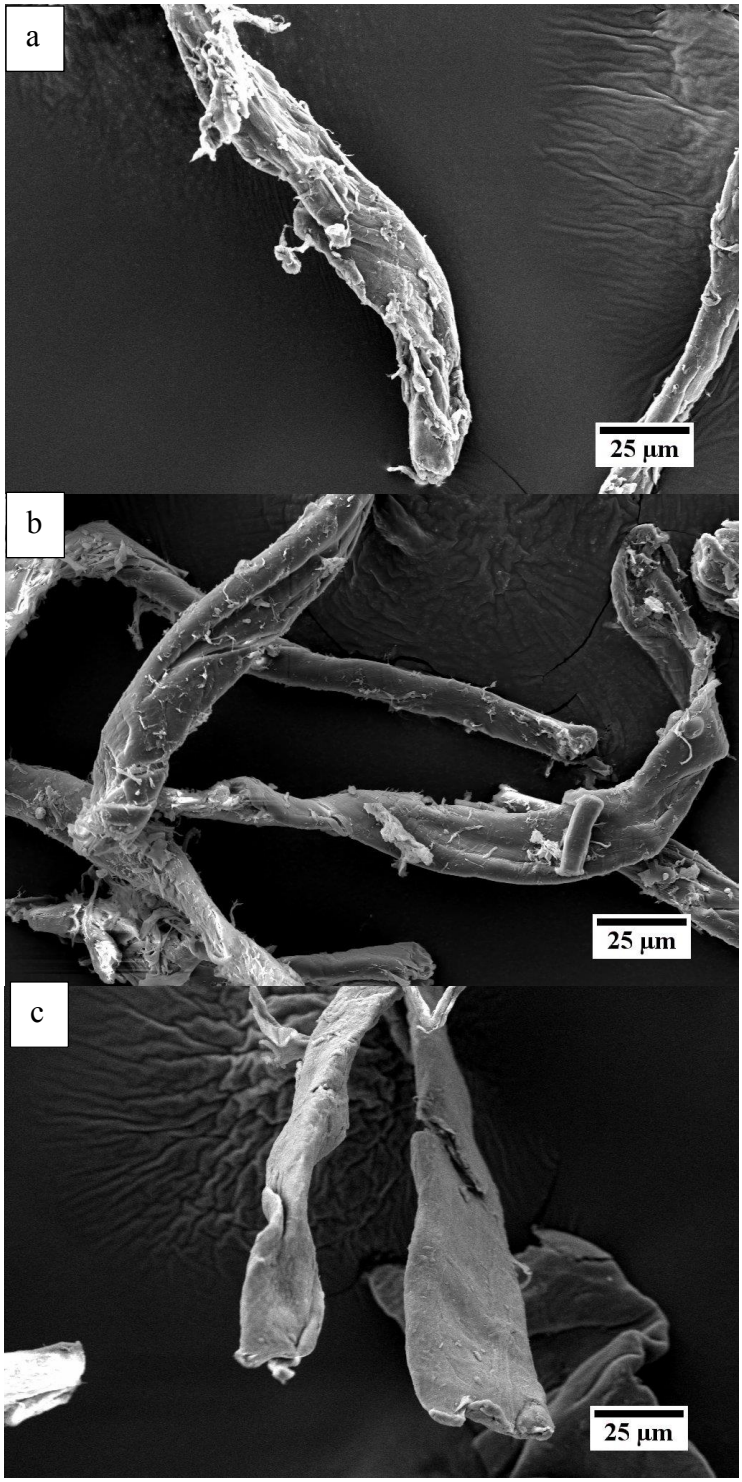

Figure 4.2. Scanning electron microscope (SEM) images of (a) filter paper, (b) cotton linter, and (c) α-cellulose at a 1.5K times magnification

Direct Blue 1 on the other hand binds to smaller pores of about 1 nm but has lower binding affinity than the orange dye. Ratio of the amounts of orange to blue dyes adsorbed by a substrate thus indicates the pore size distribution of the substrate with higher values indicating larger pores [4, 44]. Here, a modified Simon's staining method was used to determine cellulose accessibility and pore size distribution by measuring total orange plus blue dye adsorption and orange to blue adsorption (O/B) ratio, respectively, for all materials as shown in Figure 4.3. Maximum dye adsorption was measured by loading biomass with a range of dye concentrations and obtaining an adsorption curve for each substrate. The trend for maximum orange plus blue dye adsorption for the model substrates was found to be as follows: filter paper > α-cellulose > Avicel > cotton linter > Sigmacell. Even though filter paper had a low O/B ratio this substrate showed very high WRV and total dye adsorption indicative of its high cellulose surface area compared to those for the rest of the materials. Thus filter paper is expected to have higher cellulose accessibility leading to higher enzyme adsorption and therefore, higher digestibility compared to those for the other materials. α-cellulose also showed high total dye adsorption but showed the lowest WRV as well as lowest O/B ratio which may negatively affect biological digestion of this substrate. Interestingly, Avicel showed the highest O/B ratio of 1.26 (opposed to 0.9-0.95 for other materials), as seen in Figure 4.3(b), indicating the presence of larger pores in the substrate and therefore, greater cellulose accessibility. Cotton linter and Sigmacell both showed low total dye adsorption and low O/B ratio and therefore, are expected to show low digestibility.

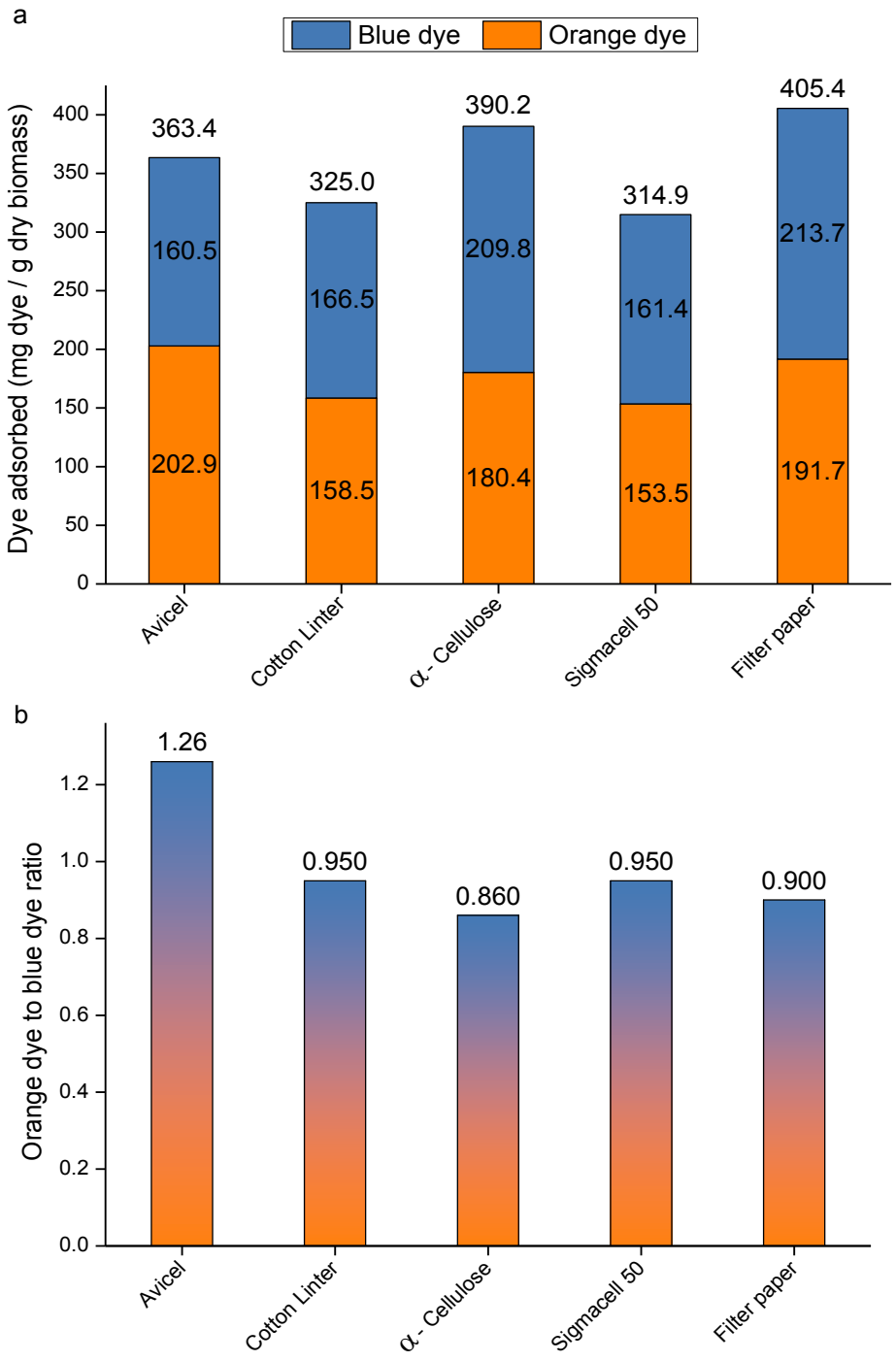

Figure 4.3. Cellulose accessibility measured via Simons' staining **(a)** total orange dye plus blue dye adsorption and **(b)** orange dye to blue dye adsorption ratio for Avicel, Sigmacell 50, cotton linter, filter paper, and α-cellulose based on maximum dye adsorption determined by adsorption isotherms with a range of dye concentration

Solid state nuclear magnetic resonance (SSNMR) and X-ray diffraction (XRD) techniques were employed to determine crystallinity of cellulose from Avicel, Sigmacell, filter paper, cotton linter, and α-cellulose and the crystallinity indices (CrI) for all materials are reported in Table 4.1. Both techniques showed the same trend for CrI of all materials. Cotton linter showed the highest CrI which is expected to affect further biological digestion negatively. α-cellulose, in contrast, had the lowest CrI and therefore, is expected to be highly digestible by both fungal enzymes and *C. thermocellum*. Similar CrI values have been reported for some of these substrates elsewhere [45]. The effect of substrates with different crystallinities on digestion performance has not been studied extensively for *C. thermocellum*. In contrast a direct relation between cellulose crystallinity and the rate of hydrolysis of cellulose by fungal enzymes has been consistently reported [46, 47]. Cellulases preferentially attack amorphous regions over crystalline regions of cellulose leading to an increase in crystallinity in the initial stages of hydrolysis [34]. *Trichoderma reseei* Cel7A enzyme is negatively affected by increasing cellulose crystallinity [35]. Further, the effectiveness of enzymes adsorbed onto the surface of cellulose is shown to depend on initial cellulose crystallinity [13]. However, effect of cellulose crystallinity on enzymatic digestion of cellulose has been contentious in literature with some reports showing an insignificant effect of cellulose crystallinity on cellulose digestion [12, 23]. Further, crystallite size of the cellulose structure for each material type influences its crystallinity and surface area. A larger crystal means reduced surface area which would decrease enzyme and water adsorption [48]. In this study however, even though both cotton linter and filter paper had higher

crystallite sizes both materials also showed higher WRV compared to the other materials. Further, an increase in crystallinity index has been observed with an increase in crystallite size of cellulose from different wood species. This is because, a larger crystallite size would mean diminished amount of surface corresponding to amorphous cellulose regions [49, 50]. This was shown to be generally true in this study with cotton linter showing the highest crystallite size and crystallinity index, whereas, α-cellulose showed the lowest values for both crystallinity index and crystallite size.

Material	Crystallinity Index		Crystallite Size (nm)	Molecular Weight		
	CrI (%) SSNMR	CrI (%) XRD		DPw	DPn	PDI
Avicel	59.4	71.3	34.3	309	71	4.35
Cotton Linter	70.9	76.0	45.1	1578	111	14.26
α-Cellulose	45.8	54.0	20.5	4389	393	11.17
Sigmacel 50	57.8	71.4	33.4	321	68	4.74
Filter Paper	62.4	68.8	40.5	4266	1008	4.23
Dried Avicel	ND	70.7	32.3	ND	ND	ND

Table 4.1. Cellulose crystallinity measured using solid state nuclear magnetic resonance (SSNMR) and X-ray diffraction (XRD) peak height techniques, crystallite size using XRD, and weight average (DPw) and number average (DPn) degree of polymerization and polydispersity index (PDI) measured using gel permeation chromatography for model cellulosic substrates. Red color indicates the most negative impact and green indicates the most positive impact of the property under consideration expected on biological digestion of cellulose. ND = Not Determined

Cellulose from filter paper and α-cellulose showed high weight average degree of polymerization (DPw) but cellulose from α-cellulose had a much lower number average degree of polymerization (DPn) and thus showed a higher polydispersity index (DPw/DPn; PDI) as reported in Table 4.1 indicating a greater molecular weight

distribution [12, 51]. Cotton linter comparatively had a lower cellulose DPn and DPw and yet much higher than those for Avicel and Sigamcell. Cotton linter had the highest PDI and therefore showed the most molecular weight distribution. Cellulose DP has long since been considered an important characteristic with a possible significant impact on cellulose digestion [22]. Higher DP would mean longer cellulose chains packed together with strong hydrogen bonds resulting in lower cellulose accessibility and digestibility [23]. High DP has been reported to have a negative impact on fungal enzymatic digestion of cellulose [52, 53]. However, a levelling off in cellulose DP after a slight decrease has been observed with insignificant overall change in DP before and after hydrolysis [11]. The effect of DP is not very well understood with there being no consensus on the extent of the impact of DP on cellulose digestion [22].

4.3.2. Fungal enzymatic hydrolysis of substrates with varying cellulose properties

Cellulosic substrates with significantly different micro-accessibility and surface characteristics were hydrolyzed using a 15 mg protein / g glucan loading of fungal enzymes and a 0.5 wt% glucan loading of each substrate with a working mass of 50 g. Substantial differences were observed in the extent of digestion achieved by fungal enzymes on Avicel, Sigmacell, filter paper, cotton linter, and α-cellulose as shown in Figure 4.4. Fungal enzymes achieved the highest glucan yield of about 90% on filter paper followed by Avicel, Sigmacell and α-cellulose, and the lowest glucan yield was achieved on cotton linter of only about 30%. High WRV (2.76 mg/g dry biomass) and total dye adsorption via Simons' staining (405.40 mg/g dry biomass) for filter paper

compared to Avicel that showed a WRV of 2.31 mg/g dry biomass and total dye adsorption of 363.40 mg/g dry biomass led to the higher digestibility of the former by fungal enzymes. This high overall cellulose surface area is essential for effective fungal enzymatic digestion of a substrate. The higher DP for filter paper compared to Avicel did not seem to affect fungal enzymatic digestion negatively suggesting a lower impact of DP on enzymatic hydrolysis than cellulose surface area.

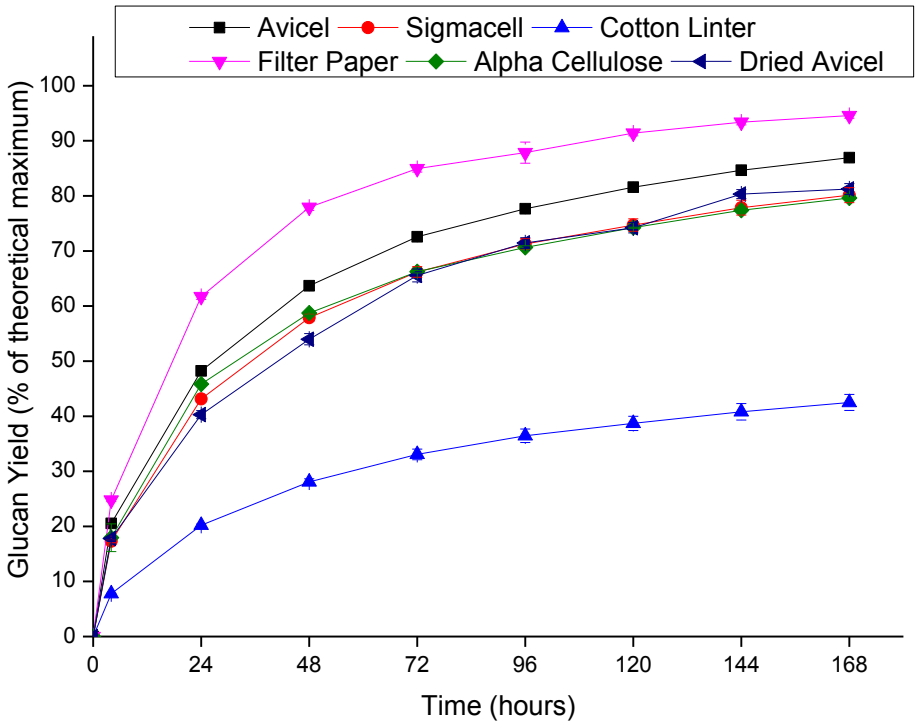

Figure 4.4. Fungal enzymatic hydrolysis glucan yield time profile on Avicel, Sigmacell 50, cotton linter, filter paper, and α-cellulose with a 0.5 wt% glucan substrate loading

Further, fungal enzymes were able to digest Avicel more effectively than Sigmacell and the only difference between Avicel and Sigmacell was in their orange dye adsorption while the blue dye adsorption was the same for both substrates. Avicel

therefore showed a much higher O/B ratio of 1.26 compared to 0.95 for Sigmacell. The larger pore sizes in Avicel were further confirmed in SEM images as shown in Figure 4.5. The Avicel particle shown in Figure 4.5(a) clearly showed more pores that were larger in size than that in the Sigmacell particle shown in Figure 4.5(b). Thus larger pore size of a substrate is essential for effective enzymatic digestion.

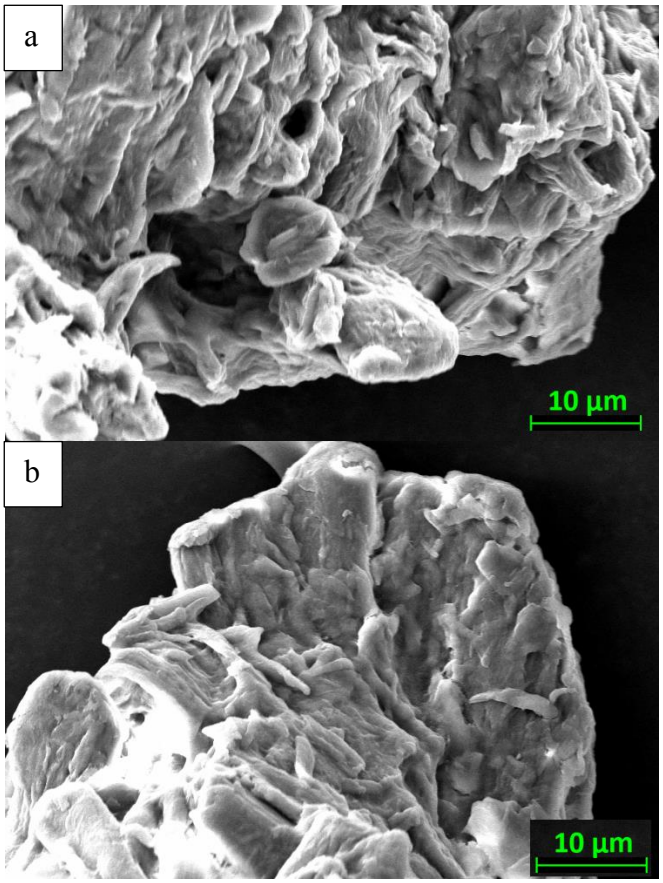

Figure 4.5. Scanning electron microscope (SEM) images of (a) Avicel and (b) Sigmacell 50 at a 5.0K times magnification

The very low CrI of 45.8% (SSNMR) and 54.0% (XRD Peak Height) measured for α-cellulose, reported in Table 4.1, contributed to its high digestibility by fungal

enzymes albeit ~7% lower than that on Avicel. Even though α-cellulose had slightly higher total dye adsorption compared to Avicel, the former also showed the lowest O/B ratio of 0.86 compared to Avicel with an O/B ratio of 1.26 again indicating the importance of larger pores in cellulose for effective fungal enzymatic digestion. α-cellulose also showed the lowest WRV of 2.14 compared to Avicel with a WRV of 2.31 mg/g dry biomass possibly contributing to the lower digestibility of the former. Further, higher cellulose DP of α-cellulose compared to Avicel may have also negatively impacted enzymatic digestion of α-cellulose. However, similar to Avicel, Sigmacell showed a higher cellulose DP than that for α-cellulose but, unlike Avicel, showed the same extent of digestion as α-cellulose. Therefore, cellulose DP did not seem to influence enzymatic digestion substantially. Overall, Sigmacell had lower total dye adsorption and higher CrI that with a negative impact on effective cellulose digestion but also had higher WRV, larger pores, and lower cellulose DP with a positive impact on cellulose digestion compared to α-cellulose. Therefore, no parameter had a more substantial impact on fungal enzymatic digestibility than other parameters for the two substrates and therefore, both Sigmacell and α-cellulose showed similar fungal enzymatic digestibility.

Cotton linter was the least amenable to fungal enzymatic digestion due to its low cellulose surface area and the presence of small pores measured via Simons' staining methods reported in Figure 4.3 and its high crystallinity reported in Table 4.1. In fact, cotton linter had the highest CrI compared to all the other materials. Even though Sigmacell had similar surface area and pore size distribution measured via Simons'

staining compared to cotton linter, the former showed much higher digestibility because lower cellulose crystallinity made Sigmacell more amenable to digestion by fungal enzymes. It is interesting to note that cotton linter showed very low digestibility even though the material had a very high WRV similar to that of filter paper indicating the importance a low crystallinity requirement of fungal enzymes for effective digestion. Typically, WRV is unable to distinguish between water retained within the biomass and the water between biomass particles [37]. Therefore, even though cotton linter showed a high WRV it is possible that the water was unable to penetrate deep in the cellulose structure, which could explain limited access of cellulose in cotton linter to enzymatic digestion. However, to further test whether WRV is an important parameter to consider during cellulose digestion, fungal enzymatic hydrolysis was performed on dried Avicel, which showed lower WRV due to potential pore collapse during rapid drying, compared to Avicel. Fungal enzymes digestion of dried Avicel was about 7% lower than that on Avicel confirming the impact of WRV on cellulose digestibility. However WRV cannot be used as an indicator of substrate digestibility by itself.

Overall, cellulose surface area and pore size measured via Simons' staining had the highest impact on substrate digestibility by fungal enzymes followed by cellulose crystallinity, WRV, and finally DP that did not influence fungal enzymatic digestion substantially. The insignificant effect of DP on overall digestion by fungal enzymes complements results reported with minimal reduction in molecular weight distribution followed by a levelling off in DP during hydrolysis suggesting that DP may be a limiting

factor in cellulose digestion only beyond a certain high molecular weight [11]. Further it seems that the digestion of cellulose really depends on the initial adsorption of cellulases onto the substrate and probably follows a peeling-off effect [12]. Cellulose surface area and pore size distribution along with cellulose crystallinity substantially influence enzyme accessibility and therefore enzyme adsorption. However, once enzymes have adsorbed onto the cellulose surface they attack the outermost layers of cellulose which once peeled off are readily hydrolyzed irrespective of cellulose DP. This explains the low impact of DP as opposed to a substantial impact of other cellulose parameters measured in this study on cellulose digestion. The insignificant effect of cellulose DP on cellulose digestion after the enzymes adsorb on the substrate also supports the synergistic mechanism of endo- and exoglucanases [12, 25]. Both endo- and exoglucanases easily digest peeled off cellulose fibers completely and synergistically. Thus, effective enzyme adsorption on cellulose is the driving force for successful enzymatic digestion of cellulose. Further, the impact of WRV on cellulose digestion supports the amorphogenesis mechanism of cellulase action [10, 12]. High WRV of the material should aid in the initial swelling of the substrate increasing substrate surface area and availability of amorphous regions on the substrate leading to effective cellulose digestion.

4.3.3. *C. thermocellum* CBP of substrates with varying cellulose properties

All materials were fermented in a 50 g working mass with a 2% v/v inoculum of *C. thermocellum* with a 0.5 wt% glucan loading of each material. Metabolites and glucose yield were measured for *C. thermocellum* CBP as a percentage of initial glucan

stoichiometrically calculated with an anhydrous correction to convert mass of glucose to glucan and is reported in Figure 4.6(a). Ethanol, acetic acid, and lactic acid were measured as the major metabolites produced by the organism. About 65% of initial glucan was attributed to the production of metabolites starting with a 0.5 wt% glucan loading of each substrate with negligible glucose accumulation. The distribution of each metabolite produced by *C. thermocellum* also did not vary significantly with substrate type. Further, unlike fungal enzymes, *C. thermocellum* showed equal solids solubilization and product formation on dried Avicel and Avicel, reported in Figure 4.7, showing no impact of substrate WRV on *C. thermocellum* fermentation. It was, thus, concluded that the substrate type and cellulose micro-accessibility did not affect digestion of the substrate by *C. thermocellum*. However, fermentations at higher substrate loadings were performed that were expected to enhance differences, if any, in digestion observed on each material by the organism. Fermentations done on 1 wt% glucan loading of substrate led to a decrease in metabolite production with a simultaneous increase in glucose accumulation suggesting inhibition of the metabolic performance of the organism while its cellulolytic ability continued as shown in Figure 4.6(b).

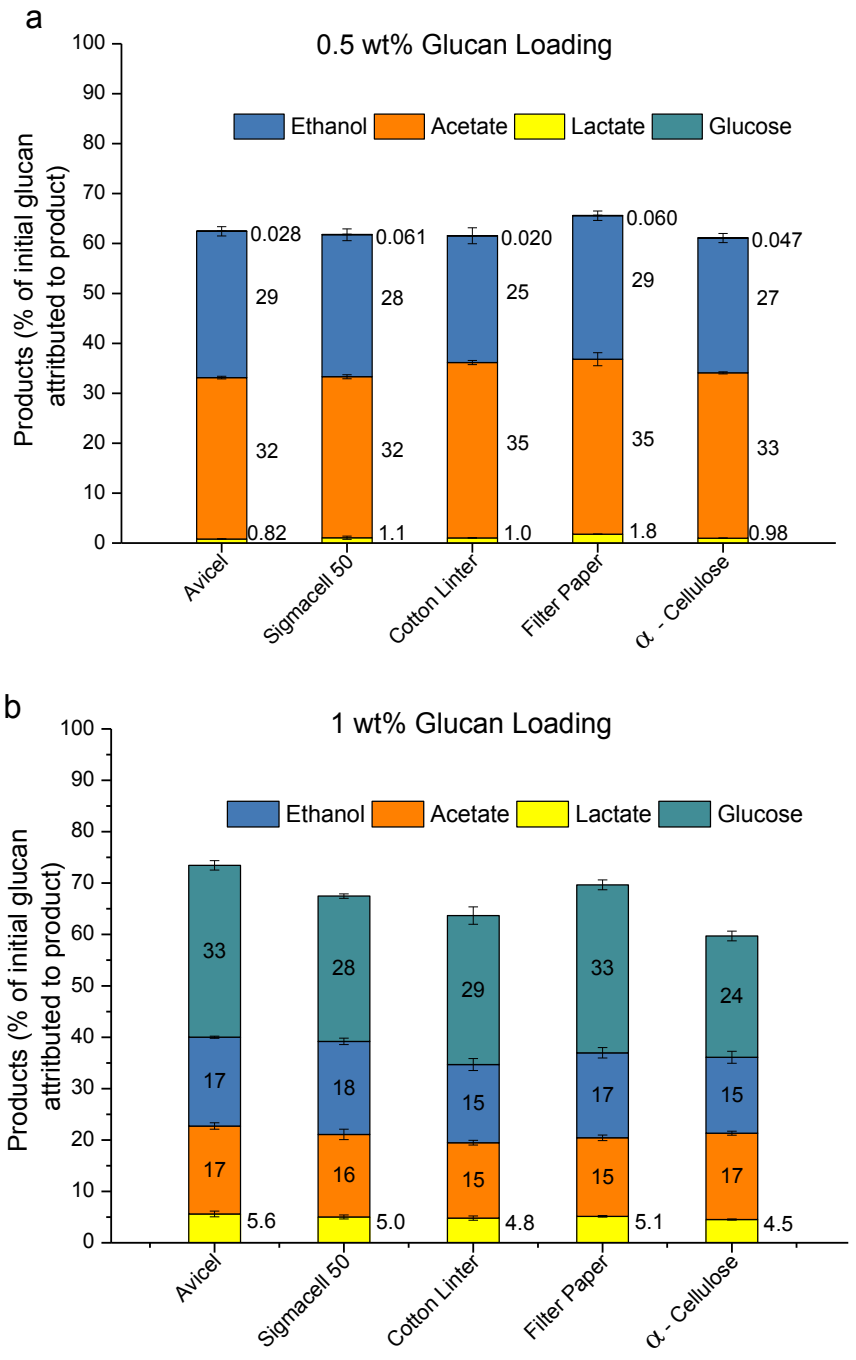

Figure 4.6. Metabolites and glucose production by *Clostridium thermocellum* after 7 days of consolidated bioprocessing on Avicel, Sigmacell 50, cotton linter, filter paper, and α-cellulose with solids loading of **(a)** 0.5 wt%, **(b)** 1 wt% glucan

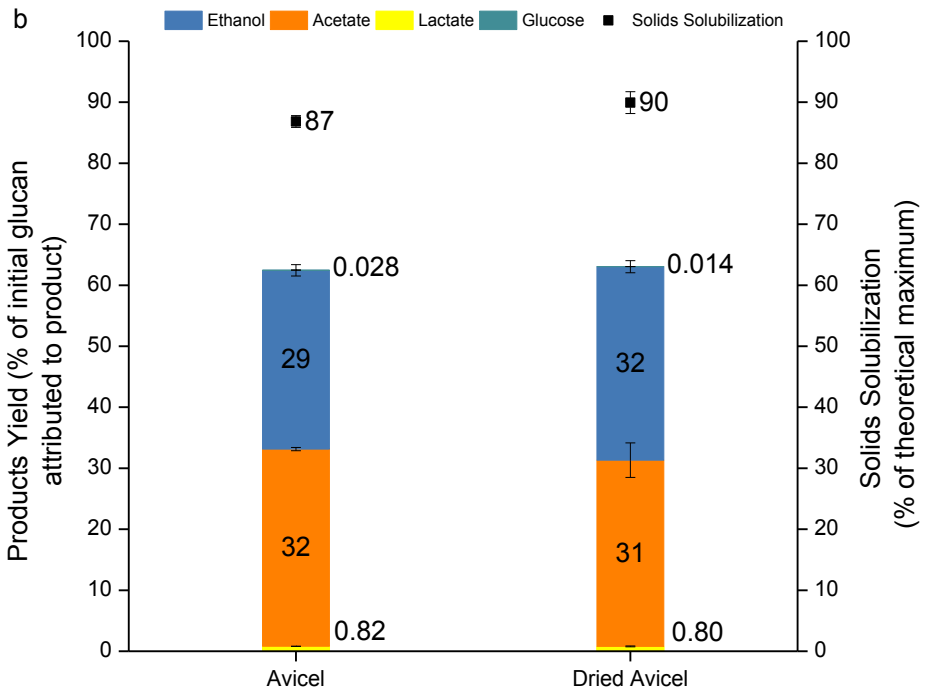

Figure 4.7. *Clostridrium thermocellum* consolidated bioprocessing solids solubilization and product yields on Avicel and dried Avicel with a 0.5 wt% substrate glucan loading

C. thermocellum inhibition, if any, should ideally affect digestion of all materials equally and any difference in digestion performance of *C. thermocellum* on different substrates should be a manifestation of substrate specific properties only. The fermentations of 1 wt% glucan of all substrates still did not reveal any significant differences in digestion of the different materials. Some differences became evident at substrate loadings of 2 and 5 wt% glucan loadings of the substrates shown in Figures 4.8 (a) and (b). Cotton linter and α-cellulose showed lower metabolites production and glucose accumulation combined compared to the other materials. This was complemented by lower solids solubilization of cotton linter and α-cellulose compared to the other materials by *C. thermocellum* shown in Figure 4.9.

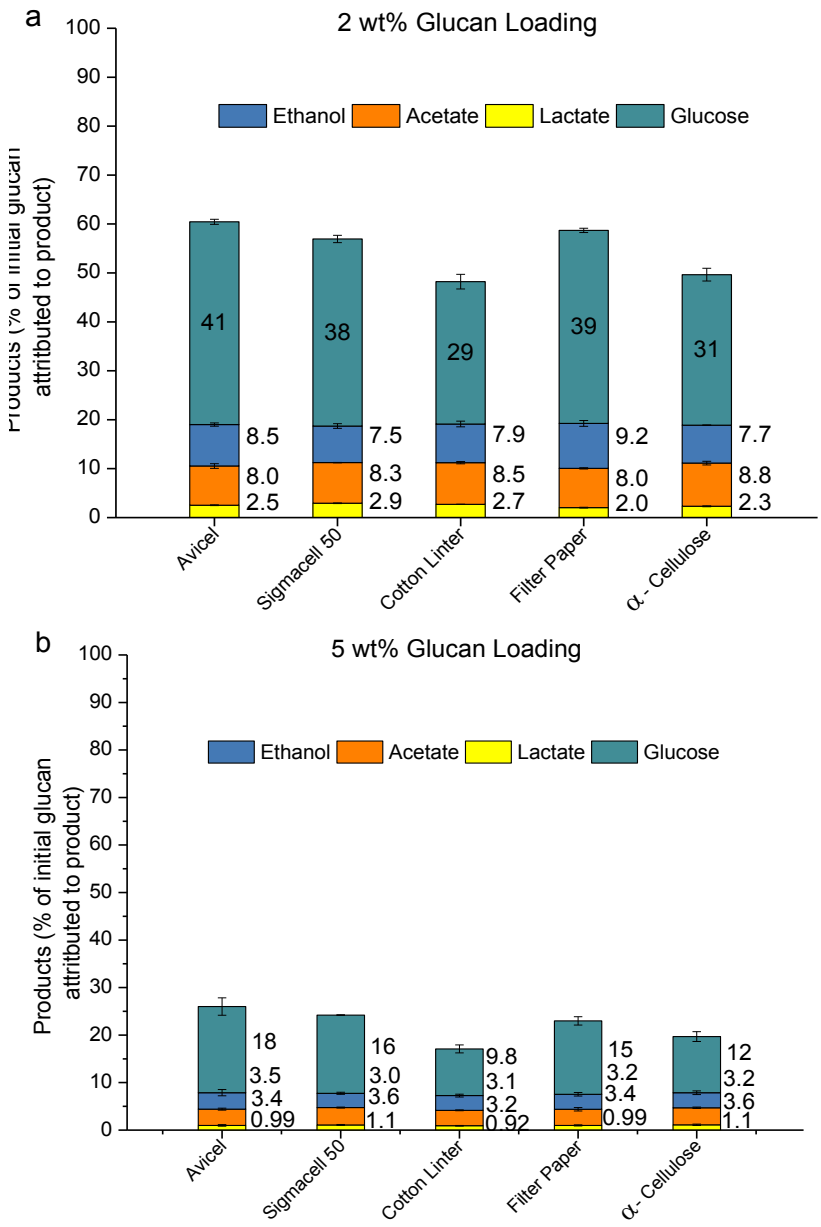

Figure 4.8. Metabolites and glucose production by *Clostridium thermocellum* after 7 days of consolidated bioprocessing on Avicel, Sigmacell 50, cotton linter, filter paper, and α-cellulose with solids loading of **(a)** 2 wt% and **(b)** 5 wt% glucan

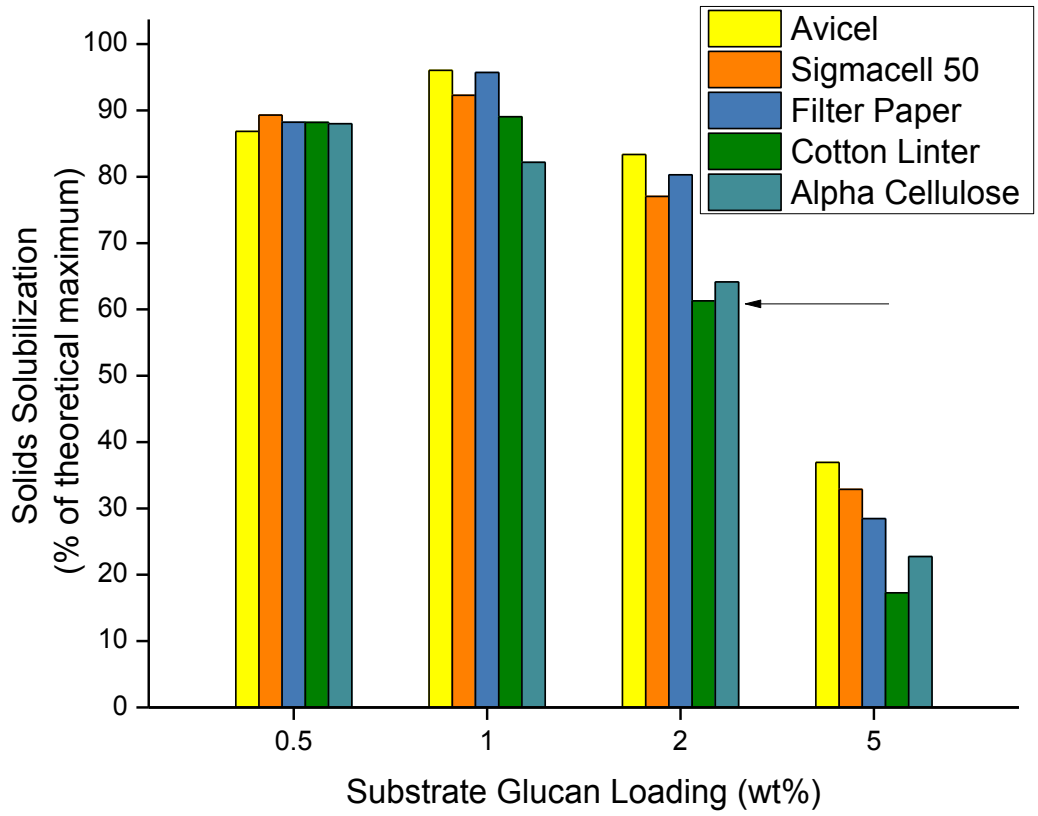

Figure 4.9. Solids solubilization by *Clostridium thermocellum* after 7 days of consolidated bioprocessing on Avicel, Sigmacell 50, cotton linter, filter paper, and α-cellulose with substrate loading of 0.5 wt%, 1 wt%, 2 wt%, and 5 wt% glucan. The arrow indicates lower solubilization by *C. thermocellum* on cotton linter and α-cellulose at high substrate loadings

Lower *C. thermocellum* digestion performance on cotton linter was similar to that observed for fungal enzymes. However, the lower digestion of cotton linter was only observed with *C. thermocellum* at a 2 wt% glucan loading of the material (and higher) and showed only a ~17% lower product formation compared to other materials at the same substrate loading. However, the negative impact of cotton linter on fungal enzymes was observed at a low 0.5 wt% glucan loading of the substrate and led to a ~57-66% lower product formation compared to other materials suggesting a much greater impact of cellulose micro-accessibility on fungal enzymes compared to *C. thermocellum*. The low

digestion of α-cellulose by *C. thermocellum* at high substrate loadings may be due to the presence of high amounts of xylan in the substrate compared to other materials that are known to be mostly pure cellulose [54-58]. *C. thermocellum* cellulosomes are known to contain xylanases that can break down xylan. However, xylose and xylo-oligomers, the breakdown products of xylan, are known to be inhibitory to *C. thermocellum* [59]. 2-5 wt% glucan loading of α-cellulose was equivalent to 0.5-1.3 wt% (~5-13 g/L) xylan loading of α-cellulose. The IC_{50} concentration of xylose or xylobiose has been determined to be 15 g/L for the M1570 strain of *C. thermocellum* with lower concentrations of both the mono- and disaccharide also inhibiting the organism [60]. Overall, *C. thermocellum* was not affected by cellulose micro-accessibility substantially and digested all materials similarly.

4.4. Conclusions:

Here, we have shown the importance of studying the impact of cellulose micro-accessibility on biological digestion by fungal enzymes and *C. thermocellum*. Cellulose micro-accessibility is expected to affect its digestion once cellulose from lignocellulosic biomass is made physically accessible to enzymes through biomass augmentation. Cellulose micro-accessibility has been shown to be influenced by cellulose crystallinity, DP, WRV, surface area, and other structural features. In this work we have related the effect of these properties on the extent of fungal enzymatic digestion compared to that by *C. thermocellum*. These substrate properties had a significant impact on the ability of fungal enzymes to digest the substrate in comparison to *C. thermocellum*. Surface area

and pore size distribution of the substrates had the greatest impact on fungal enzymatic digestion, followed by cellulose crystallinity, WRV, and DP. Cotton linter was the least digestible substrate compared to Avicel, Sigmacell, filter paper, α-cellulose, and dried Avicel due to its low surface area, smaller pore size distribution, and high crystallinity and DP. Filter paper was more digestible by fungal enzymes than Avicel due to higher surface area measured as total dye absorption via Simons' staining as well as higher swelling ability measured as high WRV for the former. Avicel had the largest pores as confirmed via SEM and by high O/B ratio leading to better digestibility of this substrate by fungal enzymes than Sigmacell and α-cellulose. Sigmacell was digested more effectively than cotton linter by fungal enzymes because of the lower cellulose crystallinity of the former. Otherwise alike, dried Avicel and Avicel were different only in their WRV. Low WRV of dried Avicel led to a less effective digestion of the material by fungal enzymes compared to Avicel also showing the impact of WRV on fungal enzymatic digestion. Filter paper had a higher cellulose DP compared to Avicel but the former was still more digestible by fungal enzymes suggesting the low impact of DP on the extent of enzymatic digestion of cellulose. Overall, fungal enzymatic digestion is driven by effective enzyme adsorption influenced by cellulose surface area, pore size, and crystallinity of the substrate. In contrast, no difference in digestion was observed on the various substrates by *C. thermocellum* at a 0.5 wt% glucan substrate loading. However, cotton linter and α-cellulose digestion by *C. thermocellum* was marginally lower than on the other substrates at 2 and 5 wt% glucan substrate loading. The lower digestion on high loadings of α-cellulose may be due the presence of high amounts of xylan, the breakdown

products of which possibly inhibited *C. thermocellum*. Overall, however, *C. thermocellum* was unaffected by cellulose micro-accessibility. Even though model substrates were used in this work to differential between the impacts of cellulose micro-accessibility from those of cellulose macro-accessibility on biological digestion, for a full understanding of cellulose micro-accessibility on digestion by fungal enzymes and *C. thermocellum* a similar thorough analysis of real world lignocellulosic biomass will be essential in the future.

4.5. Methods and materials:

4.5.1. Substrates

Avicel® PH-101 (11365, Lot No. BCBN7864V), Sigmacell Cellulose Type 50 (S5504, Lot No. SLBB7781V), cotton linter (discontinued, Lot No. 090M0144V), and α-cellulose (C8002, Lot No. 066K0076) are commercially available (Sigma-Aldrich®, St. Louis, MO) and were used as is during fermentations. Whatman™ 1 filter paper (1001-110) awas milled using a Thomas Wiley® mill (Model 3383-L20, Thomas Scientific, Swedesboro NJ) and passed through a size 40 mesh. These substrates are known to be of high purity and were assumed to have 100% glucan for calculations [54-58]. Ethanol (E1028), acetic acid (A38-212), lactic acid (L6661) used as standards for HPLC analysis were obtained from Spectrum® Chemical Mfg. Corp. (Gardena, CA), Fisher Scientific™ (Fair Lawn, NJ), Sigma-Aldrich® (St. Louis, MO), respectively. Glucose (G8270), also used as an HPLC standard, was obtained from Sigma-Aldrich® (St. Louis, MO).

4.5.2. *Clostridium thermocellum* fermentations

C. thermocellum DSM 1313 wild type was kindly provided by Prof. Lee Lynd at Dartmouth College, Hanover NH. A stock culture was grown in a 500 mL anaerobic media bottle (Chemglass Life Sciences, Vineland NJ) and stored in 5 mL serum vials at -80°C. Pellet nitrogen content was analyzed according to methods published in literature to determine the growth curve for a 500 mL and a 50 mL culture with a 2% by volume inoculum and a 5 g/L glucan Avicel loading [15, 61] reported in Figure 3.11. Seed cultures were grown with a 5 g/L glucan loading of Avicel® PH101 (Sigma Aldrich, St. Louis, MO) in a 50 mL working volume for 8-9 hours (approximately the start of exponential phase based on pellet nitrogen analysis) in Media for Thermophilic Clostridia (MTC) without trace minerals using a 2% by volume inoculum. The medium components and concentrations used are shown in Table 3.1. All solutions except the vitamins solution were sterilized by autoclaving. The vitamins solution was filter sterilized using 28 mm diameter polyethersulfone (PES) syringe filters with 0.2 μm pores (Corning® Life Sciences, Tewksbury MA). The seed culture was stored overnight in a refrigerator before using it for inoculation the next day for all experiments. Fermentations were performed in 125 mL bottles (Wheaton, Millville NJ) with varying glucan loading of cellulosic substrates in triplicates at a working mass of 50 g. Bottles containing substrate and water were purged with nitrogen. A 45 seconds application of vacuum and 14 psi nitrogen over a total of 27-30 min was used for purging. The bottles were then sterilized by autoclaving at 121°C for 35 min. All media solutions and inoculum were injected aseptically into the bottles. Fermentations were run at 60°C with a shaking speed of 180

rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD). Bottles were opened after 7 days of fermentation and liquid samples were taken to measure metabolites and simple sugars content. The liquor samples were centrifuged in 500µL capacity Ultrafree™-MC Centrifugal Devices with Durapore™ Membrane (EMD Millipore, MA USA) 15,000 rpm for 10 min. The filtered liquid solution was then analyzed by HPLC. Insoluble solids were also recovered after fermentation and rinsed thoroughly to determine solids solubilization.

4.5.3. Fungal enzymatic hydrolysis

Enzymatic hydrolysis was performed at 0.5 wt% glucan and a 15 mg protein / g glucan enzyme loading with a working mass of 50 g in 125 mL Erlenmeyer flasks in triplicates. The National Renewable Energy Laboratory (NREL) Analytical Procedure "Enzymatic Saccharification of Lignocellulosic Biomass" was followed [62] using Accellerase® 1500 cellulase (DuPont Industrial Biosciences, Palo Alto CA) enzyme cocktail. The BCA protein content of Accellerase® 1500 was reported elsewhere to be 82 mg/mL [63]. Enzymatic hydrolysis was run at 50°C and 150 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD). Flasks were allowed to equilibrate at temperature before adding the enzyme solution. 1 mL representative samples including the insoluble substrate and liquor combined were collected from each flask after 4 hours, 24 hours, and every 24 hour period thereafter for a total of 7 days. The samples were centrifuged in 1.5 mL Simport® microcentrifuge tubes (Spectrum® Chemical Manufacturing Corporation,

New Brunswick, NJ) at 15,000 rpm for 10 min and the supernatant was analyzed by HPLC.

4.5.4. Analytical procedures

Waters Alliance e2695 HPLC system (Waters Co., Milford MA) equipped with a Bio-Rad Aminex HPX-87H column and a Waters 2414 refractive index detector was used for analysis. 5 mM sulfuric acid mobile phase was eluted at a flow rate of 0.6 mL/min. Empower™ 2 software package was used for the integration of chromatograms.

4.5.5. Yield calculations

All experiments were performed in triplicates, unless otherwise specified. Error bars represent the standard deviation in the replicates. Metabolite yield was calculated as the glucan required to produce each metabolite through stoichiometry of balanced glucose to metabolite reactions. Glucose was further converted to glucan using the anhydrous correction factor (= 0.9). Solids solubilization was measured as the percentage loss of solids after 7 days of fermentations compared to solids at time zero. All yields were based on glucan loaded initially.

4.5.6. Solid state nuclear magnetic resonance

The cellulose crystallinity of samples was measured using solid-state cross polarization magic angle spinning (CP/MAS) nuclear magnetic resonance (SSNMR). The samples were moisturized and packed into 4-mm cylindrical Zirconia MAS rotors.

All the SSNMR experiments were carried out on a Bruker Avance III HD 500-MHz spectrometer operating at frequencies of 125.77 MHz for ^{13}C in a Bruker double-resonance MAS probe at room temperature. The acquisition conditions for CP/MAS experiments were as follows: a 5 μs (90°) proton pulse, 3.0 ms contact pulse, 3 s recycle delay and 4096 scans. The rotor spin rate was 8000 Hz. The cellulose crystallinity index (CrI) was determined from the areas of the crystalline and amorphous C_4 signals using the following formula:

$$\mathrm{CrI} = \frac{A_{86\text{-}92\ \mathrm{ppm}}}{A_{86\text{-}92\ \mathrm{ppm}} + A_{79\text{-}86\ \mathrm{ppm}}}$$

4.5.7. X-ray diffraction

The crystallinity indices (CrI) of cellulose samples were measured by X-ray diffraction (XRD) using a Rigaku (Tokyo, Japan) Ultima IV diffractometer with CuKα radiation having a wavelength λ(Kα1) = 0.15406 nm generated at 40 kV and 44 mA. The diffraction intensities of freeze-dried samples placed on a quartz substrate were measured in the range of 8 to 42° 2θ using a step size of 0.02° at a rate of 2°/min. The CrI of the cellulose samples were calculated according to the method described by Segal *et al*.[64] by using eq. (1) presented below:

$$CrI = \frac{I_{200} - I_{Am}}{I_{200}} \qquad (1)$$

where I_{200} and I_{Am} are the maximum and minimum intensity of diffraction at approximately 2θ = 22.4 - 22.5° and 2θ = 18.0 - 19.0°, respectively.

Scherrer's equation [65, 66] was used for estimating crystallite size:

$$\beta = \frac{k\lambda}{\tau cos\theta} \quad (2)$$

where λ is the wavelength of the incident X-ray (1.5418 Å), θ the Bragg angle corresponding to the (2 0 0) plane, β the full-width at half maximum (FWHM) of the X-ray peak corresponding to the (2 0 0) plane, τ is the X-ray crystallite size, and k is a constant with a value of 0.89 [67, 68].

4.5.8. Gel permeation chromatographic (GPC) analysis.

GPC after tricarbanilation was used to measure the weight-average molecular weight (M_w) and number-average molecular weight (M_n) of cellulose were measured by. Briefly, cellulose substrates were dried overnight under vacuum at 45°C. The dried cellulose samples were then derivatized with phenyl isocyanate in an anhydrous pyridine system. An Agilent 1200 HPLC system (Agilent Technologies, Inc, Santa Clara, CA) equipped with Waters Styragel columns (HR1, HR4, and HR6; Waters Corporation, Milford, MA) was used to perform size-exclusion separation. Number-average degree of polymerization (DP_n) and weight-average degree of polymerization (DP_w) of cellulose were obtained by dividing M_n and M_w, respectively, by 519 g/mol, the molecular weight of the tricarbanilated cellulose repeating unit.

4.5.9. Scanning Electron Microscopy (SEM)

Samples for SEM were placed on carbon tape on aluminum stubs and sputter-coated with gold. SEM was carried out on Zeiss Auriga FIB-SEM at an accelerating

voltage of 10 kV with back scatter detector at 100 to 5000 times magnification. Raw images were adjusted for brightness and contrast in ImageJ software [69]. Images were merged using Adobe Photoshop CC v. 2017.

4.5.10. Water retention value (WRV)

WRV was measured as described previously [4]

4.5.11. Simons' staining

Simons' staining was performed as described previously using the high molecular weight fraction (≥30,000 kDa) of Direct Orange 15 dye (CAS: 1325-35-5) and Direct Blue 1 (CAS: 2610-05-1) obtained from Pylam Products Company, Inc. (Tempe, AZ) [4, 42]

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Chapter 5. Glucan accessibility drives digestion of lignocellulosic biomass by *Clostridium thermocellum*

In Review at *Biotechnology for Biofuels*

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5.1. Abstract

The thermophilic anaerobic bacterium *Clostridium thermocellum* is a multifunctional ethanol producer, capable of both saccharification and fermentation, that is central to the consolidated bioprocessing (CBP) approach of converting lignocellulosic biomass to ethanol without external enzyme supplementation. Although CBP organisms have evolved highly efficient machinery for biomass deconstruction, achieving complete solubilization requires targeted approaches, one of which is pretreatment, to prepare recalcitrant biomass feedstocks for further biological digestion. Here, differences between how *C. thermocellum* and fungal cellulases respond to senescent switchgrass prepared by different pretreatment techniques reveal host-specific relationships between biomass substrate composition and its digestibility. *C. thermocellum* achieved highest sugar release from de-lignified switchgrass prepared by co-solvent enhanced lignocellulosic fractionation (CELF) and dilute alkali pretreatments demonstrating greater resilience to the presence of hemicellulose sugars than fungal enzymes. Theoretical glucan solubilization and theoretical total (glucan + xylan) sugar release was achieved after *C. thermocellum* CBP combined with CELF pretreatments of switchgrass. Further, the effect of glucan accessibility, influenced by lignin, on its solubilization by *C. thermocellum* in turn showed a direct correlation with metabolite production. The comprehensive nature of this work with comparison of four different pretreatment methods and two different biological digestion techniques is unparalleled and provides a strong platform for future work on integration of pretreatment with CBP.

5.2. Introduction

Fuel ethanol derived from abundant lignocellulosic biomass has the highest potential to alleviate the dependence on fossil petroleum in the transportation sector while also dramatically reducing associated greenhouse gas emissions [1, 2]. The conversion of lignocellulosic feedstocks into ethanol currently requires a combination of size reduction, thermochemical pretreatment, fungal enzyme production, enzymatic hydrolysis, sugar fermentation, and product recovery [3, 4]. The high capital and operating costs of these processes, especially that of production and purification of large doses of cellulolytic enzymes from *Trichoderma reesei* required to obtain sufficiently high sugar yields, challenges the economic competitiveness of cellulosic ethanol [5]. Thus, intense research is required to reduce the amount of fungal enzymes needed or eliminate their use to decrease processing costs. Consolidated bioprocessing (CBP) is an improved method that reduces enzyme-related costs by using cellulolytic microorganisms that have the ability to produce their own enzyme consortium to simultaneously hydrolyze biomass polysaccharides and ferment the released sugars into ethanol and other desirable bioproducts [6, 7]. *Clostridium thermocellum* is a particularly promising CBP cellulolytic microorganism [3, 6, 8] that is capable of nearly completely digesting cellulose materials such as Avicel on its own [9]. In contrast, even though high, only 48% of the cellulose in natural Alamo switchgrass was solubilized by *C. thermocellum* as shown in this work. Thus, cellulose accessibility to digestion in a complex substrate such as lignocellulosics remains the primary barrier to economic production of cellulosic ethanol and methods to overcome biomass recalcitrance to breakdown remain a subject of intense study [4, 10,

11]. For biological systems, recalcitrance of a certain type of biomass material has been attributed to the presence of lignin and hemicellulose in biomass interfering with cellulose macro-accessibility, the physical availability of cellulose to enzymatic saccharification [12-15]. In fact, an increase in accessibility of the substrate to saccharolytic enzymes of *C. thermocellum* via ball milling during cotreatment of switchgrass has been shown to increase solubilization [16]. Various biomass pretreatment techniques have been developed that can also prepare lignocellulosic biomass for further conversion to ethanol by targeting removal of hemicelluloses or lignin to ensure greater cellulose accessibility [11, 17, 18].

Substrate adaptive changes in *C. thermocellum* cellulosomes reported in literature suggest that the host organism adjusts the proportion of different activities in the cellulosome, such as endoglucanases, exoglucancases, xylanases, and pectinases, in response to substrate features [19]. However, a comprehensive evaluation of CBP performance on different substrates and targeted optimization of pretreatments optimized specifically for this system has not been done. A significant amount of CBP literature is focused on at most one or two pretreatment methods without optimization of pretreatment parameters to maximize either polysaccharides solubilization or ethanol production [20-23]. On the other hand, a number of past studies have reported results from application of various pretreatment types and pretreatment conditions followed by use of fungal enzymes for further digestion [18, 24-26]. Other, more focused studies attempted to elucidate the impact of lignin or hemicellulose removal on enzymatic hydrolysis by

fungal enzymes [27-30]. Along those lines, here we aim to systematically show the effect of biomass composition, specifically the presence of lignin and hemicellulose (represented by xylan), on sugar solubilization and metabolites production by *C. thermocellum*.

5.3. Results and Discussion:

5.3.1. Switchgrass pretreatments

Alamo switchgrass was selected as a fast growing energy crop rich in both pentose and hexose sugars to serve as the model feedstock for this study [31]. In addition to unpretreated switchgrass, compositionally distinct materials were prepared by subjecting switchgrass to four different pretreatment methods: hydrothermal, dilute acid, dilute alkali, and co-solvent enhanced lignocellulosic fractionation (CELF). These pretreatments were chosen based on their abilities to distinctively solubilize hemicellulose sugars (quantified as xylan) or lignin (measured as Klason-lignin or acid insoluble lignin) or both from unpretreated biomass [32, 33]. CELF pretreatment was recently developed to employ tetrahydrofuran (THF) as a miscible co-solvent in water used in combination with dilute acid to enhance biomass delignification and depolymerization [32]. Pretreatment conditions were varied over a range of reaction temperatures and times to help establish optimum conditions for maximum glucan and xylan sugar release from pretreatment (Stage 1) in combination with *C. thermocellum* CBP (Stage 2) for each pretreatment type. Sugar recovery was tracked starting from both glucan and xylan in unpretreated biomass as established elsewhere for optimization of

pretreatments in combination with fungal enzymes [34]. Total mass of glucan, xylan, and lignin found in unpretreated switchgrass and pretreated solids were tracked starting from 100 g equivalent of the unpretreated material as shown in Figure 5.1 to reveal both relative compositions of each component and mass changes after pretreatment.

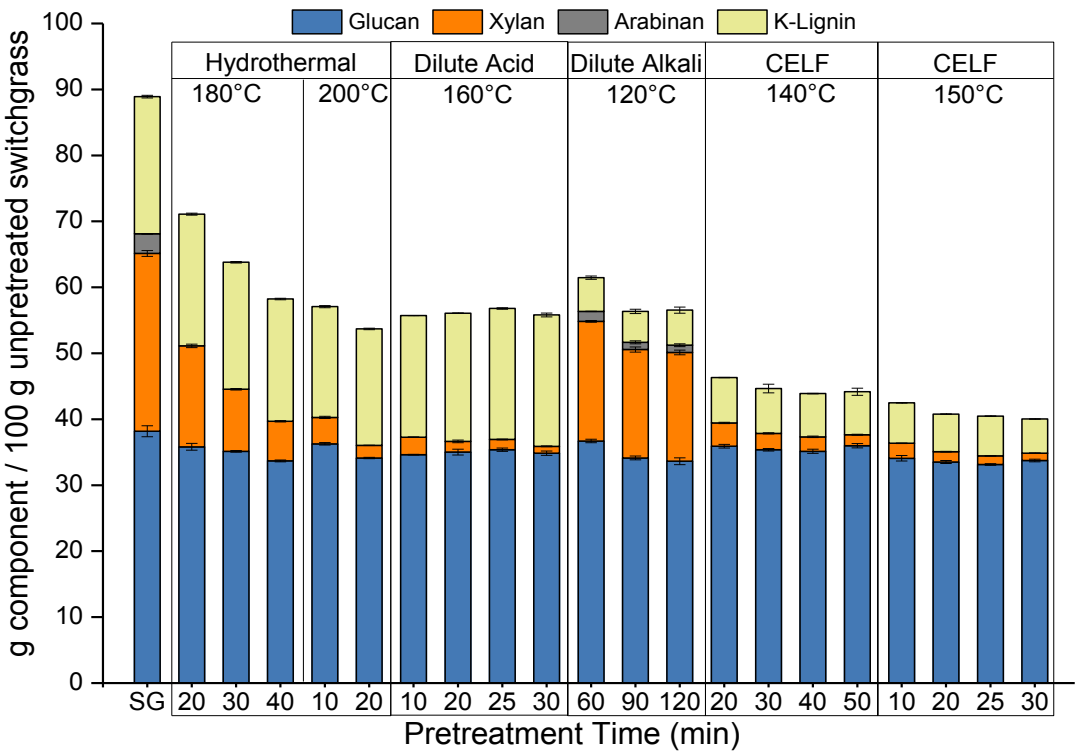

Figure 5.1. Tracking fate of components of switchgrass in solids before and after hydrothermal, dilute acid, dilute alkali, and co-solvent enhanced lignocellulosic fractionation (CELF) pretreatments adjusted to a basis of 100 g of initial unpretreated switchgrass (SG)

Overall, the solubilization of glucan during pretreatment was minimal for all pretreatment types increasing slightly with longer reaction times. For CELF pretreatment, temperature had a greater impact on glucan content in pretreated solids allowing tuning of different conditions to modify relative compositions of each component. Hydrothermal

and dilute acid pretreatments achieved high xylan removal, about 44-93% and 89-96%, respectively, while only about 5-14% lignin removal was observed. Notably, dilute acid pretreatment resulted in increased lignin in pretreated solids compared to hydrothermal pretreatment associated with the formation of pseudo lignin through polysaccharide degradation, which has been shown to inhibit enzymatic digestion [35, 36]. Aggressive lignin removal was achieved by dilute alkali pretreatment, ranging from 71-76%, while only about 30-41% xylan removal was observed. High xylan content in dilute alkali pretreated solids is expected to influence solubilization by *C. thermocellum* and fungal enzymes differently. CELF pretreatment removed about 85-96% xylan and 67-76% lignin at pretreatment conditions applied in this study. Overall, removal of either xylan or lignin or both from switchgrass, thereby increasing macro-accessibility of cellulose to enzymes, is expected to aid further biological deconstruction of pretreated solids. Although glucan composition in solids varied across hydrothermal, dilute acid, dilute alkali, and CELF pretreatments, being 49-59%, 59-60%, ~55%, 74-78%, respectively, CELF pretreated solids contained the most glucan relative to xylan and lignin and is expected to be the most highly digestible. High xylan content of hydrothermal and dilute alkali solids and high lignin content of dilute acid pretreated solids would serve to provide a wide range of compositionally distinct solids to evaluate substrate-enzyme/host effects.

5.3.2. Impact of substrate composition on *C. thermocellum* consolidated bioprocessing

Unpretreated and pretreated switchgrass were then employed in *C. thermocellum* consolidated bioprocessing to understand the effect of lignin and hemicellulose removal either independently or simultaneously on biological deconstruction. Glucan solubilization ability of *C. thermocellum* was measured on the different solids and is reported as glucan solubilization time profile for 7 days in Figure 5.2 and Figure 5.3. *C. thermocellum* solubilized unpretreated milled switchgrass effectively to achieve ~48% glucan solubilization. Even though this is a reasonably high glucan solubilization, it is still not enough to achieve an economically viable technology underlining the importance of pretreatment in assisting the biological deconstruction of biomass by *C. thermocellum*. Not only did CELF pretreatments assist *C. thermocellum* in achieving the highest glucan solubilization but also realized theoretical glucan solubilization in less time as compared to other pretreatment methods implying that physical removal of both lignin and hemicellulose from the biomass is essential for successful solubilization of switchgrass. Also, dilute alkali pretreated solids showed faster and/or higher glucan conversion than dilute acid and hydrothermal pretreatments, indicating that physical removal of K-lignin, as is evident in dilute alkali pretreatments, had a more positive impact on glucan conversion than physical removal of xylan as observed in hydrothermal and dilute acid pretreatments.

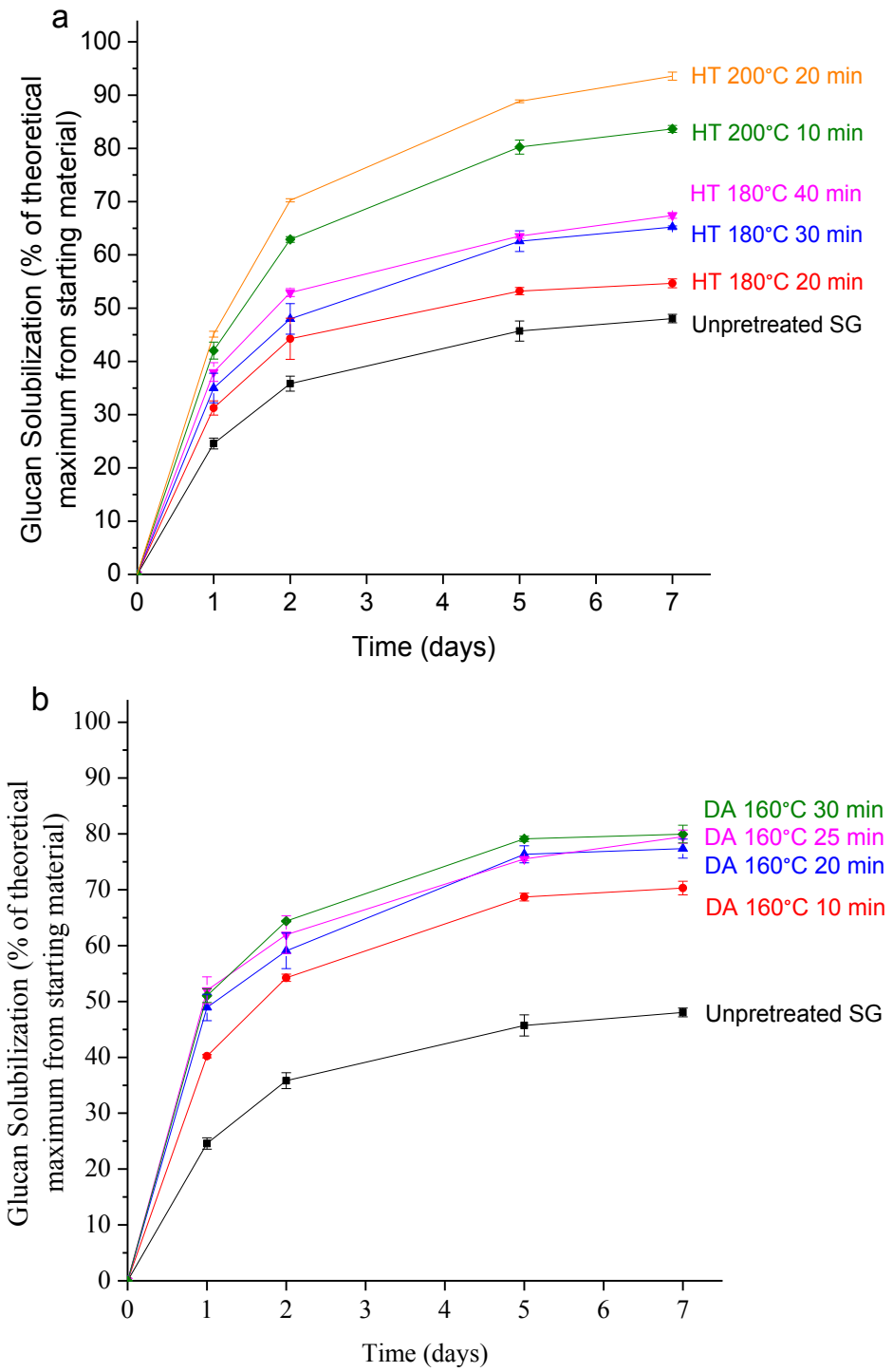

Figure 5.2. *C. thermocellum* consolidated bioprocessing (CBP) glucan solubilization time profiles for unpretreated and **(a)** hydrothermal (HT) and **(b)** dilute acid (DA) pretreated switchgrass (SG)

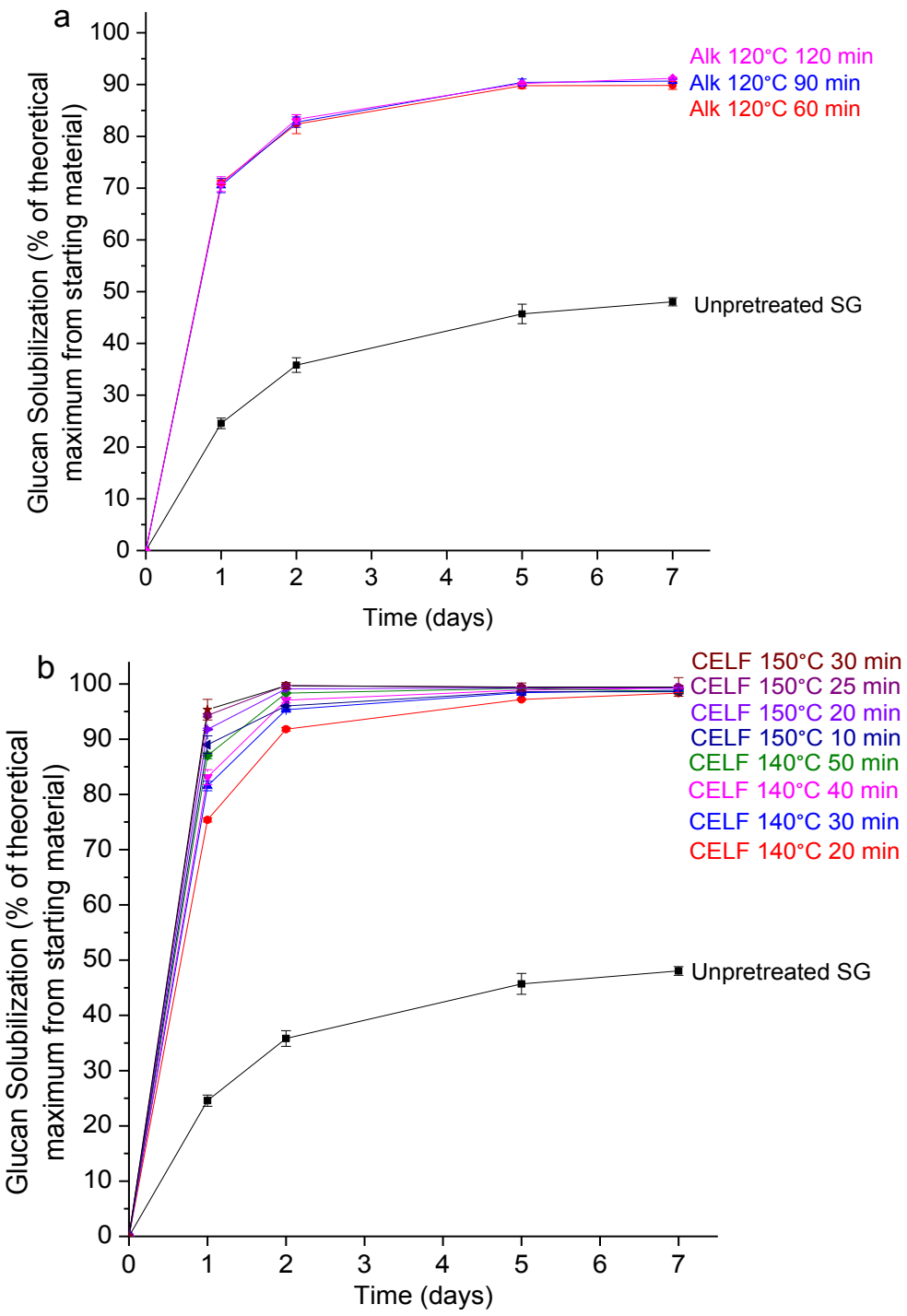

Figure 5.3. *C. thermocellum* consolidated bioprocessing (CBP) glucan solubilization time profiles for unpretreated and **(a)** dilute alkali (Alk) and **(b)** co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG)

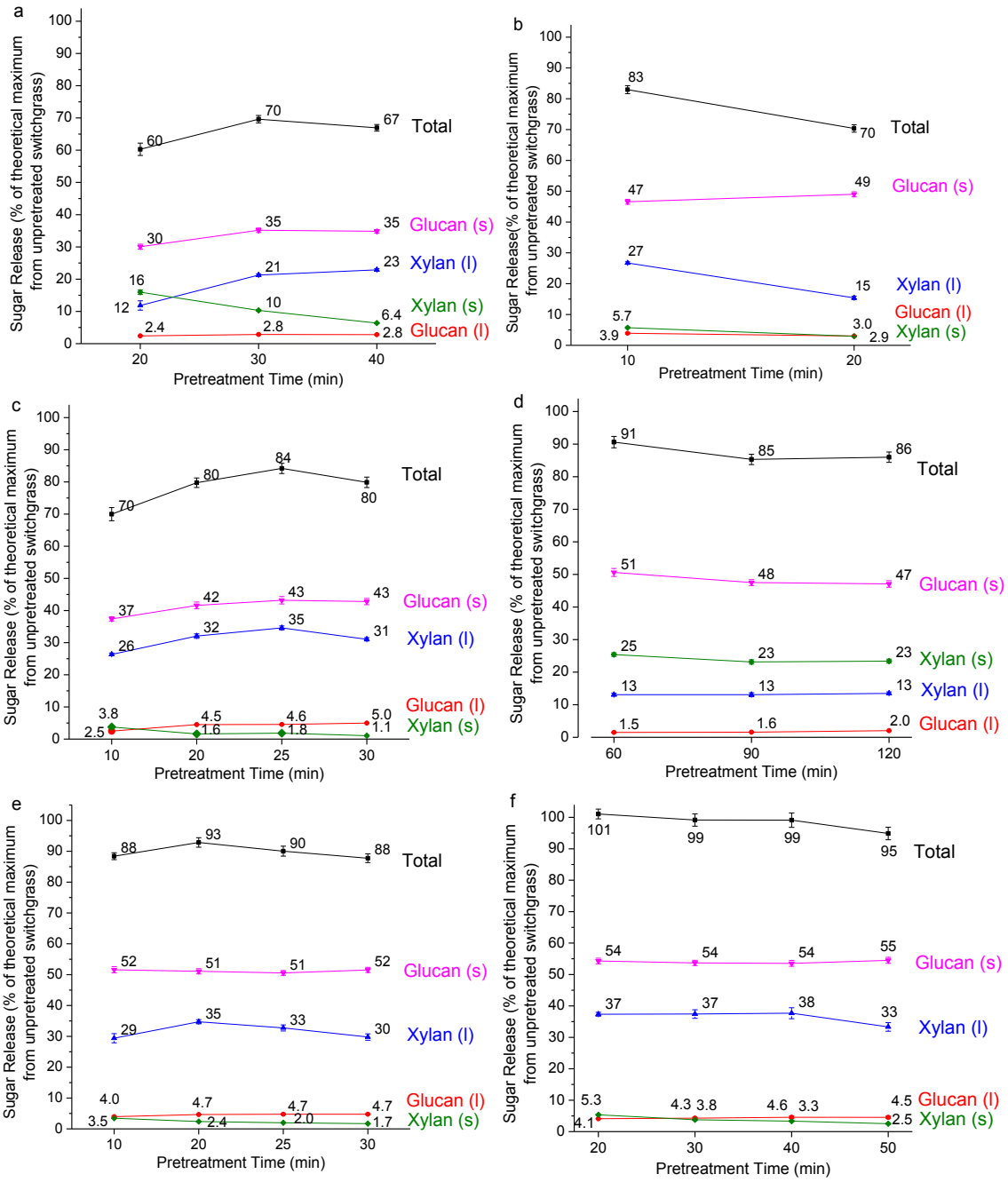

Figure 5.4. Glucan and xylan release from Stage 1 (pretreatment; designated as "l") and Stage 2 (7 days of *C. thermocellum* consolidated bioprocessing; designated as "s") for **(a)** hydrothermal (180°C), **(b)** hydrothermal (200°C), **(c)** dilute acid, **(d)** dilute alkali, **(e)** co-solvent enhanced lignocellulosic fractionation (CELF; 150°C), and **(f)** CELF (140°C) pretreatments

To evaluate different pretreatment methods fairly, each pretreatment technology was optimized for maximum total sugar release (glucan plus xylan), from pretreatment as Stage 1 combined with *C. thermocellum* CBP as Stage 2 depicted in Figure 5.4. Carbohydrates broken down during pretreatment were recovered in the pretreatment liquor as either monomers, oligomers, or degradation products thereof depending on pretreatment type and conditions. The optimization of pretreatment conditions for maximum sugar release critically depended on these Stage 1 sugar yields. These optimum conditions are harsh enough to produce biologically digestible solids but not severe enough for degradation of sugars, especially hemicellulose sugars, released during pretreatment in the liquor. Along with achieving complete solubilization of carbohydrates in lignocellulosic biomass it is essential to recover released sugars at theoretical yields for further conversion to product. The optimum conditions for hydrothermal pretreatment of switchgrass were found to be 200ºC for 10 min, dilute acid at 160ºC for 25 min, dilute alkali at 120ºC for 60 min, and CELF at 140ºC 20 min. For the sake of clarity Figure 5.5 shows total sugar release from Stage 1 (orange/yellow) and Stage 2 (light/dark green). Although different pretreatments solubilized different amounts of sugars during Stage 1, *C. thermocellum* was highly capable of releasing the remaining sugars from the solids during Stage 2. Higher glucan solubilization and total sugar release observed from CELF and dilute alkali pretreated biomass compared to those observed from dilute acid and hydrothermal pretreatments indicates that removal of lignin from biomass by pretreatment had a greater impact on digestion by *C. thermocellum* than removal of xylan and/or lignin relocation. This is further validated by the higher Stage 2 sugar release

achieved by *C. thermocellum* on delignified materials (CELF and dilute alkali pretreated solids) than on hydrothermal and dilute acid pretreated solids which are rich in lignin.

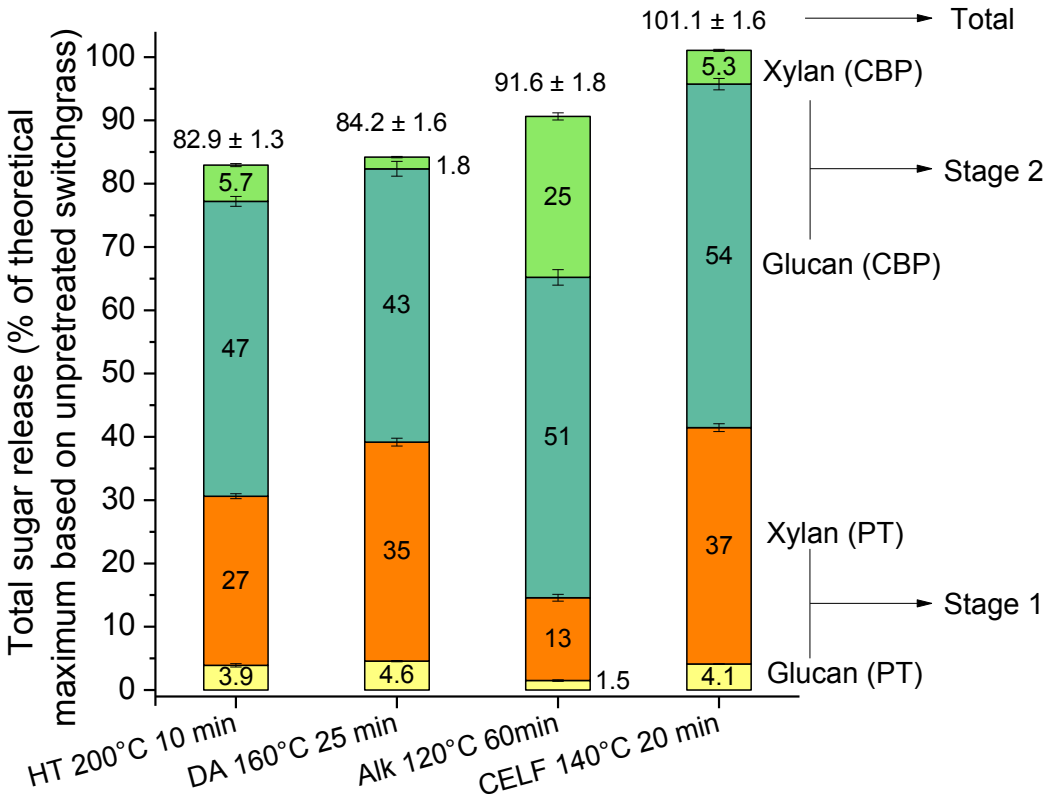

Figure 5.5. Total combined sugar (glucan plus xylan) release from Stage 1 (pretreatment, designated as "PT") and Stage 2 (7 days of *C. thermocellum* consolidated bioprocessing, designated as "CBP") for solids resulting from hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreatments at conditions that gave the highest total sugar release

The greater effect of lignin removal on enzymatic saccharification has been attributed to increased cellulose accessibility to enzymatic digestion due to the absence of lignin and a reduction in unproductive binding of enzymes to lignin as reported for fungal enzymes [37-39]. Lignin removal from the biomass may have a similar effect on biomass

deconstruction by *C. thermocellum* as well. Further, the greater effect of lignin removal on glucan solubilization by fungal enzymes has also been attributed to an increase in xylan accessibility and therefore digestibility due to the absence of lignin [40, 41]. This mechanism may apply to biomass deconstruction by *C. thermocellum* as well [42, 43]. Interestingly, high xylan release achieved during CBP contributed to the high Stage 2 sugar release observed on dilute alkali pretreated solids. *C. thermocellum* was able to break down xylan even though the wild type strain used in this work is not known to ferment xylose or xylo-oligomers [19]. Further, we decided to compare the impact of xylan removal by *C. thermocellum* on glucan solubilization by the organism from dilute alkali pretreated solids with low lignin content (~8% for solids pretreated at 120°C for 60 min) compared to hydrothermal pretreated solids with high lignin content (~27% for solids pretreated at 180°C for 20 min) as shown in Figure 5.6. Both materials had high xylan contents of 21% in hydrothermal and 27% in dilute alkali pretreated solids. The result that *C. thermocellum* removed 91% xylan from dilute alkali pretreated solids but only 68% xylan from hydrothermal pretreated solids supports the argument that lignin removal increased xylan accessibility to *C. thermocellum*. This increased xylan digestion from dilute alkali pretreated biomass by *C. thermocellum* may have contributed to the higher glucan solubilization of 90% compared to only 55% from hydrothermal pretreated solids as presented in Figure 5.6(b). This interpretation is further supported by *C. thermocellum* being able to achieve only 48% glucan solubilization from unpretreated switchgrass with high lignin content for which the organism could only remove 60% of the xylan. Further, removal of acetyl and uronic acid substitutions from biomass

hemicellulose by dilute alkali pretreatments may have also contributed to the higher cellulose and hemicellulose accessiblility to digestion [4].

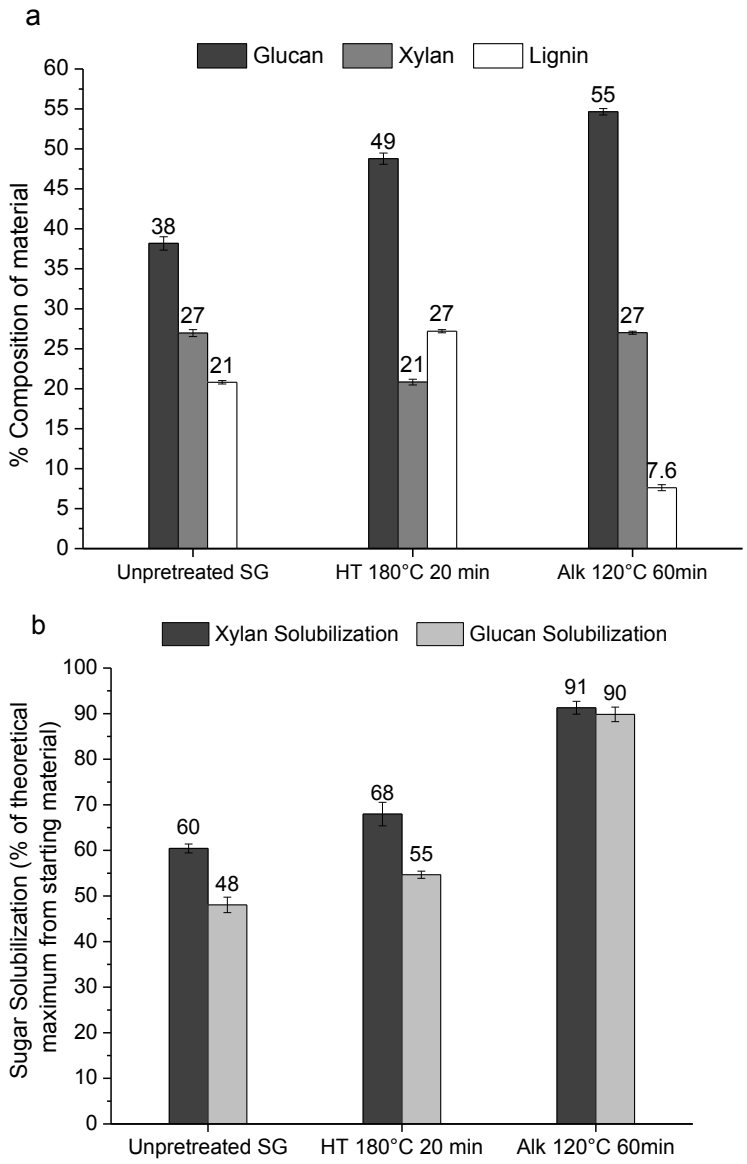

Figure 5.6. (a) % Compositions of unpretreated switchgrass (SG), hydrothermal pretreated solids at 180°C for 20 min, and dilute alkali pretreated solids at 120°C for 60 min and **(b)** corresponding glucan and xylan solubilizations by *C. thermocellum* from these materials

5.3.3. Impact of substrate composition on biological digestion

The deconstruction performance of CELF followed by CBP, was compared to deconstruction by traditional use of fungal enzymes following pretreatment by the same pretreatment methods, with results shown in Figure 5.7. For the sake of clarity, only the conditions that gave the highest total sugar release from each pretreatment coupled with CBP are shown in this Figure.

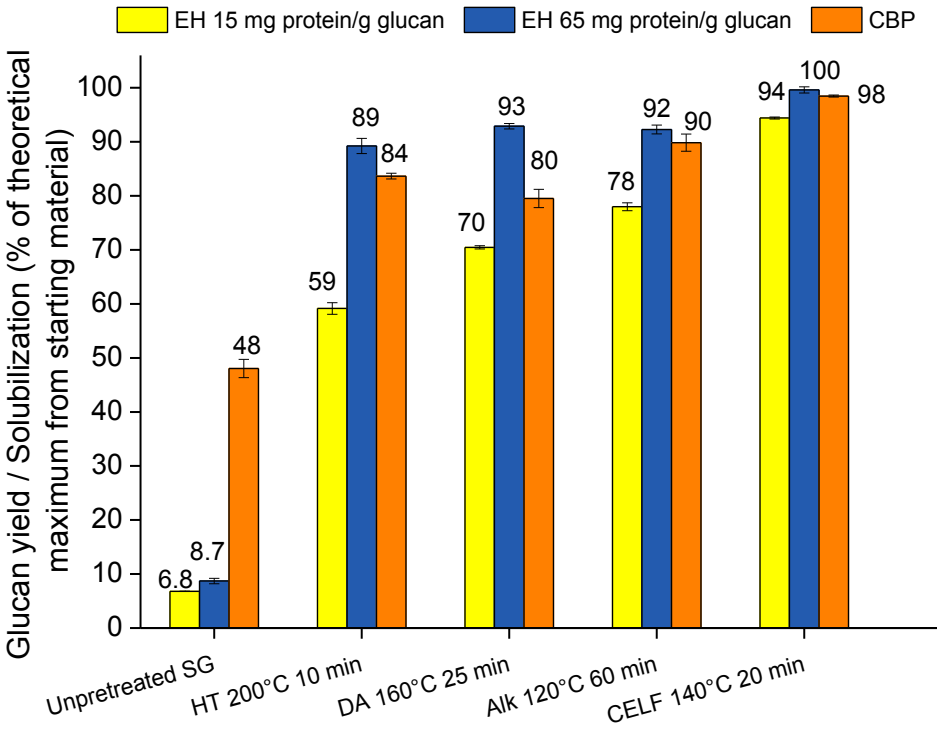

Figure 5.7. Comparison of *C. thermocellum* consolidated bioprocessing (CBP) glucan solubilizations and fungal enzymes mediated enzymatic hydrolysis (EH) glucan yields after 7 days of each biological operation for unpretreated switchgrass (SG) and solids prepared by hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreatments at conditions that gave the highest sugar release

Glucan deconstruction is measured as glucan yield in liquid for enzymatic hydrolysis and based on glucan solubilization from pretreated solids after fermentation by CBP divided by glucan initially loaded. It has been shown previously that the different methods of measuring sugar release are comparable [44]. Fungal enzymes achieved only a 7% glucan yield from unpretreated switchgrass even at a high and expensive enzyme loading of 65 mg protein / g glucan in contrast to *C. thermocellum* breaking down 48% of the glucan in unpretreated switchgrass. As a reference point, 20 mg protein / g glucan loading has been projected to cost about $1.47 / gal of ethanol [5]. Although all materials were autoclaved for sterilization prior to CBP while this was not applied prior to enzymatic hydrolysis, autoclaving conditions are far too mild to increase digestibility by *C. thermocellum* as shown in Figure 5.8. This yield difference is an important distinction between the two biological approaches and highlights the effectiveness of the *C. thermocellum* cellulosome and/or the enzyme microbe synergy observed during *C. thermocellum* digestions [45]. As discussed earlier, the better performance by CBP may be attributed to the ability of *C. thermocellum* to break down xylan in biomass to make cellulose more accessible. Others have reported synergies between xylanases and cellulases to boost hydrolysis performance on pretreated biomass by increasing cellulose accessibility either via xylan removal or increased swelling and porosity of cellulose fibers [46]. In addition, xylanases may break down the lignin carbohydrate complexes to improve substrate digestibility [47].

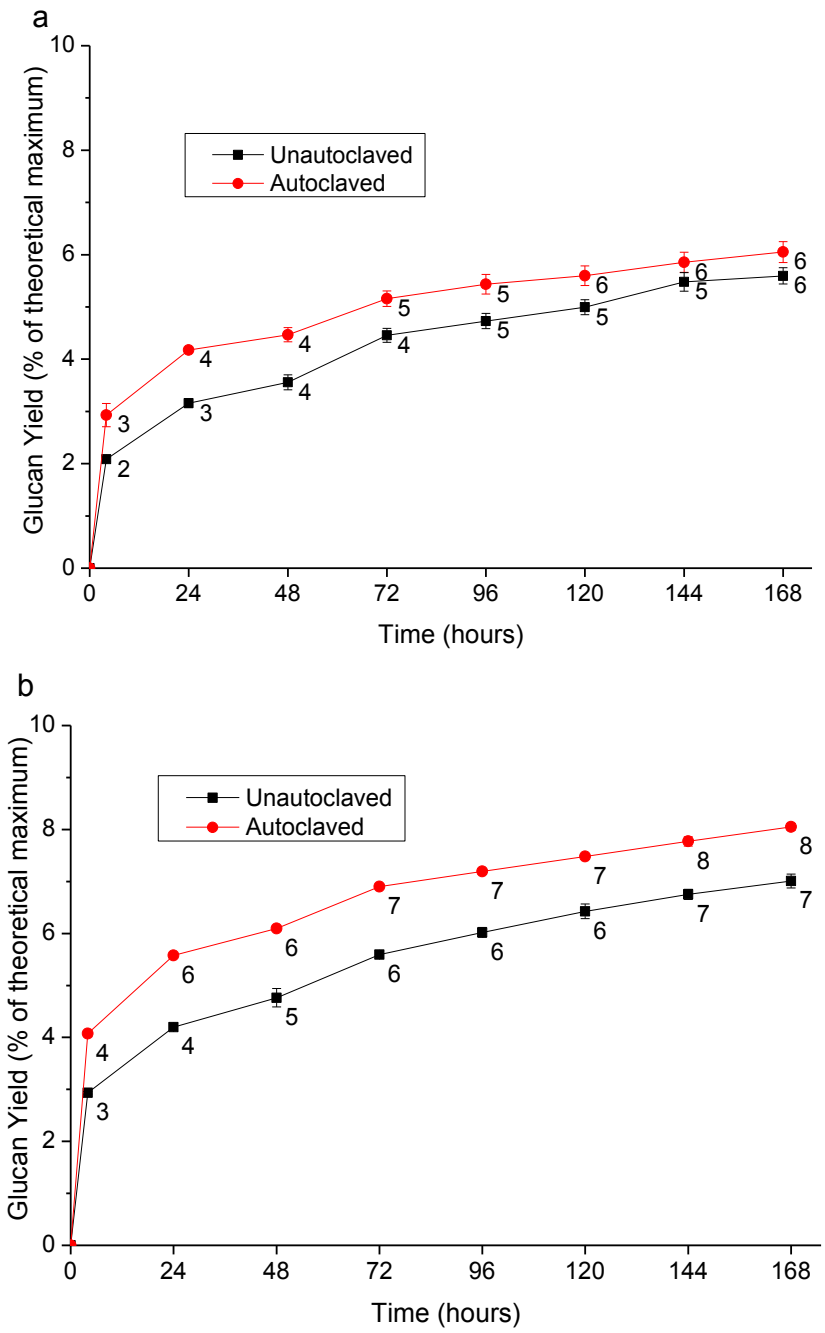

Figure 5.8. Enzymatic hydrolysis (EH) glucan yield time profile on autoclaved *vs.* unautoclaved switchgrass with **(a)** 15 mg protein / g glucan and **(b)** 65 mg protein / g glucan enzyme loadings of Accellerase® 1500.

As expected, glucan-rich material from CELF pretreated switchgrass was the most digestible irrespective of the biological system considered. Enzymatic hydrolysis at 65 mg protein / g glucan and CBP both showed almost theoretical glucan yield / solubilization from CELF pretreated solids; however, a slightly lower glucan yield resulted at 15 mg / g glucan enzyme loading. Altogether, *C. thermocellum* always performed better than fungal enzymes did at 15 mg protein / g glucan and the same as achieved by 65 mg / g glucan enzyme loadings. The presence of xylan in the substrate may have affected enzymatic hydrolysis at the lower enzyme loadings as validated by low glucan yield on hydrothermal compared to dilute acid pretreated solids at that enzyme loading. *C. thermocellum*, in contrast, showed slightly higher glucan solubilization on hydrothermal than dilute acid pretreated solids suggesting minimal impact of higher xylan present in the former on the organism. The effect of the presence of xylan in the substrate on enzymatic hydrolysis is also evident from the low glucan yield from dilute alkali pretreated solids at the low enzyme loading compared to that by *C thermocellum*. Even though enzymatic hydrolysis at the higher enzyme loading was not significantly impacted by varying substrate features, the high enzyme loading is expected to affect processing costs negatively. Overall, *C. thermocellum* CBP showed high glucan solubilization, especially on substrates with low lignin content, and is further also expected to lower fungal enzyme related processing costs.

5.3.4. Impact on glucan accessibility on metabolites production by *C. thermocellum*

The translation of glucan solubilization from biomass to production of fermentation metabolites by *C. thermocellum* is shown in Figure 5.9. Metabolites production is reported as the percentage of stoichiometric amount of glucan accounted for generation of each metabolite released based on initial glucan loaded in fermentation. 67% of initial glucan in CBP was accounted for ethanol, acetic acid, and lactic acid production by *C. thermocellum* on CELF pretreated solids, ~1% higher than metabolites production on dilute alkali, ~10% higher than on hydrothermal, and ~15% higher than on dilute acid pretreated solids and ~40% higher than metabolites production on unpretreated solids. Overall, total metabolite production correlated with an increase in cellulose macro-accessibility and solubilization on solids produced by all the pretreatments employed; higher polysaccharide solubilization translated into higher product yields. Although the amount of ethanol produced is greater from dilute alkali pretreated solids than from hydrothermal pretreated solids, the amount of acetic acid produced remains the same. This result suggests that the organism shifted its carbon flux from acetic acid to ethanol production in response to stressful conditions. A shift in carbon flux from acetic acid and ethanol production to lactic acid on CELF pretreated solids compared to metabolites produced on dilute alkali pretreated solids further confirms stressful conditions experienced by the organism. Along with direct product inhibition, pH drop due to the formation of acetic acid and other carboxylic acids could be the primary cause of these stressful conditions as shown in Chapter 3.

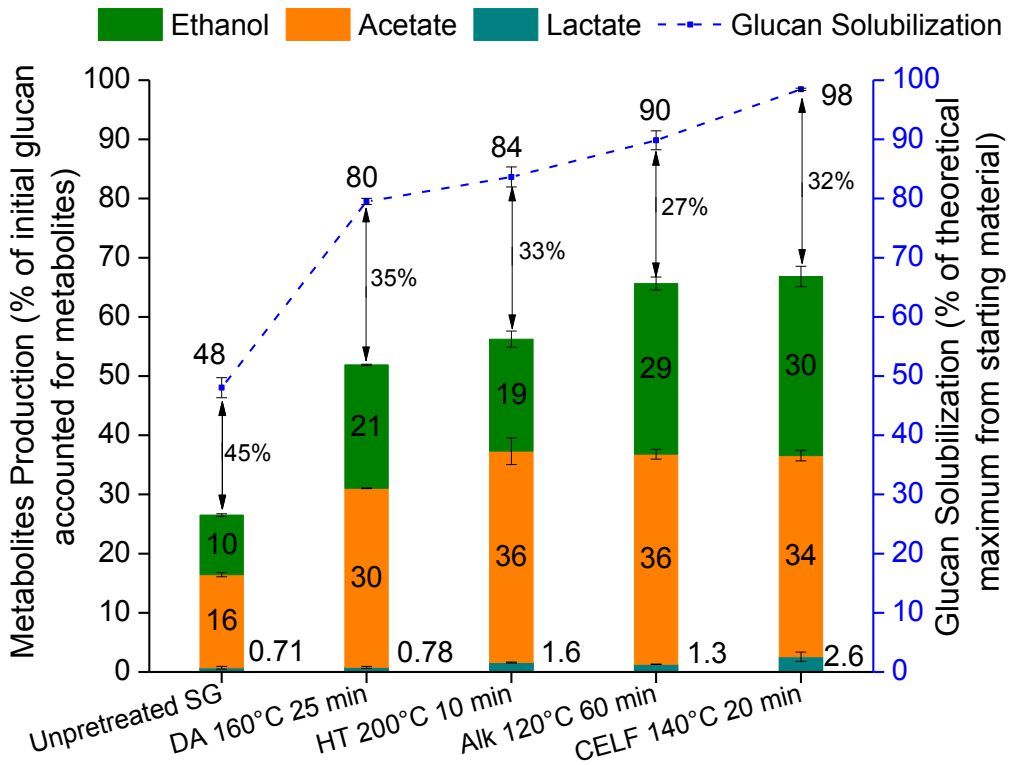

Figure 5.9. *Clostridium thermocellum* glucan solubilization and metabolite production after 7 days of fermentation on unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass. Values on the arrows indicate the percentage of solubilized glucan that is unaccounted for.

Figure 5.9 also points out that 55-73% of the solubilized glucan can be attributed to major metabolites, acetic acid, ethanol, and lactic acid; while the rest is expected to have been used for cell growth and maintenance, production of enzymes, and other products [9]. Highest fraction of solubilized glucan accounted for metabolites production by *C. thermocellum* was 73% from dilute alkali pretreated solids, suggesting that *C. thermocellum* benefits more from lignin removal than xylan removal. Although *C. thermocellum* deconstruction of CELF pretreated solids showed the greatest glucan solubilization of 98%, about 68% of this solubilized glucan was accounted for

metabolites production, 5% lower than that from dilute alkali pretreated solids. The lower utilization of solubilized glucan on CELF pretreated solids may perhaps be because the organism was inhibited by higher product concentrations, particularly that of acetic acid, and/or a drop in pH to non-optimal conditions.

5.4. Conclusions

These results provide a unique picture on how compositional differences from switchgrass prepared by four different pretreatments impact subsequent deconstruction of solids by *C. thermocellum* compared to fungal enzymatic hydrolysis. Hydrothermal, dilute acid, dilute alkali, and CELF pretreatments substantially increased glucan solubilization compared to that realized from unpretreated switchgrass. Nonetheless, *C. thermocellum* was capable of achieving ~48% glucan solubilization on unpretreated switchgrass compared to about 7% glucan yield by fungal enzymes. The dramatic difference in deconstruction performance showed that *C. thermocellum* can break down lignocellulosic biomass more effectively than fungal enzymes. The fact that dilute alkali pretreatment increased glucan solubilization by *C. thermocellum* to ~90%, higher than from solids produced by either hydrothermal or dilute acid pretreatments at reasonable conditions indicates that lignin removal from switchgrass had a greater effect on total sugar release than xylan removal and/or lignin relocation. *C. thermocellum* was not adversely affected by the presence of xylan in the biomass (~2.5 g/L during fermentation at 5 g/L or 0.5 wt% glucan loading) even though the wild type strain of the organism is not known to ferment xylose or xylooligomers [19]. Such benefits of *C. thermocellum*'s

complex cellulosome can be useful is selecting industrial scale pretreatments in the future. However, *C. thermocellum* is shown to be inhibited at higher concentrations of xylose and xylo-oligomers during fermentation at higher solid loadings [48]. Nearly theoretical and rapid deconstruction of CELF pretreated solids by *C. thermocellum* shows that glucan accessibility drives biomass digestion by the organism. Further, the trends in production of metabolites, ethanol, acetic acid, and lactic acid, by *C. thermocellum* correlated well with trends in its glucan solubilization from pretreated solids. These results suggest that CELF pretreatment combined with *C. thermocellum* CBP could serve as a useful reference point against which to measure deconstruction of lignocellulosic biomass in addition to offering a promising process of converting biomass to ethanol with high yields without added enzymes. At this point it also important point out the critical differences in processing attributes and their effects on costs associated with each pretreatment. Hydrothermal pretreatments have the advantage of not requiring neutralization or conditioning because of no use of chemicals but require higher pretreatment temperature and, therefore, pressure to produce digestible solids. Temperature, as high as 200°C, was required for hydrothermal pretreatments to achieve the same total sugar release as dilute acid pretreatments at 160°C. However, dilute acid pretreatment requires expensive reactor materials of construction due to the corrosive nature of sulfuric acid, cost of acid, challenges in mixing acid, the need for acid neutralization [4] , and sugar degradation during hydrolysate conditioning prior to fermentation. Dilute alkali pretreatments have the advantage of being effective at low temperature conditions, with only 120°C being sufficient to be effective for this study.

However, the pretreatment time is longer, ranging from 1 to 2 h in this study. Moreover, alkali is more expensive than sulfuric acid, and removal and recovery of alkali is difficult [4]. Polar aprotic solvent THF used for CELF pretreatment enhances the performance of dilute acid and can be produced sustainably from biomass [32]. Moreover, its low boiling point ~66°C makes it easy to be recovered and recycled after pretreatment. Removing THF from the liquid stream produced by CELF for recycle precipitates lignin as a solid to leave an aqueous phase rich in hemicellulose sugars. These sugars are then available for fermentation after conditioning of the liquor.

5.5. Materials and methods

5.5.1. Substrate

Five year old fully mature Alamo switchgrass harvested in January 2014 that had been chopped to an approximate size of ¾ inch was obtained from Genera Energy Inc. The composition of unpretreated milled Alamo switchgrass was determined to be 38.18 (±0.8) % glucan, 26.96 (±0.4) % xylan, 2.97 (±0.05) % arabinan, and 20.8 (±0.2) % K-lignin. This biomass was completely mixed before dividing and transferring it into multiple gallon sized bags that were stored in a freezer. The entire contents of each bag were knife milled by Thomas Wiley® mill (Model 4, Thomas Scientific, Swedesboro NJ) equipped with a 1 mm sieve. The resulting milled biomass was mixed thoroughly before each use.

5.5.2. Switchgrass pretreatment

Hydrothermal pretreatments were performed at 180°C for 20, 30, and 40 min and 200°C for 10 and 20 min. Dilute acid pretreatments were done at 160°C for 10, 20, 25, and 30 min, whereas, dilute alkali pretreatments were done at 120°C for 60, 90, and 120 min. Co-solvent enhanced lignocellulosic fractionation (CELF) pretreatments were performed at 140°C for 20, 30, 40, and 50 min and 150°C for 10, 20, 25, and 30 min. Dilute acid and CELF pretreatments used 0.5 wt% sulfuric acid, whereas dilute alkali pretreatments were with 1 wt% sodium hydroxide. CELF pretreatment employed tetrahydrofuran (THF) as co-solvent at a 1:1 volume ratio with water/dilute sulfuric acid solution. These pretreatment conditions were based on prior work and reported in the literature, [32, 49-52] and because the results followed trends consistent with prior results in most cases, the pretreatments were performed once without replicates. A 10 wt% solids loading was fed to all pretreatments based on a total of 800 g reaction mass. Before pretreatment, biomass was soaked overnight with the other ingredients at room temperature. The biomass for CELF, however, was soaked at 4°C to minimize solvent evaporation. A 1 L Hastelloy Parr reactor (236HC series, Parr Instruments Co., Moline, IL) equipped with a double stacked pitch blade impeller rotating at 200 rpm was used for all pretreatments. A 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ) maintained the pretreatment temperature within ± 2°C. The reactor temperature was measured by a K-type thermocouple probe (CAIN-18G-18, Omega Engineering Co., Stamford, CT, USA). The heat up time of the reactor contents from 30°C to the desired temperature was usually between 3-4 min. At the completion of the target pretreatment

time, the reactor was lowered into a room temperature water bath to cool its contents to ~80°C in about 2 min. The contents of the reactor were then vacuum filtered at room temperature using a glass fiber filter paper, and a sample of the pretreatment liquor was taken for analysis. The solids were thoroughly rinsed with room temperature deionized water to remove any soluble sugars, degradation products, acid/alkali, and solvents. Since the co-solvent mixture had a different density than pure water, density of the CELF pretreatment liquor was determined by weighing 25 mL of the liquor in a volumetric flask immediately after the reaction. The composition of the liquor was measured by HPLC following the standard NREL Laboratory Analytical Procedure "Determination of sugars, byproducts, and degradation products in liquid fraction process samples".[53] The sugar yields reported in the pretreatment liquor included both monomers and oligomers. The rinsed solids were subjected to further biological deconstruction using either *Clostridium thermocellum* or fungal enzymes.

5.5.3. *Clostridium thermocellum* consolidated bioprocessing

The DSM 1313 wild type strain of *C. thermocellum* employed for all experiments were kindly provided by Dr. Lee Lynd at Dartmouth College, Hanover NH. A stock culture was grown for 8.5 hours in a 500 mL anaerobic media bottle (Chemglass Life Sciences, Vineland NJ) and aseptically transferred to 5 mL serum vials for storage at -80°C. A 2% by volume inoculum of these stock cultures were used to prepare the seed cultures grown with a 5 g/L glucan loading of Avicel® PH-101 (11365, Lot No. BCBN7864V, Sigma Aldrich, St. Louis, MO) in a 50 mL working volume for 8-9 hours in Media for

Thermophilic Clostridia (MTC) without trace minerals (see Table 3.1). The pellet nitrogen content and metabolites production by *C. thermocellum* grown in this manner in a working volume of 200 mL is shown in Figure 3.11. Pellet nitrogen content was analyzed according to methods published in literature [9, 54]. The seed culture was stored overnight in a refrigerator for about the same time for each experiment before inoculation the next day. The concentrations of different media solutions prepared separately and purged with nitrogen are shown in Table 3.1. All solutions except the vitamins solution were sterilized by autoclaving, whereas the vitamins solution was sterilized by passing it through 28 mm diameter polyethersulfone (PES) syringe filters with 0.2 μm pores (Corning® Life Sciences, Tewksbury MA). Consolidated bioprocessing (CBP) was performed in 125 mL bottles (Wheaton, Millville NJ) at a 0.5 wt% glucan loading of unpretreated or pretreated biomass in triplicates at a working mass of 50 g. Bottles containing biomass and water were purged with repeated 45 seconds application of vacuum and 14 psi nitrogen over a total of 27-30 min and then sterilized by autoclaving at 121°C for 35 min. The bottles were incubated at 60°C at a shaking speed of 180 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD) after the injection of all media solutions and 2% by volume inoculum. Insoluble solids left after CBP were recovered and rinsed thoroughly. Compositional analysis was performed on these CBP residues to determine carbohydrates left in the solids from which solubilization could be calculated. The solids were sampled in triplicates at 1, 2, 5, and 7 days to determine the carbohydrate solubilization time profile. This approach of measuring solubilization was followed because sugars released in solution are immediately used by *C. thermocellum* for

fermentation to metabolites and are not left in solution as they would be for fungal enzymatic hydrolysis. The fermentation liquor was also analyzed after 7 days of fermentation to determine production of ethanol, lactic acid, and acetic acid. The liquor samples were passed through 28 mm diameter polyethersulfone (PES) syringe filters with 0.2 μm pores (Corning® Life Sciences, Tewksbury, MA) into 1.5 mL Simport® microcentrifuge tubes (Spectrum® Chemical Manufacturing Corporation, New Brunswick, NJ). These tubes were centrifuged at 15,000 rpm for 10 min and the supernatant was analyzed by HPLC.

5.5.4. Enzymatic saccharification

Unpretreated and pretreated switchgrass solids were enzymatically hydrolyzed at a loading of 0.5 wt% glucan and working mass of 50 g in 125 mL Erlenmeyer flasks in triplicates following the NREL Laboratory Analytical Procedure "Enzymatic Saccharification of Lignocellulosic Biomass" [55]. Accellerase® 1500 cellulase (DuPont Industrial Biosciences, Palo Alto CA) was used at 15 and 65 mg / g glucan protein loadings for all experiments, with the loadings based on the amount of glucan in unpretreated switchgrass as described elsewhere so as to not penalize a pretreatment for releasing more sugars before enzymatic saccharification [52, 56]. The BCA protein content of Accellerase® 1500 was reported elsewhere to be 82 mg/mL [57]. The flasks were incubated at 50°C and 150 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD) and allowed to equilibrate before adding the enzyme solution. 1 mL representative samples including the insoluble substrate and liquor were collected from each flask after

4 hours, 24 hours, and every 24 hour period thereafter for a total of 7 days or 168 hours in 1.5 mL Simport® microcentrifuge tubes (Spectrum® Chemical Manufacturing Corporation, New Brunswick, NJ). These tubes were centrifuged at 15,000 rpm for 10 min and the supernatant was analyzed by HPLC.

5.5.5. Compositional analysis of solids

Prior to analysis, unpretreated and pretreated solids were dried for two days to constant moisture content in a 40°C incubator oven. CBP residues were dried to constant weight in a 60°C oven. The compositions of unpretreated and rinsed pretreated switchgrass and consolidated bioprocessing (CBP) residues were determined in triplicates according to the standard NREL Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin in Lignocellulosic Biomass" [58]. If the amount of material was insufficient to meet the NREL specified amount, the amounts of ingredients were modified proportionately. Glucan, xylan, arabinan, Klason-lignin (K-Lignin), and ash were measured in this manner, with lignin accounting for all the acid insoluble lignin in the biomass.

5.5.6. Analytical procedures

Analysis of all the liquid samples was by a Waters Alliance e2695 HPLC system (Waters Co., Milford MA) equipped with a Bio-Rad Aminex HPX-87H column and a Waters 2414 refractive index detector. 5 mM sulfuric acid was the eluent at a flow of 0.6 mL/min. Integration of the chromatograms was by the Empower™ 2 software package.

All experiments and analysis were done in triplicates, unless otherwise specified. Error bars were calculated as the standard deviation for the triplicates, unless otherwise specified. Even though the pretreatments were only performed once the compositional analysis for all pretreated solids and pretreatment liquor was done in triplicates. Error for total sugar release was calculated based on a combination of standard deviation of glucan and xylan solubilization in CBP, glucan and xylan yields in pretreatment liquor, and glucan and xylan composition of pretreated solids following statistical rules for combining standard deviation.

5.5.7. Calculations

Sugar yield, conversion, and release were expressed in terms of the polymeric form of the sugar throughout this paper with anhydrous correction factors converting monomeric sugar concentrations measured by HPLC to the corresponding polymer carbohydrate. Thus, the amount of glucose measured via HPLC can be converted to the equivalent amount glucan by multiplying the glucose amount by 0.9, while the factors for translating xylose to xylan and arabinose to arabinan are 0.88. The glucan release from the biomass for CBP is measured in terms of glucan solubilization and that for enzymatic saccharification is measured as glucan yield as described in the main text and calculations shown below. Stage 1 sugar release refers to sugars captured in the pretreatment liquor, whereas, the Stage 2 sugar release refers to sugar solubilized by *C. thermocellum* from pretreated solids; both measurements are based on the initial amount of glucan plus xylan in unpretreated switchgrass. The term ‘sugar release’ includes both glucan and xylan in

the calculations unless specified otherwise. Stage 1 and Stage 2 sugar releases combined are termed total sugar release. All of these sugar release calculations are done based on a dry mass basis. Detailed calculations are shown below.

1. Mass of (dry) biomass to be loaded (g) =

$$(\%\ Solids\ Loading\ Fraction) \times (Total\ Reaction\ Mass\ (g))$$

2. Mass of (wet) biomass loaded in Parr reactor for pretreatment (g) =

$$\frac{(\%\ Solids\ Loading) \times (Total\ Reaction\ Mass\ (g))}{(100 - \%\ Moisture\ Content)}$$

3. Mass of total liquid to be loaded for pretreatment (g) =

$$Total\ Reaction\ Mass\ (g) - Mass\ of\ (Wet) Biomass\ Loaded (g)$$

4. Mass of liquid present before pretreatment (g) =

$$Total\ Reaction\ Mass\ (g) - Mass\ of\ (Dry) Biomass\ Loaded (g)$$

5. Acid/alkali loading (g) =

$$\frac{(Mass\ of\ Liquid\ Present\ Before\ Pretreatment) \times (\%\ Acid\ Loading)}{(\%\ Concentration\ of\ Acid\ Solution)}$$

6. Pretreatment % solids yield =

$$\frac{Mass\ of\ Dry\ Solids\ Recovered\ After\ Pretreatment\ (g)}{Mass\ of\ (Dry) Biomass\ Loaded\ before\ Pretreatment\ (g)} \times 100$$

7. Mass of liquid after pretreatment (g) =

$$Total\ Reaction\ Mass\ (g) - Mass\ of\ Dry\ Solids\ Recovered\ After\ Pretreatment (g)$$

8. Volume of liquid after pretreatment (L) =

$$\frac{Mass\ of\ Liquid\ After\ Pretreatment\ (g)}{Density\ of\ Liquid\ After\ Pretreatment\ (g/L)}$$

(Density of liquid measured for CELF pretreatment and assumed 1 g/mL for aqueous pretreatments)

9. % Stage 1 glucan or xylan release =

$$\frac{Concentration\ of\ Sugar\ Monomer\ in\ Liquid\ After\ Pretreatment\ (g/L) \times Volume\ of\ Liquid\ After\ Pretreatment\ (L) \times Anhydrous\ Factor}{Mass\ of\ Glucan\ Plus\ Xylan\ in\ Solids\ Before\ Pretreatment\ (g)} \times 100$$

10. CBP % sugar solubilization =

$$\frac{Mass\ of\ Sugar\ Polymer\ in\ Solids\ before\ CBP\ (g) - (\%\ Sugar\ Polymer\ in\ Solids\ After\ CBP \times Mass\ of\ Dry\ Solids\ After\ CBP\ (g))}{Mass\ of\ Sugar\ Polymer\ in\ Solids\ before\ CBP\ (g)} \times 100$$

11. Enzyme loading in enzymatic hydrolysis, mg protein/g glucan in unpretreated biomass =

$$mg\ Protein\ Per\ g\ Glucan\ Loaded \times \frac{Mass\ of\ Glucan\ in\ (Dry) Biomass\ Before\ Pretreatment}{Mass\ of\ Glucan\ in\ Dry\ Solids\ After\ Pretreatment}$$

12. Enzymatic hydrolysis % sugar yield =

$$\frac{Concentration\ of\ Sugar\ Monomer\ Measured\ in\ Liquid\ (g/L) \times (Working\ Volume\ \ (L) \times Anhydrous\ Factor}{Mass\ of\ Sugar\ Polymer\ before\ hydrolysis\ (g)} \times 100$$

13. % Stage 2 glucan or xylan release =

$$\frac{\%\ Sugar\ Yield\ or\ Solubilzation \times \%\ Sugar\ Polymer\ in\ Pretreated\ Solids \times Mass\ of\ Dry\ Solids\ Recovered\ After\ Pretreatment\ (g)}{Mass\ of\ Glucan\ Plus\ Xylan\ in\ Solids\ Before\ Pretreatment\ (g)} \times 100$$

14. % Total sugar release =

$$\%\ Stage\ 1\ Glucan\ Release + \%\ Stage\ 1\ Xylan\ Release + \%\ Stage\ 2\ Glucan\ Release + \%\ Stage\ 2\ Xylan\ Release$$

15. CBP metabolites production (g metabolites starting with 100 g glucan) =

$$\frac{(Metabolites\ Concentration\ in\ Liquid\ (g/L) \times Working\ Volume\ in\ Flask\ (L))}{Mass\ of\ Initial\ Glucan\ Loaded\ for\ CBP\ (g)} \times 100$$

16. CBP metabolites production (% of initial glucan accounted for metabolites)

$$\frac{CBP\ Metabolites\ Production\ (g\ Metabolite\ Starting\ with\ 100\ Glucan) \times Anhydrous\ Factor}{Stoichometric\ Factor \times Molar\ Mass\ Ratio}$$

17. Stoichiometric Factor (for a balanced glucose to metabolite reaction) =

$$\frac{Stoichiometric\ Coefficient\ of\ Metabolite}{Stiochiometric\ Coefficient\ of\ Glucose}$$

18. Molar Mass Ratio =

$$\frac{Molar\ Mass\ of\ Metabolite}{Molar\ Mass\ of\ Glucose}$$

19. CBP metabolites production (% of solubilized glucan accounted for metabolites) =

$$\frac{CBP\ metabolites\ production\ (\%\ of\ initial\ glucan\ accounted\ for\ metabolites)}{CBP\ glucan\ solubilization} \times 100$$

20. % solubilized glucan unaccounted =

$$\frac{CBP\ glucan\ solubilization - CBP\ metabolites\ production\ (\%\ of\ initial\ glucan\ accounted\ for\ metabolites)}{CBP\ glucan\ solubilization} \times 100$$

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Chapter 6. Understanding the mechanism of thermochemical and biological breakdown of switchgrass*

The contents of this chapter will be used for publication in a scientific journal in part or in full

*This chapter was completed in collaboration between University of California Riverside[1], University of Tennessee Knoxville[2], and Oak Ridge National Laboratory[3]. *C. thermocellum* fermentations and fungal enzymatic hydrolysis experimentation and experimental design, planning, and management of project was done by Ninad Kothari[1]. Simons' staining and scanning electron microscopy were performed by Samarthya Bhagia[2], solid state nuclear magnetic resonance, 2D HSQC nuclear magnetic resonance, and gel permeation chromatography were performed by Yunqiao Pu[2,3], Mi Li[2], and Chang Geun Yoo[2,3].

6.1. Abstract

Complex lignocellulosic biomass can be deconstructed for fuel ethanol production that has the highest potential to alleviate high petroleum demands for transportation. Biomass recalcitrance hinders effective deconstruction of the substrate to simpler, fermentable sugars required by microorganisms to produce ethanol. A combination of thermochemical or mechanical biomass augmentation and biological digestion techniques have been developed to aid successful biomass deconstruction. Promising pretreatment technologies, such as, hydrothermal, dilute acid, dilute alkali, and co-solvent enhanced lignocellulosic fraction (CELF) followed by fungal enzymatic and bacterial hydrolysis and fermentation have been studied for effective performance. However, achieving complete digestion of polysaccharides from biomass for theoretical recovery and utilization of sugars aimed at high ethanol yield and titers requires thorough process optimization and understanding of mechanism of biomass deconstruction. This study aims at understanding digestion of switchgrass by the above mentioned thermochemical pretreatments in combination with *Clostridium thermocellum* consolidated bioprocessing (CBP) compared to traditional fungal enzymatic hydrolysis (EH). Unpretreated and pretreated switchgrass and their CBP and EH residues were extensively characterized by a suite of analytical techniques to provide an understanding of structural changes in switchgrass occurring during deconstruction. CELF pretreated solids showed the highest accessibility measured via Simons' staining and digestibility by both fungal enzymes and *C. thermocellum* followed by dilute alkali and dilute acid / hydrothermal pretreated solids. Lignin removal from the biomass had a more positive impact on substrate

accessibility and digestibility than xylan removal. Unlike dilute alkali pretreatment, acid based pretreatments showed an increase in cellulose crystallinity in the following order: hydrothermal > dilute acid > CELF and also reduced cellulose degree of polymerization substantially. All thermochemical and biological digestion approaches led to an increase in Syringyl to guaiacyl lignin (S/G) ratio and a decrease in β-O-4 lignin interunit linkage and hydroxycinnamates content of unpretreated switchgrass. High pretreatment temperature and use of alkali as catalyst led to a decrease in G lignin during pretreatment. G lignin potentially leads to less crosslinking producing thinner cell walls that can easily be deconstructed. Both biological digestion approaches led to a decrease in either S or G lignin that was carbohydrate associated.

6.2. Introduction:

Lignocellulosic biomass cell wall structure is comprised of cellulose, hemicellulose, and lignin making up the lignocellulosic matrix [1, 2]. Cellulose and hemicellulose from the biomass can be broken down to simpler sugars that can be fermented to ethanol and other useful metabolites. However, the complex cell wall structure in plant biomass is aimed at plant survival in the environment against physical, chemical, and biological breakdown [2]. Even though ethanol production from lignocellulosic biomass has been studied extensively, biomass recalcitrance is still a hindrance that has to be overcome for effective recovery of simple, fermentable sugars [2-6]. The traditional approach of ethanol production from lignocellulosic biomass thus involves particle size reduction, biomass pretreatment, enzyme production, enzymatic

saccharification, hexose fermentation, pentose fermentation, and product recovery [4-6]. A separate enzyme production step, typically using *Trichoderma reesei*, is necessary and is also the most expensive operation in this process [7]. Biomass augmentation is therefore used to aid fungal enzymes in biomass digestion thereby reducing enzyme dosage and associated costs required for high sugar yields [5, 6, 8-14]. In contrast, consolidated bioprocessing (CBP) is a simple process that combines enzyme production, enzymatic hydrolysis, and fermentation into one operation [15-22]. *Clostridium thermocellum* is a promising native cellulolytic strategy CBP organism that can produce a complex, multi-functional cellulosome to digest lignocellulosic biomass [19, 22-25]. *C. thermocellum* is further able to utilize the simpler sugars obtained after biomass digestion to produce ethanol and other useful metabolites. However, biomass augmentation is still essential in achieving high polysaccharides solubilization and metabolite production by *C. thermocellum* [13, 14, 26-28]

A number of biomass structural features including lignin, cellulose, hemicellulose, and other glycan characteristics impact both thermochemical and biological deconstruction of biomass. These different biomass deconstruction techniques in turn affect the biomass and its properties uniquely. Unlike fungal enzymes, *C. thermocellum* is unaffected by cellulose micro-accessibility that is influenced by cellulose crystallinity, water retention value, surface area, molecular weight, etc. reported in Chapter 4. A number of other reports have shown the impact of cellulose crystallinity, degree of polymerization and other properties on the extent of biological digestion of

lignocellulosics, especially by fungal enzymes [29-37]. Further, cellulose macro-accessibility or physical availability, especially that influenced by the presence of bulk lignin, drives *C. thermocellum* digestion as reported in Chapter 5. Dumitrache et al. have shown that *C. thermocellum* ATCC 27405 was sensitive to the composition of biomass, particularly the presence of lignin, and that high syringyl to guaiacyl lignin (S/G) ratio was correlated with increased cellulose accessibility. They showed 2.9 times higher fermentation ethanol yield on *Populus* genotype with a higher S/G ratio [38]. High syringyl content was also related to high molecular weight lignin or longer lignin chains with a reduced impact on enzymatic activity [38]. Yee et al. and Fu et al. have shown that the downregulation of caffeic acid-O-methyl transferase (COMT) gene in lignin biosynthesis pathway in switchgrass led to increased digestion and ethanol production by *C. thermocellum* while requiring milder pretreatment conditions as opposed to the wild type plant biomass [39-41]. Similarly, low recalcitrant poplar natural variants SKWE 24-2 and BESC 876 with mutations in their 5-enolpyruvylshikimate-2-phosphate (EPSP) synthase gene affecting lignin biosynthesis were shown to have properties different from those of BESC standard poplar natural variant by Thomas et al [28]. Specifically, SKWE 24-2 and BESC 876 had loosely held cell wall structures, high water retention value, and high S/G ratio compared to the more recalcitrant BESC standard poplar. These properties of the low recalcitrant lines were shown to have a positive impact on glucan solubilization in the pretreated solids by *C. thermocellum.* Further, *C. thermocellum* showed increased glucan solubilization and a reduction in lignin molecular weight compared to that achieved by fungal enzymes [28]. Biomass accessibility, lignin, and

structural features of lignin have been shown to impact and be impacted by *C. thermocellum* fermentations [42, 43]. Sugar release via hydrothermal pretreatment and fungal enzymatic digestion is also affected by *Populus trichocarpa* lignin content and composition [44]. Hydroxycinnamates involved in lignin carbohydrate complexes have further been shown to increase biomass recalcitrance by increasing the proximity of lignin to polysaccharides [45]. Different lignocellulosic biomass are known to have varying lignin interunit linkages, to an extent depending on lignin composition (S,G, and H lignin), that are broken down during biomass digestion to varying degrees [43]. Further, thermochemical pretreatments affect lignin and cellulose characteristics substantially [44, 46-48].

Thus, a number of factors impact lignocellulosic biomass bulk composition and substrate properties which in turn impact biological digestion. Biomass type, species, genetic modifications, location, growth conditions, fertilizers and water used, soil salinity, harvesting and storage conditions, etc. are all expected to impact biomass that is ultimately used for ethanol production [49-57]. In light of the complexity of lignocellulosic biomass, it is important to reduce / eliminate process sensitivity to such variation in biomass structure, composition, and properties. To aid feedstock-independent process development a thorough understanding of biomass properties, the impact of different substrates and their characteristics on digestion, and overall mechanism of biomass digestion is essential. Specifically, *C. thermocellum* is known to adapt its cellulosomal composition based on the substrate it encounters to better digest the

substrate and is therefore a step toward a feedstock-independent process [25]. Further, biological digestion by fungal enzymes and *C. thermocellum* and the impact of various substrate properties on the two biological approaches are expected to be different. Therefore, here, a suite of techniques were employed to characterize unpretreated switchgrass compared to hydrothermal, dilute acid, dilute alkali, and co-solvent enhanced lignocellulose fractionation (CELF) pretreated switchgrass to determine changes in the substrate during pretreatement and the impact of substrate properties on the ability of *C. thermocellum* and fungal enzymes to digest these substrates. Further, residues left undigested after CBP and fungal enzymatic hydrolysis were characterized to be able to elucidate the biological digestion process. First, the extent of glucan digestion of unpretreated and pretreated switchgrass by both fungal enzymatic hydrolysis and *C. thermocellum* CBP was determined. We related cellulose digestion to cellulose accessibility of unpretreated and pretreated switchgrass determined via Simons' staining technique. We further compared scanning electron microscope (SEM) images of all materials including the CBP and EH residues to compare the distinctive physical changes occurring during thermochemical and biological digestion of switchgrass. Then we looked at changes in cellulose crystallinity and degree of polymerization throughout the digestion process to relate the impact of thermochemical and biological digestion on cellulose in the substrate. We also characterized lignin isolated from all materials to determine relative abundances of syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) lignin, lignin interunit linkages (β-O-4, β-β, and β-5), and hydrxyocinnamates (ferulate and *p*-coumarate) involved in lignin carbohydrate complexes (LCC). Such a

comprehensive and unrivaled characterization of a wide variety of materials was performed to reveal biomass structural changes during thermochemical and biological digestion of switchgrass with the goal of understanding the impact of these changes on the extent of digestion.

6.3. Results and discussions:

6.3.1. Impact of pretreatment on the substrate and its biological digestion

Alamo switchgrass was pretreated using four different pretreatment technologies: hydrothermal, dilute acid, dilute alkali, and CELF. These pretreatments were chosen because of their ability to produce solids with varying compositional characteristics [58, 59] also shown in previous chapters of this dissertation. These biomass treatment techniques also represent a variety in thermochemical pretreatments with the use of distinct catalysts aimed at helping us understand the mechanism of thermochemical digestion of switchgrass. Optimization of these pretreatments on switchgrass for maximum total sugar release (glucan + xylan) from pretreatment and *C. thermocellum* CBP combined was reported in Chapter 5. The percentage composition of solids produced after hydrothermal, dilute acid, dilute alkali, and CELF pretreatments of switchgrass performed at optimized conditions for maximum total sugar release are shown in Figure 6.1.

Hydrothermal and dilute acid pretreatments are acid based pretreatments that focus on hemicellulose removal as evidenced by high xylan and arabinan removal from

switchgrass after these pretreatments leaving behind solids with very low (<7%) xylan content and no arabinan. Hydrothermal and dilute acid pretreatments at conditions chosen for this work achieved 85% and 94% xylan removal, respectively, but removed only 19% and 4% lignin, respectively. Higher lignin removal during hydrothermal pretreatment was possibly due to the much higher pretreatment temperature of 200ºC employed for this pretreatment compared to 160ºC used for dilute acid pretreatment. This could be coupled with possible pseudo lignin formation during dilute acid pretreatment due to degradation of carbohydrates during pretreatment leading to an apparent increase in mass of lignin measured in the dilute acid pretreated solids.

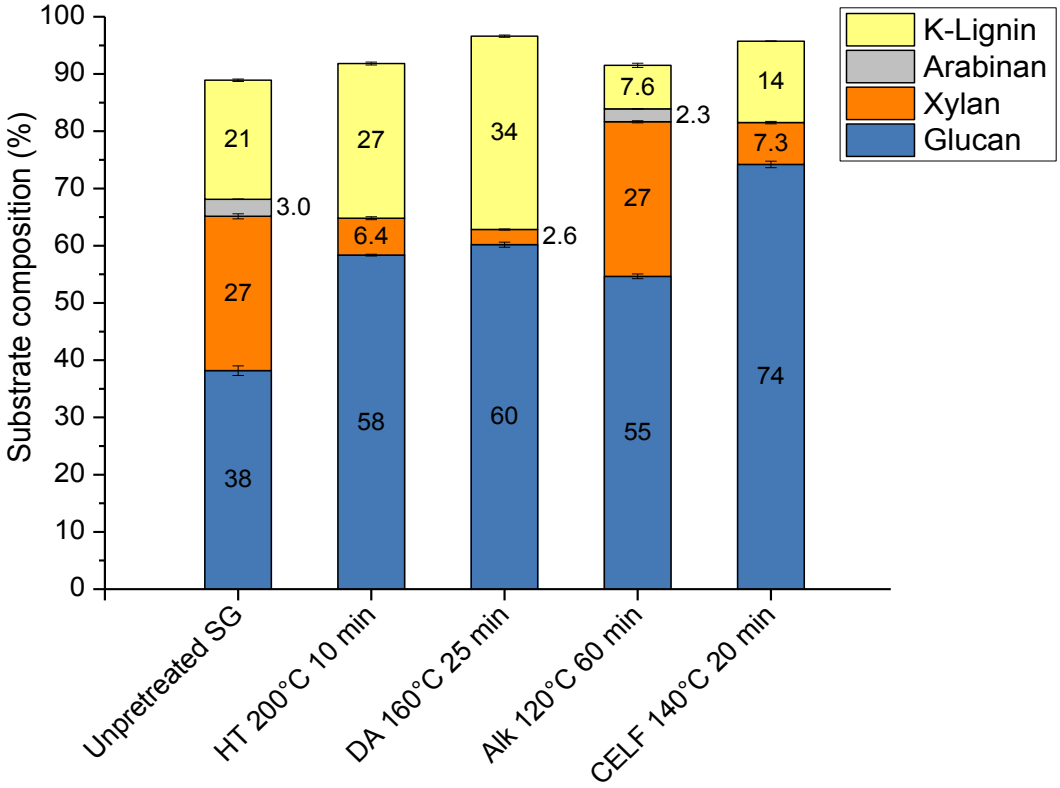


Figure 6.1. Composition of unpretreated switchgrass (SG) and solids produced by hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fraction (CELF) pretreatments of SG performed at optimized conditions for maximum sugar release for each pretreatment technology

Dilute alkali pretreatment on the other hand removed 75% lignin but removed only 32% xylan and therefore produced solids with high xylan content (~27%). CELF pretreatment removed both lignin and xylan in large quantities achieving 67% and 87% lignin and xylan removal, respectively. Thus, CELF pretreated solids showed the highest glucan content amounting to 74% glucan as opposed to 38%, 58%. 60%, and 55% glucan in unpretreated switchgrass and hydrothermal, dilute acid, and dilute alkali pretreated solids, respectively as reported in Figure 6.1 and Chapter 5. SEM images of hydrothermal and dilute acid pretreated solids showed striations and surface removal of matter when compared to unpretreated switchgrass, representing xylan removal as shown in Figure 6.2. Dilute alkali pretreated solids looked less ordered and more crumpled compared to dilute acid and hydrothermal pretreated solids representing lignin removal from switchgrass. CELF pretreated solids showed a striated structure similar to the other acid-based pretreatments along with deeper removal of matter compared to other pretreatments due to the high removal of both xylan and lignin from these solids.

By measuring the amount of Direct Orange 15 dye adsorbed by a substrate, the cellulose surface area and thus cellulose accessibility can be determined semi-quantitatively [38, 42, 53]. The high molecular fraction of Direct Orange 15 has been shown to have a high affinity to cellulose, as opposed to other components of the plant cell wall structure, and is similar to cellulases based on size [60-62]. Cellulose accessibility of unpretreated and pretreated substrates used in this study was found to be

in the following order: CELF = dilute alkali > dilute acid > hydrothermal > unpretreated switchgrass as shown in Figure 6.3.

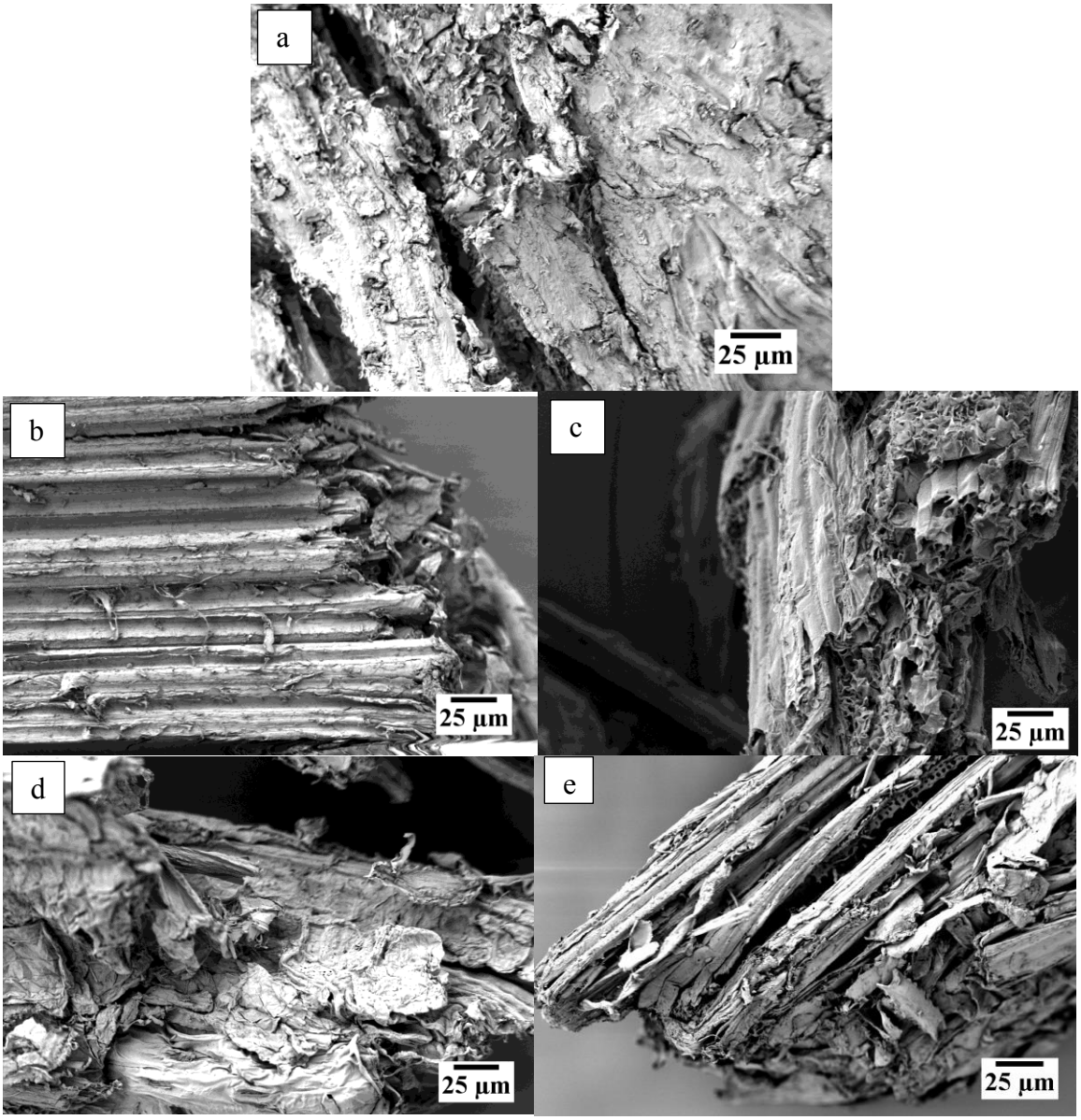


Figure 6.2. Scanning electron microscope (SEM) images of **(a)** unpretreated switchgrass and **(b)** hydrothermal (200ºC for 10 min), **(c)** dilute acid (160ºC for 25 min), **(d)** dilute alkali (120ºC for 60 min), and **(e)** co-solvent enhanced lignocellulosic fraction (CELF) (140ºC for 20 min) pretreated switchgrass

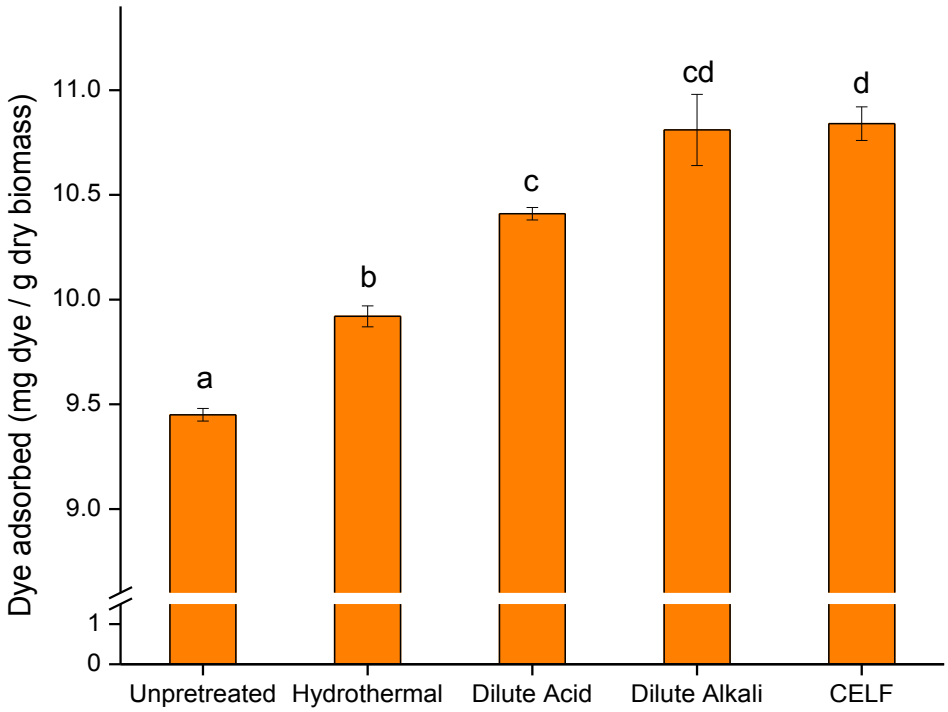


Figure 6.3. Effect of hydrothermal (200ºC for 10 min), dilute acid (160ºC for 25 min), dilute alkali (120ºC for 60 min), and co-solvent enhanced lignocellulosic fractionation (CELF) (140ºC for 20 min) pretreatments on cellulose accessibility of switchgrass measured by dye adsorption via Simons' staining method. Samples were analyzed in triplicate. A one-way ANOVA was performed at 95% significance level post-hoc using Bonferroni method with a p-value of 0.0000777 and F-statistic of 89.26. Same letter indicates no significant difference.

An increase in accessibility has also been shown for *Populus* natural variants after hydrothermal pretreatment [53]. Here, however, lignin removal after CELF and dilute alkali pretreatments affected cellulose accessibility more than xylan removal after dilute acid and hydrothermal pretreatments of switchgrass. This correlated well with fungal enzymatic hydrolysis (EH) of these substrates using 15 mg protein / g glucan enzyme loading during which final yield was found to be in the following order: CELF > dilute alkali > dilute acid > hydrothermal > unpretreated switchgrass as seen in Figure 6.4. Similarly, the presence of low lignin in dilute alkali pretreated solids was responsible for

the high cellulose accessibility compared to high lignin containing unpretreated switchgrass with low cellulose accessibility. Both dilute alkali pretreated solids and unpretreated switchgrass had similar xylan contents. The greater impact of lignin removal and thus increased cellulose accessibility was also found to be true for *C. thermocellum* CBP as reported here in Figure 6.5 and in Chapter 4. The slightly higher cellulose accessibility for dilute acid pretreated solids compared to hydrothermal pretreated solids might be due to lower xylan content in the former. Hemicelluloses are shown to similarly influence cellulose accessibility measurements via Simon's staining technique elsewhere [63, 64]. EH was negatively affected by the presence of xylan in hydrothermal pretreated solids compared to lower xylan content in dilute acid pretreated solids. Digestion by *C. thermocellum* generally showed the same trends as fungal enzymatic hydrolysis of these substrates with the exception of higher glucan solubilization of hydrothermal pretreated solids compared to dilute acid pretreated solids by *C. thermocellum* shown in Figure 6.5. This can be attributed to the presence of xylanases in the *C. thermocellum* cellulosomal system, even though the organism is not known to metabolize either xylose or xylo-oligomers, which helped the organism effectively digest hydrothermal pretreated solids with slightly higher xylan content [25].

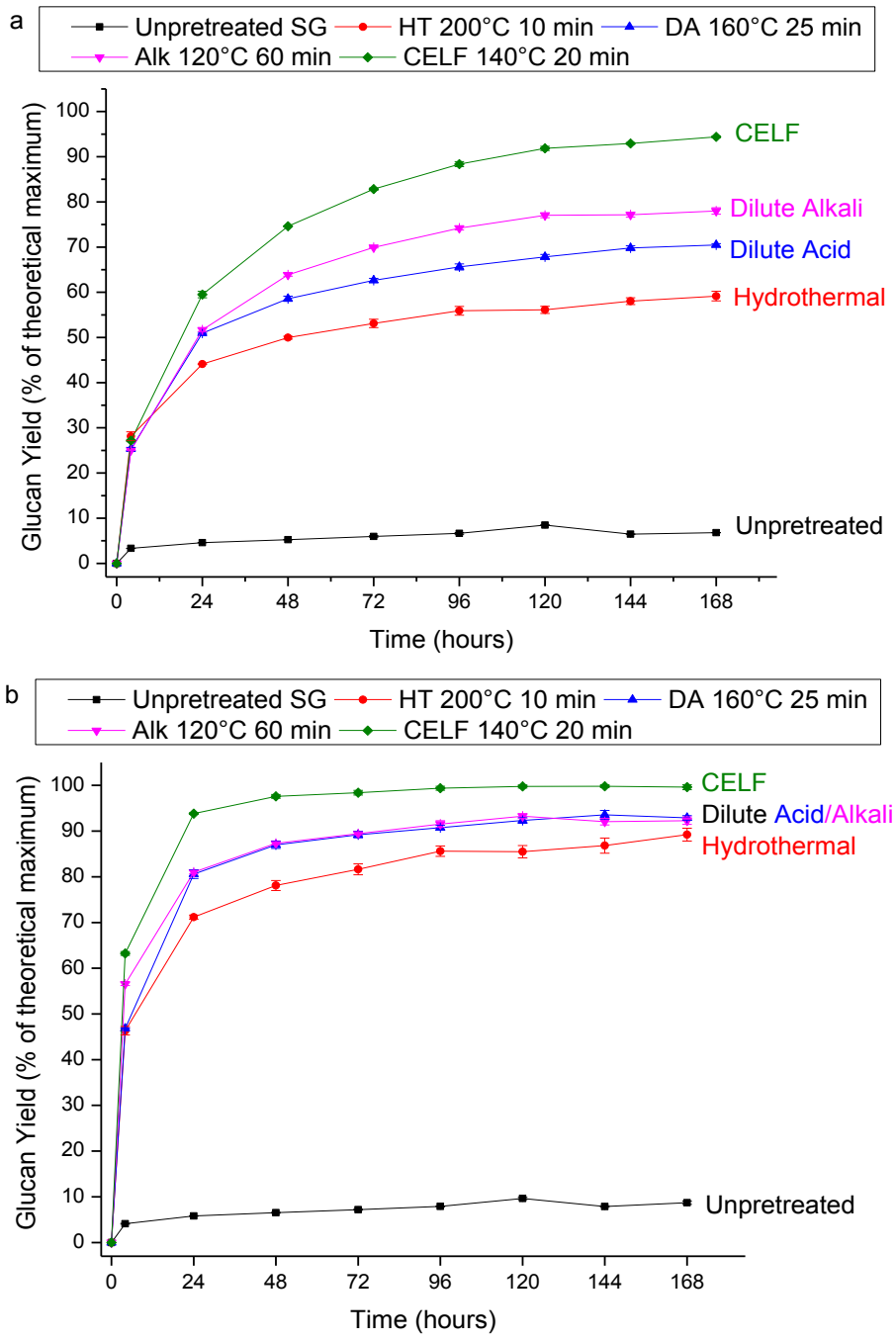


Figure 6.4. Fungal enzymatic hydrolysis glucan yield time profiles for unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG) with **(a)** 15 mg protein / g glucan enzyme loading and **(b)** 65 mg protein / g glucan enzyme loading

The biggest difference in digestion of the different substrates by the two biological approaches, EH (15 and 65 mg protein / g glucan enzyme loads) and *C. thermocellum* CBP (2% by volume inoculum), was in the digestion of unpretreated switchgrass as shown in Figures 6.4 and 6.5. *C. thermocellum* was able to achieve 48% glucan solubilization from unpretreated switchgrass within 5 days of fermentation as opposed to fungal enzymes that achieved <10% glucan yield within the first day of hydrolysis and then ceased to solubilize the substrate further even at the high enzyme loading of 65 mg protein / g glucan.

The SEM image of solid residues obtained after EH (65 mg protein / g glucan) of unpretreated switchgrass as shown in Figure 6.6(a) looks similar to that of unpretreated switchgrass itself shown in Figure 6.2(a) with minimal change. In comparison, CBP residues of unpretreated switchgrass shown in Figure 6.6(f) looks digested and smoothed out in comparison to unpretreated switchgrass. SEM images showed highly digested residues with smaller size particles left behind after both EH and CBP of CELF and dilute alkali pretreated solids compared to residues obtained after EH and CBP of hydrothermal and dilute acid pretreated solids as shown in Figure 6.6. This corroborates the overall more positive impact of lignin removal on biological digestion compared to xylan removal from switchgrass. Overall, *C. thermocellum* performed equivalently to 65 mg protein / g glucan and better than 15 mg protein / g glucan EH in terms of glucan solubilization. CBP residues of all substrates, overall, look more digested with smaller size particles compared to EH (65 mg protein / g glucan) residues as seen in SEM images

in Figure 6.6. CELF pretreated solids were the most digestible due to the high removal of both lignin and xylan from switchgrass and the residues obtained after both EH and CBP look highly digested in the SEM images.

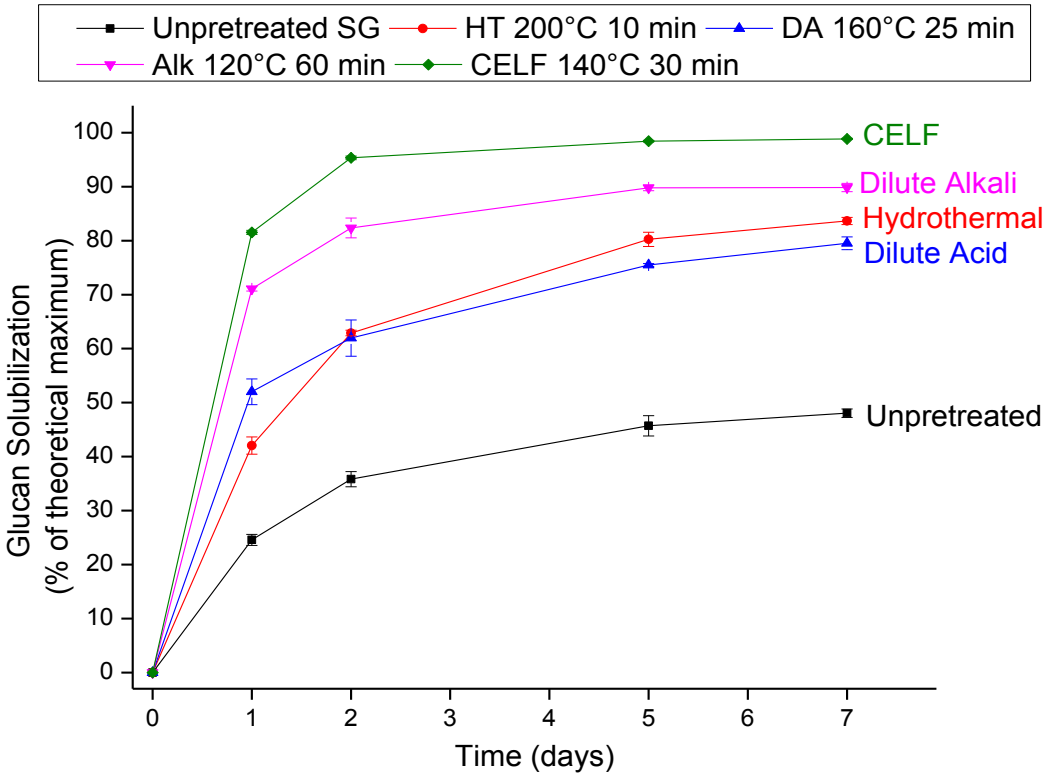
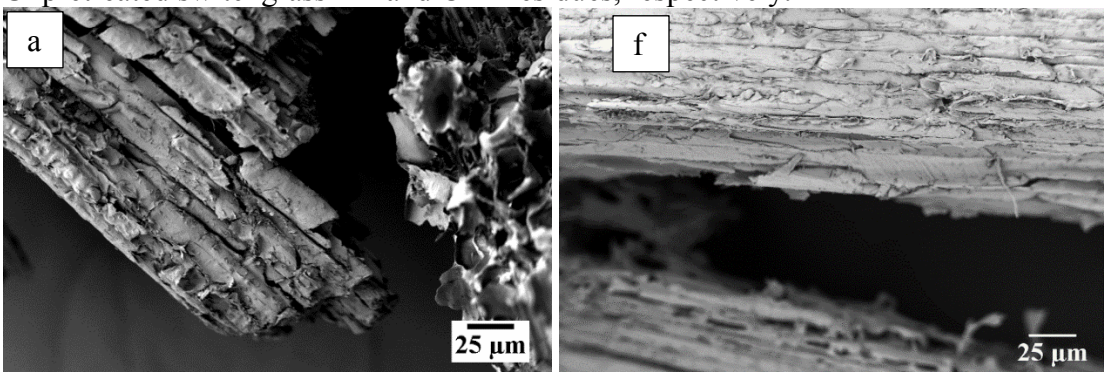
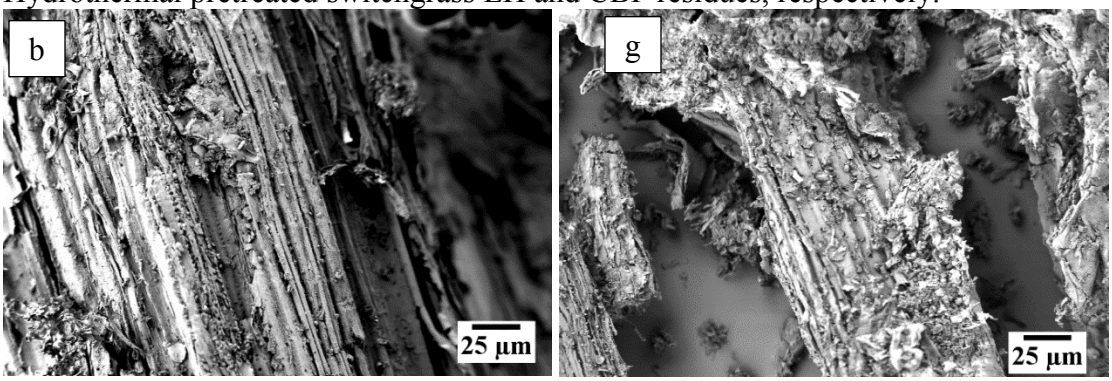


Figure 6.5. *C. thermocellum* consolidated bioprocessing (CBP) glucan solubilization time profiles for unpretreated and hydrothermal (HT), dilute acid (DA), dilute alkali (Alk), and Co-solvent enhanced lignocellulosic fractionation (CELF) pretreated switchgrass (SG)

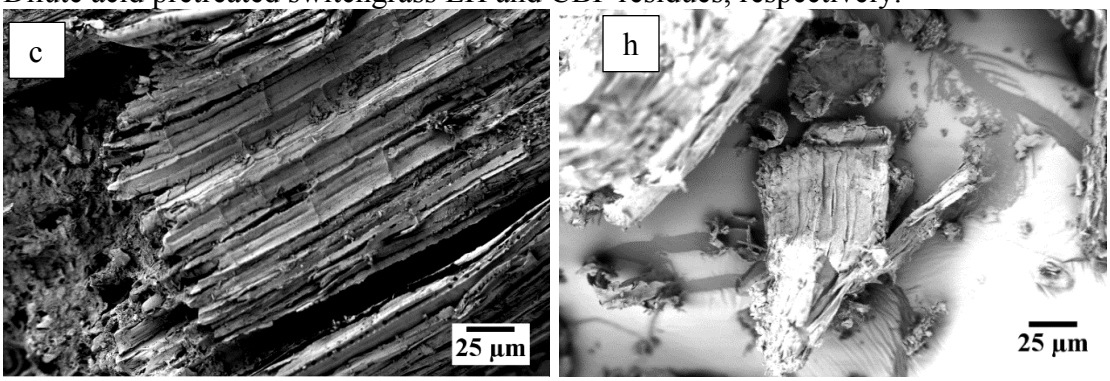
Unpretreated switchgrass EH and CBP residues, respectively:



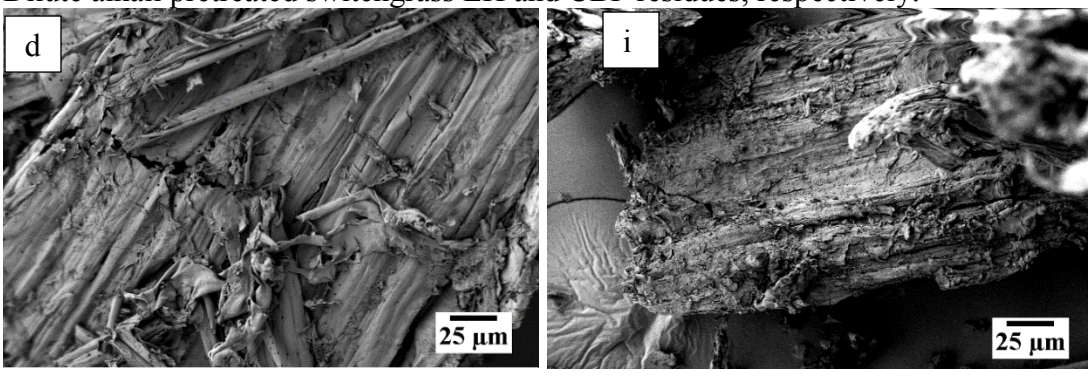
Hydrothermal pretreated switchgrass EH and CBP residues, respectively:



Dilute acid pretreated switchgrass EH and CBP residues, respectively:



Dilute alkali pretreated switchgrass EH and CBP residues, respectively:



CELF pretreated switchgrass EH and CBP residues, respectively:

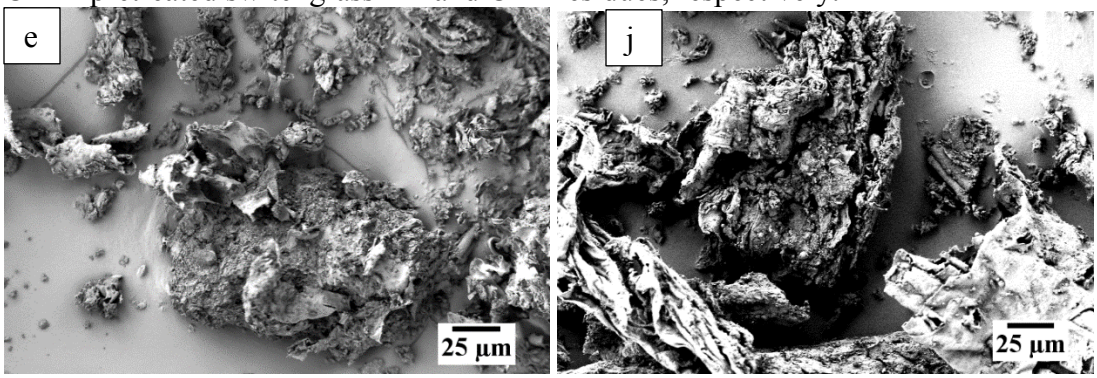


Figure 6.6. Scanning electron microscopy (SEM) images of residues recovered after 7 days of fungal enzymatic hydrolysis (EH) (65 mg protein / g glucan enzyme load) of **(a)** unpretreated switchgrass and **(b)** hydrothermal (200ºC for 10 min), **(c)** dilute acid (160ºC for 25 min), **(d)** dilute alkali (120ºC for 60 min), and **(e)** co-solvent enhanced lignocellulosic fraction (CELF) (140ºC for 20 min) pretreated switchgrass and residues recovered after 7 days of *C. thermocellum* consolidated bioprocessing (CBP) of **(f)** unpretreated switchgrass and **(g)** hydrothermal, **(h)** dilute acid, **(i)** dilute alkali, and **(j)** CELF pretreated switchgrass

6.3.2. Impact of thermochemical and biological digestion on switchgrass cellulose properties

Cellulose crystallinity was determined using solid state nuclear magnetic resonance (SSNMR) and degree of polymerization (DP) and polydispersity index (PDI) using gel permeation chromatography (GPC) of cellulose isolated from unpretreated and pretreated switchgrass (henceforth, collectively referred to as substrates) and residues recovered after CBP and EH (65 mg protein / g glucan) of the corresponding substrates (henceforth, referred to as either CBP or EH residues). All acid-based pretreatments, dilute acid, hydrothermal, and CELF pretreatments, significantly altered the overall crystallinity index (CrI) of cellulose as shown in Figure 6.7.

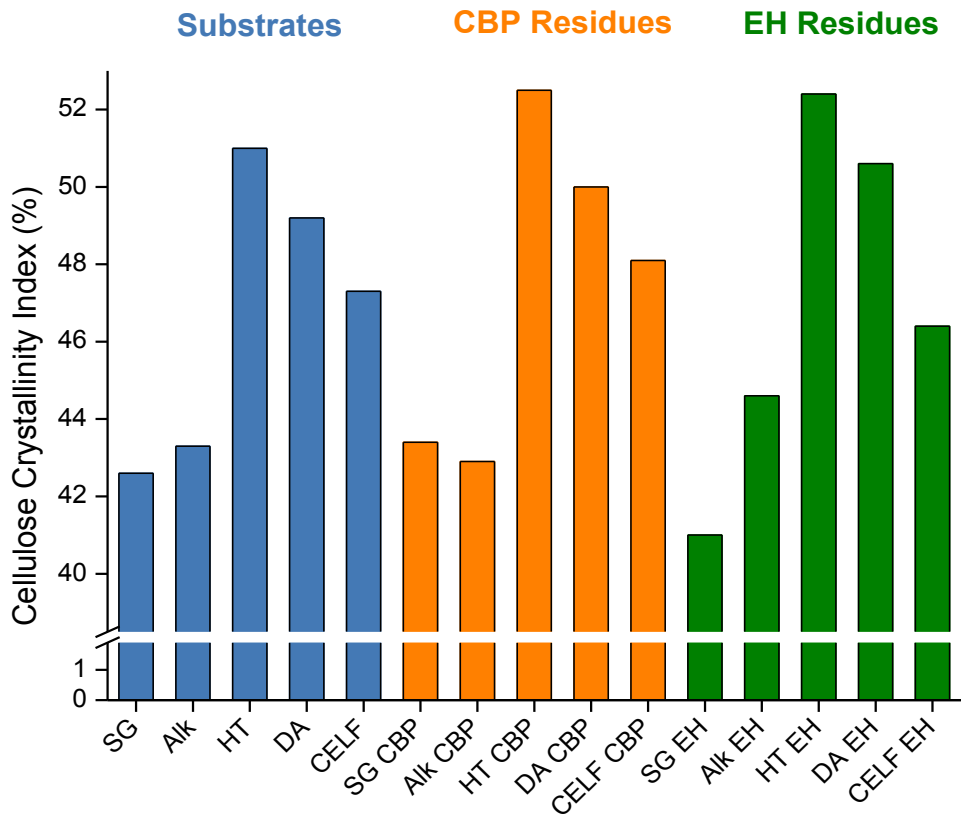


Figure 6.7. Crystallinity indices (CrI) of cellulose isolated from unpretreated and hydrothermal (HT) (200ºC for 10 min), dilute acid (DA) (160ºC for 25 min), dilute alkali (Alk) (120ºC for 60 min), and co-solvent enhanced lignocellulosic fractionation (CELF) (140ºC for 20 min) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) (65 mg protein / g glucan) residues obtained after 7 days of biological digestion

In other words, acid-based pretreatments led to digestion of some of the amorphous cellulose in switchgrass leading to an overall increase in crystallinity of cellulose in pretreated solids consistent with other reports [47, 48]. In contrast to acid-based pretreatments, there was negligible change in the CrI of cellulose from dilute alkali pretreated solids compared to CrI of cellulose from unpretreated switchgrass. Further, hydrothermal and dilute acid pretreated solids showed the highest cellulose crystallinity, which when coupled with low accessibility measured via Simons' staining hindered

effective hydrolysis, especially by fungal enzymes. On the other hand, CELF and dilute alkali pretreated solids had lower cellulose crystallinity and higher cellulose accessibility leading to better EH and CBP performance compared to that for dilute acid and hydrothermal pretreated solids. Even though dilute alkali pretreated solids showed lower CrI compared to CELF pretreated solids, the presence of high amounts of xylan in dilute alkali solids may have led to lower EH yields compared to that from CELF pretreated solids. Even though cellulose from unpretreated switchgrass had very low CrI of ~42%, fungal enzymes were able to digest <10% cellulose from this substrate. In contrast, as shown in Chapter 4, fungal enzymes effectively digest Avicel, which is a more crystalline model cellulosic substrate [33]. This shows that physically availability of cellulose or cellulose macro-accessibility has a greater impact on digestion by fungal enzymes than cellulose properties or cellulose micro-accessibility. It would, thus, be more beneficial to consider any impact of cellulose crystallinity (or other cellulose properties) on biological digestion for generally more macro-accessible substrates, such as, pretreated substrates than unpretreated substrates.

Overall, both *C. thermocellum* and fungal enzymes were not able to substantially alter overall cellulose crystallinity during hydrolysis for the residues of most substrates as seen in Figure 6.7. This has also been reported for *C. thermocellum* fermentations of two *Populus* natural variants [38]. However, the crystallinity of the residues of dilute alkali pretreated solids was increased after EH. While dilute alkali pretreatment did not preferentially digest amorphous cellulose in comparison to other pretreatment methods

leaving amorphous cellulose available for digestion during EH that fungal enzymes prefer over crystalline cellulose [29]. In comparison, *C. thermocellum* equally digested both amorphous and crystalline cellulose from dilute alkali pretreated solids causing no change in overall cellulose crystallinity for residues after CBP. Similarly, as reported in Chapter 4, *C. thermocellum* performance was not impacted substantially by cellulose properties and the organism equally digested model cellulosic substrates with varying cellulose crystallinity and other properties. In contrast, fungal enzymes were negatively impacted by high cellulose crystallinity as also reported in Chapter 4 and elsewhere [31, 33-35, 65]. Further, fungal Cel7A cellulase has been reported to be extensively impacted by high cellulose crystallinity compared to CelA from *Caldicellulosiruptor bescii*, a thermophilic, anaerobic bacteria similar to *C. thermocellum* [65].

Higher cellulose DP means longer cellulose chains with stronger hydrogen bonds reducing accessibility that has been shown to negatively impact biological digestion [1, 29, 66, 67]. While dilute alkali pretreatment did not alter cellulose crystallinity, this pretreatment also did not affect cellulose degree of polymerization to the same extent as other pretreatment technologies did as shown in Figure 6.8. All acid-based pretreatment technologies substantially decreased both the number average and weight average degree of polymerization, DPn and DPw respectively, which were further reduced during hydrolysis by both *C. thermocellum* and fungal enzymes. CELF pretreated CBP and EH residues showed the lowest cellulose DPn suggesting that CELF pretreated solids were more amenable to cellulose DP reduction than the other materials by both biological

approaches. Unpretreated switchgrass and dilute alkali pretreated residues left behind after biological digestion by both biocatalysts showed very high DP, suggesting that acid-based pretreatments that reduce DP can further aid reduction in DP by both biological approaches. An increase in cellulose DP has also been reported for *C. thermocellum* digestion of two unpretreated *Populus* natural variants [38].

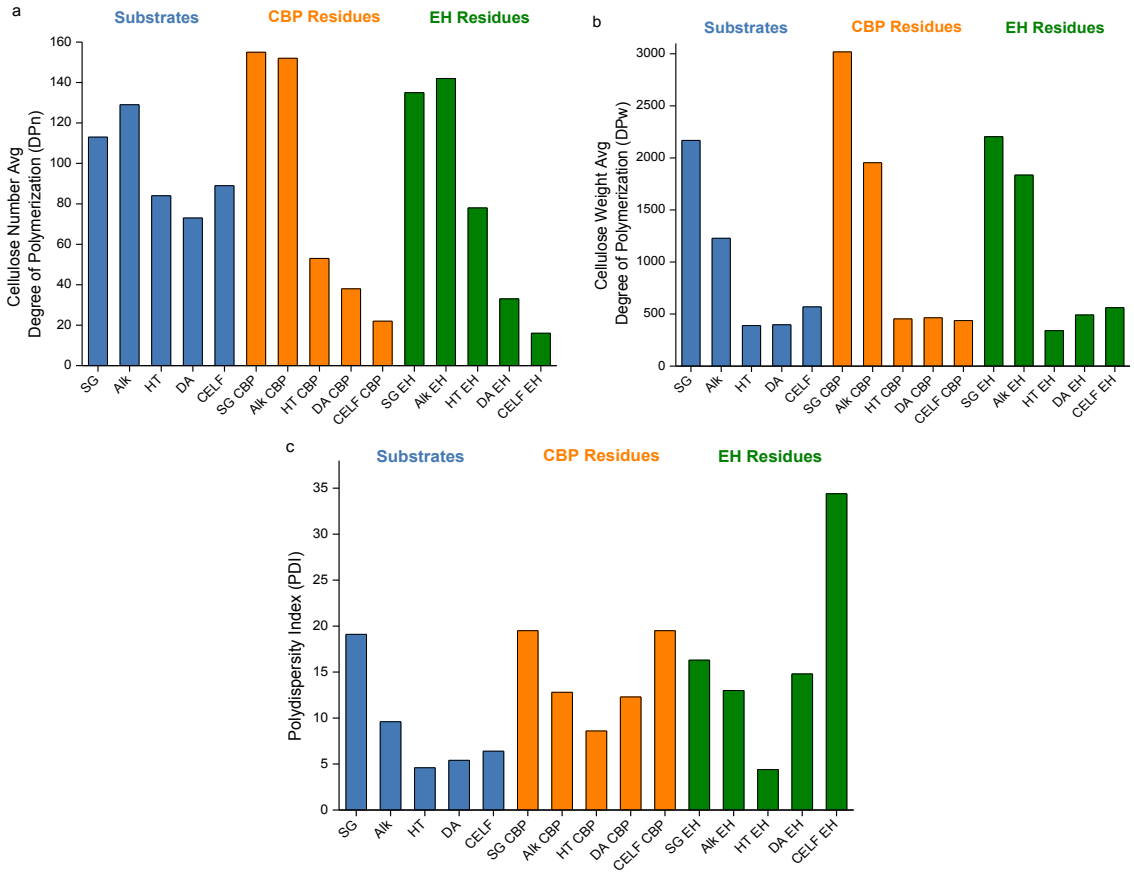


Figure 6.8. (a) Number (DPn) and **(b)** weight (DPw) average degree of polymerization and **(c)** polydispersity indices (PDI) of cellulose in unpretreated and hydrothermal (HT) (200ºC for 10 min), dilute acid (DA) (160ºC for 25 min), dilute alkali (Alk) (120ºC for 60 min), and co-solvent enhanced lignocellulosic fractionation (CELF) (140ºC for 20 min) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) residues (65 mg protein / g glucan after 7 days of biological digestion

Similar to a decrease in DPn shown here for hydrothermal pretreated switchgrass and further decrease in DPn in their EH and CBP residues, a decrease in DPn has also been reported for three hydrothermal pretreated *Populus* natural variants, BESC standard, SKWE 24-2, and BESC 876 and their EH and CBP residues compared to unpretreated materials [53]. Further, there was an increase in the polydispersity indices after biological digestion of all materials suggesting a larger decrease in DPw than DPn as shown in Figure 6.8(c). This was in contrast to a negligible change in PDI observed on unpretreated *Populus* natural variants with varying S/G ratios with different PDIs, which did not change after *C. thermocellum* fermentation [38]. However, there was no relationship between DP and extent of overall biological digestion and thus cellulose DP does not seem to be a driving factor for digestion by either biological approaches. Overall, acid-based pretreatments impacted both cellulose crystallinity and cellulose degree of polymerization, whereas, dilute alkali pretreatment did not. Further, cellulose crystallinity may affect biological digestion but cellulose DP did not seem to have an influence on the extent of biological digestion of the biomass. However, cellulose properties may only impact biological digestion of substrates that have high cellulose macro-accessibility.

6.3.3. Structural changes in lignin after thermochemical and biological digestion of switchgrass

Syringyl (S), guaiacyl (G), p-hydroxyphenyl (H) are the three monolignol units predominantly found in lignin that are polymerized from sinapyl acohol (4-(3-

hydroxyprop-1-enyl)-2,6-dimethoxyphenol) , coniferyl acohol 4-(3-hydroxy-1-propenyl)-2-methoxyphenol), and p-coumaryl alcohol (4-(3-hydroxy-1-propenyl)phenol), respectively. Composition of lignin monolignol subunuits, S, G, and H, has been proposed to affect thermochemical and biological degradation of lignocellulosics [45]. Majority of the studies report a positive impact of S/G ratio on hydrolysis by fungal enzymes as well as *C. thermocellum* [38, 44, 68]. Further, the influence of bulk lignin content on enzymatic hydrolysis has been shown to diminish at higher S/G ratios (≥ 2) suggesting the positive effect of the presence of S lignin [44]. A number of reports have speculated the reasons for better digestibility with the presence of higher amounts of syringyl lignin and therefore higher S/G ratios. Generally, S lignin has linear chains with less cross-linking compared to G lignin because C-5 position in the syringyl unit is methoxylated and therefore blocked [69]. This potentially leads to a higher occurrence of β-β (Resinol) bonds leading to less cross-linking and a lower occurrence of more stable β-5 (phenylcoumaran) and 5-5 bonds (C-5 position is blocked) [69-71]. S lignin has been reported to have lower molecular weights due to less possibility of crosslinking with β-β bonds [44, 70]. S lignin is also reported to have a high proportion of β-O-4 (β-aryl-ether) bonds that are highly reactive leading to an increased susceptibility to lignin removal during pretreatment and overall increased biomass susceptibility to enzymatic hydrolysis [72]. In contrast, multiple reports have shown minimal effect of S/G ratio on enzymatic digestion of lignocellulosics [72-74]. Others showed negative impact of high S/G on enzymatic digestion of alkaline and acid pretreated miscanthus [75, 76]. A more recent study showed that higher S/G ratio in *Populus* natural variants possibly led to longer

linear lignin chain lengths with an overall high lignin molecular weight [38]. It has also been reported that the relative amount of the labile β-O-4 bonds does not depend on S/G ratio and remains constant as long as some syringyl units are present [44]. Further, higher proportion of uncondensed lignin with high β-O-4 bonds has also been shown to negatively affect cell wall degradation [72]. Similarly, through modeling, the long chain β-O-4 containing lignin has been shown to be able to linearly orient parallel to the cellulose surface with increased interaction with cellulose as opposed to β-5 and 5-5 bonds that stiffen lignin overall causing a flat adsorption onto cellulose [77]. Thus, there is limited consensus on the significance of the impact of S/G ratio and lignin interunit linkages on biological cellulose hydrolyzability. Further, the impact of pretreatment on S/G ratio is also contentious in literature. S/G ratio has been shown to drop with hydrothermal, dilute acid, and alkaline pretreatments suggesting more susceptibility of S lignin to breakdown with higher pretreatment severities impacting S/G ratio more [44]. A faster cleavage of β-O-4 has been shown to occur during pretreatments under alkaline conditions [78]. Further, significant S lignin fragmentation has been shown after steam explosion pretreatment of miscanthus (unpretreated S/G = 1.34) and wheat straw (unpretreated S/G = 1.12). However, the same study showed that pretreatment of poplar led to more removal of G lignin (unpretreated S/G = 1.29) [79]. Similarly, another study showed an increase in S/G ratio after hydrothermal pretreatment of three *Populus* natural variants [75]. Further, a similar increase in S/G ratio after alkaline hydrogen peroxide pretreatments of switchgrass was also reported with a simultaneous substantial break

down of β-O-4 bonds [46]. Thus, there are conflicting reports on the fate of S/G ratio during thermochemical pretreatments.

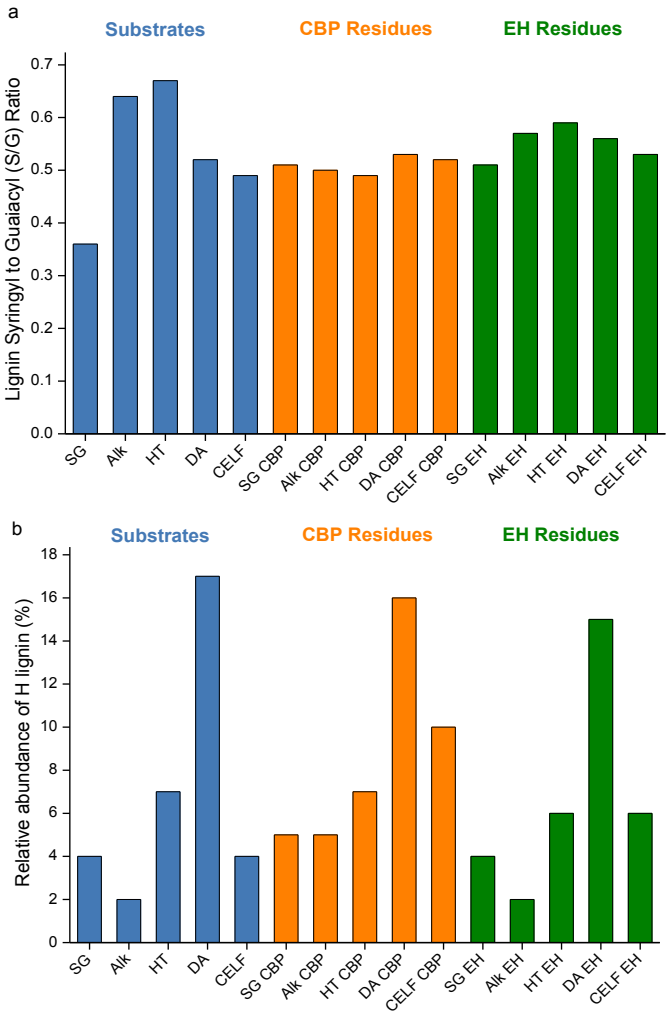


Figure 6.9. Relative abundance of (a) syringyl to guaiacyl monolignol subunit ratio (S/G) and (b) *p*-hydroxyphenyl (H) monolignol subunit content in lignin isolated from unpretreated and hydrothermal (HT) (200ºC for 10 min), dilute acid (DA) (160ºC for 25 min), dilute alkali (Alk) (120ºC for 60 min), and co-solvent enhanced lignocellulosic fractionation (CELF) (140ºC for 20 min) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) (65 mg protein / g glucan) residues after 7 days of biological digestion

In the current study, hydrothermal, dilute acid, dilute alkali, and CELF pretreatments were all responsible for an increase in S/G ratio in switchgrass as shown in Figure 6.9(a) consistent with some reports. This could be because of the presence of very low S lignin in unpretreated switchgrass (S/G ratio = 0.36) and the sheer presence of high amounts of G lignin possibly leading to more degradation of the latter during pretreatment causing an increase in S/G ratio in pretreated solids. S/G ratio was still less than 1 even in the pretreated solids. More G lignin break down with an increase in S/G ratio has been reported for dilute acid hydrolysis of wood samples from a second generation *Populus* cross (S/G ratio of unpretreated samples varied from 1.8 to 2.3) [68]. Interestingly, forage maize with lower S lignin has also been shown to lead to higher milk and meat production [80]. It was hypothesized that higher G content produced more crosslinked but thinner cell walls and is therefore easier to break down. Further, it was also speculated that cell wall with lower S lignin was less mature and less lignified leading to better chemical and enzymatic digestion [81]. Interestingly, hydrothermal and dilute alkali pretreated solids showed higher S/G ratios of 0.67 and 0.64, due to significant removal of G lignin, in comparison to other substrates as shown in Figure 6.9(a). It is important to remember that hydrothermal pretreatment achieved only 20% lignin removal, whereas, dilute alkali pretreatment achieved 75% lignin removal. However out of the respective amounts of lignin removed by both pretreatments, both pretreatments achieved G lignin removal more than S lignin removal leading to a 78% and 86% increase in S/G ratio after hydrothermal and dilute alkali pretreatments, respectively. Here, alkali seems to have catalyzed substantial G lignin removal compared

to acid catalysts, whereas, the very high temperature (200ºC) used in hydrothermal pretreatment may have contributed to high G lignin removal compared to either dilute acid or CELF pretreatments.

No direct impact of S/G ratio on glucan solubilization by *C. thermocellum* or fungal enzymes can be determined, especially since each of the materials have varying amounts of lignin and the overall lignin content itself had a stronger impact on solubilization. Interestingly, as in the case of thermochemical digestion of switchgrass, *C. thermocellum* increased the S/G ratio of unpretreated switchgrass during hydrolysis consistent with multiple reports on *C. thermocellum* [38, 43]. This has been attributed to removal of G lignin that has a less sterically hindered phenolic group than S lignin (2 methoxy groups in S lignin units compared to 1 in G lignin units induces more steric hindrance) [43]. However, since G lignin removal during biological digestion of unpretreated switchgrass was observed by both *C. thermocellum* and fungal enzymes, G lignin removed is possibly carbohydrate associated. Further higher G lignin removal than S lignin from unpretreated switchgrass by CBP and EH could just be due to the sheer presence of high amount of G lignin in unpretreated switchgrass. This could also explain the decrease in S/G ratio for dilute alkali and hydrothermal pretreated solids after both CBP and EH. Higher presence of S lignin in these substrates could be why S lignin breakdown observed was higher compared to G lignin breakdown, especially if this is only a manifestation of carbohydrate related lignin breakdown. S/G ratio of all CBP residues was around 0.5, whereas, those of all EH residues varied from 0.5-0.6. S/G ratio

of EH residues of all substrates followed the same trend as that of the substrates themselves (hydrothermal > dilute alkali > dilute acid > CELF > unpretreated switchgrass), whereas, the substrates lost this trend after *C. thermocellum* fermentation with residues showing equal S/G ratios.

H-lignin, which has not been studied as extensively as the other monolignol subunits of lignin, is negatively correlated to biomass recalcitrance and therefore, positively to enzymatic hydrolysis yields [45]. H lignin is reported to negatively impact cellulose crystallinity improving cellulose digestion in wheat and rice samples [82]. Further, H lignin is shown to be more reactive than S lignin using density functional theory [83]. High sugar yields were obtained from H-rich Arabidopsis mutant as opposed to G- or S-rich mutants [83]. Here, Figure 6.9(b) shows that dilute alkali and CELF pretreatments that reduce lignin content also reduced H lignin content specifically. In contrast, dilute acid and hydrothermal pretreatments showed a relative increase in H lignin content possibly due to higher removal of G lignin as shown by an increase in S/G ratio after pretreatment. Substrates with high lignin content, i.e. unpretreated switchgrass and hydrothermal and dilute acid pretreated solids, showed an increase in solubilization by both *C. thermocellum* and fungal enzymatic hydrolysis with an increase in H lignin content in the substrates. Thus, for substrates with high lignin content, the presence of higher H lignin could possibly lead to an improvement in the extent of biological digestion.

Further, as expected, β-O-4 interunit linkage was the most common in unpretreated switchgrass and all pretreatments were able to breakdown β-O-4 and β-β bonds as shown in Figure 6.10. While all pretreatments reduced the relative abundance of β-O-4 bonds in switchgrass, dilute acid and hydrothermal pretreatments caused a substantial reduction (73%) of this type of interunit linkage. Both hydrothermal and dilute acid pretreatments do not remove a lot of lignin from switchgrass as seen in this work but they are still able to cause a significant change in the lignin structure, possibly due to the use of high temperatures. Most of the lignin is still left behind in dilute acid and hydrothermal pretreated solids with a simultaneous reduction in β-O-4 bonds, which suggests condensation and possible redeposition of lignin in the substrate [13]. Thus lignin, once isolated, from hydrothermal and dilute acid pretreated solids would not have a native structure impacting downstream lignin valorization and utilization. Even though, dilute alkali and CELF pretreatments remove a lot of lignin from the biomass, lignin left in the solids seems relatively untouched and shows similar relative abundance of interunit linkages as unpretreated switchgrass. It would be interesting to recover and characterize the broken down lignin from the dilute alkali and CELF pretreatment liquor. Even though CELF pretreatment utilizes a 0.5 wt% sulfuric acid solution, similar to dilute acid pretreatment, THF used in the former has been shown to prevent aggregation of lignin. Without the presence of THF lignin aggregation is expected in aqueous environments leading to recondensation of lignin as in the case of dilute acid pretreatment [84]. Further, as expected, strong β-5 bonds were not broken down during any pretreatment and the relative abundance of this type of interunit linkage increased possibly due to reduction in

the relative abundance of other types of linkages. Both *C. thermocellum* and fungal enzymes caused a 15% reduction in the relative abundance of β-O-4 bonds during hydrolysis of unpretreated switchgrass with no substantial change in abundance of β-β and β-5 bonds. However, there was a slight increase in the relative abundance of all interunit linkages after hydrolysis of most pretreated substrates using either biological approach. The increase in relative abundance of β-O-4 (~19-30%) and β-5 (~30%) linkages after EH of dilute acid and hydrothermal pretreated solids was especially high.

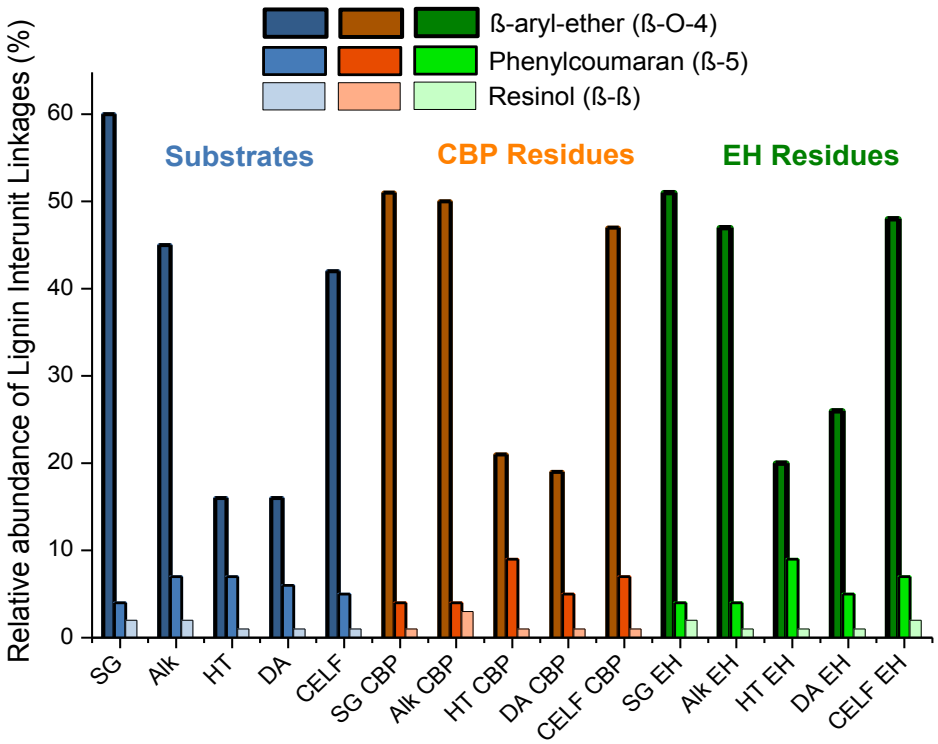


Figure 6.10. Relative abundance of interunit linkages in lignin isolated from unpretreated and hydrothermal (HT) (200ºC for 10 min), dilute acid (DA) (160ºC for 25 min), dilute alkali (Alk) (120ºC for 60 min), and co-solvent enhanced lignocellulosic fractionation (CELF) (140ºC for 20 min) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) (65 mg protein / g glucan) residues after 7 days of biological digestion

6.3.4. Fate of Lignin-carbohydrate linkages during digestion of switchgrass

Hydroxycinnamates, namely ferulates (FA) and *p*-coumarates (pCA), are common in grasses that are part of lignin-carbohydrate complexes (LCCs) [45, 85]. Bifunctional *p*-Coumaric and ferulic acids form ester linkage from their carboxyl group or an ether linkage from their phenolic groups [86]. Both ferulic and *p*-coumaric acids can cross link with lignin and hemicellulose by esterification of their carboxylic groups to arabinose in arabinoglucuronoxylan and etherification of the hydroxyl group to phenyl hydroxyls in lignin. Ferulic bridges are common in grasses, unlike wood LCCs, and are sometimes referred to as "lignin/phenolic carbohydrate complexes" [87].The carbohydrate part of LCCs in grasses are composed predominantly of arabino-4-O-methylglucuronoxylan [86]. LCCs draw lignin closer to polysaccharides and thus increase overall biomass recalcitrance [45]. Alkali treatments break the ester linkages freeing carbohydrates from lignin leaving behind hydroxycinnamic acids and their residues [87]. A number of alkaline pretreatment technologies have been reported to cleave FA and pCA to increase biomass digestibility [46, 88-90]. The ether linkages can be broken down through acid catalyzed reactions while the ester linkage may remain intact [87, 91, 92]. Here, dilute alkali pretreatment was able to break the ester bonds of both FA and pCA leading to a sharp decrease in their relative abundance as shown in Figure 6.11 consistent with other reports. Even though both FA and pCA were reduced substantially by acid based pretreatments, FA was removed in larger quantities than pCA. CELF pretreatment especially showed low removal of pCA (34%) compared to dilute acid pretreatment with 60% reduction in pCA relative abundance. Overall, all pretreatments reduced the

amounts of hydroxycinnamates present in switchgrass, thus reducing recalcitrance. Even though *C. thermocellum* has been shown to have ferulic acid esterases and to produce *p*-coumaric acid in the fermentation broth, the organism was the least effective in hydroxycinnamates removal compared to all other thermochemical and biological digestion techniques [93]. Generally, there was an increase in FA and pCA after hydrolysis of pretreated substrates by both CBP and EH. Hydrothermal pretreated solids were the most amenable to reduction in FA after both CBP and EH.

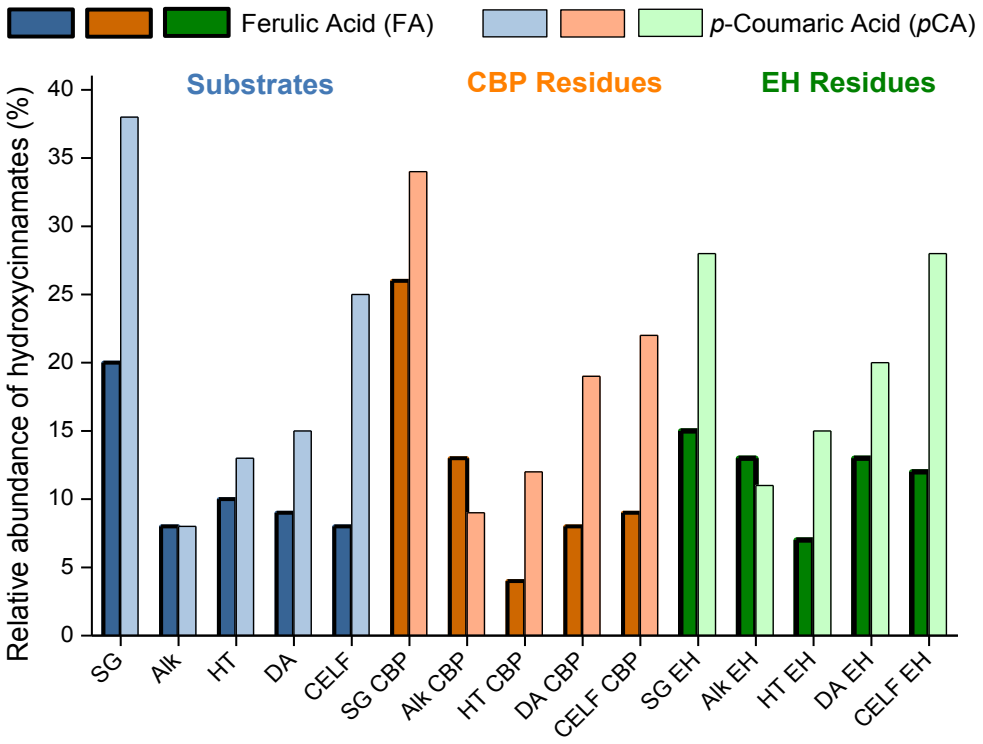


Figure 6.11. Relative abundance of hydroxycinnamates in unpretreated and hydrothermal (HT) (200ºC for 10 min), dilute acid (DA) (160ºC for 25 min), dilute alkali (Alk) (120ºC for 60 min), and co-solvent enhanced lignocellulosic fractionation (CELF) (140ºC for 20 min) pretreated switchgrass (SG) and their corresponding *C. thermocellum* consolidated bioprocessing (CBP) and fungal enzymatic hydrolysis (EH) (65 mg protein / g glucan) residues after 7 days of biological digestion

6.4. Conclusions:

This study is a comprehensive work on understanding the mechanism of switchgrass deconstruction using four different thermochemical pretreatment technologies and two different biological digestion approaches. Each of these deconstruction technologies utilize unique chemical or biological catalytic systems that affect the biomass in different ways. Overall, we tried to elucidate the process of thermochemical and biological breakdown of switchgrass, the structural changes that occur in the biomass during digestion, and the impact of the structural changes on the overall digestibility of the substrate. We showed that CELF pretreatment produced the most accessible substrate, measured via Simons' staining, and was also the most digestible substrate by both CBP and EH. CELF and dilute alkali pretreatments that removed more lignin from switchgrass produced solids with higher accessibility and digestibility compared to solids produced from dilute acid and hydrothermal pretreatments that removed more xylan from switchgrass. *C. thermocellum* was overall able to digest all substrates more effectively compared to fungal enzymes, solubilizing more glucan and as also corroborated by smaller particle sizes of material observed in SEM images of CBP residues compared to EH residues. Acid based pretreatments affected cellulose properties more than dilute alkali pretreatment, CBP, or EH. A sharp increase in cellulose CrI was observed after all acid based pretreatments due to the deconstruction of amorphous cellulose more than crystalline cellulose in switchgrass. Amongst the pretreated solids, hydrothermal and dilute acid pretreated solids had the highest crystallinity and the lowest accessibility measured via Simons' staining and were

therefore the least digested. Even though dilute alkali pretreated solids had much lower CrI than CELF pretreated solids, the higher amount of xylan in the former led to lower digestibility. Fungal enzymes increased CrI of dilute alkali pretreated solids slightly after hydrolysis because of their preference to breakdown amorphous cellulose more than crystalline cellulose, unlike *C. thermocellum* that did not cause any change in the CrI after fermentation. Acid based pretreatments caused a decrease in cellulose DP, which was further reduced after CBP and EH. In contrast, dilute alkali pretreatments did not reduce cellulose DP and there was negligible change in DP of cellulose in dilute alkali pretreated residues obtained after CBP and EH. An increase in DP after biological digestion of unpretreated switchgrass was observed and has been shown before.

Both thermochemical pretreatments and biological digestion led to an increase in S/G ratio of lignin from unpretreated switchgrass attributed to more G lignin removal than S lignin during biomass deconstruction. This provides evidence to a certain degree for the hypothesis that G lignin potentially leads to the formation of more cross linked lignin with lower molecular weight and thinner cell walls. Lignin with more G and less S monolignol units is speculated to be less lignified making the biomass overall more susceptible to digestion. Further, G lignin removal was higher with the use of alkali as a catalyst or at higher pretreatment temperatures. Lignin reduction was observed during both CBP and EH which was assumed to be carbohydrate associated. G lignin removal from unpretreated switchgrass that has low S/G ratio, whereas, S lignin removal from dilute alkali and hydrothermal pretreated solids that have high S/G ratio was observed. H

lignin proportion in substrates with overall higher lignin content was shown to impact digestibility of the substrates. All thermochemical and biological digestions techniques used in this work led to a decrease in β-O-4 lignin interunit linkage. Hydrothermal and dilute acid pretreatments reduced the β-O-4 bonds more than other digestion techniques. However, since both hydrothermal and dilute acid pretreatments do not remove a lot of lignin and simultaneously reduced the β-O-4 linkage substantially, the lignin is thought to have condensed and redeposited. The three interunit linkages, β-O-4, β-β, and β-5, increased after CBP and EH of pretreated solids. All thermochemical and biological digestion techniques reduced hydroxycinnamates content from unpretreated switchgrass substantially, *C. thermocellum* being the least effective. However, overall, hydroxycinnamates content does not seem to impact biological digestion substantially.

6.5. Materials and methods:

6.5.1. Substrate

Chopped Alamo switchgrass (~3/4 inch) was obtained from Genera Energy, Inc. The switchgrass was harvested in January 2014 and was fully mature at five years old. This biomass was thoroughly mixed and sorted into multiple gallon sized bags and stored in a freezer. Thomas Wiley® mill (Model 4, Thomas Scientific, Swedesboro NJ) (knife mill) was used to mill the entire contents of each bag and passed through a 1 mm sieve. The milled biomass was mixed thoroughly before each use. The composition of the biomass was determined to be 38.18 (±0.8) % glucan, 26.96 (±0.4) % xylan, 2.97 (±0.05) % arabinan, and 20.8 (±0.2) % K-lignin

6.5.2. Thermochemical pretreatments

Pretreatment conditions previously determined best for maximum total sugar release (glucan + xylan) from pretreatment and *C. thermocellum* CBP combined were used in this study and are listed as follows: hydrothermal pretreatment at 200°C for 10 min, dilute acid pretreatment at 160°C for 25 min, dilute alkali pretreatment at 120°C for 60 min, and co-solvent enhanced lignocellulosic fractionation (CELF) pretreatment at 140°C for 20 min. Pretreatments were performed as described previously in Chapter 5. Briefly, all pretreatments were performed at a 10 wt% solids loading with a total reaction mass of 800 g in a 1 L Hastelloy Parr reactor (236HC series, Parr Instruments Co., Moline, IL). A 0.5 wt% sulfuric acid solution was used during dilute acid and CELF pretreatments. While dilute acid pretreatment was performed in an aqueous solution, CELF pretreatment utilized tetrahydrofuran (THF) as co-solvent in water at a 1:1 by volume ratio. Dilute alkali pretreatment was done with a 1 wt% sodium hydroxide solution. The Parr reactor was equipped with a double stacked pitch blade impeller that was set at 200 rpm. A 4 kW fluidized sand bath (Model SBL-2D, Techne, Princeton, NJ) was used to maintain the pretreatment temperature within ± 2°C which was measured using a K-type thermocouple probe (CAIN-18G-18, Omega Engineering Co., Stamford, CT, USA). At the completion of the target pretreatment time, the reactor was lowered into a room temperature water bath to cool its contents, which were then vacuum filtered at room temperature using a glass fiber filter paper. The solids were thoroughly rinsed with room temperature deionized water to remove any soluble sugars, degradation products, acid/alkali, and solvents.

6.5.3. *Clostridium thermocellum* consolidated bioprocessing

Clostridium thermocellum DSM 1313 was kindly provided by Prof. Lee Lynd at Dartmouth College, Hanover NH. Stock culture was prepared and growth curve using pellet nitrogen content was determined as described previously (see Figure 3.11). Seed cultures were grown on 5 g/L glucan loading of Avicel® PH-101 (Sigma Aldrich, St. Louis, MO) in 50 mL volume for 8-9 hours in Media for Thermophilic Clostridia (MTC) without trace minerals (see Table 3.1) with a 2% by volume inoculum. The vitamins solution was sterilized by passing it through 28 mm diameter polyethersulfone (PES) syringe filters with 0.2 μm pores (Corning® Life Sciences, Tewksbury MA), whereas, the other media solution were autoclaved. Fermentations were performed in 125 mL bottles (Wheaton, Millville NJ) with a 0.5 wt% glucan loading of substrates and a working mass of 50 g. Bottles containing biomass and water were purged with nitrogen to maintain anaerobic conditions and then autoclaved for sterilization. A repeated 45 seconds application of vacuum and 14 psi nitrogen over a total of 27-30 min was used to purge the bottles. Fermentations were performed at 60°C at a shaking speed of 180 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD) with a 2% by volume inoculum. Insoluble solids left after CBP were recovered and rinsed thoroughly. Compositional analysis was performed on the residues to determine glucan solubilization. Residues recovered from multiple replicates were provided to Prof. Arthur Ragauskas' laboratory group for further characterization.

6.5.4. Enzymatic hydrolysis

Accellerase® 1500 cellulase (DuPont Industrial Biosciences, Palo Alto CA) was used at 15 and 65 mg / g glucan protein loadings for enzymatic hydrolysis. These loadings were based on the amount of glucan in unpretreated switchgrass so as to not penalize a pretreatment for releasing more sugars before enzymatic hydrolysis as described elsewhere [94, 95]. The BCA protein content of Accellerase® 1500 was 82 mg/mL as reported elsewhere [96]. Hydrolysis was performed following the NREL Laboratory Analytical Procedure "Enzymatic Saccharification of Lignocellulosic Biomass" [97]. Briefly, 125 mL Erlenmeyer flasks containing 0.5 wt% glucan substrate with a working mass of 50 g were incubated at 50°C and 150 rpm in a Multitron Orbital Shaker (Infors HT, Laurel MD). Flasks were allowed to equilibrate before adding the required enzyme solution. Representative samples were collected from each flask after 4 hours, 24 hours, and every 24 hour period thereafter to determine glucan yield. The samples were centrifuged and the supernatant was analyzed by HPLC. Insoluble residues from multiple replicates were recovered, washed, and provided to Prof. Arthur Ragauskas' laboratory group for further characterization.

6.5.5. Compositional analysis of solids

NREL Laboratory Analytical Procedure "Determination of Structural Carbohydrates and Lignin in Lignocellulosic Biomass" [98] was followed to determine the composition of unpretreated, and pretreated switchgrass and their CBP and enzymatic hydrolysis residues. Solids were dried to moisture content < 10% in either a 40ºC or 60ºC oven prior

to analysis. The amounts of ingredients required for analysis were modified proportionately if the amount of material being analyzed was insufficient to meet the NREL specified amount. Percent composition of glucan, xylan, arabinan, Klason-lignin (K-Lignin, acid insoluble lignin), and ash were determined for each material

6.5.6. Sugar analysis

A Waters Alliance e2695 HPLC system (Waters Co., Milford MA) was used for analysis of all liquid samples. Bio-Rad Aminex HPX-87H column and a Waters 2414 refractive index detector were used. Mobile phase was a 5 mM sulfuric acid solution eluted at 0.6 mL/min. Integration of the chromatograms was by the Empower™ 2 software package.

6.5.7. 2D Heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR)

For lignin characterization, whole cell wall NMR analysis was conducted as described previously [99]. Briefly, the samples were ball-milled using Retsch PM 100 at 600 rpm for 2 hours. About 50 mg of the ball-milled sample was loaded in a 5 mm NMR tube with 0.4 mL of DMSO-d_6/HMPA-d_{18} (4:1, *v/v*) and sonicated for 2 hours. Two-dimensional ^{1}H-^{13}C HSQC NMR experiments were conducted at 300ºK using a Bruker Avance-III 500 MHz spectrometer with a 5 mm cryogenically cooled probe and a Bruker pulse sequence (hsqcetgpspsi2.2). The spectra were measured with spectral width of 12 ppm in F2 (^{1}H) dimension with 1024 time of domain and 166 ppm in F1 (^{13}C) dimension

with 256 time of domain, a 1.0-s delay, a $^{1}J_{C–H}$ of 145 Hz, and 128 scans. Relative abundance of lignin subunits, hydroxycinnamates, and interunit linkages were estimated by volume integration of contours in HSQC spectra.

6.5.8. Solid-state NMR

All the residues were filtered through 417 Filter paper (VWR Inc.) and the residue retained were freeze-dried. One portion of the dried residue was used to isolate cellulose. The cellulose isolation and cellulose crystallinity measurement was conducted according to literature [100, 101]. In detail, the isolated cellulose samples were stored in a sealed container to prevent moisture loss. The NMR samples were prepared by packing the cellulose into 4-mm cylindrical Zirconia MAS rotors. Cross polarization magic angle spinning (CP/MAS) NMR analysis of cellulose was carried out on a Bruker Advance-400 spectrometer operating at frequencies of 100.59 MHz for ^{13}C in a Bruker double-resonance MAS probe head at spinning speeds of 10 kHz. CP/MAS experiments utilized a 5 μs (90°) proton pulse, 1.5 ms contact pulse, 4 s recycle delay, and 4000 scans. The cellulose crystallinity index (CrI) was determined from the areas of the crystalline and amorphous C_4 signals using the following formula:

$$\mathrm{CrI} = \frac{A_{86\text{-}92\ \mathrm{ppm}}}{A_{86\text{-}92\ \mathrm{ppm}} + A_{79\text{-}86\ \mathrm{ppm}}}$$

6.5.9. Gel permeation chromatography (GPC)

The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of cellulose were measured by GPC after tricarbanilation. Briefly, the

isolated cellulose (see solid-state NMR) was collected and dried under vacuum at 45°C overnight. The dried cellulose samples were then derivatized with phenyl isocyanate in an anhydrous pyridine system prior to GPC analysis. Size-exclusion separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc, Santa Clara, CA) equipped with Waters Styragel columns (HR1, HR4, and HR6; Waters Corporation, Milford, MA). Number-average degree of polymerization (DP_n) and weight-average degree of polymerization (DP_w) of cellulose were obtained by dividing M_n and M_w, respectively, by 519 g/mol, the molecular weight of the tricarbanilated cellulose repeating unit.

6.5.10. Scanning Electron Microscopy (SEM)

Samples for SEM were placed on carbon tape on aluminum stubs and sputter-coated with gold. SEM was carried out on Zeiss Auriga FIB-SEM at an accelerating voltage of 10 kV with back scatter detector at 100 to 5000 times magnification. Raw images were adjusted for brightness and contrast in ImageJ software [102]. Images were merged using Adobe Photoshop CC v. 2017.

6.5.11. Simons' staining

Simons' staining was performed as described previously using the high molecular weight fraction (≥30,000 kDa) of Direct Orange 15 dye (CAS: 1325-35-5) [53, 61]

6.6. References

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Chapter 7. Summary and General Conclusions

7.1. Summary

A combination of chemistry and biology is the future of the manufacturing industry. The industrial revolution saw an increase in production capacity of the world to meet all human needs. A direct consequence of improved fulfilment of human needs was the population boom starting from the mid-1700s. With population growth came increased demands for commodities and energy. It was not until the 1960s and 1970s that people started realizing the exhaustible nature of fossil fuels and the impact they have on the environment. Fossil fuels alone cannot meet the present and future human needs and even if fossil fuels could achieve all human needs it would be at the expense of the environment. It is high time that we move away from the industrial era towards a sustainable era that takes social, environmental, and economic aspects of life in this world on Earth into account. With this in mind, it makes sense to take advantage of Nature on Earth for a more sustainable production industry to meet our needs. Specifically, this research focuses on utilization of switchgrass, an energy crop, for the production of a sustainable and renewable fuel, ethanol, to replace petroleum and alleviate and meet human energy demands. Further, learning from the carbon cycle and organisms such as *C. thermocellum* in the guts of rumen and soil, this research presents an avenue for an effective ethanol production process. Switchgrass, a lignocellulosic biomass, is complex and its cell wall structure and composition depends on a number of environmental factors. This points to an immediate need for a feedstock-independent process for ethanol production. Further, *C. thermocellum*, even though promising, cannot efficiently produce enough ethanol from lignocellulosic biomass to meet human energy

demands. This dissertation aims to tackle the recalcitrance barrier of switchgrass, overcome this barrier through thermochemical pretreatments, and improve sugar release and metabolite production by *C. thermocellum* through a rigorous process development approach. The findings from this dissertation provide critical insights into the importance of better understanding how to best combine various types of biomass with different structural features, biomass pretreatments, and CBP for effective ethanol production.

C. thermocellum CBP for ethanol production has shown great promise by eliminating the separate expensive step for enzyme production and reducing the number of operations compared to the traditional approach of ethanol production. A lot can be learned from the behavior of *C. thermocellum* in nature where it is found in guts of rumen, soil, hot springs, etc. and lives in a complex, natural co-culture system depending on cross-feeding for vitamins and on other organisms as electron sinks. *C. thermocellum*'s cellulosome is a multi-enzyme, multi-functional complex for effective deconstruction of cellulose, hemicellulose, and other components of lignocellulosic biomass. The organism metabolizes cellobiose and cellodextrins over glucose, saving ATP required for transportation per hexose, giving it an advantage in a complex, natural environment with other organisms that require glucose for metabolic activity. *C. thermocellum* is a prime candidate for native cellulolytic strategy of CBP and its metabolism has been genetically altered to improve ethanol production and tolerance. Even though *C. thermocellum* possesses an effective cellulolytic system and has been provided with an improved metabolic system for ethanol production, the organism has

not been studied extensively on real world lignocellulosic biomass. Biomass augmentation techniques, such as pretreatment and cotreatment, are still required to aid *C. thermocellum* digestion of lignocellulosic biomass. Comprehensive and systematic *C. thermocellum* fermentation development and optimization in conjunction with biomass augmentation technologies for maximum sugar release and ethanol production is crucial for a successful industrial use of *C. thermocellum* CBP for ethanol production.

7.2. Key developments of this dissertation

The overall goals of this research were to: define process configurations to maximize sugar release from pretreatments and *C. thermocellum* CBP combined and understand mechanisms underlying chemical and biological deconstruction of lignocellulosic biomass. This research showed that *C. thermocellum* was more effective compared to fungal enzymes in breaking down almost all the polysaccharides left in solids resulting from switchgrass pretreatments with make markedly different changes in the composition of the substrate. Further, *C. thermocellum* CBP realized 100% glucan solubilization and 100% glucan plus xylan release when combined with CELF pretreatment that removed extensive amounts of both lignin and hemicellulose from switchgrass. This result is unparalleled in literature and has never been shown for any combination of biomass digestion techniques. Further bulk composition of switchgrass seems to be the driving factor for *C. thermocellum* digestion of the substrate while substrate structural characteristics had a low impact on *C. thermocellum*. This is in stark contrast to digestion by fungal enzymes that are clearly impacted by substrate

characteristics, especially cellulose properties. Further, bulk lignin removal from switchgrass was a more effective approach to reducing recalcitrance than removal of xylan from switchgrass for both biological systems. The systematic approach followed in this work was necessary in order to study the integration of pretreatments with CBP and understand the process of switchgrass deconstruction by these processes.

Specifically, Chapter 3, impact of cellulose loading on *C. thermocellum* cellulolytic and metabolic performance, showed that increases in cellulose loading led to metabolic performance inhibition of the organism due to increased product formation and pH change. A substrate loading of 0.5 wt% glucan was determined to be the best for *C. thermocellum* flask fermentations that showed no inhibition of the organism. This substrate loading was used to study substrate effects on fermentations and discern substrate impacts on *C. thermocellum* from effects of underlying metabolic features of the fermentation system. *C. thermocellum*'s metabolic performance was affected before its cellulolytic ability was during fermentations with inhibitory substrate loadings. A drop in *C. thermocellum* metabolites yield, accounting for ethanol, lactic acid, and acetic acid, from 62% of glucan for 0.1-0.5 wt% glucan loadings of Avicel to about 40% at 1 wt% glucan was observed. This was accompanied by an increase in glucose accumulation at 1 wt% glucan loading of Avicel because the solids solubilization by *C. thermocellum* was >90%, which further revealed inhibition of metabolic performance. While pH change and acetic acid were shown to be inhibitory to *C. thermocellum* at high substrate loadings, the high carbon to nitrogen (C/N) ratio at high substrate loadings may have also impacted *C.*

thermocellum fermentations. If MOPS buffer is not metabolized by the organism, ammonium chloride is the major nitrogen source in the medium (see Table 3.1) used for *C. thermocellum* fermentations. 1.5 g/L NH_4Cl used during *C. thermocellum* fermentations amounts to ~0.4 g/L of nitrogen available for the organism. *C. thermocellum* was able to utilize at least 0.18 g/L of the available nitrogen after 12 hours of fermentation based on the pellet nitrogen growth profile of the organism shown in Figure 3.11. Thus, half the nitrogen provided to *C. thermocellum* through the medium is quickly utilized by the organism during fermentation possibly leading to nitrogen limitation during the rest of the fermentation. This would create a C/N ratio imbalance especially at the higher substrate loadings possibly leading to stressful conditions for the organism. Therefore, future work on impact of high substrate loadings on *C. thermocellum* will need to be studied while keeping the C/N ratio constant.

Chapter 4, impact of cellulose properties on *C. thermocellum* and fungal enzymatic saccharification, demonstrated that cellulose digestion by fungal enzymes is substantially impacted by cellulose properties, whereas *C. thermocellum* digestion of cellulose is not affected by the cellulose properties. Cellulose properties that influence fungal enzyme adsorption onto cellulose, such as surface area, pore size distribution, and crystallinity, had the greatest effect on fungal enzymatic digestion of cellulose. These results further suggest that the *C. thermocellum* cellulolytic system is more effective and efficient compared to fungal enzymes in cellulose digestion as the former is less sensitive to properties of cellulose while showing higher digestion.

Chapter 5, glucan accessibility drives digestion of lignocellulosic biomass by *C. thermocellum*, indicated that theoretical glucan solubilization and total sugar release from switchgrass was achieved using a CBP and CELF pretreatment combination. Further, lignin removal had a greater impact than xylan removal from switchgrass for achieving higher glucan solubilization and total sugar release. The effect of glucan accessibility, influenced by lignin, on its solubilization by *C. thermocellum* in turn also showed a direct correlation with metabolite production. The comprehensive nature of this work with comparison of four different pretreatment methods and two different biological digestion techniques is unparalleled and provides a strong platform for future work on integration of pretreatment with CBP.

Chapter 6, understanding the mechanism of thermochemical and biological breakdown of switchgrass, explored the mechanisms underlying processes used for the breakdown of switchgrass and the impact of switchgrass structural features on the digestion process itself. Unpretreated and pretreated switchgrass and their CBP and EH residues were extensively characterized by a suite of analytical techniques to provide an understanding of structural changes in switchgrass occurring during thermochemical and biological deconstruction. CELF pretreated solids showed the highest accessibility measured via Simons' staining and digestibility by both fungal enzymes and *C. thermocellum* followed by dilute alkali and dilute acid / hydrothermal pretreated solids. Unlike dilute alkali pretreatment, acid based pretreatments showed an increase in cellulose crystallinity in the following order: hydrothermal > dilute acid > CELF and also

reduced cellulose degree of polymerization substantially. All thermochemical and biological digestion approaches led to an increase in syringyl to guaiacyl lignin (S/G) ratio and a decrease in β-O-4 lignin interunit linkage and hydroxycinnamates content of unpretreated switchgrass. High pretreatment temperature and use of alkali as catalyst led to a decrease in G lignin. G lignin potentially leads to less crosslinking producing thinner cell walls that can easily be deconstructed. Both biological digestion approaches led to a decrease in either S or G lignin that was carbohydrate associated.

7.3. Recommendations for future research

While the work reported in this dissertation is extensive and systematic, the results reported are specific to switchgrass and *C. thermocellum* DSM 1313 and can be extrapolated to other types of biomass and bacterial strains only to a certain extent. In Chapter 3, inhibition of *C. thermocellum* was shown at higher substrate loadings that will be typically necessary in an industrial process. Future work on understanding *C. thermocellum* fermentations at high substrate loadings will need to be performed while keeping the C/N ratio constant to avoid nitrogen limitation during fermentation. The ethanol and acetic acid tolerance of the organism were also reported to be insufficient for a large scale process. Thus, future *C. thermocellum* genetic modification to improve ethanol yield and titers while improving ethanol tolerance of the organism and reducing the production of by-products is necessary. A number of genetic systems have been developed for *C. thermocellum* modification to eliminate acetate, lactate, hydrogen, and formate production and improve ethanol formation but these strains need to be

extensively screened on real world lignocellulosic biomass. Even though *C. thermocellum* has an effective cellulosome to digest cellulose, it is still limited in achieving theoretical sugar release from lignocellulosics. The work reported in this dissertation lays a foundation for future integration of thermochemical pretreatment techniques, required for effective biomass digestion, with fermentation using genetically modified *C. thermocellum* strains. However, thermochemical pretreatments utilize a wide range of chemicals and produce a number of polysaccharide and lignin degradation products as reported in Chapter 5. Limited use of chemicals and efficient recovery of harsh and expensive chemicals will be necessary while using pretreatments. The cost of using such chemicals will have to be weighed in comparison to the incomplete utilization of polysaccharides from biomass without pretreatment for ethanol production by *C. thermocellum*. A thorough techno-economic analysis of the process would be beneficial. Further, the utilization of hemicellulose sugars, which are released in the liquor during most pretreatments, for ethanol production is vital. Effective detoxification steps will be needed for the removal of degradation products in the liquor and utilization of hemicellulose sugars. Chapters 4 and 6 discuss the impact of substrate structural features on thermochemical and biological digestion of the substrate and in turn discuss the changes occurring in the biomass during these digestion techniques. These chapters point out that lignocellulosic biomass digestion is too complex to be controlled by any one single biomass structural feature. Even though we applied a suite of extensive analytical techniques on substrates and residues left behind after *C. thermocellum* fermentation and fungal hydrolysis, we were unable to definitively point out the mechanism of biomass

digestion or point to a few factors that substantially impacted digestion. But what is unique to *C. thermocellum* is that this organism is able to adjust its cellulosomal composition based on the substrate it encounters for effective digestion allowing for variation in biomass structural features to a certain extent. Similar work on different lignocellulosic biomass would provide a better understanding of the mechanism of biomass digestion for effective sugar release. Further, a comparison of *C. thermocellum* with other promising CBP organisms, such as, *Caldicellulosiruptor bescii*, and *Clostridium phytofermentans* will also be very useful.