Processing of lignocellulosics feedstocks for biofuels and co-products via consolidated bioprocessing with the thermophilic bacterium, *Clostridium thermocellum* strain DSMZ 1237

by

Valery B. Agbor

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Supervisory Committee

Dr. David B. Levin (Department of Biosystems Engineering, University of Manitoba)
Supervisor

Dr. Nazim Cicek (Department of Biosystems Engineering, University of Manitoba)
Departmental member

Dr. Ying Chen (Department of Biosystems Engineering, University of Manitoba)
Departmental member

Dr. Michael Butler (Department of Microbiology, University of Manitoba)
Departmental Member

Dr. Alex Berlin (Department of Biosystems Engineering, University of Manitoba)
Adjunct Member

Dr. Jay J. Cheng (Department of Biological and Agricultural Engineering, North Carolina State University)
External Examiner

Abstract

Processing of lignocellulosic biomass for transportation fuels and other biocommodities in integrated biorefineries has been proposed as the future for emerging sustainable economies. Currently bioprocessing strategies are all multi-step processes involving extensive physicochemical pretreatments and costly amounts of exogenous enzyme addition. Consolidated bioprocessing (CBP), or direct microbial conversion, is a strategy that combines all the stages of production into one step, thus avoiding the use of expensive pretreatments and exogenous enzymes that reduce the economic viability of the products produced. With a growing trend towards increased consolidation, most of the reported work on CBP has been conducted with soluble sugars or commercial reagent grade cellulose. For CBP to become practical fermentative guidelines with native feedstocks and purified cellulose need to be delineated through specific substrate characterization as it relates to possible industrial fermentation. By carefully reviewing the fundamentals of biomass pretreatments for CBP, a comparative assessment of the fermentability of non-food agricultural residue and processed biomass was conducted with *Clostridium thermocellum* DSMZ 1237. Cell growth, and both gaseous and liquid fermentation end-product profiles of *C. thermocellum* as a CBP processing candidate was characterised. Batch fermentation experiments to investigate the effect of cellulose content, pretreatment, and substrate concentration, revealed that higher yields were correlated with higher cellulose content. Pretreatment of native substrates that increased access of the bacterial cells and enzymes to cellulose chains in the biomass substrate were key parameters that determined the overall bioconversion of a given feedstock to end-products. The contribution of amorphous cellulose (CAC) in different biomass substrates subjected to the same pretreatment conditions was identified as a novel factor that contributed to differences in bioconversion and end-product

synthesis patterns. Although the overall yield of end products was low following bioaugmentation with exogenous glycosyl hydrolases from free-enzyme systems and cellulosome extracts. Treatment of biomass substrates with glycosyl hydrolase enzymes was observed to increase the rate of bioconversion of native feedstocks in biphasic manner during fermentation with *C. thermocellum*. A "quotient of accessibility" was identified as a feedstock agnostic guideline for biomass digestibility.

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List of Abbreviations

AC Alpha cellulose

ADF Acid detergent fibre

ATP Adenosine triphosphaye

AMP Adenosine Monophosphate

AOAC Association of Analytical Communities

BCC Beverage cup cellulose

CAC Contribution of amorphous cellulose

CBP Consolidated bioprocessing

CBM Carbohydrate binding module

eCBP Engineered consolidated bioprocessing

rCBP Ruminant consolidated bioprocessing

DMC Direct microbial conversion

GC Gas chromatography

GH Glycosyl hydrolase

HH Hemp hurds

HHD Hemp hurds double

HHQ Hemp hunds quadruple

HF Hemp fibre

HPLC High performance liquid Chromatograph

NDF Neutral detergent fibre

SHF Separate hydrolysis and fermentation

SSF Simultaneous saccharification and fermentation

SSCF Simultaneous saccharification and co-fermentation

PFC Purified flax cellulose

PHC Purified hemp cellulose

PRC Purified rice cellulose

PWC Purified wood cellulose

QA Quotient of Accessibility

XRD X-ray diffraction

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Dedication

I would like to dedicate this thesis primarily to my darling wife Helen Agbor. For her wonderful virtue of bringing out the best in me and for working hard at home with our Kids while I work my way through the maze of graduate school. You are my true blessing and I thank you. And secondarily to my parents, Ashu Rose my sweet mom, Rose Ngombabi, my step-mom and Charles Tambong Agbor, my Dad, for all their hard work and support to help me walk the path of the erudite.

Chapter 1

Biomass Pretreatment and Consolidated Bioprocessing for Biocommodities (CBP)

This chapter is based on the following literature review publications:

1) Agbor, VB, Cicek N, Sparling R, Berlin A, Levin DB. 2011. Biomass pretreatment: fundamentals toward application. Biotechnology advances 29, no. 6, 675-685;

2) Levin DB, Agbor VB, Carare C, Cicek N, Sparling R. 2014. Biomass Pretreatment for Consolidated Bioprocessing (CBP). Chapter 47, *In*, "Advances in Biorefineries: Biomass and waste supply chain exploitation", KW Waldron (Ed.). Woodhead Publishing, Cambridge, UK. ISBN-13: 978 0 85709 521 3.

My contributions: As may be seen above, I was the first author on the 2011 paper, and although I am listed as the second author of the 2014 paper, I made a significant contribution to the writing of this paper.

1.1 Abstract

Both biomass pretreatment and the subsequent bioprocessing strategy used employed determines the cost of the final feedstock biocommodity produced. This chapter reviews various types of physico-chemical pretreatment for lignocellulosic biomass. Different types of pretreatment methods and process conditions are described, and the effects of each method on the biomass are discussed. The different configurations of biomass processing are presented, the rational for consolidated bioprocessing (CBP), models of CBP and organism development for CBP are addressed to highlight the increasing trend towards consolidated bioprocessing as a pathway to low cost biorefining.

1.2 Introduction

The depletion of "sweet crude" oil reserves around the world and the increasing global effort to reduce dependence on petroleum-based fuels has intensified the development of biofuels as transportation and industrial fuels. The costs of production, transportation, preconditioning and pretreatment, and subsequent conversion of these feedstocks via microbial fermentation ultimately determines the costs of biomass-based ("cellulosic") fuels. The cost of biomass production drives the final cost of energy production from any given feedstock (Chandra et al., 2007; Lynd et al., 2002), but pretreatment of the biomass is the second most expensive unit cost in the conversion of lignocellulose to ethanol and other chemicals (NREL, 2002).

Industrial bioethanol production is advancing beyond grain-based ethanol production because of the energy limitations and economic / environmental concerns associated with sugar / starch-based ethanol production (Brown, 2006; Groom et al., 2008; Searchinger et al., 2008; Simpson et al., 2008). With the annual global energy demand predicted to increase to 17 billion tonnes of oil by 2035, it is evident that fuels derived from lignocellulosic biomass are an attractive and less expensive alternatives for local biofuel production, compared to sugars-, starch-, or oil-based feedstocks with higher economic value.

Lignocellulosic biomass is the most abundant organic material in nature with 10-50 billion tonnes annual worldwide production (Claassen et al., 1999). Lignocellulosic biomass consists of tightly knit polymers (cellulose, hemicelluloses, lignin, waxes, and pectin) synthesized by plants as they grow. Figure 1.1 shows inter-relationship between the 3 major plant polymers targeted by the biorefinery.

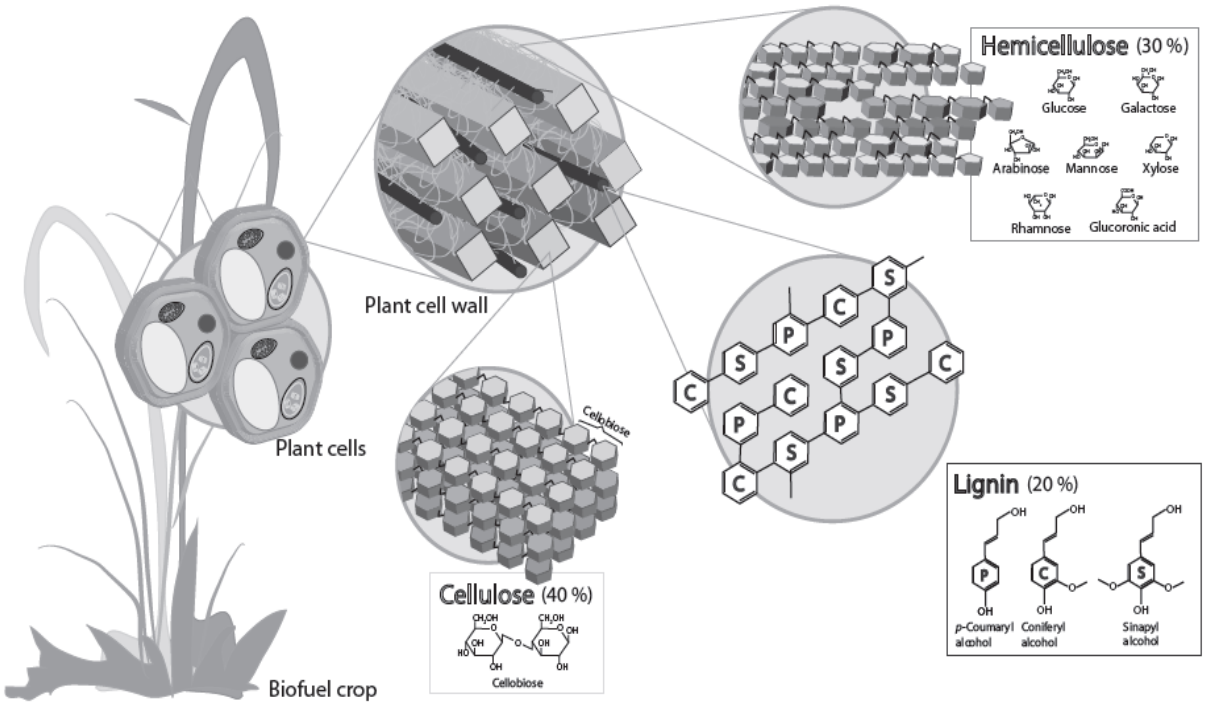


Figure 1.1 Plant Biomass polymers (cellulose, hemicelluloses and lignin). The, source of polymers, the inter-relatedness in the heteromatrix and the monomeric units of the polymers from a woody plant showing approximate percentages (Levin et al., 2013).

As a consequence of its complex and highly order structure, lignocellulosic biomass is inherently recalcitrant to bioprocessing and requires deconstruction by various pretreatments to release sugars within the biopolymers for fermentation. The term “pretreatment” was coined to describe any process that converts lignocellulosic biomass from its native form (which is recalcitrant to hydrolysis with cellulases) to a form that is more digestible by hydrolytic enzymes (Lynd et al., 2002). Pretreatments range from simple size reduction to more advanced biological or physico-chemical processes designed to improve the digestibility of the biomass. Table 1.1 summaries the various physical pretreatments and their effects on biomass structure, while Table 1.2 presents a summary of the major physico-chemical pretreatments. A combination of physical and physico-chemical pretreatments is often used to improve the digestibility of lignocellulosic biomass (Agbor et al., 2011).

Pretreaments that modify the biomass composition to make it more accessible vary from neutral, to acidic, to quite alkaline (Table 1.2). Dilute acidic pretreatments will hydrolyze the mostly hemicelluloses, leaving cellulose and lignin intact. Alkaline pretreatments will solubilise less hemicellulose and lignin than acidic pretreatments, but will alter the structural / chemical nature of the lignin, producing a hydrated cellulose product, mixed with hemicelluloses and lignin. Solvent-based pretreatment such as Organosolv pretreatments will solubilise almost all of the hemicellulose, precipitate the lignin, and leave behind a purer cellulose mesh (Merino and Cherry, 2007; Mosier et al., 2005; Zhao et al., 2009).

Table 1.1 Various physical pretreatments and their effects on biomass structure.

Method	Particle size (mm)	Main effect on biomass	References
Coarse size reduction	10-50	Increase in available surface area	(Cadoche and López, 1989; Palmowski and Muller, 1999)
Chipping	10-30	Decrease heat and mass transfer limitations	(Palmowski and Muller, 1999)
Grinding	0.2-2	Shearing, Reduce particle size, degree of polymerisation and cellulose crystallinity	(Agbor et al., 2011; Sun and Cheng, 2002)
Milling (Disk, hammer and ball milling)	0.2-2	Shearing, Reduce particle size, degree of polymerisation and cellulose crystallinity	(Zhua et al., 2009)
Use of microwaves	No change	Effects compaction of biomass and specific energy required for Compression when used with a chemicals e.g. NaOH and water	(Kashaninejad and Tabil, 2011)
Use of gamma rays	No change	Cleave β-1,4 glycosidic bonds, thus increasing surface area and decrease cellulose crystallinity	(Takacs et al., 2000)

Table 1.2 Summary of physico-chemical pretreatment methods.

Method	Process conditions	Mode of action	References
Steam pretreatment or Steam explosion	Involves rapidly heating biomass with steam at elevated temperatures (190–240 ^{o}C) and pressures between 0.7 and 4.8 Mpa with residence times of 3–8 min followed by explosive decompression as the pressure is released.	Hemicellulose hydrolysis is thought to be mediated by the acetic acid generated from acetyl groups associated with hemicellulose and other acids released during pretreatment The pressure is held for several seconds to a few minutes to promote hemicellulose hydrolysis and then released.	(Mcmillan, 1994) (Mosier et al., 2005) (Weil et al., 1997)
Liquid hot water pretreatment	Optimally operated between 180-190 ^{o}C e.g. for corn stover) and at low dry matter- about (1-8 %) content, leading to more poly and oligosaccharide production. Temperature of 160-190 ^{o}C are used for pH controlled LHW pretreatment and 170-230 ^{o}C have been reported depending on the severity of the pretreatment.	Hemiacetal linkages are cleaved by hot water, liberating acids during biomass hydrolysis, which facilitates the breakage of ether linkages in the biomass.	(Bobleter, 1994) (Wyman et al., 2005)
Dilute acid pretreatment	Dilute sulfuric acid is mixed with biomass to increasing the accessibility to the cellulose in the biomass by solubilising hemicellulose. The mixture is heated directly with the use of steam as in SP, or indirectly via the vessel walls of the reactor.	The substrate is heated to the desired temperature in an aqueous solution and pretreated using preheated sulfuric acid (concentrations of < 4 wt %) in a stainless-steel reactor. In this pretreatment the dilute acid releases oligomers and monomeric sugars by affecting the reactivity of the biomass carbohydrate polymers.	(Esteghalian et al., 1997) (Torget et al., 1990)

Ammonia fibre / freeze explosion , Ammonia recycle percolation and Soaking aqueous ammonia	By bringing biomass in contact with anhydrous liquid ammonia at a loading ratio of 1:1 to 1:2 (1-2 kg of ammonia/ kg of dry biomass) for 10-60 min at 60-90 ^{o}C and pressures above 3 MPa, or (150- 190 ^{o}C) for a few minutes	The chemical effect of ammonia or ammonia under pressure causes the cellulosic biomass to swell, thus increasing the accessible surface area while decrystalizing cellulose as the ammonia penetrates the crystal lattice to yield a cellulose-ammonia complex.	(Alizadeh et al., 2005) (Kim and Lee, 2005) (Mittal et al., 2011)
Organosolv pretreatment	Organosolv pretreatments are conducted at high temperatures (100-250 ^{o}C) using low boiling point organic solvents (methanol and ethanol) or high boiling point alcohols (ethylene glycol, glycerol, tetrahydrofurfuryl alcohol) and other classes of organic compounds.	Pretreatments with organic solvents extract lignin and solubilised hemicelluloses by hydrolyzing the internal lignin bonds, as well as the ether and 4-O-methylglucuronic acids ester bonds between lignin and hemicellulose and also by hydrolyzing glycosidic bonds in hemicellulose, and partially in cellulose depending on process conditions.	(Thring et al., 1990) (Zhao et al., 2009)
Lime pretreatment **and** **Wet oxidative pretreatment**	Conducted over a wide temperature range 25-130 ^{o}C using 0.1 g $Ca(OH)_2$ / g biomass at low pressures. Treatment of biomass with air, water and oxygen at temperatures above 120 with or without a catalyst such as an alkali.	Solubilise hemicelluloses and lignin by deactylation and partial delignification. Oxidative factors come into play when oxygen is introduced at high pressures.	(Chang et al., 1997) (Chang et al., 2001) (Galbe and Zacchi, 2007)
Carbon dioxide explosion pretreatment	Involves the use of supercritical carbondioxide at high pressures (1000-4000psi) at a given temperature up to 200 ^{o}C for a few minutes.	Carbonic acid formed from the penetration of carbon dioxide into wet biomass at high pressure helps in hemicelluloses hydrolysis while the release of the pressure results in	(Kim and Hong, 2001); (Zheng et al.,

		disruption of biomass.	1995)
Ionic-Liquid Pretreatment	Using ionic liquids at temperatures < 100 ^{o}C as non-derivatising solvents to effect dissolution of cellulose .	Ionic liquids disrupt the three dimensional network of lignocellulosic components by competing with them for hydrogen bonding.	(Moultrop et al., 2005) (Zavrel et al., 2009)
Fractionation Solvents	Cellulose and organic-solvents lignocellulose fractionation	Solvents such as phosphoric acids, sulphite or ionic liquids enable disruption of fibrillar structure of biomass and effecting cellulose crystallinity.	(Zhu et al.,2009)

1.3 Cellulose

The relative abundance of cellulose, hemicellulose, and lignin are *inter alia,* key factors in determining the optimum energy conversion route for each type of lignocellulosic biomass (Mckendry, 2002). Cellulose is the main constituent of plant cell wall conferring structural support and is also present in bacteria, fungi, and algae. When existing as an unbranched, homopolymer, cellulose is a polymer of ß-D-glucopyranose moieties linked via ß-(1-4) glycosidic bonds with well documented polymorphs (Figure 1.2). The degree of polymerization of cellulose chains in nature ranges from 10000 glucopyranose units in wood to 15000 in native cotton. The repeating unit of the cellulose chain is the disaccharide cellobiose as oppose to glucose in other glucan polymers (Desvaux, 2005; Fengel and Wegener, 1984). The cellulose chains (20-300) are a grouped together to form microfibrils, which are bundled together to form cellulose fibres. The cellulose microfibrils are mostly independent but the ultrastructure of cellulose is largely due to the presence of covalent bonds, hydrogen bonding and Van der Waals forces. Hydrogen bonding within a cellulose microfibril determines ‘straightness’ of the chain but interchain hydrogen bonds might introduce order (crystalline) or disorder (amorphous) into the structure of the cellulose (Laureano-Perez et al., 2005).

1.4 Hemicellulose

Hemicellulose is the second most abundant polymer (20-50 % of lignocellulose biomass) and differs from cellulose in that it is not chemically homogeneous. Hemicelluloses are branched, heterogenous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose) and acetylated sugars. They have lower molecular weight compared to cellulose and branches with short lateral chains that are easily hydrolysed (Fengel and Wegener, 1984; Saha, 2003).

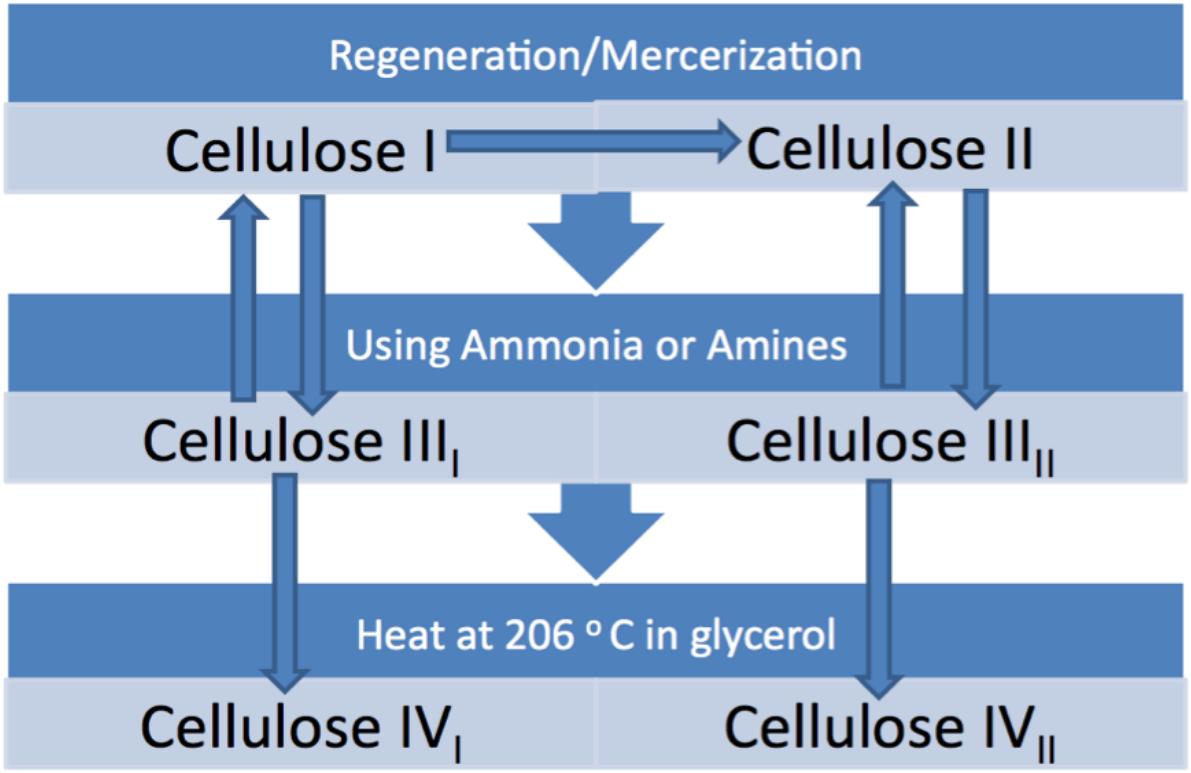


Figure 1.2 Interconversion of polymorphs of cellulose. Cellulose I, is native or natural cellulose. Cellulose II obtained from cellulose I by solubilisation in a solvent followed by precipitation by dilution in water (i.e. regeneration) or by mercerization, which involves the swelling of native cellulose fibres in concentrated sodium hydroxide upon removal swelling agent. Cellulose III_1 and III_{11} are formed by treatment with ammonia or amines from celluloses I and II, respectively. Polymorphs IV_1 and IV_{11} are derived from III_1 and III_{11} respectively by heating to 206 ^{o}C in glycerol (O'Sullivan, 1996)

Hemicelluloses differ in composition. Hemicelluloses in agricultural biomass, like straw and grasses, are composed mainly of xylan, while softwood hemicelluloses contain mainly glucomannan. In many plants xylans are heteropolysaccharides with backbone chains of 1,4-linked ß-D-xylopyranose units. In addition to xylose, xylan may contain arabinose, glucuronic acid, or its 4-*O*- methyl ether, acetic acid, ferulic and p-coumaric acids. Xylan can be extracted easily in an acid or alkaline environment while extraction of glucomannan requires a stronger alkaline environment (Balaban and Ucar, 1999; Fengel and Wegener, 1984).

Among the key components of lignocellulosics, hemicelluloses are the most thermo-chemically sensitive (Hendricks and Zeeman, 2009; Levan et al., 1990). Hemicelluloses within plant cell walls are thought to 'coat' cellulose-fibrils and it has been proposed that at least 50% of hemicellulose should be removed to significantly increase cellulose digestibility. Nevertheless, severity parameters must be carefully optimized to avoid the formation of hemicellulose degradation products such as furfurals and hydroxymethyl furfurals which have been reported to inhibit the fermentation process (Palmqvist and Hahn-Hägerdal, 2000a; Palmqvist and Hahn-Hägerdal, 2000b). For this reason, pretreatment severity conditions are usually a compromise to maximize sugar recovery and depending upon what type of pretreatment method is used hemicellulose could be obtained either as a solid fraction or a combination of both solid and liquid fractions (Chandra et al., 2007).

1.5 Lignin

Lignin is the third most abundant polymer in nature. It is present in plant cell walls and confers a rigid, impermeable, resistance to microbial attack and oxidative stress. Lignin is an amorphous heteropolymer network of phenyl propane units (p-coumaryl, coniferyl and sinapyl alcohol) held together by different linkages (Hendricks and Zeeman, 2009). Lignin is generally

accepted as the ‘glue’ that binds the different components of lignocellulosic biomass together, thus making it insoluble in water. Because of its close association with cellulose microfibrils, lignin has been identified as a major deterrent to enzymatic and microbial hydrolysis of lignocellulosic biomass (Avgerinos and Wang, 1983). Chang and Holtzapple showed that biomass digestibility is enhanced with increasing lignin removal (Chang and Holtzapple, 2000). In addition to being a physical barrier, the detrimental effects of lignin include: i) non-specific adsorption of hydrolytic enzymes to ``sticky´´ lignin; ii) interference with, and non-productive binding of cellulolytic enzymes to lignin-carbohydrates complexes; and iii) toxicity of lignin derivatives to microorganisms.

Different feedstocks contain different amount of lignin that must be removed via pretreatment to enhance biomass digestibility. The lignin is believed to melt during pretreatment and coalesces upon cooling such that its properties are altered; it can subsequently be precipitated (Brownell and Saddler, 1987; Converse, 1993; Lynd et al., 2002). Delignification (extraction of lignin by chemicals) causes biomass swelling, disruption of lignin structure, increases in internal surface area, and increased accessibility of cellulolytic enzymes to cellulose fibers. Although not all pretreatments result in substantial delignification, the structure of lignin may be altered without extraction due to changes in the chemical properties of the lignin. The pretreated biomass becomes more digestible than the raw biomass even though it may have approximately the same lignin content as non-pretreated biomass.

1.6 Process configurations for biofuel production

Industrial scale cellulosic biofuels production requires efficient, low cost processes that will ensure economic viability. The current paradigm for bioprocessing of lignocellulosic biomass into bioethanol involves a four-step process: (i) cellulase production; (ii) hydrolysis of

polysaccharides; (iii) fermentation of soluble cellulose hydrolysis products; and (iv) fermentation of soluble hemicellulose hydrolysis products (Lynd et al., 2002). These processes have been segmented in different combinations over time to design different configurations or processing strategy to reduce the cost of biofuels production as shown in Figure 1.3 below.

Separate Hydrolysis and fermentation (SHF) is a four-stage process with a separate biocatalyst for each. Simultaneous saccharification and fermentation (SSF) combines hydrolysis and fermentation of hexose (C6) sugars, without the pentose (C5) sugars, while simultaneous saccharification and co-fermentation (SSCF) combines cellulose hydrolysis and fermentation of both hexose and pentose sugars in one step. Currently, majority of pilot studies and industrial processes have proceeded with separate saccharification and fermentation options for bioprocessing featuring enzymatic hydrolysis e.g. bioethanol production.

Consolidated bioprocessing (CBP) on the other hand combines cellulase production and substrate hydrolysis and fermentation of the hydrolysate (both hexose and pentose sugars) in one step, thus saving the cost of investing in a multi-step process (Lynd, 1996; Lynd et al., 2002; Xu et al., 2009). Of all the reported technological advances to reduce processing costs for cellulosic ethanol, CBP has been estimated to reduce production costs by as much as 41% (Lynd et al., 2008). A techno-economic evaluation for bioethanol production from softwood (spruce), hardwood (salix), and an agricultural residue (corn stover), concluded that the process configuration (SSF) had greater impact on the cost reduction compared to the choice of substrate (Sassner et al., 2008). Hence, direct microbial conversion (DMC) or CBP of lignocellulosic biomass utilizing bacteria, may be considered a preferred method for ethanol or biological hydrogen production (Carere et al., 2008; Levin et al., 2006).

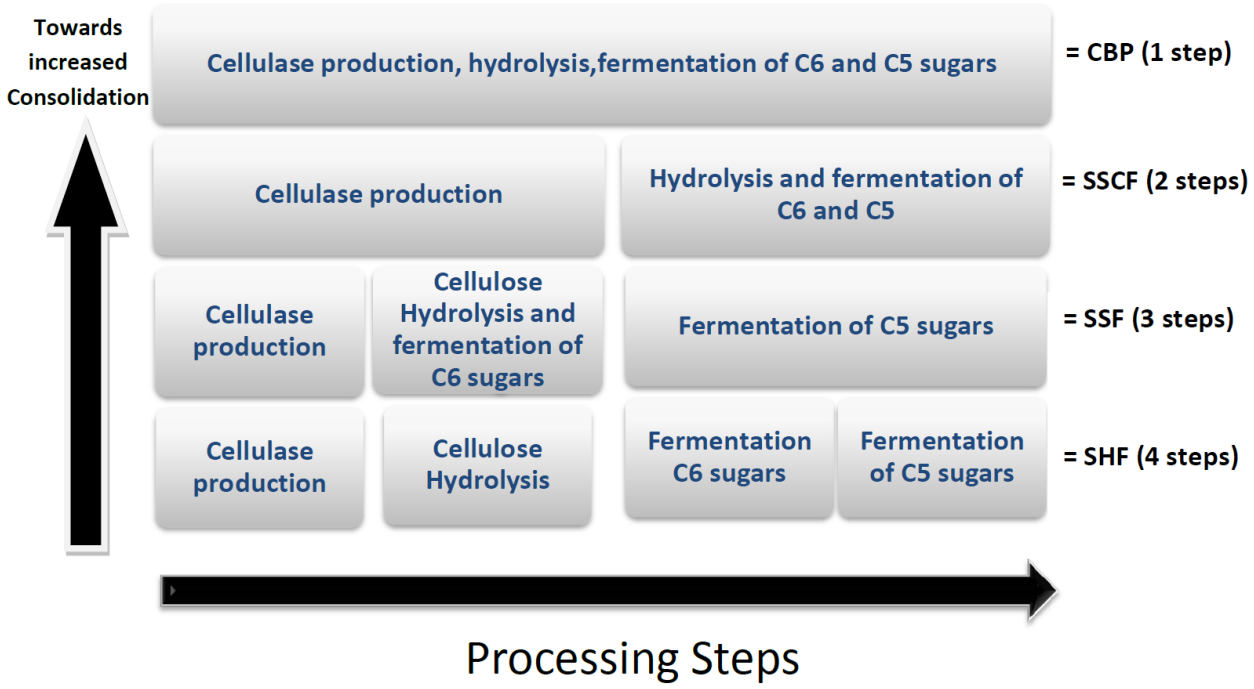


Figure 1.3 Evolution of biomass processing strategies featuring enzymatic hydrolysis. The horizontal arrow indicates the four primary treatment processes involved with cellulosic ethanol production. The vertical arrow indicates steps taken towards increased consolidation. SHF: separate hydrolysis and fermentation, SSF: simultaneous saccharification and fermentation, SSCF: simultaneous saccharification and co-fermentation, CBP: consolidated bioprocessing (adapted from Lynd et al., 2002).

Although CBP offers the greatest potential for reducing the costs of biofuel and co-product production, a great deal remains to be realised in the development of microbial biocatalysts that can utilise cellulose and other fermentable sugars to produce the products of interest with yields that are industrially relevant. In the rest of this chapter, we discuss the rationale for, and models of, CBP as well as strategies for development of microbial biocatalysts that may improve yields of desired products from CBP-based biorefineries.

1.7 The rationale for CBP

Bioenergy use is propagated by incentives and successful policy interventions that have been enacted to select and encourage industrial development of renewable energy sources. Bioethanol continues to be the dominant biofuel with increasing use as a transportation fuel in Brazil, USA, Canada, and the European Union (EU). The routine processes (SHF, SSF, and SSCF) for industrial ethanol production all involve a dedicated cellulase production step. Avoiding this step has been noted to be the largest potential energy saving step. For a given amount of pretreated biomass, it has been shown that CBP offers cost saving benefits that are not associated with the routine methods, even at the lowest amount of cellulase loading required to produce fermentation products. However, typical CBP yields of ethanol and other end-products are much lower than obtained via traditional processes (Lynd et al., 2008; Lynd et al., 2005).

Hydrogen (H_2), on the other hand, is considered a clean energy carrier, having a gravimetric energy density of 122 KJ g^{-1} and water as the only by product of combustion. Currently H_2 is produced via energy intensive, environmentally harmful processes such as catalytic steam reformation of methane, nuclear or fossil fuel-mediated electrolysis of water, or by coal gasification (Carere et al., 2008; Levin et al., 2004). Biological H_2 production via anaerobic fermentation using cellulosic substrates is an attractive process because: (i) it is less

energy intensive; (ii) utilizes a simple process design and feedstock processing; and (iii) has the potential to utilise agricultural or agri-industrial by-product streams (Levin et al., 2006; Sparling et al., 1997; Valdez-Vazquez et al., 2005). However, rates and yields of H_2 by direct microbial conversion, are low, and increasing the rates and yields remains a challenge for biological H_2 production by CBP (Levin et al., 2009).

1.8 Models of CBP

The trend in industrial bioprocessing for biofuels and other industrial products, such as lactic acid, glutamic acid, n-butanol, and pinene (Hasunuma et al., 2012) is toward increased consolidation of the different process steps (as described in Figure 10.2). Lessons drawn from nature and the industry can help further research and development of CBP for biofuels and co-products, given that CBP seeks to mimic natural microbial cellulose utilisation for industrial applications.

1.8.1 Ruminant or natural CBP

Many animals and insects have evolved to feed on and digest raw biomass. Some well known cellulolytic bacteria have been isolated from these organisms as their natural habitat e.g. *Ruminococcus albus* and *Clostridium termitidis* in habit the gut of ruminants and termites, respectively. By taking a close look at the highly developed ruminal fermentation of cattle (i.e. ruminant CBP = rCBP), Weimer et al. (2009), proposed that breakthroughs developed by ruminants and other already existing anaerobic systems with cellulosic biomass conversion, can guide future improvements in engineered CBP (eCBP) systems. Comparing the journey of the feed through the bovine digestive tract to the transformation process of a cellulosic feedstock in a biorefinery, Weimer et al. (2009) suggest that the sliding, longitudinal movement of bovine rumination is a better physical pretreatment than conventional grinding. This is because it results

in substantial increase in surface area of the plant material available for microbial attack resulting in ``effective fibre´´ properties similar to burr mills. Burr mills consume two thirds of the energy required by hammer mills, and although they have been considered less efficient in the grinding of grain, they could be efficient in the milling of lignocelllulosic biomass for CBP as a result of the ``effective fibre´´ properties generated (Weimer et al., 2009b).

A great amount of effort is invested chewing the feed into a fine physically pretreated state. The feed is masticated while it is moist, another strategy supported by recent studies which show that milling after chemical treatment will significantly reduce energy consumption, reduce cost of solid-liquid separation requirements, and reduce the energy required for mixing pretreated slurries (Zhu and Pan, 2010; Zhua et al., 2009). Another similarity is the fact that ruminal microflora (*R. albus, R. flavefaciens and fibrobacter succinogens*) found in high numbers in the rumen are capable of rapid growth on cellulose using cellulosomal complexes similar to the well characterised cellulosomes of *Clostridium thermocellum or C. phytofermentans* that are being investigated for eCBP (Lynd et al., 2002; Weimer et al., 2009b). Thus, the limitation of rCBP could be explored to develop better operating parameters for eCBP. In summary, it appears that ruminants have developed an efficient and elegant physical pretreatment process that could provide insight in developing a physical pretreatment process tailored for industrial CBP, as well as serve as a model for eCBP.

1.8.2 Engineered CBP

Unlike rCBP, eCBP seeks to utilise pure cultures of specialist native cellulolytic bacteria, or recombinant cellulolytic bacteria, to convert cellulose via direct fermentation to value-added end-products. Aerobic or anaerobic microorganisms could be used for eCBP, however the use of a separate aerobic step for cell growth is not envisioned because it is not a characteristic feature

of CBP. In eCBP, much attention is dedicated to the strategic development of cellulolytic and hemicellulolytic microorganisms both for substrate utilisation and end-product formation. Among the many bacteria that have been considered as CBP-enabling microorganisms, anaerobic bacteria such as *C. thermocellulum,* C. *phytofermentans* , and the aerobic yeast *S.accharomyces cerevisiae* have been the most investigated as potential eCBP enabling microorganisms. Figure 1.4 compares natural and engineered bioprocessing by differentiating the different unit operations.

1.9 Microorganisms, enzyme systems and bioenergetics of CBP

1.9.1 CBP Microorganisms

Based on substrate utilization, carbohydrate hydrolyzing species represent a wide-range of specialist and non-specialist microbes, and specialized microbes capable of utilizing cellulose or hemicellulose-derived sugars are preferentially selected as CBP-enabling microorganisms. Cellulolytic bacteria belong to the Phyla Actinobacteria, Proteobacteria, Spirochates, Thermotogae, Fibrobacteres, Bacteriodes, and Firmicutes but approximately 80% of the cellulolytic bacteria are found within the Firmicutes and Actinobacteria (Bergquist et al., 1999). Many of these bacteria isolated from soil, insects, ruminants, compost, and sewage have the natural ability to hydrolyse cellulose and/or hemicellulose with the majority of the reported bacteria belonging to the Phylum Firmicutes, and are within the Class Clostridia and the Genus *Clostridium*. For example, *Clostridium thermocellum* is a gram positive, acetogenic, obligate anaerobe with the highest known grown rate on crystalline cellulose and the most investigated as a potential CBP-enabling bacteria (Lynd et al., 2002; Xu et al., 2009).

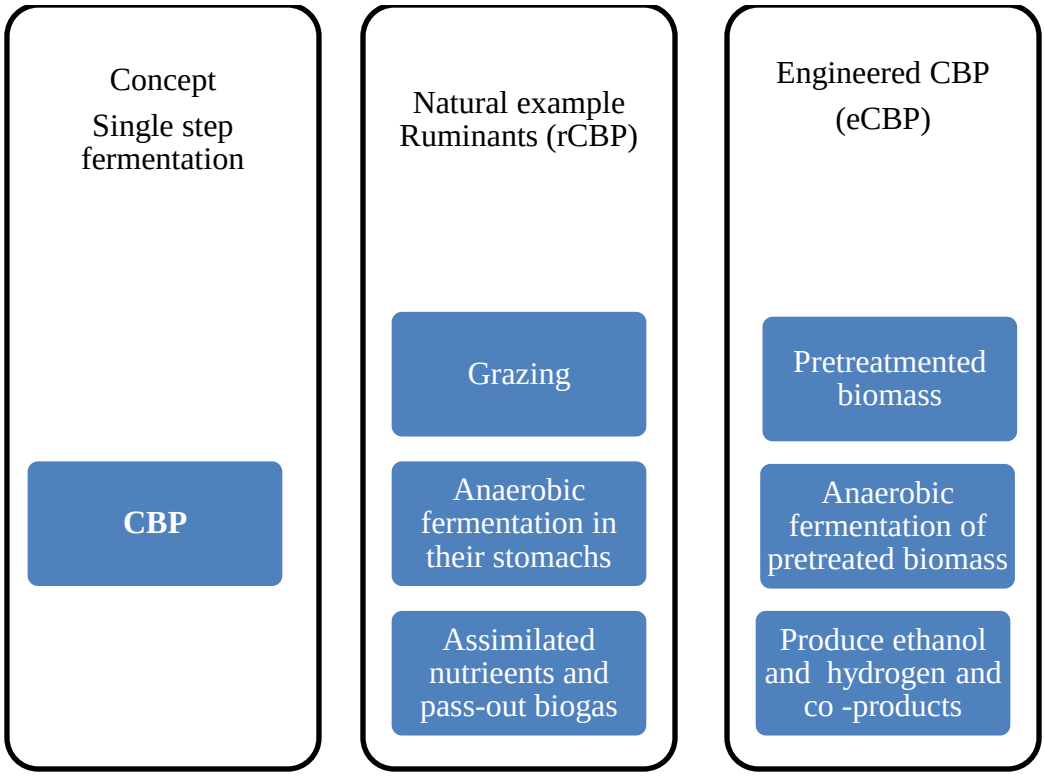


Figure 1.4 Comparison of natural and engineered consolidated bioprocessing by differentiating the different unit operations adapted from Weimer and co-workers (Weimer et al., 2009b).

Although cellulolytic bacteria belong to aerobic or anaerobic group of bacteria, for large scale CBP, anaerobiosis is advantageous because of oxygen transfer limitations that are avoided when using anaerobic bacteria (Demain et al., 2005). However, because of the increasing tendency to consolidate steps for bioethanol production, *S. cerevisiae* and the fungus *Tricoderma reesei* are being investigated as CBP enabling candidates for bioethanol production (van Zyl et al., 2007; Xu et al., 2009). Other candidates into which saccharolytic systems have been engineered for CBP include *Zymomonas mobilis* , *Escherichia Coli,* and *Klebsiela oxytoca* (van Zyl et al., 2007).

1.9.2 Carbohydrate active enzyme systems

To utilize plant biomass for growth, microorganisms produce multiple enzymes that hydrolyse the cellulose, hemicellulose, and pectin polymers found in plant cell walls (Warren, 1996). As a class, these carbohydrate active enzymes are referred to as glycoside hydrolases (GHs). Extracellular GHs can be secreted freely into the environment surrounding the cell (non-complex GH systems) or they can be cell-associated in large enzyme complexes (cellulosomes). Gycoside hydrolases that specifically target cellulose include: i) Endoglucanases, (1,4 ß-D-glucan-4-glucanohydrolases, which cleave random internal amorphous sites of a cellulose chain producing cellulo-dextrins of various lengths and thus new chain ends; ii) Exoglucanases (including 1,4-ß-D-glucanohydrolases or cellodextrinases and 1,4-ß-D glucan cellobiohydrlases, or simply cellobiohydrolase), which act in a processive manner on either the reducing and non-reducing ends of cellulose chains liberating either D-glucose (glucanohydrolase) or D-cellobiose (cellobiohydrlase) or shorter cellodextrins; and iii) ß-glucosidase (ß-glucoside glucohydrolases), which hydrolysze soluble cellodextrins and cellobiose to glucose. The ability of cellulases to

hydrolyse ß-1,4 glycosidic bonds between glucosyl residues distinguishes cellulase from other glycoside hydrolases (Lynd et al., 2002).

1.9.3 Non-complex glycoside hydrolase systems

Non-complex systems consist of secreted glycoside hydrolases and generally involve fewer enzymes. Aerobic fungi of the genera *Trichoderma* and *Aspergillus* have been the focus of research for non-complex cellulase systems. *Trichoderma reesei,* which is the most researched non-complex cellulase system, produces at least two exoglucanases (CBHI and CBHII), five endoglucanases (EGI, EGII, EGIII, EGIV, and EGV) and two ß-glucosidases (BGLI and BGLII) that act synergistically in the hydrolysis of polysaccharides. However, CBHI and CBHII are the principal components of the *T. reesei* cellulase system representing 60% and 20%, respectively, on a mass basis of the total protein produced (Lynd et al., 2002). The ability to produce and secret over 100 g of cellulase per litre of culture has established *T. reesei* as a commercial source of cellulase enzymes (Xu et al., 2009).

1.9.4 Complex glycoside hydrolase systems

Some anaerobic cellulolytic microorganisms possess a specialised macromolecular complex of carbohydrate active enzymes known as the cellulosome. First described for *C. thermocellum* by Lamed et al. (1983), the cellulosome is an exocellular, multi-component complex of GHs that mediates binding to lignocellulosic biomass and subsequent hydrolysis of the cellulose and hemicellulose polymers (Carere et al., 2008; Lamed et al., 1983). Functionally, cellulosomes are assembled on the cell walls of bacteria and enable concerted enzyme activity by minimizing distances of enzyme substrate interactions and optimizing synergies among the catalytic components, thus enabling efficient hydrolysis of the polymers and uptake of the hydrolysis products (Lynd et al., 2002).

Although cellulosome composition can differ in the number and variety of GHs from one species to another, they generally consist of catalytic components attached to a glycosylated, non-catalytic scaffold protein that is anchored to the cell wall. In *C. thermocellum*, the anchor protein, known as the cellulose integrating protein (CipA), or 'scalffoldin', is a large (1850 amino acid long and 2-16 MDa) polypeptide, which is anchored to the cell wall via type II cohensin domains. The *C. thermocellum* cellulosome contains 9 GHs with endoglucanase activity (CelA, CelB, CelD, CelE, CelF, CelG, CelH, CelN, and CelP), 4 GHs which exhibit exoglucanase activity (CbhA, CelK, CelO, CelS), 5-6 GHs which exhibit xylanase activity (XynA, XynB, XynV, XynY, XynZ), 1 enzyme with chitinase activity (ManA), and 1-2 with lichenase activity (LicB). CelS, the major exoglucanase, and Cel A, the major endoglucanase associated with the *C. thermocellum* cellulosome generate oligocellulodextrins containing two (cellobiose) to five (cellopentose) glucose residues (Demain et al., 2005; Lynd et al., 2002). The cellulosomes of some strains of *C. thermocellum* have been shown to degrade pectin probably via pectin lyase, polygalacturonate hydrolase, or pectin methylesterase activities. Other minor activities include ß-xylosidase, ß-galactosidase, and ß-mannosidase (Demain et al., 2005; Lynd et al., 2002). These modules have dockerin moieties that can associate with the cohesion domains of the scaffoldin to form the cellulosome, as shown in Figure 1.5.

Cellobiose phosphorylase, which hydrolyses cellobiose and longer chain oligocellulodexrins to glucose and glucose-1-phospahte via substrate level phosphorylation, and cellodextrin phosphorylase, which phosphorylates ß-1,4-oligoglucnas via phosphorolytic cleavage, have also been associated with the *C. thermocellum* cellulase system. Unlike the fungal cellulases, the celllosome of *C. thermocellum* is able to completely solubilize crystalline cellulose such as Avicel, a characteristic referred to as Avicelase or 'true cellulase activity

(Demain et al., 2005). Moreover, the *C. thermocellum* cellulase system results in the oligosaccharide hydrolysis products that are different from those of aerobic cellulolytic fungi like *T. reesei*, which generates cellobiose as the primary hydrolysis product (Zhang and Lynd, 2005).

1.9.5 Mode of action

Polysaccharide hydrolyzing enzymes such as cellulases and xylanases are modular proteins consisting of at least two domains: i) the catalytic module; and ii) the carbohydrate binding module (Gilkes et al., 1991; Horn et al., 2012). Common features of most GH systems that effects binding to cellulose surface and facilitate hydrolysis are the carbohydrate binding module (CBMs), which are known to have the following functions: i) CBMs play a non-catalytic role by "sloughing-off" cellulose fragments from the surface of cellulosic biomass by disrupting the non-hydrolytic crystalline substrate (Lynd et al., 2002); ii) they help concentrate the enzymes on the surface of the substrate (i.e. the proximity effect or phase transfer); and iii) they help in substrate targeting / selectivity. CBMs specific for insoluble cellulose are categorized as Type A CBMs, which interact with crystalline cellulose and Type B, which interact with non-crystalline cellulose (Arantes and Saddler, 2010a).

Recent studies that have focused on bacterial and fungal GHs have identified two GH families that have flat substrate-binding surfaces with the capability of cleaving crystalline polysaccharides via an oxidative reaction mechanism that depends on the presence of divalent metal ions and an electron donor. These two families are the Family 33 Carbohydrate Binding Module (CBM33) proteins identified in bacteria and Family 61 Glycoside Hydrolases from fungi (Horn et al., 2012; Vaaje-Kolstad et al., 2010). CBM33 and GH61 GHs bind to cellulose via their flat CBM substrate binding sites, which disrupt the orderly packing of the crystalline

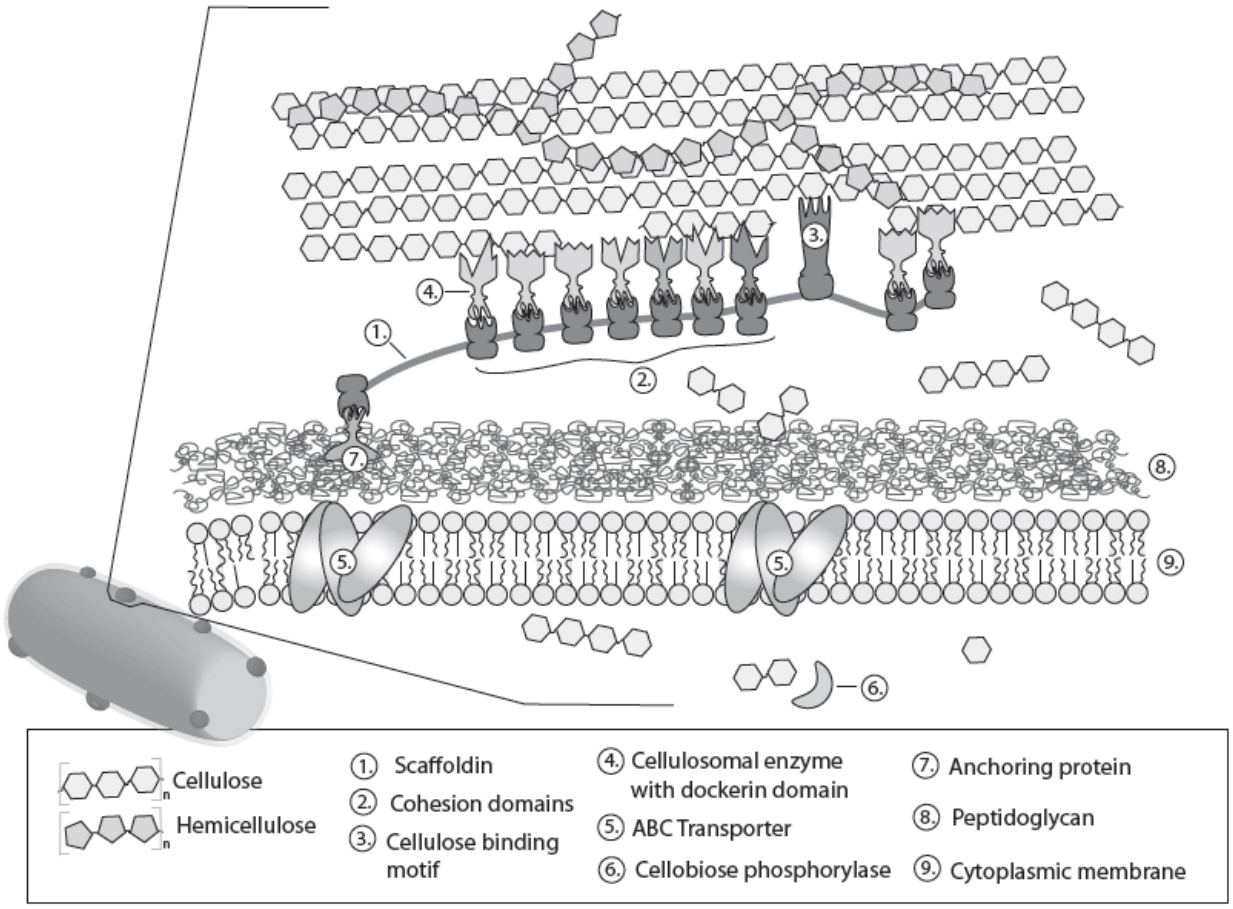


Figure 1.5 Structural representation of the cellulosome as a macromolecular enzyme complex on the surface of a cellulolytic bacteria , displaying the various components of a complex cellulase system (Levin et al., 2013).

cellulose chains, creating accessible points by both introducing cuts in the polymer chains and by generating charged groups at the cut sites.

Endoglucanases and exoglucanses act synergistically. The endoglucanases generate new reducing and non-reducing ends for exoglucanases, which in turn release soluble cellodextrins and cellobiose that are converted to glucose by ß-glucosidase (Horn et al., 2012; Kostylev and Wilson, 2012; Wood and McCrae, 1979). However, for GHs to efficiently hydrolyze cellulosic biomass, they must first be able to access the cellulose chains that are tightly packed in microfibrils trapped within a heteropolymer matrix (Arantes and Saddler, 2010a; Horn et al., 2012). Factors that increase accessibility have been identified and intensely investigated (Arantes and Saddler, 2010a; Arantes and saddler, 2010b; Horn et al., 2012; Jeoh et al., 2007; Reese, 1956).

1.10. Bioenergetics of CBP

The bioenergetics of CBP will differ with the type (aerobic, aerotolerant, or anaerobic) and number (pure, co-, or mixed-cultures) of microorganisms being considered as CBP-enabling agents. This was thought to be even more challenging for anaerobes from a bioenergetic standpoint given that ATP available from catabolism is used to support both cell growth and cellulase production (Lynd et al., 2002). An assessment of the bioenergetic benefits associated with growth on cellulosic substrates in terms of net cellular energy currency (ATP, ADP, or AMP) available for growth and cellulase production is vital for an eCBP-enabling microorganisms.

A comprehensive bioenergtic model validating the bioenergetic feasibility of employing *C. thermocellum* on crystalline cellulose was reported by Zhang and Lynd (2005), who determined that *C. thermocellum* assimilates oligo-cellodextrins (G2-G6) of mean chain length

of n ≈ 4 (where n = degree of polymerisation of glucose (G) moieties). The oligo-cellulodextrins are imported into the cell and then cleaved by substrate level phosphorylation by cellodextrin- and cellobiose-phosphorylases. Phosphorylation results in cleavage of ß-glucosidic bonds releasing glucose and glucose-6-phosphate, that undergo glycolysis via the Emden-Meyerhoff pathway to generate ATP. Assimilation of oligo-cellodextrins with an average of 4.2 glucose units more than compensates for higher ATP expended on cellulase synthesis when *C. thermocellum* is grown on cellulose compared to cellobiose (Zhang and Lynd, 2005). Thus, the anaerobic fermentation of cellulose using *C. thermocellum* as a CBP-enabling microorganism is bioenergetically feasible, without the need for added saccharolytic enzymes.

1.11 Organism development

Although CBP of cellulosic biomass offers great potential for lower cost biofuels and fermentation products, robust, industrial microorganisms capable of both high rates of substrate conversion and high yields of the desired fermentation end-products are not available. Desirable characteristics of CBP-enabling microorganisms include production of highly active GH enzymes for rapid substrate hydrolysis, transport and utilisation of the resulting hydrolytic products, high product selectivity and yield. Considerable efforts are underway to identify natural isolates with the desired characteristics and/or to develop stains with the desired characteristics via genetic engineering. The former strategy involves using naturally occurring cellulolytic microorganisms to improve end-product properties related to product yield, tolerance and titre. A classical approach is to metabolically influence end-product yield and solvent tolerance in anaerobic cellulolytic Clostridia. The latter strategy involves the use of genetic engineering of non-cellulolytic microorganisms. The best example of this is the engineering of *S. cerevisiae*, which naturally exhibits high product yields and solvent tolerance, to express a

heterologous GH system that enables it to hydrolyze cellulose/hemicellulose or utilize sugars derived from hemicellulose hydrolysis (Lynd et al., 2005; Lynd et al., 2002). The vast majority of R&D towards organism development is focused on either bacteria or yeast as primary candidates for CBP-enabling microbe. However, the use of cellulolytic non-unicellular fungi as CBP has also been proposed (Xu et al., 2009). This strategy that can be classified under the native cellulolytic strategy from the proponents of CBP. Figure 1.6 show the organism development strategies and commonly employed CBP enabling microorganisms.

1.11.1 Metabolic engineering

Bailey (1991) defined metabolic engineering (ME) as the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with recombinant DNA technology. Metabolic engineering tools being used in the quest for the development of ethanologenic and currently hydrogenic microorganisms include: i) mutagenesis via homologous recombination involving the mutation of a target gene that encodes a native protein to down regulate the expression of another protein or results in the synthesis of an undesired or inactive protein; ii) Heterologous gene expression as a means manipulating the metabolic fluxes towards the synthesis of a desired end product. This is the most likely metabolic engineering strategy amenable to biofuels and over expression of enzyme catalysis flow past forks to the desired end product is a common strategy (Carere et al., 2008). Pyruvate overflow in *Clostridium cellulolyticum* was established to be as a result of inability of pyruvate-ferredoxin oxido-reductase to metabolise pyruvate to acetyl-CoA, resulting in reduced cell growth and increase lactate. However, heteologous expression of pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis* into *Clostridium cellulolyticum* resulted in 93% acetate,

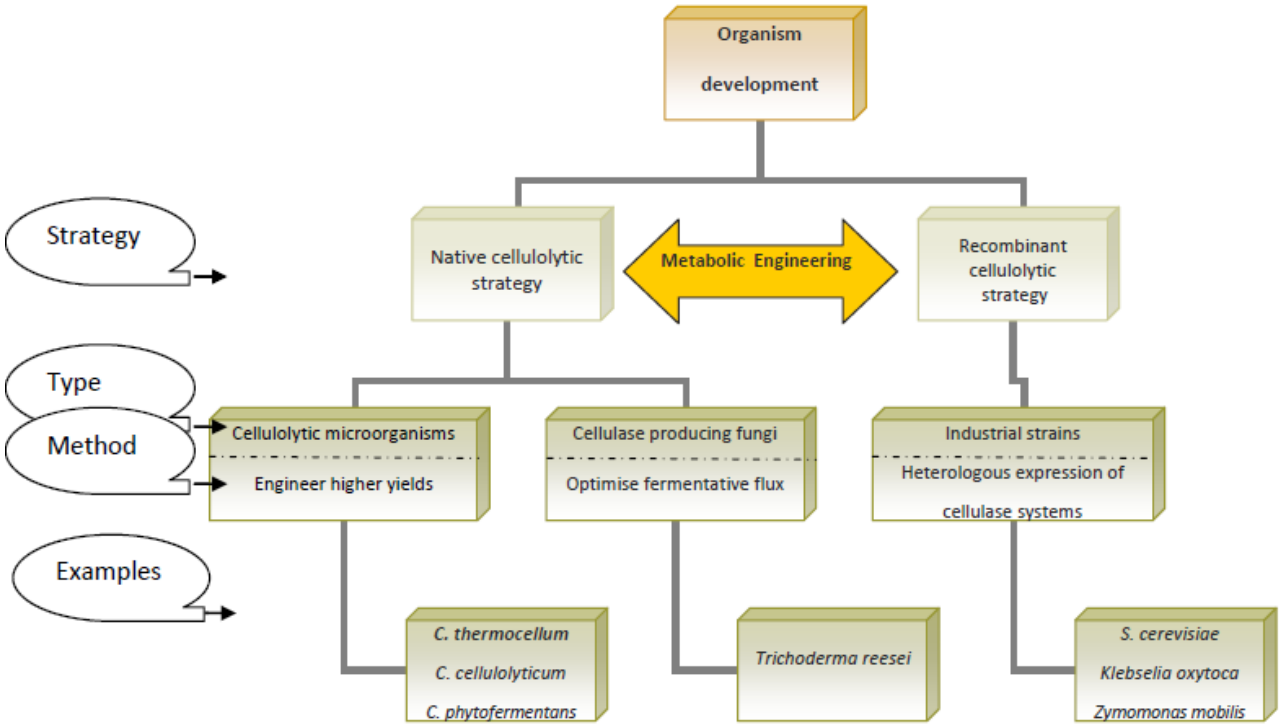


Figure 1.6 Organism development strategies employed in research development for a consolidated bioprocessing enabling microorganism.

53% ethanol, and hydrogen yield increased by more than 75%, demonstrating that cellulose fermentation can be improved by using genetically engineered strains of cellulolytic Clostridia (Guedon et al., 1999); iii) Antisense RNA (asRNA) attempts in redirecting metabolic flow by targeting the same genes as in mutagenesis but instead of completely abolishing protein activity as in mutagenesis, asRNA aims explicitly at down-regulating the expression of a native protein by inhibiting translation due to duplex RNA structure blocking the ribosome binding site or rapid degradation of mRNA by RNases specific for RNA duplex or by the inhibition of mRNA transcription due to premature termination. By so doing asRNA avoids potential lethal mutations and can be used to inducibly repress expression of proteins by using inducible promoters for asRNA. This strategy was used to reduce levels of enzymes responsible for butyrate formation in *Clostridium acetobutylicum* demonstrating that asRNA can be used to down-regulate specific protein, thus redirecting metabolic flux (Carere et al., 2008; Desai and Papoustakis, 1999).

1.11.2 Natural versus engineered GH systems

The development of bacteria and fungi for CBP has focused mostly on the use of microorganisms that naturally express GH systems to hydrolyze cellulose / hemicelluloe and synthesize products of interest from the hydrolysis products. Metabolic engineering of anaerobic cellulolytic bacteria has been the primary approach for enhancing the yields of the desired products so that they can meet the requirements of an industrially consolidated bioprocess. Gene transfers systems, electrotransformation protocols, and recombinant strains with enhanced product synthesis profiles have been described for both *C. cellulolyticum* and *C. thermocellum*.

Previous studies have shown that cellulose utilisation by the mesophilic *C. cellulolyticum* is strongly dependant on initial cellulose concentration which ultimately affects carbon flow distribution leading to end-products. The cessation of early growth was as a result of pyruvate

overflow during high carbon flux (Desvaux et al., 2000; Guedon et al., 1999). Increased level of less reduced metabolite; ethanol and lactated were observed with high levels of carbon flux, whereas at a low carbon flux, pyruvate is oxidised preferentially to acetate and lactate, thus showing an innate capability to balance carbon and electron flow or generation reducing equivalents (Guedon et al., 1999). However, a decrease in the accumulation of pyruvate accumulation at high carbon flux was achieved by heterologous expression of pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) from *Zymomonas mobilis* in a shuttle vector pMG8. Growth of recombinant strain resulted in 150% increase in cellulose utilisation, 180% in crease in dry cell weight, 48% decrease in lactate production, 93% increase in acetate and 53% increase in ethanol over the wild-type *C. cellulolyticum*, proving that genetically engineered strains could be used to greatly increase yields of cellulose fermentation by *C. cellulolyticum* (Guedon et al., 2002).

To show the potential of using *C. thermocellum* as robust platform organism for CBP, Argyros et al., (2011) constructed a mutant with novel genetic engineering tools that allow for the creation of unmarked mutations while using a replicating plasmid. A counter selection strategy was used to delete genes for lactate dehydrogenase (Ldh) and Phosphotransacetylase (Pta) resulting in a stable strain with 40:1 ethanol selectivity and a 4.2-fold increase in ethanol yield over the wild-type strain (Argyros et al., 2011).

Expression of heterologous cellulases in non-cellulolytic microorganisms that are known to possess desired product formation characteristics such as faster sugar consumption, higher ethanol yield and high resistance to ethanol and fermentation inhibitors has also been accomplished (Hasunuma and Kondo, 2012). For example genes encoding endoglucanse II (EGII) cellobiohydrolase II (CBII) from *T. Reesei* and beta-glucosidase BGL1 from *Aspergilus*

aculeatus were integrated into the chromosome of wine yeast strain using a single vector conferring resistance to antibiotics G418. The mutant strain was able to hydrolyse corn stover cellulose and produced ethanol without the addition of exogenous saccharolytic enzymes (Khramtsov et al., 2011). Significant advances related to recombinant enzyme expression support the potential of *S. cerevisiae* as CBP host, and the number of genes expressed is not probably as important as the metabolic burden and stress responses associated with such high-level expression (van Zyl et al., 2007).

Heterologous expression of cellulolytic enzymes for the development of a cell surface which provides display of cellulolytic enzymes or cellulases that are secreted is currently being investigated in other non cellulolytic, ethanologenic bacteria such as *E. coli, Zymomonas mobilis* and *Klebsiella oxytoca* to enable growth and fermentation of pretreated lignocellulosic biomass (Jarboe et al., 2007; van Zyl et al., 2007)

1.12 Benefits and constraints of CBP

CBP is a less energy intensive method and a potential low cost route for the production of cellulosic ethanol, as well as other industrially important products, because of the avoided cost of exogenous enzymes required for cellulose hydrolysis in SHF, SSF and SSCF (Lynd et al., 2008; Weimer et al., 2009b; Xu et al., 2009). The saccharification and fermentation steps in SHF and SSF have large differences in operating temperatures which complicate development of pilot and industrial scale processes compared to CBP, which is conducted in single vessel at a single optimized temperature. CBP offers simplification of the total operation process for ethanol production from cellulosic biomass, compared to SHF and SSF (Hasunuma and Kondo, 2012; Hasunuma et al., 2012). CBP also has the added benefit of requiring minimal pretreatment of lignocellulosic biomass because pretreated feedstocks for CBP do not need to be completely

saccharifide with large volumes of costly exogeneous enzymes,. Thus, the cost of pretreatment, which is a key bottle neck in lowering the net cost of production of cellulosic bioethanol, is kept very low (Agbor et al., 2011; Xu et al., 2009).

Although suitable for the production of high value products and low cost fuels, the quest for a suitable industrial CBP-enabling microorganism limits the impact of the process technology design for industrial purposes compared to sequential step processes. While the use of industrial yeast and bacterial strains used in conventional SHF and SSF processes is well established, the use of natural and/or engineered microorganisms in CBP is not yet mature and hence industrial implementation has been slow. However, research interest in CBP is growing and production of different products via CBP are under investigation (Hasunuma and Kondo, 2012; Lynd et al., 2008; Xu et al., 2009).

1.13 Research Objectives

The main objective of this work is to characterise and evaluate the fermentability of processed and pretreated, local, non-food cellulosic feedstocks (biomass) to be used for direct cellulose fermentation for production of biofuels and co-products. The long-term objective of this research is to identify and evaluate the changes in biomass properties following pretreatment and processing that will enhance biofuel (ethanol and hydrogen) production via direct microbial conversion by fermentation. Understanding the substrate characteristics of potential feedstocks for biorefining will enhance the synthesis of biofuels and co-products generated for a given substrate as we explore the fermentation options available to the integrated biorefining industry.

The short-term objectives of my research are to characterise local feedstocks, such as hemp fibres and hurds, flax shives and fines, rice and wheat straw, and wood, for their physical,

and physico-chemical pretreatment requirements and evaluate biofuel production from these feedstocks by fermentation. The specific objectives of my research were:

Objective 1) Quantify the rate of conversion of pretreated versus raw cellulosic substrates by *Clostridium thermocellum* during the fermentation process;

Hypothesis: pretreatment will improve substrate conversion rates, and end-product yields and profiles of *C. thermocellum* cultured under CBP conditions

Objective 2) Identify feedstock properties that affect the quantification of aqueous (ethanol and organic acids) and gaseous (hydrogen and carbon dioxide) fermentation products synthesized by *Clostridium thermocellum* using pretreated and raw cellulosic substrates.

Hypothesis: End-product profiles will differ, depending on the type, source, and nature of the substrate /microorganism.

Objective 3) Carry out enhanced direct microbial conversion or bioaugmented Consolidated Bioprocessing (bCBP) with exogenous enzymes using pretreated, processed and raw cellulosic substrates.

Hypothesis: enhanced CBP of lignocellulosic biomass can attain higher yields as result of increase accessibility and combine enzyme activity.

1.14 Thesis structure

Chapter 1 is an introductory review base on the review article "Biomass Pretreatment: fundamentals toward application" authored by Agbor et al. (2011) and the Book Chapter "Biomass Pretreatment for Consolidated Bioprocessing (CBP)", by Levin et al. (2014). Chapter 2 presents the characterization and fermentation of different fractions of hemp biomass in as single-step process (otherwise known as direct microbial conversion or consolidated bioprocessing). The effects of cellulose contents and structure on the rate and yield of end-

products of fermentation with *C. thermocellum* are highlighted. Chapter 3 presents data on different lignocellulosic feedstocks that are subjected to the same or similar type of physicochemical pretreatment, their rates of hydrolysis, and subsequent conversion as a function of the perentage of amorphous cellulose. In this chapter we describe the 'contribution of amorphous cellulose' (CAC) as a novel factor that is correlated with the efficiency of microbial conversion to fermentation end-products (i.e. yields of H_2 and CO_2). Chapter 4 reports the use of exogeneous glycosyl hydrolases to increase accessibility to cellulose within agricultural residue in biomass during fermentation, resulting in higher end-product yields in a shorter time. Our data suggest that bioaugmention with a minimal dosage of exogenous glycosyl hydrolases could improve the rate of end-products of DMC or CBP for hydrogen production. Finally, chapter 5 encapsulates the research contribution of this thesis, how the findings reported herein may be used to further research on biofuels, and how the results may be useful to bioprocessing for biofuel production.

Chapter 2.

Consolidated bioprocessing of agricultural hemp residues for hydrogen and ethanol production: The effect of cellulose content and structure on end-product synthesis patterns and efficiency of substrate conversion by *Clostridium thermocellum*.

This Chapter is based on the following publication: Agbor V, Zurzolo F, Blunt W, Dartiailh C, Cicek N, Sparling R, Berlin A, Levin DB. 2014. Single-step fermentation of hemp residues for hydrogen and ethanol production. Biomass and Bioenergy, 64, 62-69.

2.1 Abstract

Biofuels are being used as a supplement, additive, or alternative to petroleum-derived transportation fuels. To investigate the potential of using hemp biomass as a feedstock for lignocellulosic biofuels production, fermentation end-product synthesised by *Clostridium thermocellum* in one-step batch fermentation reactions using purified hemp cellulose (PHC), hemp fibres (HF), and hemp hurds (HH), were compared to reagent-grade α-cellullose (AC). Exponential phase growth and production rates on PHC were comparable to those observed with AC. Net production of ethanol for AC (8.47 mM) was slightly higher than PHC (6.56 mM), but significantly higher than HF (5.48 mM) and HH (3.52 mM), while the final hydrogen yield was comparable for AC (12.70 mM), PHC (11.01 mM), HF (10.91 mM), and HH (4.72mM). End-product yields were dependent on the intrinsic cellulosic content as well as the presence of other polymers present in substrate biomass. Rates of ethanol and hydrogen production were similar in early log phase, but varied in mid-log, and accessibility to cellulose was shown to determine gaseous yields and end-products of metabolism. Our data suggest that pretreatments that improve the accessibility of the cellulolytic bacterium, *C. thermocellum,* to cellulose fibres have a greater

effect on increasing the yield of fermentation products because of the associated changes in the cellulose structure than simply increasing the concentration of the substrate.

2.2 Introduction

Lignocellulosic biomass is the most abundant organic material in nature with 10-50 billion tonnes annual worldwide production and in excess of 180 million tonnes of available cellulosic feedstock per year from agricultural resources (Demain et al., 2005; Galbe and Zacchi, 2007). Although biofuels can be produced from grains, current renewable fuel standards mandate a 96.4% increase in efficient and 'advanced biofuels', the majority of which can be derived from cellulosic biomass (Sannigrahi et al., 2010). Unlike the corn ethanol industry, which faces many challenges including the "food versus fuel debate" (Agbor et al., 2011; Brown, 2006; Sun and Cheng, 2002), cellulosic ethanol from biomass can utilize a variety of lignocellulosic feedstocks, ranging from different types of soft and hard woods, including short rotation trees, such as poplar and willow, to agricultural residues from food crops, such as corn stover and cereal straws, to dedicated lignocellulosic energy crops, i.e. switch grass, miscanthus (Perlack and Wright, 2005). In this study, we have investigated the potential of using residues of industrial hemp, *Canabis sativa*, as substrate for bioethanol and biohydrogen production.

As an industrial plant, hemp fibre has many applications in the textile industry. Hemp seeds are used for the production of neutraceuticals and food products, while the hurds and fibre have some paper and composite material applications (karus and Vogt, 2004; MAFRI, 2011). Large scale cultivation of hemp offers great potential as a feedstock for biorefineries, and studies in Sweden have investigated the use of hemp biomass for bioethanol and biogas production (Kreuger et al., 2011a; Kreuger et al., 2011b). In the United States during 1920s, 25% of

Standard oils sales in the Midwest were from the ethanol derived from hemp biomass (Biofuels, 2008).

As an energy crop, hemp biomass has the following advantages: i) *C. sativa* is a multi-use crop that can generate a variety of higher-value co-products as well as biofuels, which is essential for economic sustainability; ii) it can be grown on marginal land compared to other energy crops grown on agricultural land; iii) it requires little fertiliser, thus minimizing the use of nitrogen-based fertilizers; an essential factor for its Life cycle analysis or LCA as a feedstock; iv) it removes smaller amounts of nutrients from the soil compared with other crops, v) hemp plants are resistant to pest and disease, vi) it grows faster than most energy crops, and vii) produces higher energy yield per acre per year than corn, sugarcane, and flax (Hempline, 2008; karus, 2000; karus and Leson, 1994).

Industrial hemp cultivation in Canada is viewed as a new crop that can compliment prairie crop rotations by breaking traditional crop cycles and increasing profits (MAFRI, 2011). Thus, there is a growing interest for high volume local cultivation of the industrial hemp as a multifunctional industrial crop with a huge supply of residual biomass after cultivation and processing. The residues from hemp cultivation and decortication constitute a mixture of fragments of hemp fibre and hurds (hemp core). We investigated the bioprocessing of this rich source of cellulosic biomass in a one-step fermentation process, otherwise known as consolidated bioprocessing (CBP) (Carere et al., 2008; Lynd et al., 2005; Lynd et al., 2002; Magnusson et al., 2008; van Zyl et al., 2007; Weimer et al., 2009a; Xu et al., 2009).

We have compared production of fermentation end-products by *Clostridium thermocellum* in single-step batch fermentations using purified hemp cellulose (PHC), hemp fibre (HF), and hemp hurds (HH), versus reagent grade α-cellulose (AC). Previous studies have

shown that *C. thermocellum, a* gram-positive, thermophilic, cellulolytic bacterium, has great potential for effective bioconversion of low-value cellulosic feedstocks because of its ability to grow well on both amorphous and crystalline cellulose (Islam et al., 2009; Levin et al., 2009; Levin et al., 2006; Lynd et al., 2005; Lynd et al., 2006; Lynd et al., 2002). In this study, the growth and end-product synthesis profiles from using residues of industrial hemp cultivation and processing have been reported to elucidate the quality of hemp biomass as potential substrate for cellulosic biorefineries.

2.3 Materials and methods

2.3.1 Substrates

The hemp (*Cannabis sativa)* used in this study was obtained from the Emerson Hemp Distribution Company, Emerson, Manitoba, Canada. Residue from this industrial hemp is made up of the hemp fibres and hemp hurds. Some of the raw hemp residue was subjected to a physico-chemical pretreatment (Organosolv®) process to solubilise hemicellulose and extract lignin. The biomass was pretreated with 50% ethanol, for 55 min, at 195 ^{o}C, using 1.5% acid as catalyst, at a 10:1 Liquid: Solid hemp biomass ratio (Pan et al., 2005; Pan et al., 2006). We refer to this water insoluble substrate derived from the Lignols' Organosolv® pretreatment as purified hemp cellulose (PHC). We also separated the fibres in the raw hemp from the hurds to represent two substrate streams from a decortication facility rich in hemp fibres (HF) and hemp hurds (HH) as shown on Table 2.1. All substrates were added to fermentation reactions at 2 g/L (20 mg). However, the amount of "available cellulose" in each substrate was different, because the amount of cellulose in each substrate was different (Table 2.2).

All test substrates (PHC, HF and HH in Table 2.1) were air dried and milled using a Retsch Rotor Beater mill SR200 equipped with a 34 mesh and 0.5mm aperture sieve. All the

milled samples were transferred into Ziplock bags and stored at room temperature. Alpha (α-) cellulose (C8002) obtained from Sigma Aldrich (Saint Louis, USA) was used as positive control.

Table 2.1 Polymer composition for alpha cellulose and industrial hemp fractions obtained from Emerson Company Manitoba, on a percent dry matter basis.

		Substrate type			
Analysis	**Method used**	**Alpha cellulose**	**Purified hemp**	**Hemp hurds**	**Hemp fibre**
Moisture (%)	AOAC930.15	6.33	5.1	6.5	6.5
Dry Matter (%)	AOAC930.15	93.77	94.9	93.5	93.5
Crude Protein	AOAC984.13	0.01	0.9	1.1	2.0
ADF (%)	ANKOM Method 5: 08-16-06	86.54	86.4	74.5	81.8
NDF (%)	ANKOM Method 6: 08-16-06	98.51	91.3	92.3	88.4
Ash (%)	AOAC942.05	0.00	4.3	1.0	2.1
Soluble Crude Protein (%)	Roe et al., 1990	<0.01	<0.05	0.1	0.4
Cellulose (%)	Cellulose = ADF - Lignin	86.43	78.9	57.7	78.5
Hemicellulose (%)	Hemicellulose = NDF -ADF	11.97	4.9	17.8	6.6
Lignin (%)	ANKOM Method 08/05	0.11	7.4	16.8	3.3

Each assay was run in duplicate and was repeated if % error was > 3 % (Roe et al., 1990).

Table 2.2 Total initial mass (mg) and total available cellulose mass (mg) in substrates used in Balch tube (growth curve) and serum bottle (end-point) experiments.

Experiment	**Total initial substrate mass (mg)**	**Volume of culture (mL)**	**Total initial cellulose mass (mg)**					
Balch tubes			**AC**	**PHC**	**HF**	**HH**	**HHD***	**HHQ***
(Growth curve experiments)	20	10.1	17.3	16	16	12	-	-
Serum bottles (End-point experiments)	100-400	50.5	86	79	79	58	116	232

* test was not included during growth curve experiments in Balch Tubes.

All test samples were over 93% TS. To test for substrate utilisation, a soluble fraction from raw hemp was obtained by autoclaving Balch tubes (27 mL; Bellco Glass Inc.,) containing 2 g/L (0.2% w/v) substrate concentrate of residual hemp biomass in 1191 and extracting the supernatant. The supernatant was used to test for growth of *C. thermocellum* on the soluble fraction derived from the substrate. The filtered supernatant was transferred into new tubes, sterilised by autoclaving and subjected to CBP with *C. thermocellum.* Negative controls were established using 1191 medium inoculated with *C. thermocellum* but without any substrate.

2.3.2 Bacteria culture and media preparation

C. thermocellum strain 1237 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany) and cultured in 1191 medium (Islam et al., 2006). DSMZ 1237 is equivalent to *C. thermocellum* strain 27405 available at the America Type Culture Collection (ATCC). For each experiment, cells used for inoculation were cultured in a 1 L Corning bottle that was used to inoculate all batch reactors used in growth experiments to ensure reproducibility. All chemicals and reagents used were obtained from Fisher Scientific unless otherwise stated. Yeast extract was obtained from Biobasic Inc (bacteriological grade). Serial subcultures of *C. thermocellum* were used to prepare and maintain fresh cultures in 1191 media containing the 2 g/L α-cellulose. Per litre of milliQ water, 1191 medium contained KH_2PO_4, 1.5 g; Na_2HPO_4, 3.35 g; NH_4Cl, 0.5 g; $MgCl_2.6H_2O$, 0.18 g; yeast extract, 2.0 g; resazurin from Sigma Aldrich, Saint Louis, USA (0.25 mg/ml), 2.0 ml; 10× vitamin solution, 0.50 ml; 10× mineral solution, 1.0 mL. Vitamin supplements and mineral elixir solution were prepared according to a previously described by Islam and Co-workers (Islam et al., 2006).

2.3.3 Experimental procedure

Balch tubes and serum bottles (Wheaton Science, obtained from Fisher Scientific), with working volumes of 27 mL and 127 mL respectively, were used for all tests. Two grams per litre of substrate were used for the experiments performed in Balch tubes containing 9 mL of 1191 media. In order to maintain an anaerobic environment all the tubes were air-sealed with butyl-rubber stoppers and crimped with aluminium seals and then gassed and degassed for four (1:4) cycles with 100% nitrogen. Each tube was reduced with 0.1 ml of sterile 2mM Na_2S reducing solution (Daniels et al., 1986) and then autoclaved. The same procedure was used with serum bottles but with a starting volume of 45 ml of 1191 media and 0.5 mL of 2mM Na_2S while varying the substrate concentration from 2 g/L to 4 and 8 g/L for HH as a native test substrate . For each experimental set, batch cultures were inoculated from a single fresh growing 1 L culture bottle at exponential phase with 10 % of the volume of media in test vessel. All batch experiments were conducted in three biological replicates. Triplicates of each experimental time point were collected for analyses according to predetermine time points interval of 3 hours or more. Batch experiments were repeated 7 times with different time intervals. To determine the amount of end-products carried over into tests samples, zero hour time point were collected immediately after inoculation. Growth curve experiments were conducted in Balch tubes bottles while end-point tests were carried-out in serum bottles.

2.3.4 Cell growth and pH measurements

Cell-growth was determined based on protein analyses using the Bradford method (Bradford, 1976). Culture aliquots of 1 mL were sampled into 1.5 mL microcentrifuge tubes (Fischer scientific) and centrifuge (Legend Micro21R, Sorvall) 10,000 x g for 10 min to separate the pellets from the supernatant. The supernatant were transferred to new 1.5 mL

microcentrifuge tubes and stored at -20 ^{o}C for other end-product analyses. The pellets were resuspended in a 0.9 % (wt/vol) sodium chloride (NaCl) and then centrifuge for 10 min at 10,000 x g. The supernatant was decanted and 1 mL of sodium hydroxide (NaOH) was added. The samples were vortex to re-suspend the pellet and boiled for 10 min in water to solubilize any remaining proteins. After cooling, the samples were later centrifuge at 10,000 x g for 5 min and the supernatants were collected for Bradford assay (Bradford, 1976) using bovine serum albumin (BSA, Sigma Aldrich) as standards. Optical densities at 595 nm (Power Wave, XS, BIO-TEK) were measured after kinetic reads were stabilized and protein concentration was calculated based on the BSA standard curve with R^2 values > 98 %. A Beckman Coulter pH meter (pHi 410) equipped with a ThermoOrion triode probe was used to measure the pH of contents in each vessel.

2.3.5 End-product analyses and substrate consumption

The concentration of soluble end-products was measured in the supernatant for each batch experiment. The concentration of ethanol in the supernatant of Balch tubes for the growth curve experiment was measured using a more sensitive UV-Test kit (R-Biopharm AG, Darmstadt, Germany). The amount of NADH produced by alcohol dehydrogenase measured spectophotometrically at 340 nm, was used to determine the ethanol content in the supernatant. Organic acids and ethanol for serum bottles were measured using a Waters HPLC system equipped with a Bio-Rad Aminex HPX-87 column (300 x 7.8 mm) equipped with a refractive detector (Waters, 2414) at 45 ^{o}C, using 5 mM sulphuric as mobile phase at a flow rate of 0.6mL/min.

2.3.6 Gas analyses

Percentage gas production (H_2 and CO_2) was measured using an SRI gas chromatograph (SRI model instruments, Model 8610C) outfitted with a thermal conductivity detector, a stainless steel molecular sieve 13x packed column (3.2 mm x 1.8m) connected in series with a stainless steel silica gel packed column (3.2 mm x 1.8) to separate H_2 and CO_2 respectively using Argon as carrier gas. Final gas compositions were quantified by correcting for temperature, pressure, volume and aqueous phase concentrations estimated according to the solubility of the gases in water (Sander, 1999). The fraction of CO_2 in equilibrium with bicarbonate was also taken in consideration (Darrett and Drisham, 1995).

2.3.7 X-ray diffraction (XRD)

XRD analysis was conducted using a Siemens D5000 powder diffractometer (Siemens AXS, Inc.,Madison, WI) equipped with Cu radiation (Cu K-alpha lambda = 1.5418 Å) and operated at 40kV and 40mA. The receiving and anti-scatter slits were set to 1.0 mm. The samples were mounted in a depressed 'well' on a zero-background quartz plate and pressed into the well with a frosted glass slide. Scans were obtained from 8 to 42 degrees two-theta using a step-width of 0.05 degree two-theta and a dwell time of 1 s/step.

The ‘contribution of amorphous cellulose’ was calculated as:

$CI = [(I_{002} - I_{AM}) \div I_{002}] \times 100$.....(equation 1) (Segal et al., 1959).

Crystallinity index measured by X-ray diffraction (CI) is a parameter that has been used to describe the relative amount of crystalline material in cellulose to allow rapid comparison of samples (Hall et al., 2010; Park et al., 2009; Segal et al., 1959). Although this method is useful for comparing relative difference between cellulosic samples, its weaknesses lies in the fact that it is not very accurate for the determination of CI values because the I_{AM} value can be

significantly underestimated, resulting in over estimation of the CI. Also, only the highest peaks are used in the calculations despite the presence of other crystalline peaks (Park et al., 2010). Finally, the peaks in a cellulose diffractogram vary considerably in their width, so height comparisons alone cannot be expected to provide a rational estimate of cellulose Crystalinity (Garvey, 2005; Park et al., 2010). For these reasons, the relative height to the minimum ratio can only be taken as a rough approximation of the contribution of amorphous cellulose to the cellulose diffraction spectrum (Park et al., 2010).

2.3.8 Dry mass measurements

Dry mass was measured by emptying the entire contents of balch tubes unto pre-dried and weighed filter papers (Whatman #1) and the liquids extracted by vacuum extraction (Gas vacuum pumps DOA-704-AA). Filters where again dried at 105 ^{o}C using Isotemp Oven from Fisher scientific for 2 hours after which the dry mass measured.

2.3.9 Scanning electron microscopy (SEM)

Samples were fixed in-situ with gluteraldehyde, treated with osmium, then critical-point dried on gold-coated nuclear pore paper. The dried samples were placed (with the pore paper) on a stub with double sided carbon tape. Imaging conditions are on the images for X10,000 and X20,000 magnifications and they were taken in a JEOL JSM-7000F SEM.

2.4. Results

Balch tube experiments where conducted to observe growth characteristics, while serum bottle experiments were carried-out to measure metabolic flux and end-product distribution over time.

2.4.1 Cell growth characteristics

In these experiments, α-cellulose served as a standardized, reagent grade substrate with a known composition (on a dry mass basis) with zero ash content as shown in Table 2.1 (Sigma

Aldrich, Saint Louis, USA; product # C8002). The hemp-derived substrates were dry-milled with a 0.5 mm sieve (34 mesh) screen to produce substrate particles with physical characteristics (surface area, degree of polymerization, and crystalinity (Agbor et al., 2011) similar to α-cellulose. It has been shown that further reduction of biomass particle size below 40 mesh (0.4 mm) had little effect on the rates and yields of biomass hydrolysis (Chang and Holtzapple, 2000; Hendricks and Zeeman, 2009).

Initial experiments indicated that no biomass production was observed when *C. thermocellum* was grown on the soluble fraction extracted from hemp biomass (data not shown) therefore, subsequent experimental negative controls were established using 1191 medium plus inoculum only (i.e. no cellulosic substrate). At a substrate concentration of 2 g/L, cell growth (based on total extracted protein) was observed after an initial lag of approximately 8 hours (Figure 2.1). Maximum protein concentration was reached when cells reached stationary phase, typically after 60 hours. Figure 2.1 shows the trend in biomass produced during growth on PHC, HH, HF and AC. Absorbance values of the Bradford assays used to determine total protein content from cell extracts were subject to substrate-specific differences in colour and crude protein. Minimal growth was observed in cultures containing 1191 medium without any substrate, and this growth was as a result of carryover from inoculums or due to the yeast extract in the 1191 medium.

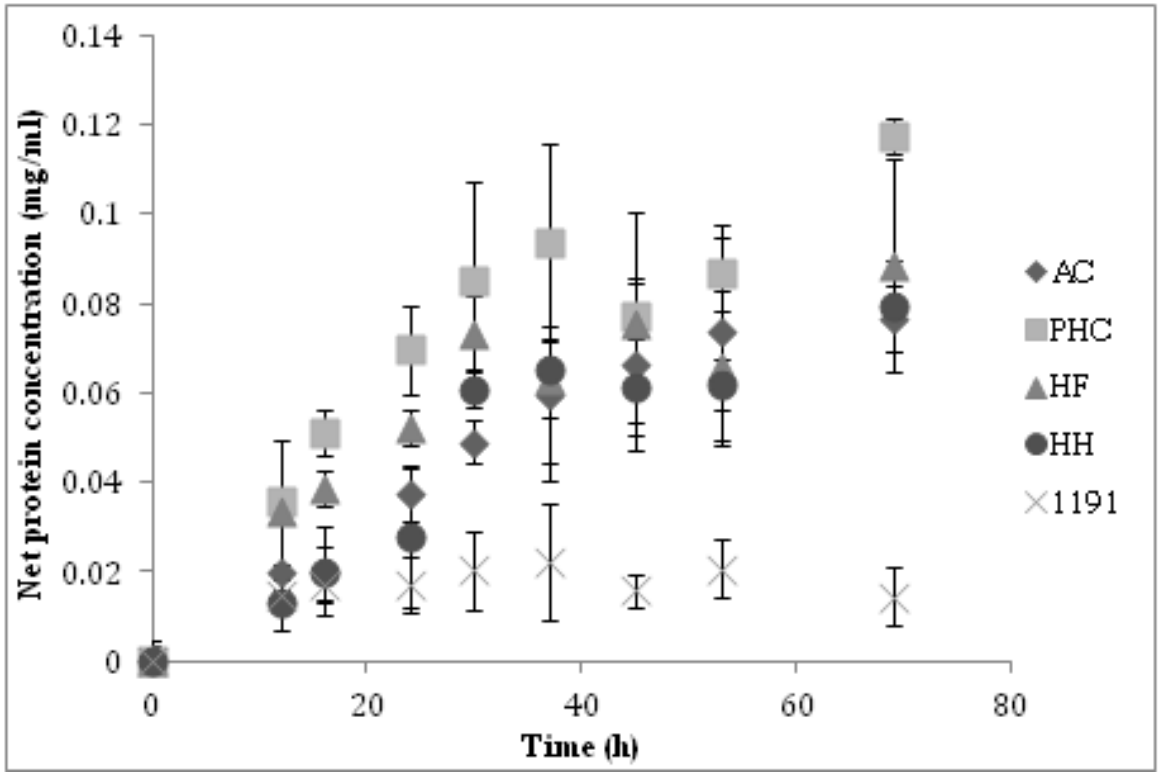


Figure 2.1 Growth characteristics of *C. thermocellum* in 27 mL Balch tube batch reactions (10.1 mL working volume) containing α-cellulose(AC), purified hemp cellulose (PHC), hemp fibre (HF), hemp hurds (HH), and 1191 medium (as a negative control). Each data point represents the average of three biological replicates. Error bars are the standard deviations (SD) between independent replicates.

2.4.2 Gas production

Figures 2.2 and 2.3 show production of H_2 and CO_2, respectively, by *C. thermocellum* cultured on 2 g/L of each substrate (HH, HF, PHC, and AC) in 1191 medium. Gas production followed the cell growth patterns (Figure 2.1). The initial production pattern was the same for all four substrates, but as growth proceeded into early log phase, the rate of H_2 and CO_2 decreased significantly for HH, while production rates of H_2 and CO_2 were comparable for AC, PHC, and HF. A possible explanation for why the production rates were similar in the beginning is that they were most likely as result of carryover from the initial inoculum, which were cultured in 2 g/L of α-cellulose and also contained yeast extract. As expected, gas production tapered off as the cells entered stationary phase. Net production in Balch tubes is summarised in Table 2.3, while Figure 2.3 shows the end-point concentrations of H_2 and CO_2 at 53 h in serum bottles (50.5 mL). Gas production (Figure 2.2A) was highest for the reagent grade α-cellulose. Lower amounts of H_2 and CO_2 were produced from PHC, HF, and HHQ, while HHD and HH produced the lowest amounts. Raw data from three biological replicates were used to calculate the means and standard deviations for each data point.

2.4.3 Soluble end-products

The major fermentation end-products produced by *C. thermocellum* growing on all four substrates were acetate, lactate, formate, and ethanol. Ethanol synthesis profiles followed the cell growth patterns observed with all feedstocks. Ethanol concentrations produced were similar for all substrates from 0 to 25 h (until mid-log phase), but production increased there-after in cultures grown on AC, PHC, and HF, but not in cultures containing HH (Figure 2.5). Net production of ethanol per volume of culture for AC (8.47 µmol/ml) was higher than those of PHC (6.56 µmol/ml), HF (5.48 µmol/ml), and HH (3.52) (Table 2.3).

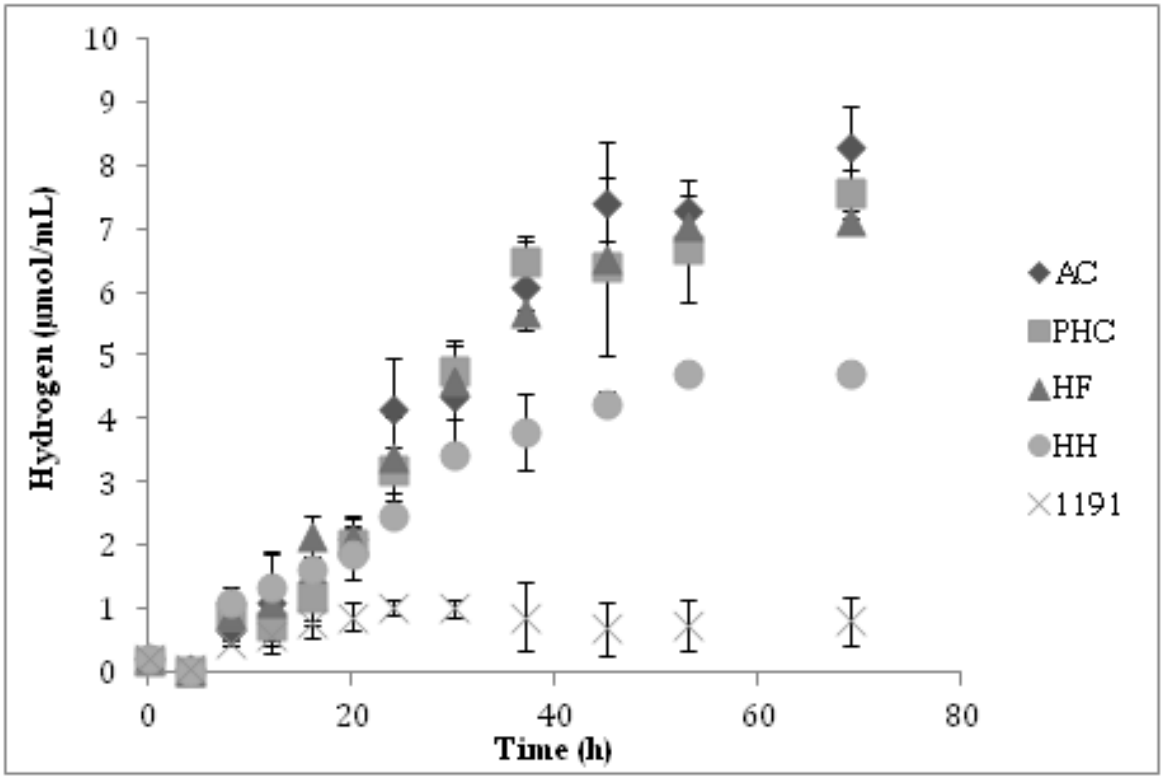


Figure 2.2 Gaseous fermentation end-product: Hydrogen (H_2) synthesis by *C. thermocellum* in 27 mL Balch tube batch reactions (10.1 mL working volume) by *C. thermocellum* in cultures containing 2 g/L of α-cellulose (AC), purified hemp cellulose (PHC), hemp fibre (HF), hemp hurds (HH), and 1191 medium (negative control). Each data point is the average of three biological replicates and errors bars are the SD.

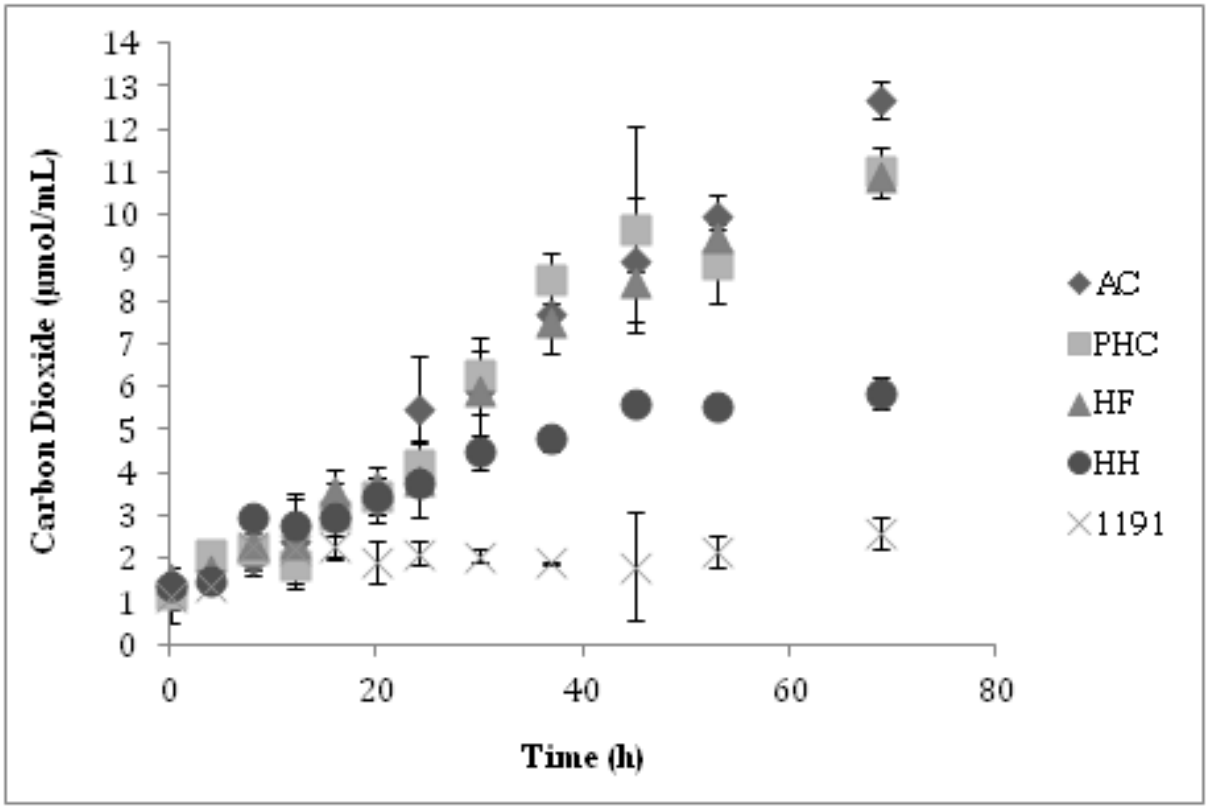


Figure 2.3 Carbon dioxide (CO_2) production by *C. thermocellum* in cultures containing 2 g/L of α-cellulose (AC), purified hemp cellulose (PHC), hemp fibre (HF), hemp hurds (HH), and 1191 medium (negative control). Each data point is the average of three biological replicates and errors bars are the SD.

Table 2.3 Summary of net productions for hydrogen (H_2), ethanol (C_2H_5OH), average of final pH recorded, oxidised and reduced metabolites and a carbon balance of one and two carbon metabolic end-products of *C. thermocellum* growth on 2 g/L of test substrates AC (α-cellulose), purified hemp cellulose (PHC), hemp fibre (HF), hemp hurds (HH).

Parameter	**Substrate**			
	AC	**PHC**	**HF**	**HH**
Net H_2 Production (µmol/ml)	8.04	7.37	6.87	4.48
Net C_2H_5OH Production (µmol/ml)	8.47	6.56	5.48	3.52
Rates of H_2 production (µmol/ml/h)	0.21	0.27	0.24	0.16
Rates of C_2H_5OH production (µmol/ml/h)	0.09	0.212	0.070	0.05
Final pH	6.36	6.59	6.50	6.89

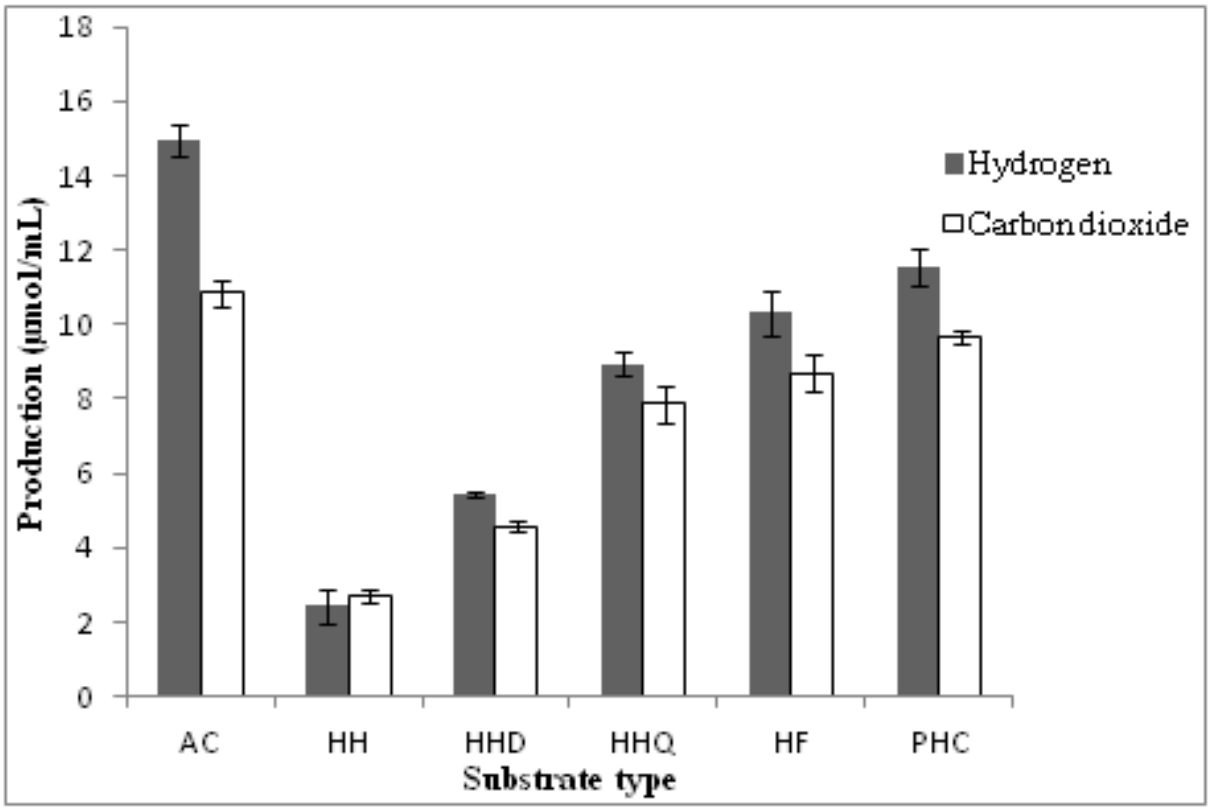


Figure 2.4 Hydrogen and carbon dioxide production after 53 hours of *C. thermocellum* growth on test substrates in 150 mL serum bottles (50.5 mL working volume) containing α-cellulose (AC), purified hemp cellulose (PHC), hemp fibre (HF), hemp hurds (HH), hemp hurds with double the dry mass (HHD), hemp hurds with quadruple the dry mass (HHQ). Each data point is the average of three biological replicates and errors bars are the SD.

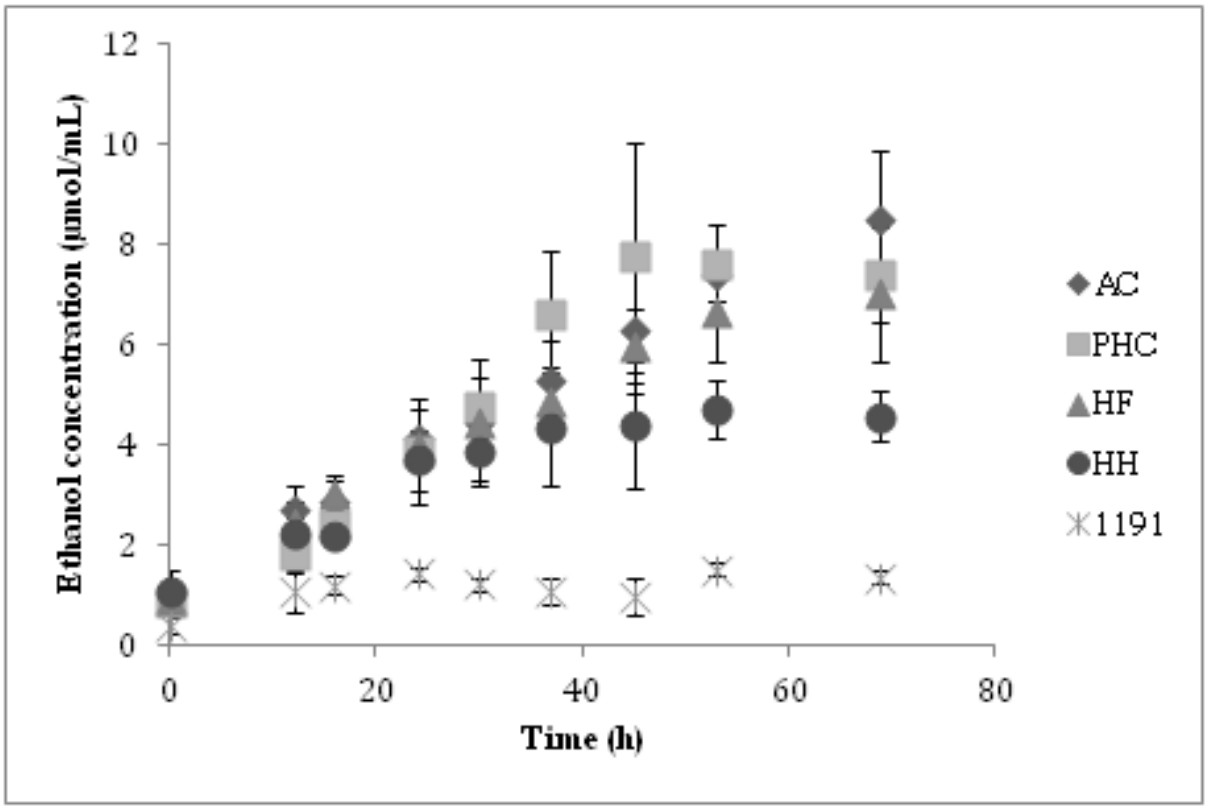


Figure 2.5 Ethanol production of *C. thermocellum* in 27 mL Balch tube batch reactions (10.1 mL working volume) containing 2 g/L of α-cellulose (AC) purified hemp cellulose (PHC), hemp fibres (HF), hemp hurds (HH) and 1191 media control. Each data point is the average of three biological replicates and errors bars are the SD.

Figure 2.6 shows the fermentation end-point concentrations of ethanol, organic acids, and the final pH at 53 h in cultures grown in serum bottles. A linear increase in the end-point ethanol concentration was observed as the loading of HH was increased from 2 g/L (HH) to 4 g/L (double the HH concentration = HD), and to 8 g/L (quadruple the HH concentration = HHQ), and the end-point concentration of ethanol produced by HHQ was not significantly different from those of HF and PHC. Acetate and formate were the major organic acids produced, although low concentrations of lactate were observed at 53 h in all the fermentations reactions. Lower concentrations of acetate and formate were observed in HH compared to AC, PHC, and HF. Organic acid production was lowest in the HH after 53 hours. The final pH observed for HH (7.04), was higher than the final pHs observed for AC (6.41), PHC (6.58), and HF (6.62) (Table 2.4).

The amount of substrate consumed by *C. thermocellum* in 10.1 mL Balch tube cultures. The amount of substrate consumed was calculated as the grams of substrate consumed = Initial substrate mass – mass of residue left after 70 hours (including cells and substrate biomass). This analysis indicates that *C. thermocellum* consumed approximately the same amount of cellulose in the AC (0.017 g), HF (0.016 g), and PHF (0.014 g) substrates, but significantly less cellulose in the HH (0.006 g) substrate. However, the yield of end-products per gram cellulose of substrate, did not improve with the increase in cellulose content or concentration of HH (Table 2.4). The ratio of single-carbon (C1) to double-carbon (C2) end-products revealed the partitioning of carbon by *C. thermocellum* in the fermentation reactions (Table 2.4). The C1/C2 ratios ranged from 0.73-0.99 (Tables 2.4) with more products/carbon accounted for with purified substrates (AC and PHC) than with unprocessed feedstocks, such as HF, HH, HHD, and HHQ. However, the C1/C2

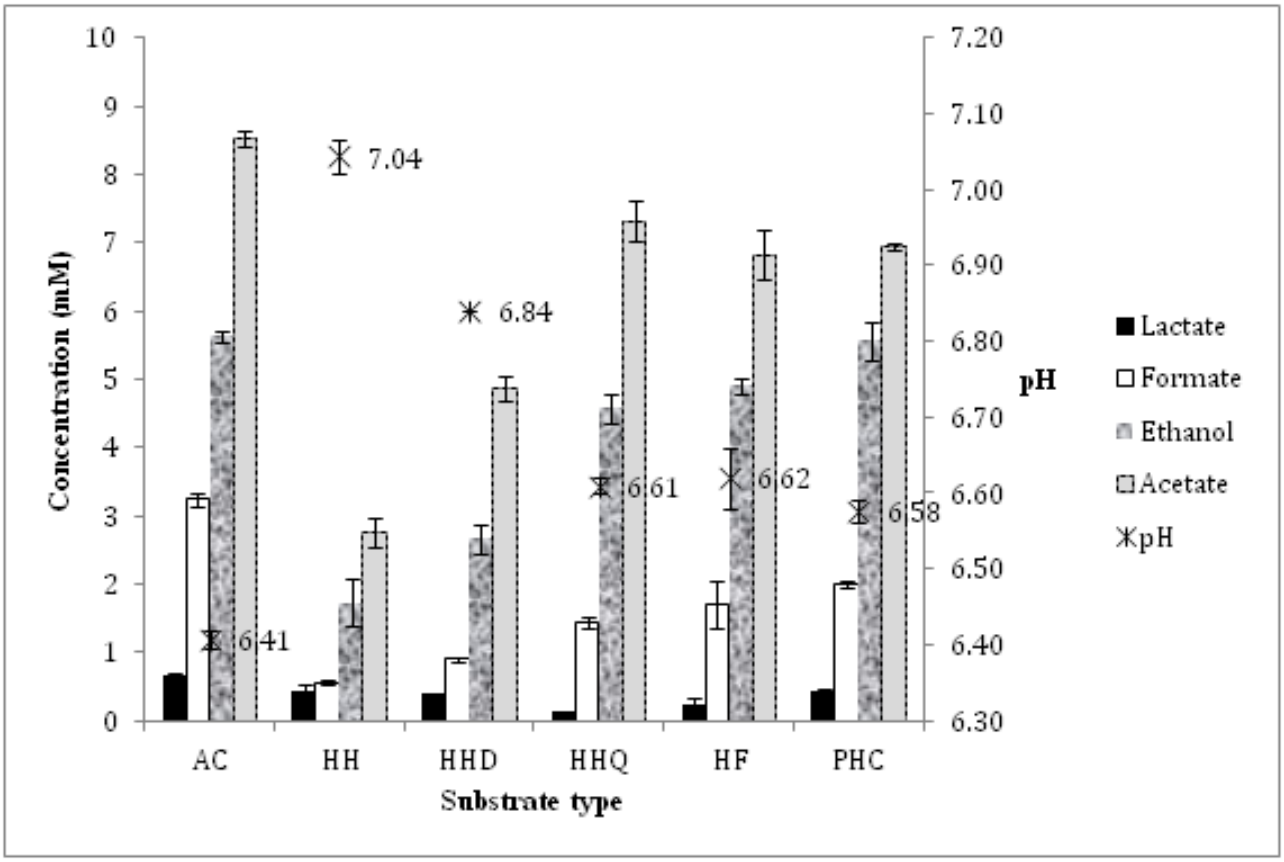


Figure 2.6 Ethanol and organic acid production after 53 hours of *C. thermocellum* growth on test substrates in 150 mL serum bottles (50.5 mL working volume) containing α-cellulose (AC), purified hemp cellulose (PHC), hemp fibre (HF), hemp hurds (HH), hemp hurds with double the dry mass (HHD), and hemp hurds with quadruple the dry mass (HHQ). Each data point is the average of three biological replicates and errors bars are the SD.

Table 2.4 Summary of substrates converted to end-products from 0.02 g biomass at end of growth in Balch tubes. The pH, redox and carbon balance (C1/C2) and distribution after 53 hours of *C. thermocellum* growth on test substrates: AC (α-cellulose), purified hemp cellulose (PHC), hemp fibre (HF), hemp hurds (HH), hemp hurds double the dry mass (HHD), hemp hurds quadruple the dry mass (HHQ) in serum bottles with a working volume of 50.5 mL.

Parameter		**Substrate**					
		AC	**PHC**	**HF**	**HH**	**HHD**	**HHQ**
Substrates converted to end-products (g)		0.017	0.014	0.016	0.006	N.D.	N.D.
C1/C2		0.99	0.94	0.88	0.73	0.73	0.78
O/R		0.95	0.94	0.95	1.03	0.93	0.95
Final pH		6.41 ± 0.01	6.58 ± 0.02	6.62 ± 0.04	7.04 ± 0.02	6.84 ± 0.00	6.61 ± .01
Acetate/Ethanol		1.51	1.25	1.39	1.60	1.83	1.60
Specific yield (mMol / g cellulose)	H_2	174.07 ± 0.42	147.19 ± 0.47	131.29 ± 0.59	42.12 ± 0.46	47.29 ± 0.06	38.72 ± 0.32
	C_2H_5OH	3.58 ± 0.09	3.90 ± 0.27	3.43 ± 0.12	1.64 ± 0.01	1.26 ± 0.21	1.09 ± 0.22

N.D. These substrates were not used for the growth curve experiment.

balances for HH, HHD, and HHQ suggest an overestimation of C2 products (acetate and ethanol) or an underestimation of C1 products (carbon dioxide and formate). The O/R ratios for all substrates indicate that all oxidized and reduced end-products were accounted for (Table 2.4).

2.4.5 XRD analysis and the contribution of amorphous cellulose

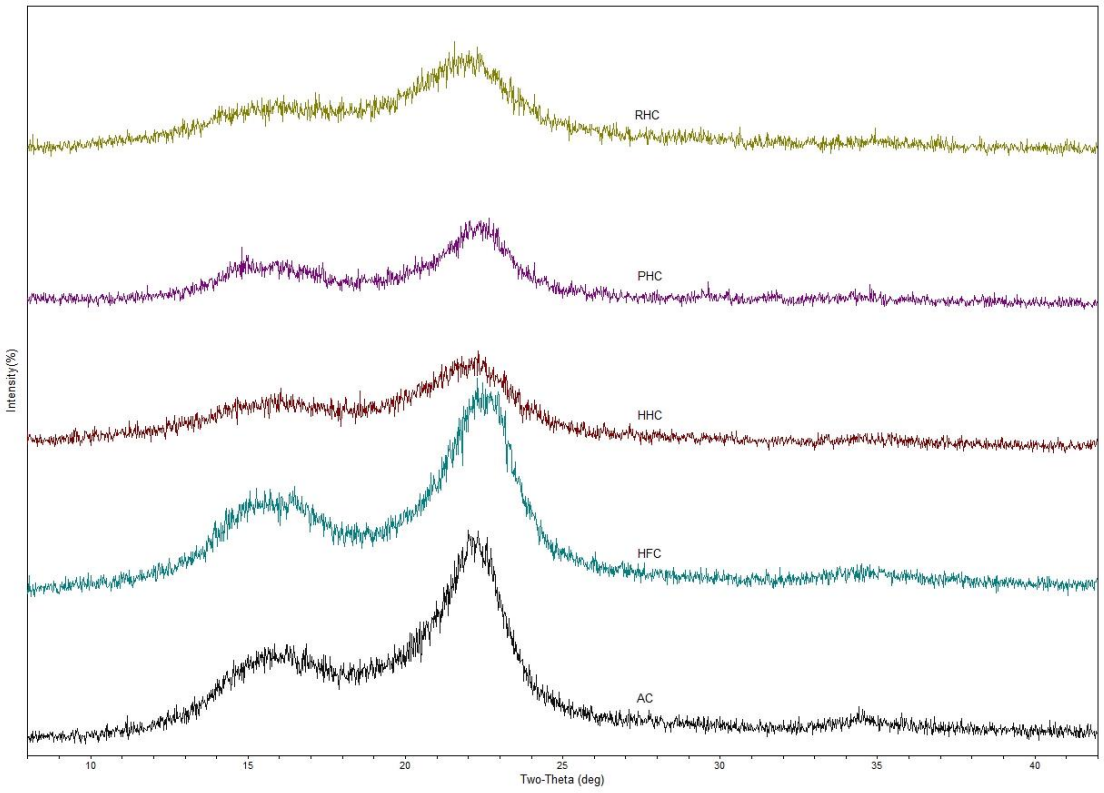
Figure 2.7A shows that all the test substrates absorbed with the same Two-Theta, meaning there was no significant variation in the cellulose type. However, a closer examination of the XRD diffractogram patterns revealed differences in the contribution of amorphous cellulose (CAC, as calculated using equation 1). The calculated CAC values for PHC were 75% while the CAC values for HF and HH were 44% on this diffractogram. And there is also a slight shift in the Two-Theta from 22 to 22.5 as illustrated in Figure 2.7b.

2.5 Discussion

Analyses of biomass polymer composition (conducted at the University of Saskatchewan, Feedstock and Innovation Institute, and reported on a dry mass basis) showed less available cellulose in HH compared to PHC, HF, and AC. Acid detergent fibre (ADF) was used to determine the portions of the biomass consisting of cellulose and lignin, while neutral detergent fibre (NDF) measured the total cell wall composition of the 3 major plant polymers: hemicelluloses, cellulose, and lignin. The difference between NDF and ADF represents the amount of hemicellulose, while ADF minus the lignin represents the approximate cellulose composition, expressed as percentage of the dry mass (Table 2.1).

Substrate conversion by *C. thermocellum* is dependent on the binding of bacterial cells and their cellulosomes, to cellulose fibres (Figure 2.8 Aand B) to effect hydrolysis and uptake (Lynd et al., 2006; Lynd et al., 2002), and thus cellulose accessibility becomes a rate determining

A


RHC
PHC
HHC
HFC
AC
Intensity(%)
Two-Theta (deg)
10
15
20
25
30
35
40

B

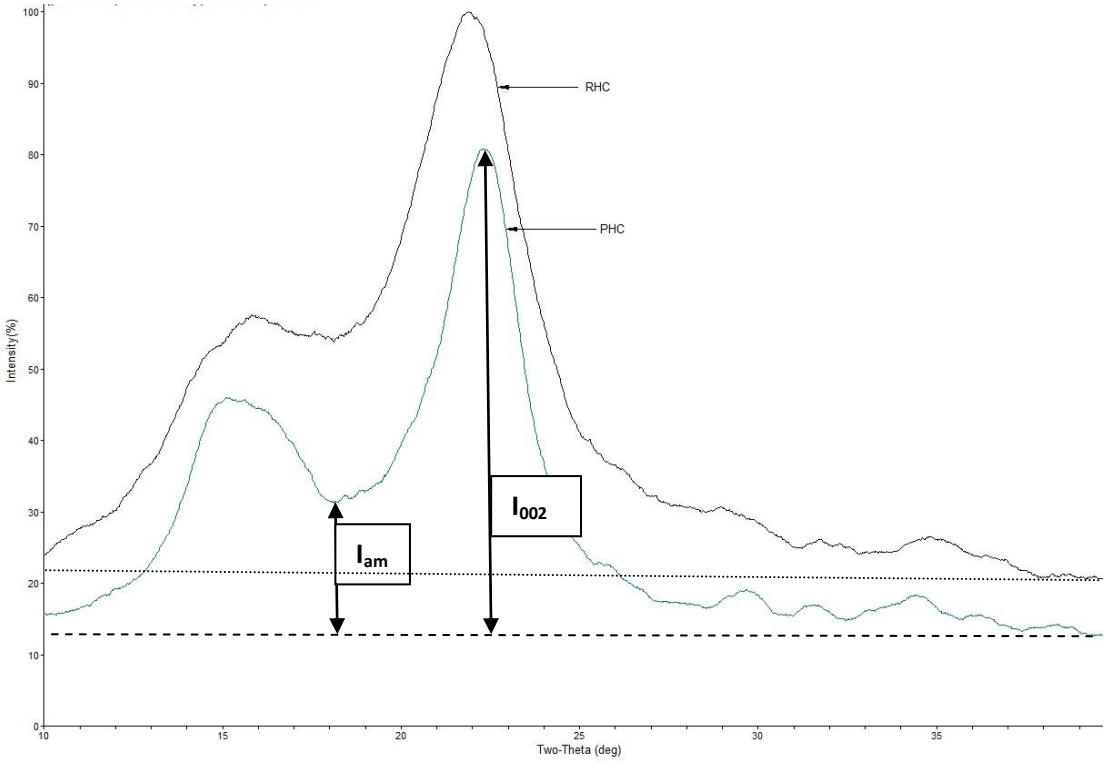

RHC
PHC
I_{002}
I_{am}
Intensity(%)
Two-Theta (deg)
0
10
20
30
40
50
60
70
80
90
100
10
15
20
25
30
35

Figure 2.7 X-ray diffractograms plots of pretreated and untreated substrates. A) Non-scaled super-imposed X-ray diffractograms of α-cellulose (AC), Hemp fibre (HF) cellulose, Hemp hurd (HH) cellulose, Purified hemp cellulose (PHC) and Raw hemp cellulose (RHC); B) A scaled super-imposed X-ray diffractograms plots of PHC and RHC.

factor (Arantes and Saddler, 2010a; Jeoh et al., 2007). This explains the slower growth rate of *C. thermocellum* on insoluble substrates relative to soluble substrates, such as cellobiose, observed previously (Islam et., 2006; Lydn et al., 2005). Our data indicate that *C. thermocellum* generates as much cell biomass during growth on 2 g/L of PHC as on reagent grade AC (Figure 2.1). Although the optical density measurements (A_{595}) of the protein data (total protein extracted from cell biomass at each time point), as determined by Bradford assays, had a high degree of variability, the yield of end-products (H_2, CO_2, and ethanol) confirm the growth differences of *C. thermocellum* on the different substrates. Significantly higher yields of fermentation end-products were observed in fermentation reactions with AC (purified reagent grade cellulose), PHC, and HF, compared to HH.

The growth of *C. thermocellum* on cellulosic substrates relies on the accessibility of its enzyme components to the cellulose in the biomasss to produce cellodextrins that are transported across the cell membrane and metabolised to produce end products such as hydrogen, carbon dioxide, ethanol and organic acids (Carere et al., 2008; Lynd et al., 2005; Lynd et al., 2006; Lynd et al., 2002). Although the net amount of hydrogen and ethanol produced was highest for AC (Table 2.3), the rates sustained during the exponential phase were comparable AC and PHC for both hydrogen and ethanol compared to any other substrates. Organosolv pretreatment generates purified cellulose fibres by solubilizing hemicelluloses, precipitating lignin, and extracting waxes and plant proteins (Agbor et al., 2011; Pan et al., 2005; Pan et al., 2006; Zhao et al., 2009). The absence of these polymers in PHC creates more binding sites for the bacteria and their catalytic components.

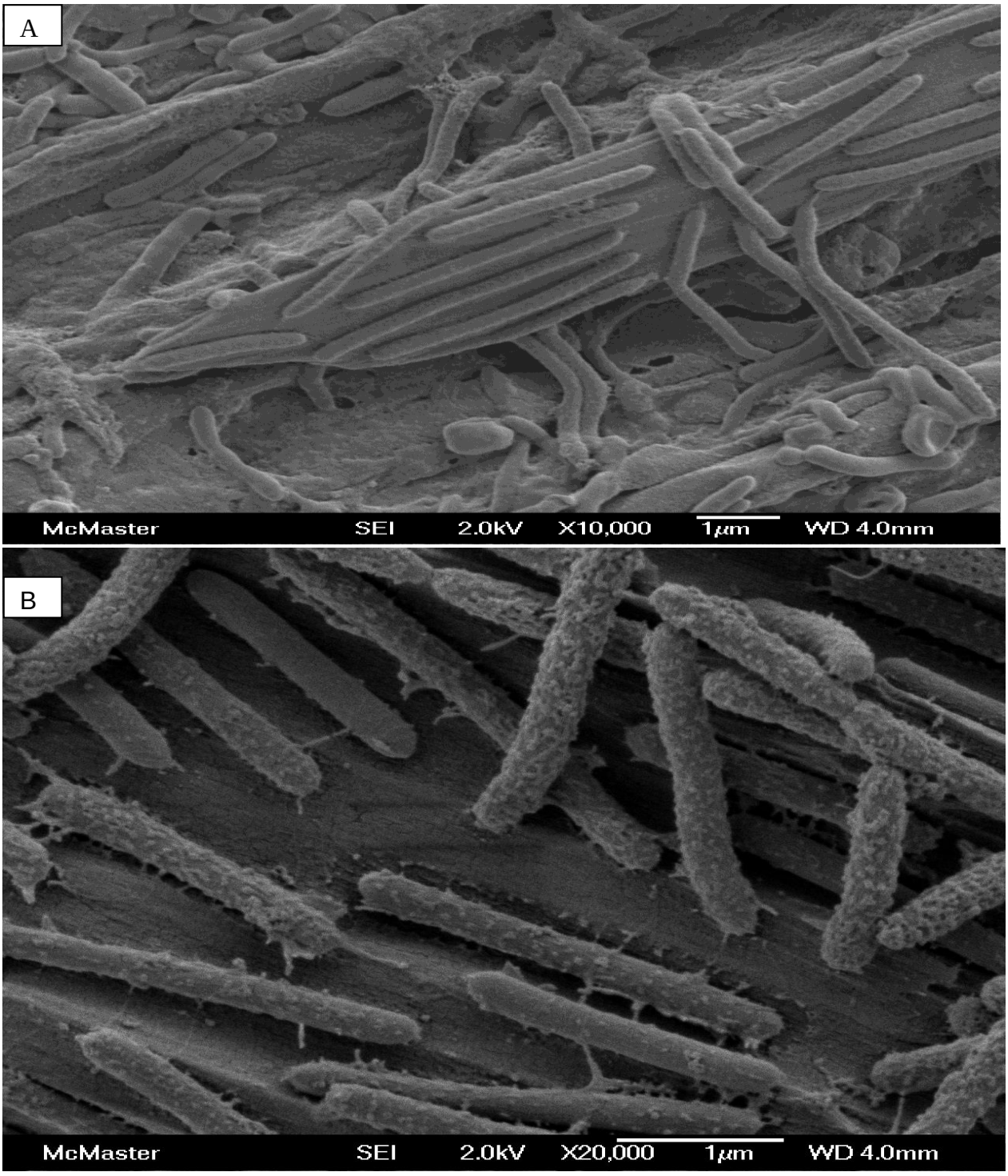


Figure 2.8 Scanning electron micrographs of *C. thermocellum* on purified cellulose fibers derived from Organosolv treated hemp biomass. a) 10,000 X magnification; b) 20,000 X magnification.

The cellulose content of PHC and HF were approximately equal (78.9% and 78.5%, respectively), while their hemicellulose (4.9% and 6.6%, respectively,) and lignin (7.3% and 3.3%, respectively) contents were not greatly different (Table 2.1). The cellulose, contents of HH, however, was significantly lower (57.7%), and the hemicellulose and lignin contents were significantly higher (17.8% and 16.8%) than PHC and HF (Table 1). Given the right conditions, when cellulose is processed, it is converted from its native form in biomass (cellulose I) to a more digestible cellulose polymorph i.e. cellulose II-IV (O'Sullivan, 1996). Our X-ray diffraction data shows that, pretreated biomass (in this case of AC and PHC) is more digestible than raw biomass (HF), even though they may have approximately the same cellulose content (Agbor et al., 2011).

When experiments were carried-out in serum bottles, and the amount of dry mass measured for HH was double and quadrupled to investigate the effect of increasing cellulose concentration and accessibility on the yield of end-products. The production of ethanol and hydrogen increased significantly as the concentration of HH increased (Figures 2.2 to 2.5). Doubling and quadrupling the concentration of HH to increase the available cellulose to (116 and 232 mg available cellulose in HHD and HHQ, respectively than the cellulose available in AC (86.43 mg available cellulose), resulted in increases in end-product titres.

However, no significant increase in the specific yields of ethanol and hydrogen per gram cellulose were observed for HHD and HHQ (Table 2.4). Moreover, although the cellulose concentration increased in HHD and HHQ, the final yield of hydrogen and ethanol did not equal the production observed with 2 g/L of AC and PHC, even though the available cellulose content was lower than in HHD and HHQ. Presumably, the specific yields of hydrogen and ethanol were limited because accessibility to the cellulose fibres was impaired by the presence of

hemicellulose and lignin polymers in the substrate. Increasing the accessibility of HH by removing lignin, hemicelluloses, pectins and wax via pretreatment results in a cellulose fraction that is highly amorphous and accessible, producing higher yields as observed in PHC.

Amorphous or less-ordered cellulose is more chemically reactive and has different physical properties such as chain spacing and order. Thus, a high CAC value will therefore imply that the substrate structurally has many more amorphous regions within substrate microfiber and will be digested at a faster rate than one with a lower CAC value. Amorphous cellulosic material is more accessible to water, exogenous enzymes, and bacterial catalytic components than crystalline or less amorphous cellulosic material and therefore hydrolysed at a much faster rate (Fan et al., 1980; Hall et al., 2010). Our data and that of Mittal et al. (Mittal et al., 2011) indicate that the amorphous cellulose content of the pretreated feedstocks affected subsequent hydrolysis and fermentation of the biomass underscoring the importance of the amorphous cellulose content along other parameters that influence bioconversion of cellulosic biomass.

2.6 Conclusion

Clostridium thermocellum is able to utilise processed and unprocessed residual fractions of hemp biomass derived from local hemp cultivation for hydrogen and ethanol production in a single-step process. Data reported in section 2.4 shows that purified hemp cellulose, derived as a by-product of the Lignol Organosolv® process, as well as raw cellulose rich hemp fibre, result in greater production of fermentation end-products compared with native hemp hurds in a consolidated bioprocess. End-product yields were based on their instrinsic cellulosic content as well as the presence of polymers present in plant biomass. Removal of lignin, hemicelluloses,

pectins, and wax in PHC resulted in a highly reactive cellulose that can be used directly as feedstock for biological ethanol and hydrogen production in a one-step fermentation process.

Chapter 3

Processing of cellulosic feedstocks for ethanol and hydrogen production.

This chapter is based on the following publication: Agbor V, Blunt W, Cicek N, Sparling R, Berlin A, Levin DB. 2011. Processing of celluylosic feedstocks for ethanol and hydrogen production. Journal of Science and Technology for Forest Products and Processes, 1, 54-61

3.1 Abstract

The number of steps preceding fermentation of lignocellulosics hydroxylates is crucial in determining the final cost of a biocommodity. We have investigated consolidated bioprocessing (CBP) of purified cellulose from forest wood (PWC), hemp (PHC), flax (PFC), rice straw (PRC), and beverage cup cellulose (BCC) compared to reagent-grade α-cellullose (AC) for ethanol and hydrogen production using *Clostridium thermocellum*. Net production of hydrogen and ethanol was highest for PWC (10.63 µmol/mL and 7.50 mM) compared to AC (8.54 µmol/mL and 6.57 mM) used as positive control. The contribution of amorphous cellulose (CAC) of PWC was 74.2 %,, while the CAC of Ac was 69.0 %. PHC and PFC, with CACs of 67.0 % and 59.8 %, respectively, also produced lower and amounts of hydrogen (7.64 mM and 7.22mM, respectively) and ethanol (5.76 mM and 5.19 mM, respectively). Rates observed during the exponential phase of growth on PWC, were comparable to those observed with AC, PHC and PFC given the same pretreatment. End-product yields were dependent on the intrinsic cellulose content and the proportion of amorphous cellulose in each test substrate. Rates of hydrogen and ethanol production were similar in early log phase but varied in both mid-log and late log phases. Overall, pretreated and processed forest wood cellulose (PWC, AC and BCC) had higher CACs and produced higher yields than pretreated agricultural residues (PHC, PFC, and PRC).

3.2 Introduction

Currently, the USA and Brazil are the leading producers of bioethanol, generating approximately 26 and 19 billion litres (L) respectively, and accounting for 89% of world's fuel ethanol production. Corn ethanol is capped at 57 billion litres in the USA, but the Renewable Fuel Standard established by the USA Energy Bill is set at 136 billion litres by 2022. The remainder (79 billion litres) is expected to come from "advanced biofuels", most of which are derived from lignocellulosic biomass (Sannigrahi et al., 2010).

In North America, ethanol and hydrogen derived from cellulosic biomass could be potential commercial energy carriers for transportation or portable power. As biofuels plants become biorefineries, achieving low-cost route processes for industrial-scale production of ethanol or hydrogen from cellulosic biomass is crucial for investor and public endorsement. So far, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and simultaneous saccharification and co-fermentation (SSCF) have been used for industrial ethanol production (Lynd, 1996; Sassner et al., 2008), while hydrogen has been produced primarily by electrolysis of water, gasification of oil / methane, and steam-reformation of natural gas (Levin et al., 2004; Nath and Das, 2004). Current bioprocessing strategies for ethanol production are yet to be cost competitive to natural gas (Galbe and Zacchi, 2007; Lynd, 1996; Lynd et al., 1996; Lynd et al., 2008), while all current methods for hydrogen production are energy intensive (Levin et al., 2004).

Direct microbial conversion (DMC), also referred to as direct cellulose fermentation or consolidated bioprocessing (CBP), is a less energy intensive method of biological hydrogen production (Carere et al., 2008; Das and Veziroglu, 2001; Levin et al., 2004) and a potential low cost route for cellulosic ethanol production (Lynd et al., 2008; Weimer et al., 2009a; Xu et al.,

2009). Even though CBP offers the greatest potential for the lowest cost route for biofuels, much remains to be elucidated with respect to: i) feedstock quality; ii) extent of pre-treatment; and iii) the fermentability of the pretreated biomass. Although CBP of lignocelluloses is an attractive alternative to current industrial methods for ethanol (i.e. SHF, SSF, SSCF) and hydrogen production, the switch to using lignocellulosic feedstocks as substrate requires characterisation and bioprocess evaluation of pretreated feedstocks used as a source of carbon. In this chapter we have assessed the fermentability of five test cellulosic feedstocks in comparison to each other and to commercial reagent grade α-cellulose (used as a positive control). The Objective of this work was to assess the fermentability (ease of converting) of different types of similarly pretreated cellulosic biomass by *Clostridium thermocellum* in a single-step fermentation process.

3.3 Materials and Methods

3.3.1 Substrate

Test substrates included: 1) organosolv pretreated hemp cellulose (PHC) originating from Emerson hemp company, Manitoba (MB); 2) organosolv pretreated wood cellulose (PWC) from spruce pulp harvested in British Columbia (BC), from Lignol Innovations in Burnaby, BC; 3) organosolv pretreated flax shive cellulose (PFC) generously provided by Schweitzer Mauduit Canada Inc. in Winkler, (MB); 4) subcritical carbon dioxide-pretreated rice straw cellulose (PRC) from Mitsubishi Heavy Industries Japan; and 5) beverage cups cellulose from Tim Hortons Canada (BCC).

Organosolv pretreatment was conducted with 50% ethanol, for 55 minutes (min), at 195 ^{o}C, using 1.5% acid as catalyst, at a 10:1 Liquid: insoluble biomass ratio using Lignol Organosolv® process (Pan et al., 2005; Pan et al., 2006). The subcritical carbon dioxide extraction pretreatment was conducted hydrothermally by Mitsubishi Heavy Industries, Ltd.

Japan. We refer to these water insoluble substrates as purified rice cellulose (PRC), purified wood cellulose (PWC), purified hemp cellulose (PHC), purified flax cellulose (PFC), and beverage cup cellulose (BCC).

Compositional analyses of the biomass substrates to determine moisture content, dry matter, acid detergent fibre (ADF), neutral detergent fibre (NDF), ash, soluble protein, cellulose, hemicellulose, and lignin were conducted at the at the University of Saskatchewan, Feeds Innovation Institute, and reported on a dry mass basis (see Table 3.1).

A Retsch Rotor Beater mill, SR200 equipped with a 34 mesh and 0.5 mm aperture sieve was used in milling the substrates. All the milled samples were transferred into Ziplock bags and stored at room temperature. Alpha (α)-cellulose (C8002) obtained from Sigma Aldrich (Saint Louis, USA) was used as positive control. All substrates were added to fermentation reactions at 2 g/L substrate loading. Negative controls were established using 1191 medium inoculated with *Clostridium thermocellum* but without any substrate.

Table 3.1 Polymer composition for all test substrates on a percent dry matter basis.

Parameter (%)	Method Used	Purified Wood	Purified Hemp	Purified Flax	Purified Rice Straw	Milled Beverage Cups
Moisture content	AOAC 930.15	4.67	5.06	4.57	9.98	4.38
Dry matter	AOAC 930.15	95.33	94.94	95.43	90.02	95.62
Crude protein	AOAC 984.13	0.37	0.87	0.53	2.79	0.27
ADF	ANKOM Method 5: 08-16-06	93.40	86.36	91.40	49.49	91.47
NDF	ANKOM Method 6: 08-16-06	95.57	91.30	97.30	64.79	98.01
Ash	AOAC 942.05	0.10	4.31	0.54	15.55	0.50
Soluble crude protein	Roe et al., 1990	0.20	<0.05	<0.05	0.60	0.08
Cellulose	cellulose = ADF - lignin	91.27	78.94	77.03	38.61	86.28
Hemicellulose	hemicellulose = NDF - ADF	2.17	4.94	5.90	15.30	6.54
Lignin	ANKOM 08/05	2.13	7.42	14.37	10.88	5.19

Each assay was run in duplicate and was repeated if % error was > 3 % (Roe et al., 1990).

3.3.2 Microorganism and Media

Clostridium thermocellum strain 1237 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany) and maintained in by culturing in 1191 medium (Islam et al., 2006). DSMZ 1237 is equivalent to *C. thermocellum* strain 27405 available at the America Type Culture Collection (ATCC). For each experiment, cells used for inoculation were cultured in a 1 L Corning bottle that was used to inoculate all batch reactors used in growth experiments to ensure reproducibility. Chemicals and reagents used were obtained from Fisher Scientific unless otherwise stated. Yeast extract was obtained from Biobasic Inc (bacteriological grade). Serial subcultures of *C. thermocellum* were used to prepare and maintain fresh cultures in 1191 media containing the 2 g/L α-cellulose. Per litre of milliQ water, 1191 medium contained: KH_2PO_4, 1.5 g; Na_2HPO_4, 3.35 g; NH_4Cl, 0.5 g; $MgCl_2.6H_2O$, 0.18 g; yeast extract, 2.0 g; resazurin from Sigma Aldrich, Saint Louis, USA (0.25 mg/ml), 2.0 ml; 10× vitamin solution, 0.50 ml; 10× mineral solution, 1.00 mL. Vitamin supplements and mineral elixir solution were prepared according to a previously described procedure in Islam et al. (Islam et al., 2006).

3.4 Experimental procedure

Batch experiments in Balch tubes and serum bottles, with working volumes of 27 mL and 127 mL respectively (Wheaton Science, obtained from Fisher Scientific), were used for all tests. For all experiments performed, 2 g/L of substrate loading was used in vessels containing 9 mL or 45 mL of 1191 media. All the tubes were air-sealed with butyl-rubber stoppers and crimped with aluminium seals and then gassed and degassed for four (1:4) cycles with 100% nitrogen in order to maintain an anaerobic environment. Before sterilization, each tube was reduced with 0.1 mL of sterile 2mM Na_2S reducing solution (Daniels et al., 1986) and then autoclaved. A similar

procedure was used with serum bottles but with a starting volume of 45 mL of 1191 media and 0.5 mL of 2 mM Na_2S. A fresh culture of *C. thermocellum* at exponential phase in a 1 L corning bottle was used to inoculate each experimental set, with 10 % of the volume of media in test vessel. All batch experiments for each substrate were conducted in three biological replicates. And triplicates of each experimental time-point were collected for analyses according to predetermine interval of 3 hours or more. The amount of end-products carried over into tests samples was determined by collecting a zero hour time point immediately after inoculation. All growth curve tests were conducted in Balch tubes while end-point tests were carried-out in serum bottles.

3.5 Analytical Procedures

Substrate polymer composition for moisture, dry matter, crude and soluble protein, lignin, acid and neutral detergent fibre previously described by Mani and co-workers (Mani et al., 2006) (Table 3.1). Gas analysis for serum bottles was conducted using an Agilent 7890 GC (gas chromatography) equipped with an TCD and FID using argon carrier gas, a gas sample valve with a 0.25 cc loop, a split inlet and two PLOT columns in series, a PLOT Q 30 m x 0.53 mm ID and a PLOT molecular sieve column 30 m x 0.53 mm ID. While gas production in the Balch tubes were analysed using an SRI gas chromatograph (SRI model instruments, Model 8610C) outfitted with a thermal conductivity detector, a stainless steel molecular sieve 13x packed column (3.2 mm x 1.8m) connected in series with a stainless steel silica gel packed column (3.2 mm x 1.8) to separate H_2 and CO_2 respectively using Argon as carrier gas. Total gas compositions were quantified by correcting for temperature, pressure, volume and aqueous phase concentrations estimated according to the solubility of the gases in water (Sander, 1999). The amount of carbon dioxide in equilibrium with bicarbonate was also taken in consideration

(Darrett and Drisham, 1995). Aqueous products such as organic acids and ethanol were measured with a Waters HPLC equipped with an HPX-Aminex 87 column (Bio-Rad Laboratories, Hercules, CA California) maintained at 45 ^{o}C with HPLC grade water as mobile phase maintain at flow of 0.6 mL/min.

X-ray diffraction analyses (XRD) was conducted using a Siemens D5000 powder diffractometer (Siemens AXS, Inc.,Madison, WI) using Cu radiation (Cu K-alpha lambda = 1.5418 Å) and operated at 40kV and 40mA. The anti-scatter and receiving slits were set to 1.0 mm. Samples were mounted in a depressed 'well' on a zero-background quartz plate and pressed into the well with a frosted glass slide. Scans were obtained from 8 to 42 degrees two-theta using a step-width of 0.05 degree two-theta and a dwell time of 1 s/step.

The contribution of amorphous cellulose in the test substrates was determine according to a method originally proposed by Segal et al., (Segal et al., 1959) for the measurement of cellulose crystallinity but now provides empirical measurements that allow rapid comparison of cellulosic samples by taking rough approximations of the contribution of amorphous cellulose (CAC) to the cellulose diffraction spectrum (Park et al., 2010).

$$CAC = \left[\frac{I_{002} - I_{AM}}{I_{002}}\right] * 100 \quad \text{.....(1)}$$

3.6 Results and Discussion

The composition of biomass substrates used in the fermentation reactions is shown in Table 3.1. All test substrates had > 90 % dry matter content. Acid detergent fibre (ADF) was used to determine the portions of the biomass consisting of cellulose and lignin, while neutral detergent fibre (NDF) measured the total cell wall composition of the three major plant polymers: hemicelluloses, cellulose, and lignin. The difference between NDF and ADF represents the amount of hemicellulose, while ADF minus the lignin represents the approximate

cellulose composition, expressed as percentage of the dry mass (Table 3.1). The results show less available cellulose following pretreatment in PRC, PFC, PHC compared to PWC and BCC. Alpha (α-)-cellulose was used as a reagent grade substrate with a well defined composition.

X-ray Diffraction analysis of biomass substrates that were pretreated using Lignol Organosolv® process to solubilise hemicellulose and extract lignin, resulted in a cellulose allomorph that is identified between 22 and 23 2 theta (θ) following the 002 peak like the α-cellulose used as positive control (Figure 3.1). Hence, the cellulose content of each Organosolv® extracted test substrate contained the same transition cellulose allomorph or polymorph as the positive control (α-cellulose), providing a good basis for comparison. Previous studies have shown that a shift from cellulose I to cellulose III can be observed by following the position of the 002 peak as it shifts from $2\theta = 23$ to $2\theta = 21$ for Cellulose III, which is known to be a more digestible cellulose allomorph compared to native cellulose or cellulose I with a similar crystallinity index (CI) (Mittal et al., 2011; O'Sullivan, 1996; Park et al., 2010; Park et al., 2009).

The diffractograms shown in Figure 3.2 suggest that PWC has the highest contribution of amorphous cellulose (CAC ≈ 74.2 %) followed by AC (CAC ≈ 69 %), PHC (CAC ≈ 67%), and PFC (CAC = 59.8 %) after pretreatment and milling. CAC values were calculated from the ratio of the height of the 002 peak and the height of the minimum (I_{AM}) between the 002 peak and the 101 peak shown on Figure 3.2 using Eq.1 (Park et al., 2010; Park et al., 2009). Amorphous cellulosic material is more accessible to water, exogenous enzymes, and bacterial catalytic components than crystalline or less amorphous cellulosic material, and therefore hydrolysed at a much faster rate (Fan et al., 1980; Hall et al., 2010). Previous studies agree that cellulase digestibility of pretreated biomass is limited by accessibility to the cellulose fibres

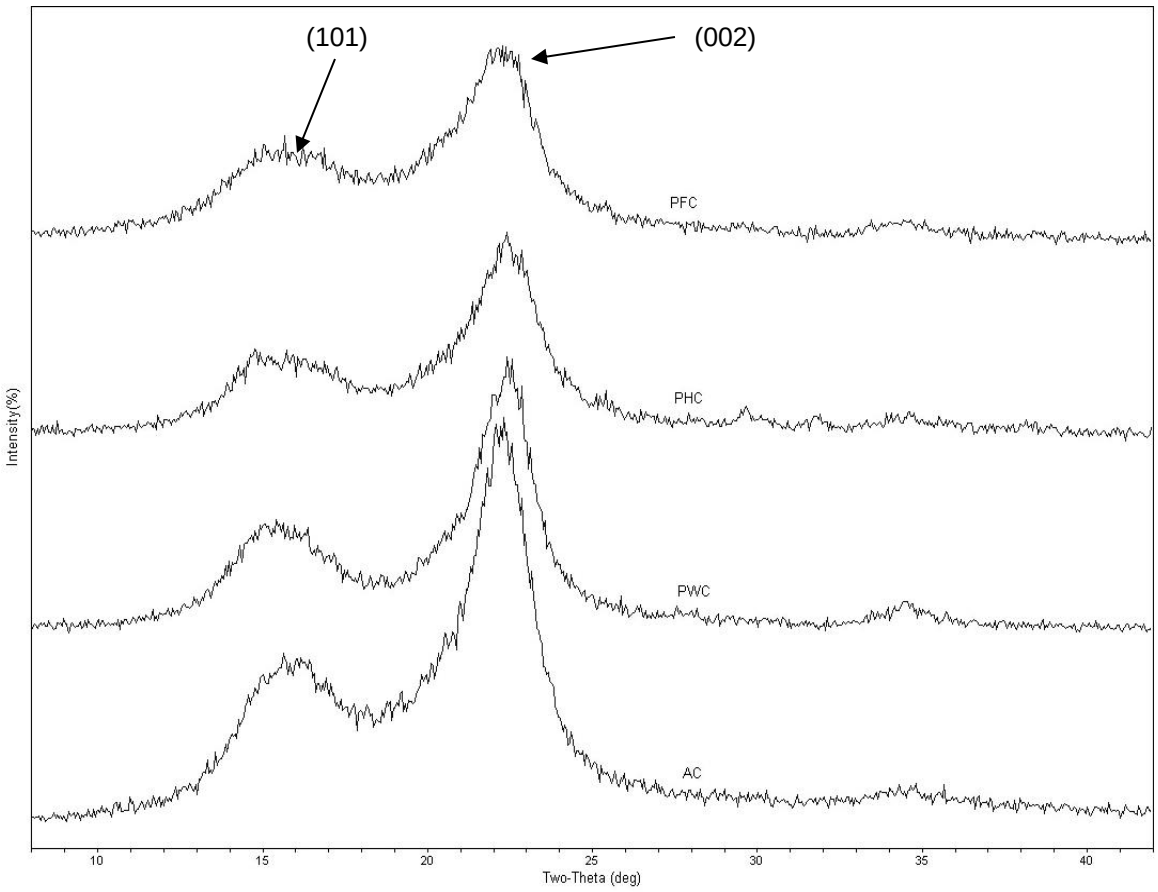


Figure 3.1 Non-scaled, super-imposed X-ray diffractograms of α-cellulose (AC), purified wood cellulose (PWC), purified hemp cellulose (PHC), and purified flax cellulose (PFC) showing the diffraction pattern of the cellulose in test substrates and control detected at 2 theta (θ) ≈ 22.3.

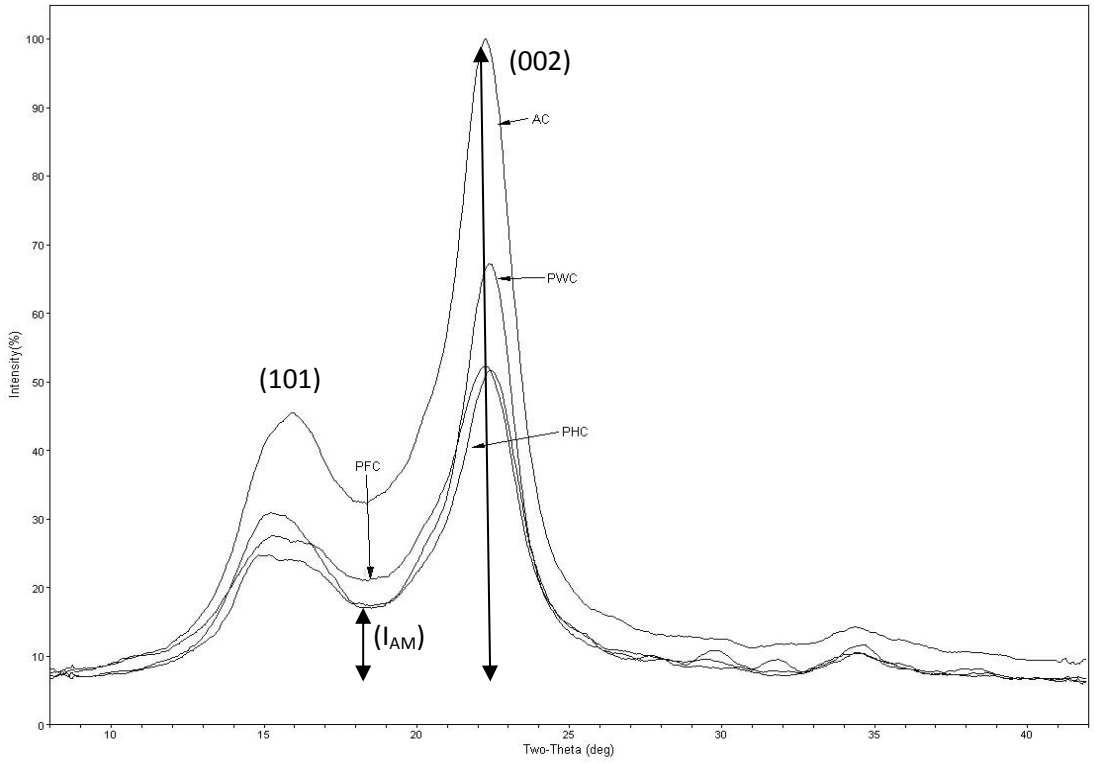


Figure 3.2 Scaled, super-imposed X-ray diffractograms of α-cellulose (AC), purified wood cellulose (PWC), purified hemp cellulose (PHC), and purified flax cellulose (PFC) showing the diffraction pattern of the cellulose in test substrates and control detected at 2 theta (θ) ≈ 22.3.

(Converse, 1993; Desai and Converse, 1997; Hendricks and Zeeman, 2009; Jeoh et al., 2007; Laureano-Perez et al., 2005; Silverstein et al., 2007).

Batch growth experiments shown in Figures 3.3 and 3.4 suggest that *C. thermocellum* was able to convert the cellulose present in test substrates to end-products during all phases of growth. More hydrogen was produced from the more amorphous and cellulose rich PWC and AC substrates compared to PHC and PFC substrates. The rate of hydrogen production was variable during the exponential phase with highest rate observed in PWC followed by AC, PHC and PFC, as shown on Figure 3..

End-products synthesized during growth on 2 g/L of each substrate were compared at 50 hours post-inoculation. Figure 3.6 shows the gaseous end-products, with PWC producing the highest hydrogen concentrations. More hydrogen production was observed in fermentation reactions containing the forest-derived cellulosic products (PWC, AC, and BCC) compared to the agricultural residues (PHC, PFC and PRC). Ethanol was the soluble end-product produced in the greatest amount. Figure 3.7 shows that more ethanol was produced in fermentation reactions containing PWC and PHC substrates than in fermentations containing AC, BCC, PFC, and PRC substrates.

Profiles of organic acids synthesized by *C. thermocellum* in fermentation reactions containing each of the test substrates indicates that acetate was produced in the greatest amount in all test vessels, followed by formate and lactate. There was a concomitant decrease in pH associated with organic acid synthesis, ranging from pH 6.53 to 7.04, with the pH dropping lower in fermentation reactions containing higher concentrations of organic acids. The final pH was lowest in fermentation reactions containing PWC (pH 6.53) and highest in reactions containing PRC (pH 7.04), indicative of the amount cellulose that had been converted to

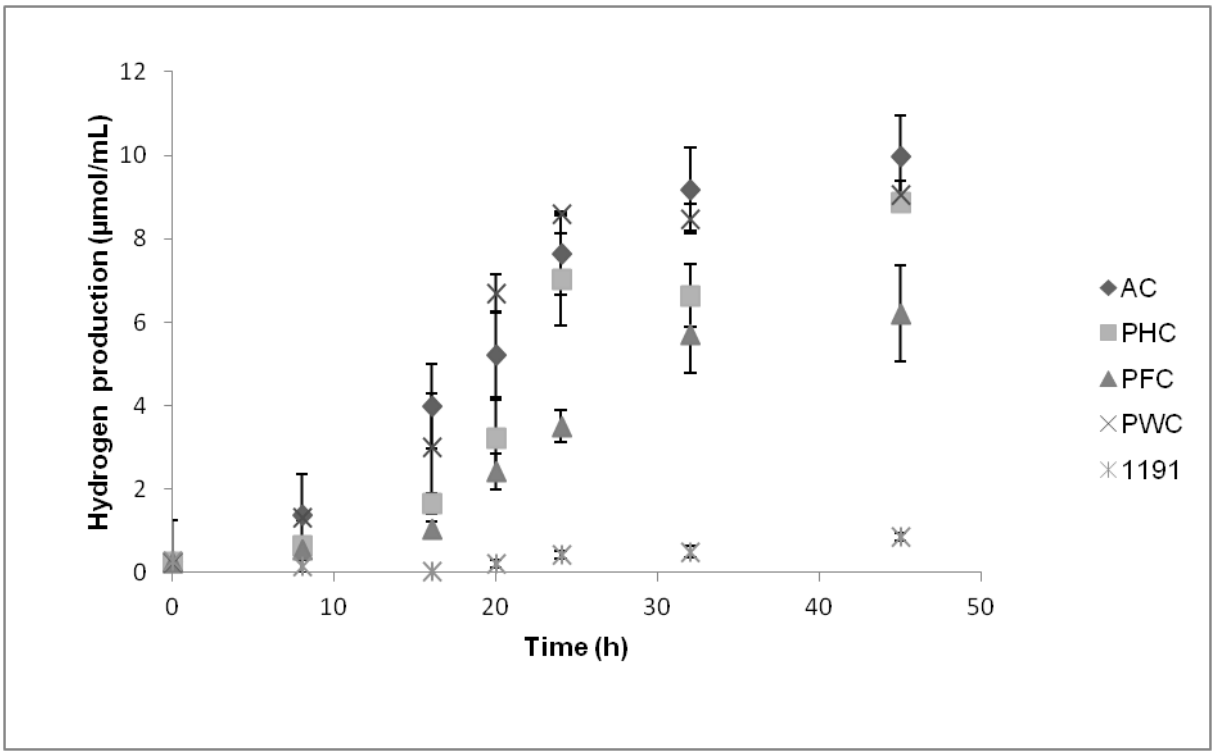


Figure 3.3 Concentrations of hydrogen (H_2) synthesized by *C. thermocellum* from 2 g/L of α-cellulose (AC), purified wood cellulose (PWC), purified hemp cellulose (PHC), and purified flax cellulose (PFC). Each data point is the average of three biological replicates and error bars are associated the Standard deviations.

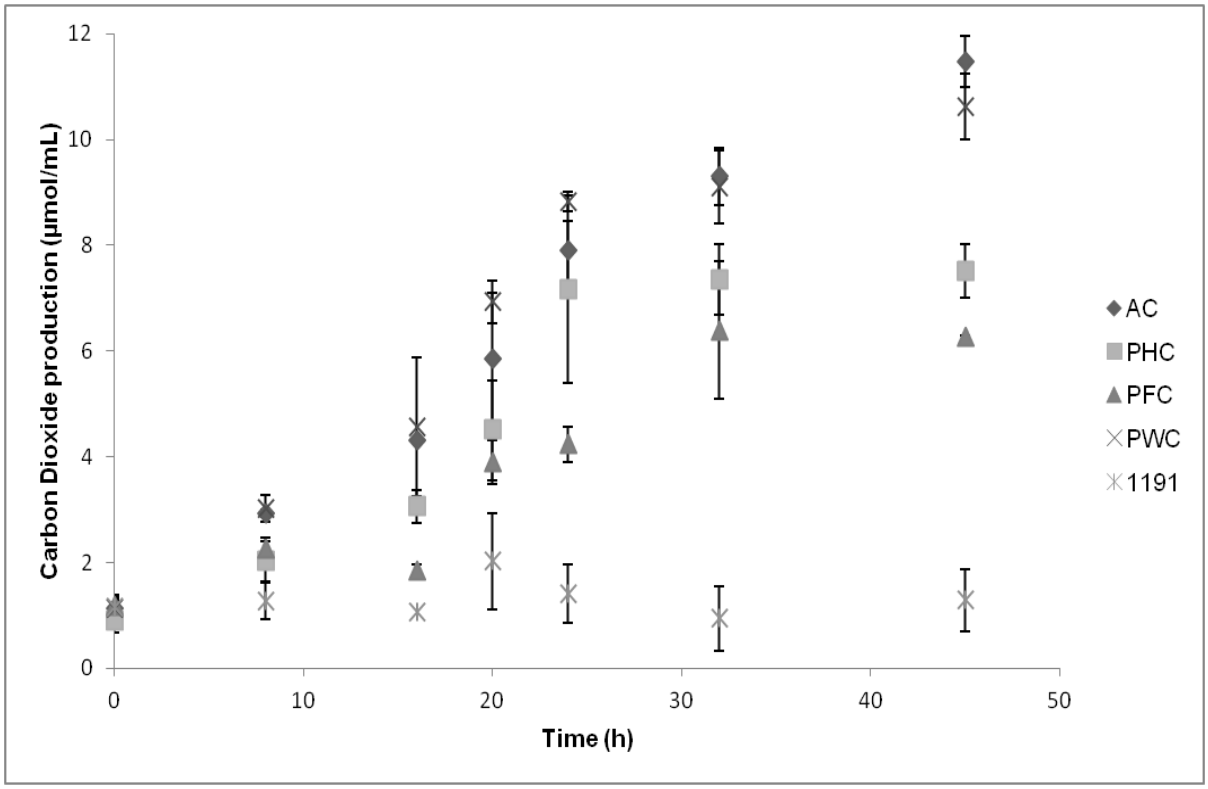


Figure 3.4 Concentrations of carbon dioxide (CO_2) synthesized by *C. thermocellum* from 2 g/L of α-cellulose (AC), purified wood cellulose (PWC), purified hemp cellulose (PHC), and purified flax cellulose (PFC). Each data point is the average of three biological replicates and error bars are associated the Standard deviation.

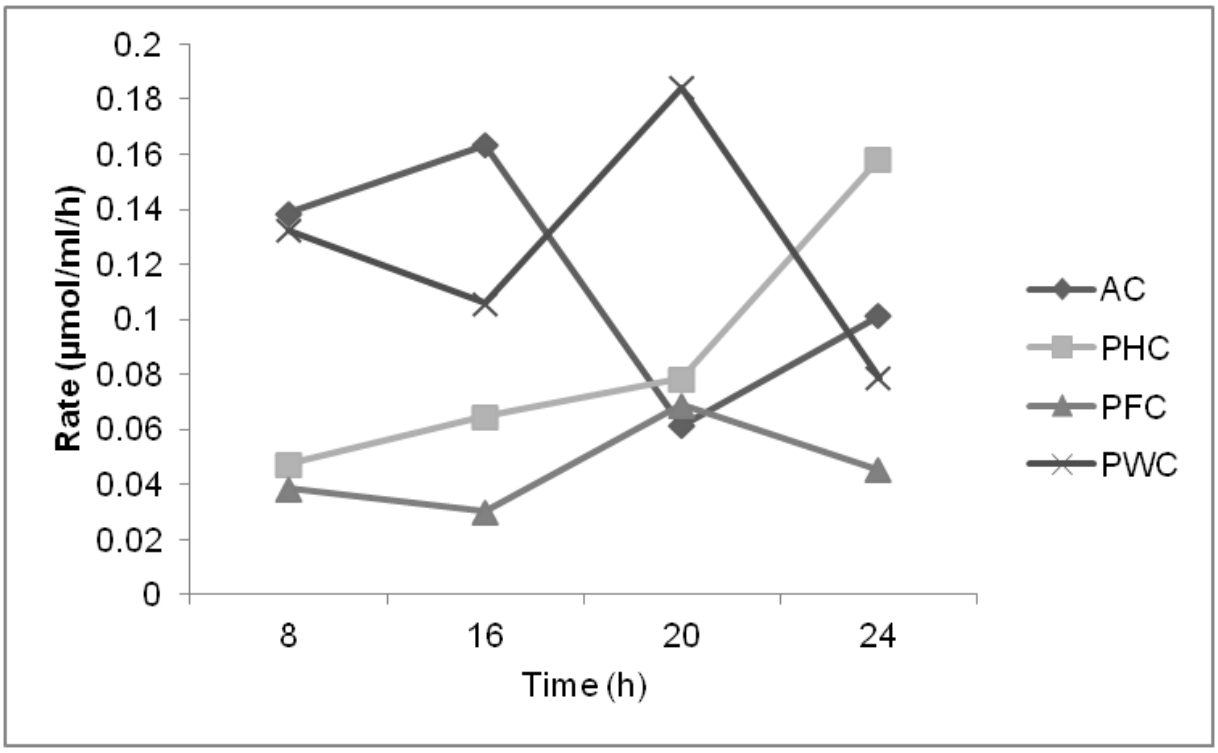


Figure 3.5 Rates of hydrogen production by *C. thermocellum* from 2 g/L of α-cellulose (AC), purified wood cellulose (PWC), purified hemp cellulose (PHC), and purified flax cellulose (PFC) during the exponential phase of growth.

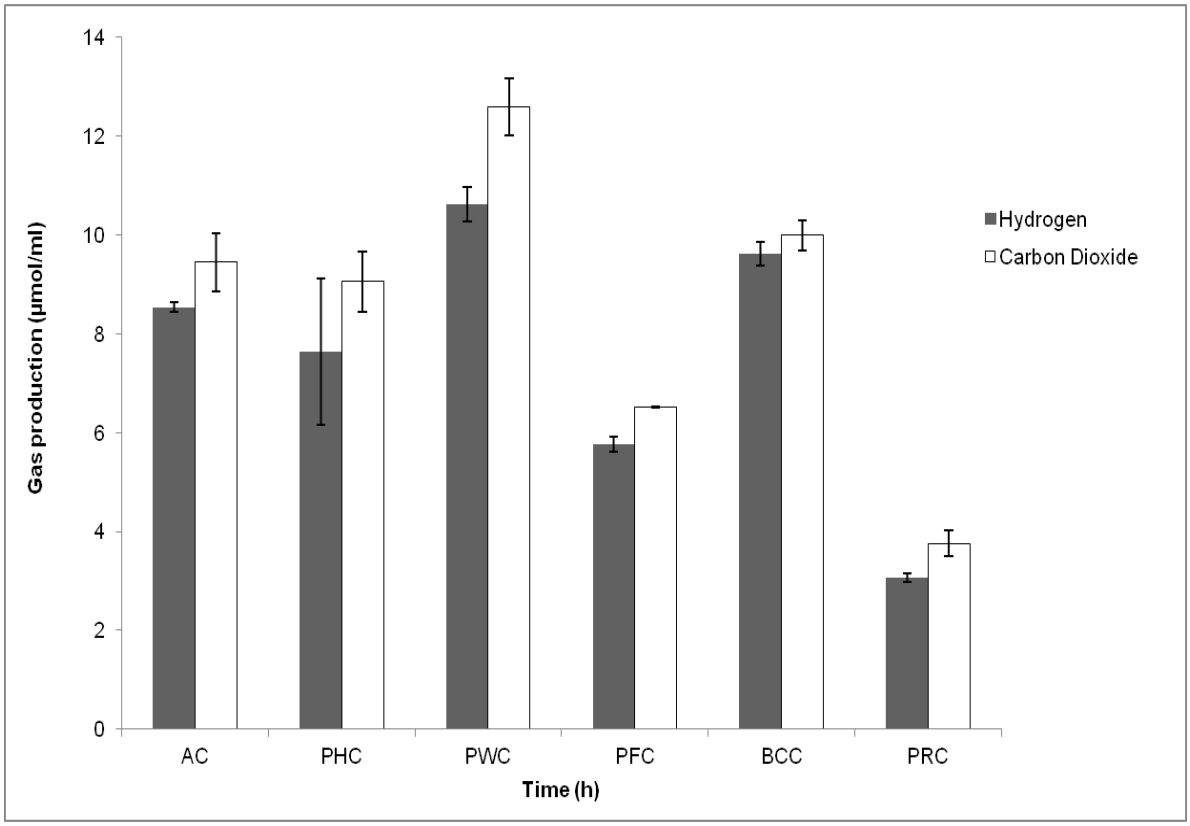


Figure 3.6 Hydrogen and carbon dioxide production after 50 hours of *C. thermocellum* growth on test substrates: AC (α-cellulose), purified wood cellulose (PWC), purified hemp cellulose (PHC), purified flax cellulose (PFC), beverage cup cellulose (BCC), and purified rice cellulose (PRC). Each bar is the average of three biological replicates and error bars are associated the Standard deviation.

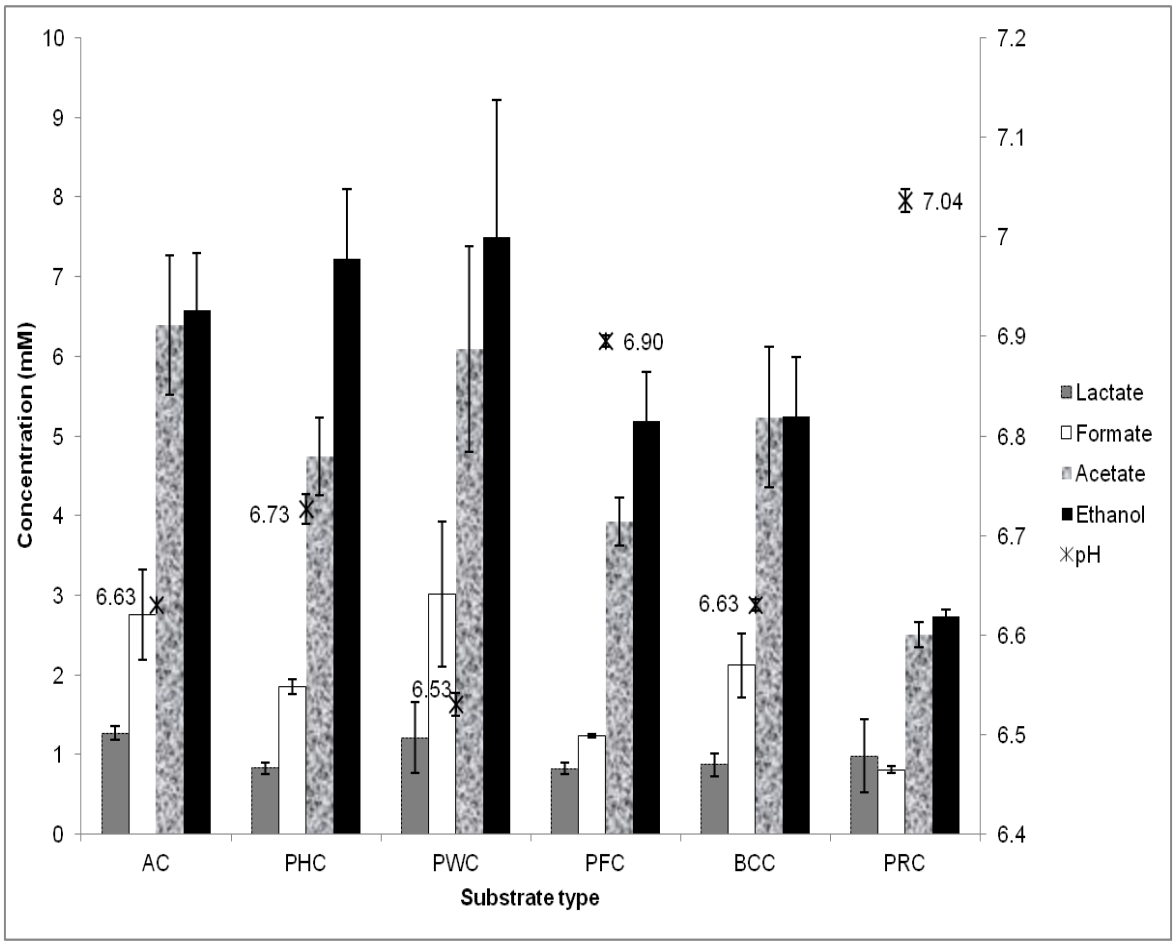


Figure 3.7 Ethanol and organic acid production after 50 hours of *C. thermocellum* growth on test substrates: AC (α-cellulose), purified wood cellulose (PWC) purified hemp cellulose (PHC), purified flax cellulose (PFC), beverage cup cellulose (BCC) and purified rice cellulose (PRC). Each bar is the average of three biological replicates and error bars are associated the Standard deviation.

end-products. Little or no production was observed in 1191 medium inoculated with *C. thermocellum*. This may be due carryover during inoculation or as result of yeast extract in 1191 medium acting as a carbon source.

The final concentrations of end-products synthesized during growth varied, based on the innate cellulose content of each substrate. Fermentation reactions containing PWC had the highest yield of ethanol and hydrogen compared to BCC, PHC, PFC, PRC and AC (Table 3.2). End-product concentrations synthesized in fermentation reactions containing Organosolv® pretreated substrates (Figures 3.3 and 3.4) correlated with the CAC values of the cellulose. PWC, which had the highest estimated CAC value, displayed the highest production of gaseous and soluble end-products, compared with cellulose from agricultural residues (PHC and PFC) and AC. However, the specific yield of ethanol per gram of cellulose was highest for PHC, compared to other test substrates.

Acetate to ethanol ratios (Table 3.2) indicate that *C. thermocellum* were beyond the late exponential phase of growth when the experiments were terminated. Acetate:ethanol ratios between 1.05 - 1.3 have been reported for the late exponential phase (Islam et al., 2009; Lynd et al., 1989). Average amounts of formate produced increased with increased cellulose availability (i.e. increased CAC), but acetate:formate ratios were higher for PFC, PRC, and PHC (purified cellulose agricultural residues) compared to PWC.AC and BCC (Table 3.2) suggestive of a shift in metabolism due to cellulose availability.

Table 3.2 Summary of hydrogen (H_2) and ethanol (C_2H_5OH), yields, average of final pH recorded, acetate:ethanol ratios, acetate:formate ratios, and specific yields of H_2 and C_2H_5OH per gram cellulose for *C. thermocellum* grown on 2 g/L of test substrates AC (α-cellulose), purified wood cellulose (PWC) purified hemp cellulose (PHC), purified flax cellulose (PFC), purified rice cellulose and milled Tim Horton cups (BCC).

Parameter	**Substrate**					
	AC	**PWC**	**PHC**	**PFC**	**PRC**	BCC
H_2 Yield (µmol/mL)	8.54	10.63	7.64	5.76	3.07	9.62
C_2H_5OH Yield (mM)	6.57	7.50	7.22	5.19	2.74	5.24
Final pH	6.63 ± 0	6.53 ± 0.01	6.73 ± 0.02	6.90 ± 0.01	7.04 ± 0.01	6.63 ± 0.01
Acetate/Ethanol	0.97	0.81	0.66	0.76	0.92	1.00
Acetate/Formate	2.32	2.02	2.55	3.17	3.10	2.47
Specific Yield of H_2 (µmol/mL/g cellulose)	92.59	116.39	96.76	74.83	79.47	111.46
Specific yield of C_2H_5OH (mMol/g cellulose)	1.55	1.79	1.99	1.47	1.54	1.32

3.7 Conclusion

Lignocellulosic biomass that is pretreated physically, chemically, or physicochemically result in unique cellulose allomorphs, or polymorphs, with unique properties that vary in their digestibility. Even when different lignocellulosic feedstocks are subjected to the same type of physicochemical pretreatment (in this case Lignol Organosolv® pretreatment) generating the same allomorph, our data indicate that the cellulose content of the pretreated feedstock and the content of amorphous cellulose affect subsequent hydrolysis and fermentation of the biomass to end-products. When subjected to pretreatments that extract lignin, solubilise hemicelluloses, reduce crystallinity, and degree of polymerisation, (in this case achieved by both physical pretreatment, i.e hammer milling, and physicochemical pretreatment), purified cellulose from forest biomass (PWC, AC and BCC) and agricultural residues (PHC, PFC and PRC) could provide hollow-reactive cellulose, needed for the production cellulosic biofuels in biorefineries.

Chapter 4

Enhance bioprocessing of lignocellulosic biomass: The effect of employing exogenous glycosyl hydrolases and the cellulosome in direct microbial conversion with *Clostridium thermocellum*

This chapter is based on the data for a manuscript in preparation: Agbor V, Blunt W, Moreira D, Cicek N, Sparling R, Berlin A, Levin DB, that will be submitted to Biomass and Bioenergy

4.1 Abstract

We have investigated enhanced consolidated bioprocessing of agricultural residues by adding cellulases, xylanases, and cellulosome extracts from the supernatant of *Clostrium thermocellum* cultures for differential hydrolysis of structural biomass polymers. The objective of this experiment was to determine if the addition of exogenous enzymes could increase access of the bacterium and/or its glycosyl hydrolase enzymes to cellulose chains in the substrate, and this enhance rates and/or yields of hydrogen or ethanol Between 0.5 and 5 mg enzyme / g cellulose was added to fermentation reactions containing substrate cellulose at a concentration of 2 g/L. Pretreatment of hemp biomass with a small amount of Cellic CTec and NS50012 (< 0.5g enzymes/g cellulose) resulted in a higher rate of end-product accumulation in the test reactor compared to the control reactor. Moreover, cellulosome extracts prepared from *C. thermocellum* cultures enhanced the yield of fermentation compared to commercial glycosyl hydrolases, and a rapid doubling of the hydrogen concentration generated in the test reactor compared to the control at enzyme loadings of < 0.5 g enzyme /g cellulose was observed. These results suggest that a minimal dosage of exogenous enzymes can disrupt the structure of lignocellulosic biomass sufficiently to enhance product synthesis during direct cellulose fermentation.

4.2 Introduction

Global demand for energy is predicted to increase by 54 % by 2025 and it is anticipated that undifferentiated advanced cellulosic biofuels will bridge the gap between current levels of bioethanol production (60 billion) and the expected 135 billion L mandated by the US Renewable Fuel Standard for 2022 (Sannigrahi et al., 2010). The high cost of production of lignocellulosic biofuels is as a result of the process configuration and very large volumes of enzyme cocktails (cellulases, and hemicellulases; 10-25 % per kilogram biomass) required for separate hydrolysis and fermentation |(SHF), simultaneous saccharification and fermentation (SSF), or simultaneous saccharification and co-fermentation (SSCF) (Lynd et al., 2008; Merino and Cherry, 2007).

Hydrogen has emerged as a clean energy fuel with a very high gravimetric energy density (122 KJ/g). Currently hydrogen produced via fermentation of lignocellulosic biomass is very attractive, as it is less energy intensive compared to electrolysis of water, gasification, and catalytic steam reformation of methane. However the yields of hydrogen produced via dark fermentation are low and need to be greatly improved (Carere et al., 2008; Levin et al., 2011; Levin et al., 2009).

Pretreated biomass consist of a disrupted structural plant polymers consisting of cellulose fibers that are coated with residual lignin and hemicelluloses (Zhu et al., 2009). Recovery of sugars for fermentation require disruption of the complex plant cell wall structure. Acid pretrements are effective against hemicelluloses, whereas alkaline pretreatments effect mostly cellulose crystallinity and only slightly affect lignin structure (Agbor et al., 2011; Levin et al., 2013). This implies that microbial conversion of the pretreated biomass can only be as effective as pretreatment used to expose the sugars within the cellulose and/or hemicellulose polymers.

Two stage pretreatments using acid and alkali to target hemicelluloses and lignin components biomass have been investigated (Rezende et al., 2011) and the mapping of lignin distribution in pretreated sugarcane bagasse showed that acid pretreatments cause accumulation of lignin in the external border of cell wall. Alkali pretreatment was effective in removing lignin from the middle of bagasse fibers, but was not effective in removing lignin from the surface of the bagasse fibers (Coletta et al., 2013). Thus, access of cellulase enzymes to cellulose fibres can limit the efficiency of enzymatic hydrolysis, even in pretreated biomass, and efficient cellulase hydrolysis of pretreated biomass is dependent on the effectiveness of the pretreatment to sufficently disrupt the biomass structure (Arantes and Saddler, 2010; Jeoh et al., 2007).

In designing an engineered CBP (eCBP) based on model of biomass processing after a model observed ruminants animals, in which a combination of mastication (representing Physical pretreatment), hydrolytic enzymes, and bacteria enable efficient extraction of sugars for metabolism by the animal (Weimer et al., 2009a). Based on that concept of eCBP, we evaluated the effectiveness combining physical pretreatment (milling) with minimal enzymatic hydrolysis and microbial conversion to increase rates of substrate conversion and fermentation end-product.

In this Chapter, we report batch fermentation experiments performed in serum bottles and bioreactors with milled cellulose that was subjected to partial hydrolysis by various glycosyl hydrolase enzymes, followed by fermentation by *C. thermocellum*. The glycosyl hydrolase Celluclast, and the commercial enzyme cocktail Cellic Ctec (Novozymes), were used to generate cellodextrins from the pretreated cellulose. NS50012 (Novozymes), which is an enzyme complex with a dominant xylanase activity, was used to solubilize residual hemicelluloses. We also tested the use of extracts containing cellulosomes and other hydrolytic factors such as the yellow affinity substance, from supernatants of stationary phase *C. thermocellum* cultured on α-

cellulose. Optimization of the differential hydrolysis process to enhance the yields of end-products via CBP are the highlights of this chapter.

4.3 Materials and methods

4.3.1 Microorganisms, media, substrates and enzymes

For details of the microrganijsms used, media preparation, substrates, and enzymes, see Chapter 2, Section 2.3.2, and Chapter 3, Section 3.3.2. The native substrates used in these studies were residues from local industrial cultivation. Hemp biomass was obtained from the Emerson Hemp Company, Emerson, Manitoba. Cattail biomass was a gift from the International Institute for Sustainable Development in Winnipeg, Manitoba, collected from the Nettlie Marsh area of Lake Winnipeg, Manitoba. Pretreated rice straw was obtained from Mitsubitshi Heavy, Industries Japan. The enzymes used in the experiments were generously supplied by Novozymes, Bagsvaerd, Denmark, while cellulosome extracts were prepared from cultures of *C. thermoellum* and concentrated using Amicon Ultra-15 Centrifugal Filter Units from Millipore USA. This procedure was adapted from (Bayer and Lamed, 1992).

4.3.2 Experimental procedure

4.3.2.1 Experiments conducted in serum bottles

Hydrolysis was conducted in Shake flask experiments at 5 mg/cellulose loading using New Brunswick shaker at 50 ^{o}C and 150 rpm over 4 to 24 hours. Hydrolysis was carried out by addition of the enzymes (cellulase, xylanase, and cellulosome extract) to flasks containing the substrate suspended in sodium citrate solution prior the fermentation. Prior to addition of the enzymes, aliquots of 100 mL sodium citrate solution (1M, pH 5.0) plus substrate were added to baffled 250 mL Erlenmeyer flasks and warmed to 50 ^{o}C in a water bath. The substrate type and amount was different in each test condition. The water insoluble solids was analysed for dry

matter content and used in fermentation with *C. thermocellum* in serum bottles (Wheaton Science) with a working volume of 127 mL. A cellulose loading of 2 g/L was used in all in serum bottle experiments. To maintain an anaerobic environment for fermentation, the bottles were sealed with butyl rubber stoppers and crimped aluminum seals. The bottles were then gassed and degassed for four cycles (of 1 min gas and 4 min degas) with 95% N_2 and 5% argon (Ar). Gas measurements in serum bottles were conducted using an Agilent 7890A gas chromatograph equipped with a TCD and an FID using argon carrier gas, a gas sample valve with a 0.25 cc loop, a split inlet and two PLOT columns in series, a PLOT Q (30 m x 0.53 mm ID) and a PLOT molecular sieve column (30 m x 0.53 mm ID), maintained at 60 ^{o}C, with HPLC grade water as mobile phase maintain at a flow rate of 0.6 mL/min.

4.3.2.2 Experiments conducted in 7 L bioreactors

Hydrolysis and fermentation experiments were also conducted in 7 L bioreactors with a 3 L culture volume. The reactors are equipped with a gas and liquid sampling ports, sterilizable gel electrode pH probe, axial-flow impellers, L-shaped spargers, 3 baffles, an electric heating jacket, and liquid-cooled condenser (Applikon Biotech, California, USA). Three litres of 1191 medium were prepared and added to both the test and control bioreactors. The reactors were then autoclaved at 121 ^{o}C for 60 minutes (min). The pH of both the test and control the reactors with hemp biomass was adjusted to 5.2, and enzymes CTec and NS50012 (5 % substrate loading) were added. The reactors were sparged overnight (18 hours) with 95% nitrogen (N_2), followed by an additional incubation period of 6 hours (for a total hydrolysis period of 24 hours). After hydrolysis, the pH was raised to 7.2 and both reactors were inoculated with 10% v/v of log-phase *C. thermocellum* that had been cultured on 2 g/L α-cellulose in corning bottle.

Gas production in the bioreactors was measured (off-line) using an SRI gas chromatograph (Model 8610C) capable of measuring 200 - 500 ppm outfitted with a thermal conductivity detector, a stainless steel molecular sieve 13 x packed column (3.2 mm X 1.8 m) connected in series with a stainless steel, silica gel packed column (3.2 mm X 1.8 m) with Argon as carrier gas. On-line gas measurements were conducted using a mass spectrometry-based Titration and Off-gas Analyzer (TOGA) that has been previously demonstrated for on-line monitoring (Gapes and Keller, 2009; Gapes et al., 2003; Pratt et al., 2003).

Protein concentrations were determined using the Bradford assay (Bradford, 1976), while end-products were measured by HPLC from 1 mL samples taken from serum bottles, or 10 mL samples taken from the bioreactors every four hours following inoculation. Aqueous products, such as organic acids and ethanol, were measured with a Waters HPLC equipped with an HPX-Aminex 87 column (Bio-Rad Laboratories, Hercules, CA).

4.4 Results and Discussion

4.4.1 Serum bottle experiments

Serum bottle experiments were conducted to assess the effect glycosyl hydrolases in a small scale before scaling-up to a bioreactor. Bradford assays revealed that the cellulase preparation had a greater protein concentration than the xylanases (NS50012) and cellulosome extracts (Figure 4.1A and 4.1B). Cellulosome extracts prepared after 60 hours of *C. thermocellum* growth were more concentrated than extracts prepared after 30 hours of *C. thermocellum* growth. This could be as result of increase cell growth over time and also as result of the continued release of cellulosome into the culture medium as growth on the cellulose fibers proceeded.

Preliminary hydrolysis for 24 hours conducted with cattail and rice straw biomass produced a WIS (water insoluble solid) with compositions that reflected the initial cellulose content. Fermentation of different substrates after enzymatic hydrolysis by *C. thermocellum* generated different hydrogen and carbon dioxide concentrations after 53 hours (Figure 4.2). Enzymatic hydrolysis of cattail biomass in shake-flasks for different lengths of time, followed by fermentation of the WIS in a serum bottles (closed batch reactions) also generated different amounts of hydrogen and carbon dioxide (Figure 4.3). When cellulase (Celluclast) was used for hydrolysis, the amount of gaseous production decreased with increasing hydrolysis time from 8 to 16 hours. This may be attributable to the breakdown oligossacharides to monosaccharides. Previous studies have shown that *C. thermocellum* efficiently metabolizes cellulodextrins (G2-G6), but exhibits limited growth on glucose, and does not metabolize and grow on xylose (Lynd et al., 2005; Zhang and Lynd, 2005).

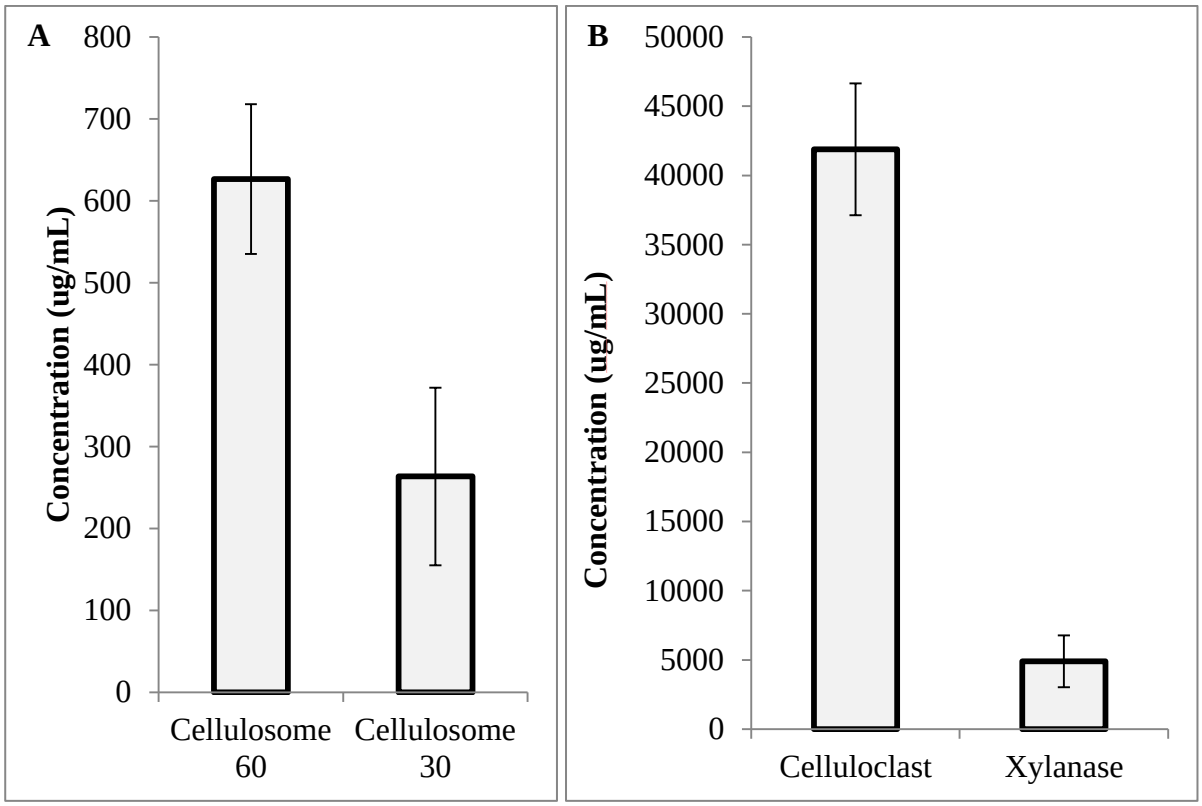


Figure 4.1 Protein concentrations of glycosyl hydrolase enzymes used in the experiments. A). Cellulosome extracts prepared after 60 and 30 hours of *C. thermocellum* growth; B) Celluclast (cellulases) cocktail and NS50012 (xylanases).

Table 4.2 Dry matter content in cattail and rice straw incubated with cellulose and/or xylanase enzymes, compared with controls (no enzyme hydrolysis), expressed as a percentage of the normal weight (weight as received).

Substrate + enzyme	Normal weight (g)	Dry weight (g)	Percentage dry weight
Cattails control	0.50	0.11	21.6
Cattails + cellulase	0.59	0.13	22.1
Cattails+ xylanase	0.57	0.14	24.0
Cattails+ cel+xyl	0.58	0.13	22.9
Rice straw control	0.55	0.15	26.3
Rice straw +cellulase	0.59	0.16	26.6
Rice straw + xylanase	0.59	0.15	25.4
Rice straw +cel+xyl	0.56	0.15	27.3

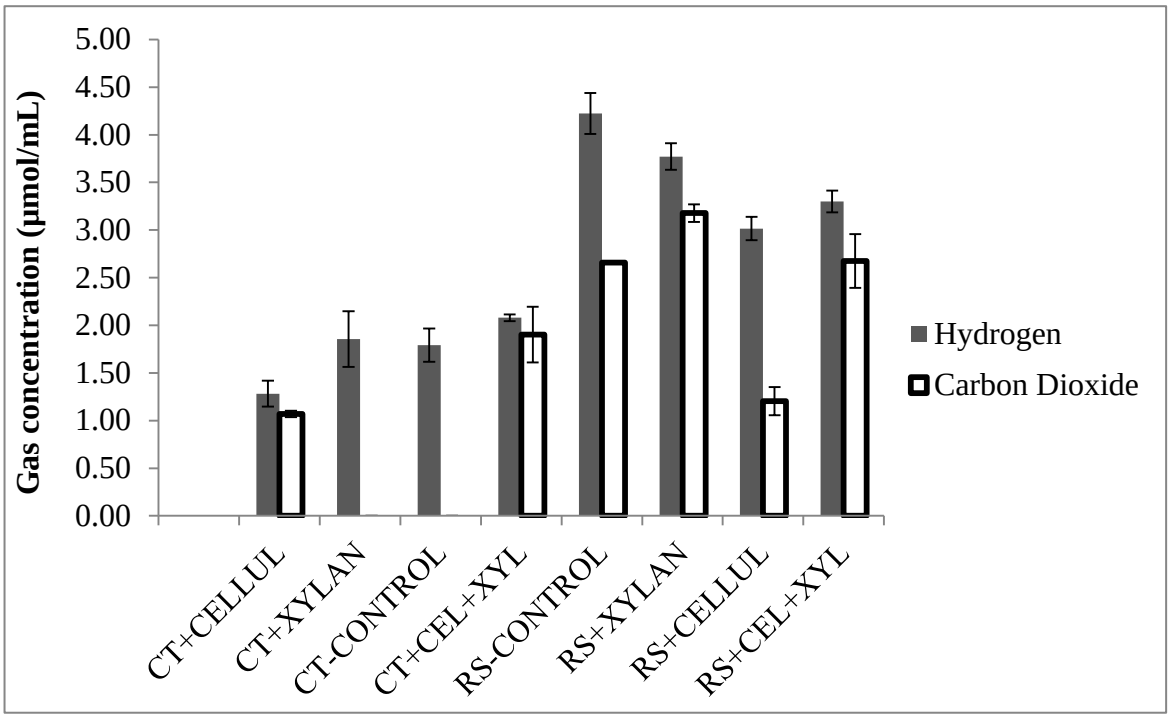


Figure 4.2 Hydrogen and carbon dioxide production from the WIS of cattails (CT) and rice straw (RS) at 2 g/L substrate loading. CT+ CELLUL = cattails with cellulases, CT+XYLAN = cattails plus xylannases, CT- CONTROL = cattails without enzymes, CT+CEL+XYL = Cattails with cellulase and xylanase, RS-CONTROL = rice straw with no enzymes, RS+XYLAN = rice straw with xylanases, RS+CEL = rice straw with Cellulases, RS+CEL+XYL = rice straw with cellulases and xylanases.

Table 4.3 Dry matter content in rice straw incubated with cellulose and/or xylanase enzymes, or cellulosome extracts, compared with controls (no enzyme hydrolysis), expressed as a percentage of the weight of substrate as received.

Substrate + enzyme	Normal weight (g)	Dry weight (g)	Percentage dry weight
Rice straw control	0.52	0.18	35.14
Rice straw + cellulase 8h	0.52	0.18	34.32
Rice straw + cellulase 16h	0.56	0.20	35.04
Rice straw + xylanase 8h	0.53	0.19	35.7
Rice straw + xylanase 16h	0.57	0.20	34.85
Rice straw + cellulase + xylanase 8h	0.52	0.18	35.28
Rice straw + cellulase and xylanase 16h	0.54	0.19	34.74
Rice straw +cellulosome 4h	0.54	0.18	33.44
Rice straw + cellulosome 8h	0.52	0.18	34.15
Rice straw + cellulosome 16h	0.54	0.18	32.52

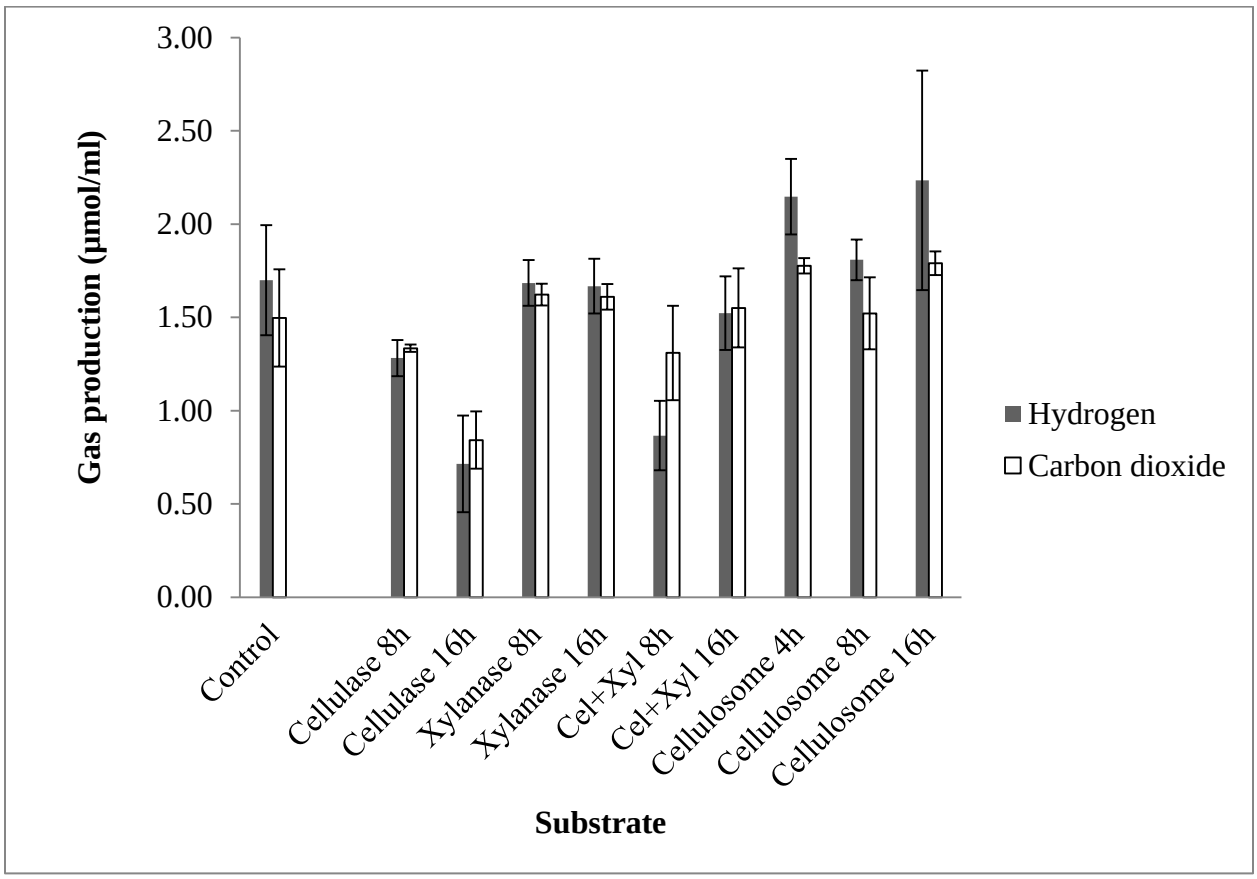


Figure 4.3 Gas production by *C. thermocellum* in fermentation reactions containing ricestraw biomass pretreated with 5 mg protein / g cellulose. Control substtrate was not treated with enzymes. Cellulase 8h = cattails that was hydrolysed for 8h with cellulases, Cellulase 16 = cattails that was hydrolysed for 16h with cellulases, Xylanase 8h and 16h is = cattailas the was hydrolysed for 8 and 16 hours. Cel+Xyl 8h = cattails that was hydrolysed with both cellulase and xylanase for 8 and 16 hours. Cellulosome 4h, 8h, and 16h = cattials that were hydrolysed with cellulosome extract for 4, 8 and 16 hours respectively.

Incubation of the substrates with xylanases for 6 to 8 hours did not appear to effect the amount of gaseous end-products generated by fermentation. Neither xylanases nor cellulases enhanced fermentation end-product synthesis, compared with the control. When cellulases and xylanases were used together. the amount of hydrogen and carbon dioxide produced decreased with increasing hydrolysis time from 8 to 16 hours (Figure 4.3). Although fermentation of the hydrolyzed substrates did not produce as much hydrogen as the control for most of the test conditions, substrates hydrolyzed by cellulosome extracts produced slightly greater amounts of hydrogen than the control with no enzymes added.

Hydrolysis with cellulosome extracts before fermentation with *C. thermocellum* yielded higher hydrogen concentrations compared to the other glycosyl hydrolases after 8 to 16 hours of hydrolysis. This may be atributable to hydrolysis of cellulose and hemicellulose polymers to oligocellulodextrins, which are the preferred substrate for *C. thermocellum*. In addition, the multiple hydrolytic components of the cellulosome make it more efficient in the deconstruction of lignocellulosic biomass compared glysosyl hydrolases, which target a specific polymer. Unlike these specific glycosyl hydrolases, the complex of enzymes in the *C. thermocellum* cellulosome includes cellulases (endo and exo-), hemicellulases, and carbohydrate esteraces that are bound to the cellulosome intergrating protein or scaffoldin (Schwarz, 2001; Shoham et al., 1999) and act synergistically as complex thus making the cellulosome more efficient at the degration of the heteromatrix of cellulose, hemicellulose, pectin, wax and lignin, in cattail biomass.

Previous studies agree that cellulosomal enzymes are modular proteins (40 to 180 KDa) with one or more catatlytic modules that enable them to attached to cellulose fibers via cellulose binding domains (CBMs) and rapidly deconstuct crystaline and non-crystaline cellulose (Bayer

and Lamed, 1992; Bayer et al., 1994; Lynd et al., 2006; Schwarz, 2001; Shoham et al., 1999). Recently the discovery of CBMs and glycosyl hydrolase family 61 (GH61s) oxidative cleavage of cellulose by copper monooxygenases reveals how cellulases bind to cellulose chains with their flat substate binding sites and disrupt cellulose fiber packing. This creates access points not only by introducing breaks in the polymer chain, but also by generating a charged group (Horn et al., 2012). After this action the classical endo/exo scheme of cellulase hydrolysis is engaged.

4.4.2 Bioreactor experiments

When fermentation was conducted in a 7 L bioreactor, the test reactor containing substrate that had been pretreated with hydrolytic enzymes showed a rapid gas production compared to the control reactor (Figure 4.4). Both hydrogen (Figure 4.4A) and carbon dioxide (Figure 4.4B) were synthesized at a higher rate in the fermentation reaction containing 5% Cellic CTec and NS50012 treated hemp biomass. This data suggests that pretreatment of hemp biomass with Cellic CTec and NS50012 disrupted the biomass structure sufficiently to allow increased accessibility to cellulose chains within the biomass, resulting in a rapid growth of *C. thermocellum*, as indicated by gas production.

Even though the test reactor showed a higher hydrogen production rate compared with the control, the final amount of hydrogen produced was approximately the same. This implies that given enough time the hydrolytic machinery of *C. thermocellum* can access the cellulose within the biomass and the bacterium will grow and synthesize end-products to the same extent. The more rapid growth and hydrogen synthesis observed in the fermentation reaction with cellulosome pretreated biomass suggest that the cellulosome enzymes were able to "pave the way" for the bacterium by generating readily available cellodextrins before inoculation. The data log from the on-line monitoring of gas produced by the TOGA system during the

fermentation reaction shows that the results obtained from off-line measurement (manual injection) using the SRI GC correlated with online measurements (Figure 4.5).

Analyses of the aqueous phase fermentation end-products (organic acids and ethanol) revealed that the concentrations of acetate, lactate, and ethanol in the test reactor containing enzyme pretreated hemp biomass were double the concentrations of these end-products observed in the control reactor (Figure 4.6). Similar amounts of formate produced in the test and control reactor and the pH in test and control reactors were also the same. As observed for gas production, rates of organic acid and ethanol synthesis by *C. thermocellum* were higher in fermentation reactions containing hemp biomass pretreated with the Cellic CTec and NS50012 (Figure 4.7).

Although all substrate tested were similar as being agricultural residues, end-product yields were higher in test conditions with pretreated feedstocks like rice straw compared to cattails or hemp.

A) **B)**

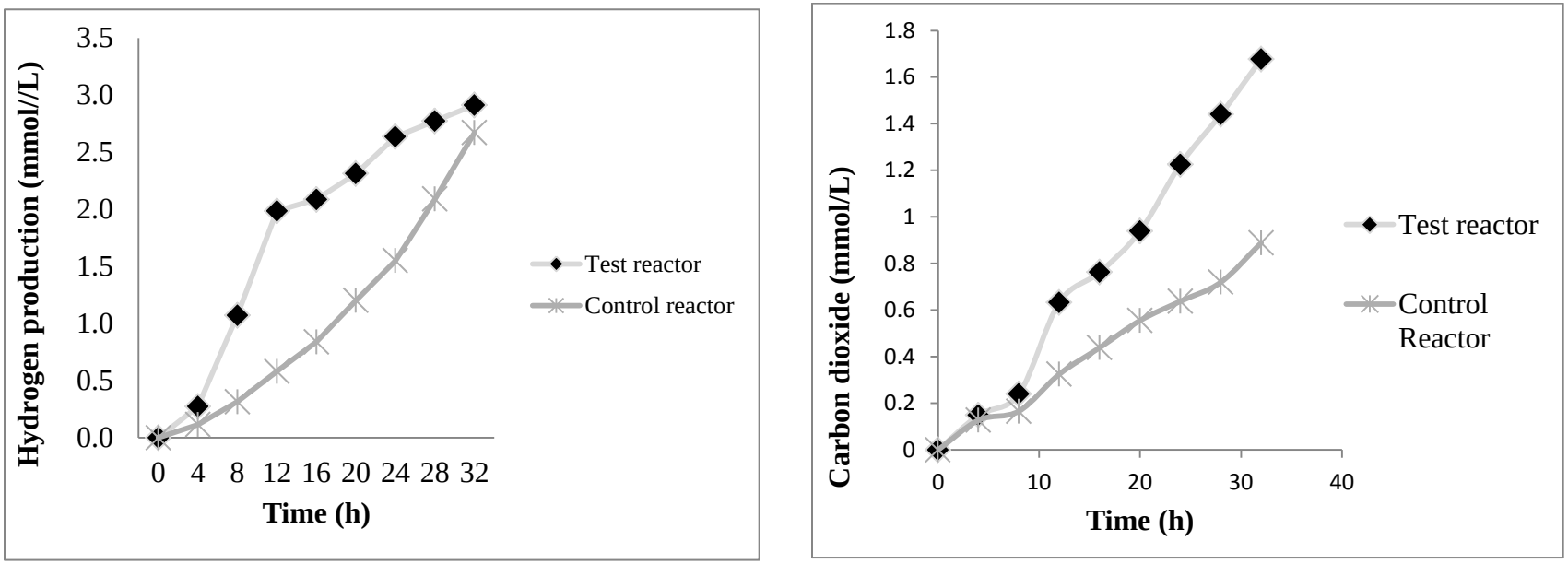


Figure 4.4 Gas production by *C. thermocellum* in fermentation reactions containing 2 g L^{-1} hemp cellulose. A) Hydrogen and B) Carbon dioxide synthesis by *C. thermocellum* cultured with pretreated with Cellic CTec and NS50012 over 32 hours (Test reactor) compared to fermentation reactions containing untreated substrate (Control reactor).

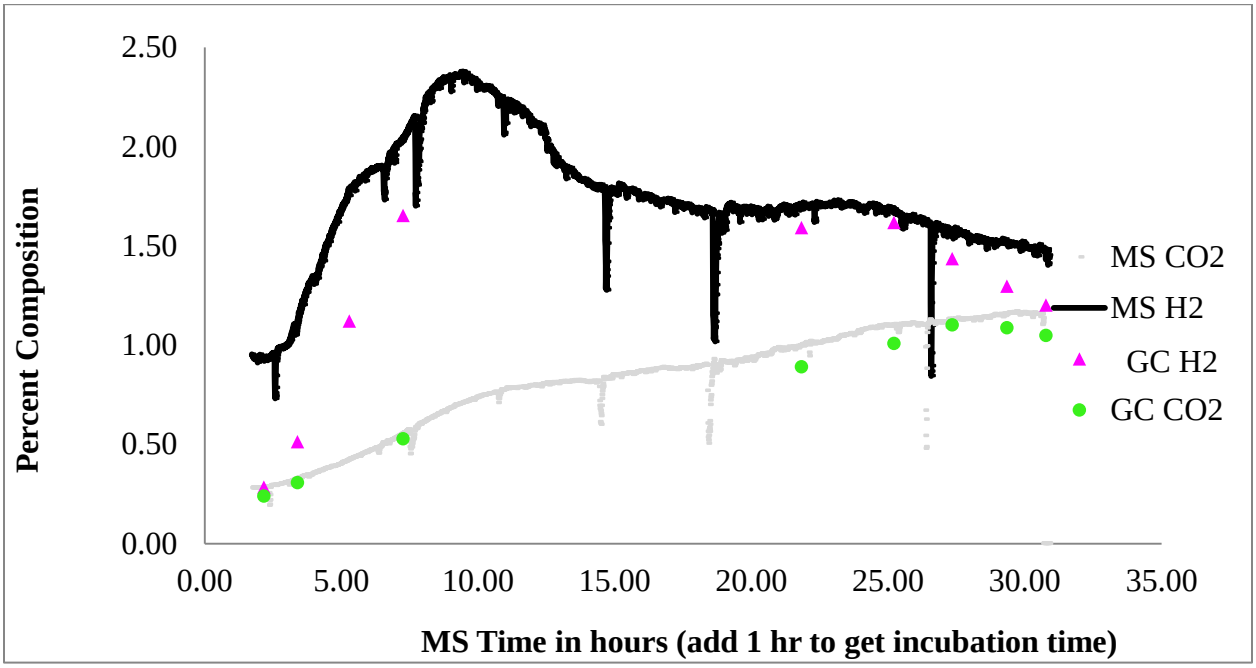


Figure 4.5 Correlation between off-line and on-line analyses of hydrogen and carbon dioxide synthesis by *C. thermocellum* cultured on 2 g/L hemp biomass pretreated by enzymatic hydrolysis. The triangle and circle represent the percentage hydrogen (H2) and carbon dioxide measure with the SRI gas chromotagrah while the black and grey lines represent hydrogen and carbon dioxide measured by mass spectrometer using the titration off-gas analyser (TOGA).

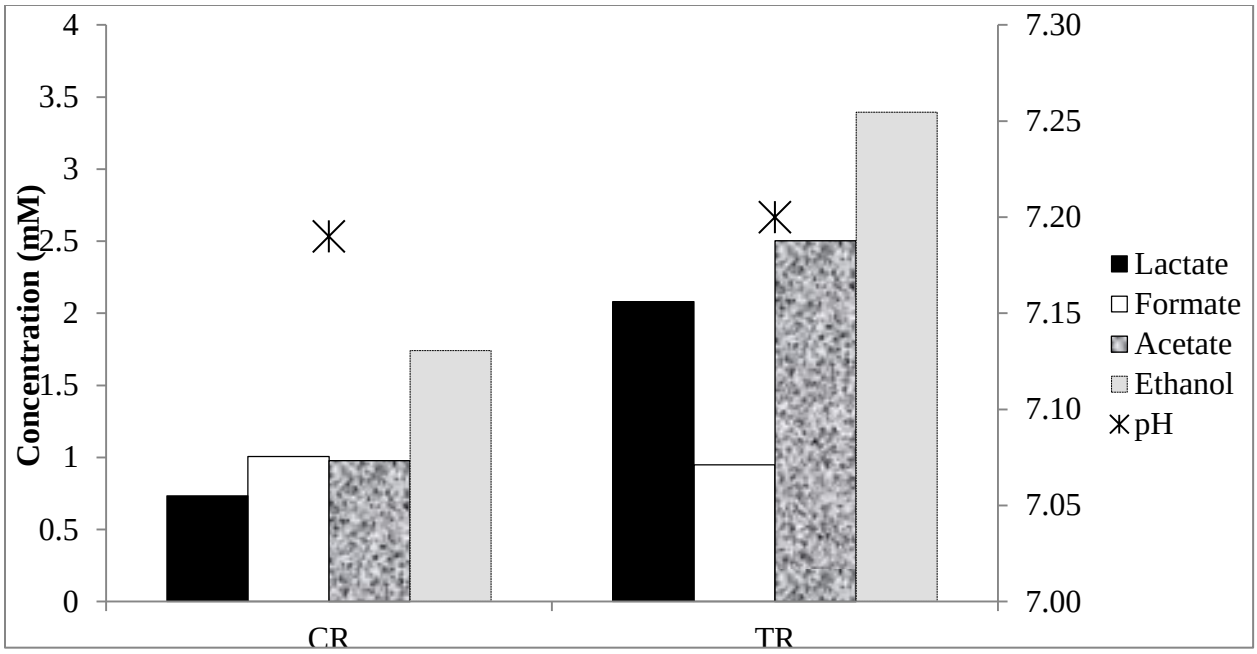


Figure 4.6 Organic acids (acetate, lactate, formate) and ethanol concentrations synthesized by *C. thermocellum* after 28 hours in fermentation reactors containing 2 g/L hemp cellulose pretreated by enzyme hydrolysis (TR) compared to the Control reactor (CR) containing untreated hemp biomass.

A) **B)**

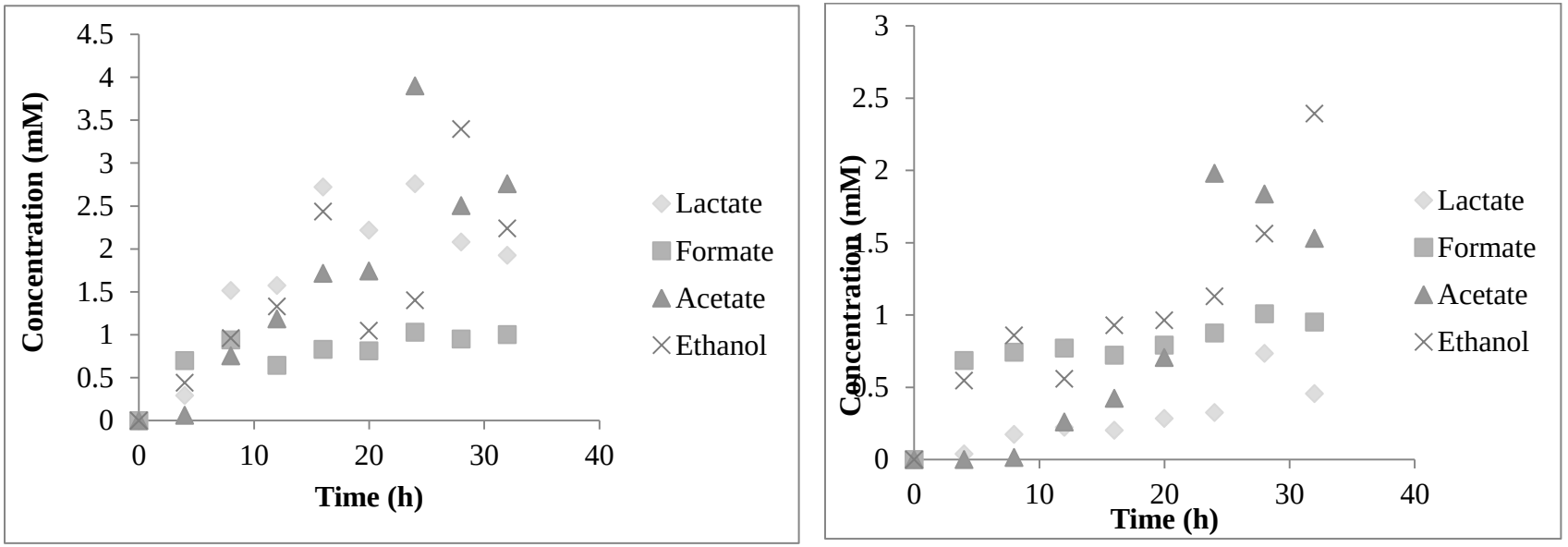


Figure 4.7 Synthesis of organic acids (acetate, lactate, formate) and ethanol by *C. thermocellum* in fermentation reactions containing A) 2 g/L hemp cellulose pretreated with enzymes (Test Reactor); and B) 2 g/L untreated hemp cellulose (Control reactor).

4.5 Conclusion

The use of exogenous commercial glycosyl hydrolases to pretreat cellulosic biomass prior to fermentation by by *C. thermocellum* did not enhance the conversion lignocellulosic biomass in small scale serum bottle experiments. However, the effect of synergy between free glycosyl hydrolases and the macromolecular complex of *C. thermocellum* resulted in a biphasic growth pattern observed in bioreactors resulting in rapid rates observe in test reactor compared to the control. As such commercial glycosyl hydrolases used to pretreat cellulosic biomass and endogenous, cellulosome-associated glycosyl hydrolases produced by *C. thermocellum* were observed to boost the rate of conversion of lignocellulosic biomass during fermentation with *C. thermocellum*. Pretreatment of hemp biomass with a small amount of Cellic CTec and NS50012 (< 0.5g enzymes/g cellulose) resulted in a higher rate of end-product accumulation in the test reactor compared to the control reactor. This may be attributed to increased access to cellulose fibres within the biomass following synergistic hydrolytic action of both free hydrolases and the cellulosome bound hydrolytic units. The hydrolytic products are metabolised by *C. thermocellum* to produce hydrogen, carbon dioxide, organic acids, and ethanol. Our data indicate that non-celluloytic glycosyl hydrolase enzymes may be used to explore synergy and increase the rate of bioconversion of native feedstocks and reduce energy intensive and often severe physicochemical pretreatment methods used to extract sugars for fermentation. This will reduce upstream processing costs for lignocellulosic biofuel production. Notwithstanding, more research with more thermostable hydrolytic enzymes will advance our knowledge and ability to use them optimally in bioprocessing of lignocellulosic biomass.

Chapter 5

Summary of research achievements and contributions, and future prospects.

5.1 Achievements and contributions

Biomass processing for cellulosic biofuels will be the cornerstone for industries eager to benefit from a bio-based economy. With a growing trend towards increased consolidation, improvements in direct microbial conversion of lignocellulosic to value-added products are essential to realize the full potential of the integrated biorefinery. Based on the objectives and hypothesis of my research, we have achieved the following:

Objective 1**:** Quantify the rate of conversion of pretreated versus raw cellulosic substrates by *Clostridium thermocellum* during CBP.

Hypothesis: Pretreatment will improve substrate conversion rates, and end-product yields and profiles of C. thermocellum cultured under CBP conditions.

We have established that the gram positive, cellulolytic bacterium, *C. thermocellum* is able to utilise processed and unprocessed residual fractions of hemp biomass derived from local hemp cultivation for hydrogen and ethanol production in a single-step process. Our findings have shown that purified hemp cellulose, derived as a by-product of the Lignol Organosolv® process, as well as raw cellulose rich hemp fibre, result in greater production of fermentation end-products compared with native hemp hurds in a consolidated bioprocess. End-product yields were based on their instrinsic cellulosic content as well as the presence of polymers present in plant biomass. Simply increasing the amount substrate fermented did not compensate for the need to increase accessibility to cellulose by physicochemical pretreatment. Removal of lignin, hemicelluloses, pectins, and wax in PHC resulted in a highly reactive cellulose that can be used

directly as feedstock for biological ethanol and hydrogen production in a one-step fermentation process. This objective has been fully achieved.

Objective 2: Identify feedstock properties that affect the quantification of aqueous (ethanol and organic acids) and gaseous (hydrogen and carbon dioxide) fermentation products synthesized by *Clostridium thermocellum* using pretreated and raw cellulosic substrates.

Hypothesis: End-product profiles will differ, depending on the type, source, and nature of the substrate.

We have established that when different lignocellulosic feedstocks are subjected to the same type of physicochemical pretreatment, the rate of hydrolysis and subsequent utilisation depends on both the innate cellulosic content and the content of amorphous cellulose in the biomass after pretreatment. An underlying assumption of this work was that the biomass used as substrate contains the same cellulose allomorph if it is subjected to the same type of pretreatment. Through this is objective, we investigated a novel parameter in biomass processing, the contribution of amorphous cellulose (CAC) and correpated this parameter with the efficiency of microbial conversion. When different biomass samples were subjected to different pretreatment methods, there was a direct correlation between the CAC values and digestibility of the biomass. In other words, the CAC values could be used to evaluate the effects of different physical pretreatments of lignocellulosic substrates with particle sizes below 40 mesh (0.400 mm), because further reduction beyond this particle size does not affect subsequent digestibility.

Objective 3: Carry out enhanced Consolidated Bioprocessing (eCBP), with exogenous enzymes to augment microbial conversion using pretreated, processed, and raw cellulosic substrates.

Hypothesis: eCBP of lignocellulosic biomass can attain higher yields as result of increase accessibility and combine enzyme activity.

We demonstrated that the progressive hydrolytic action of Cellic Htec and the enzyme complex NS50012 improved the rate of end-product formation during fermentation of native hemp biomass with *C. thermocellum*, in bioreactors, and that the hydrolytic activity of cellulosome components had a significant impact on the rate and yield of fermentation end-products synthesized compared to other other glycosyl hydrolases (cellulases and hemicellulases) in a closed system. Thus, a minimal dosage of non-celluloytic glycosyl hydrolase enzymes could be used to reduce the high severities employed during physico-chemical pretreatemt of lignocellulosic feedstocks that result in increased costs of biofuels production.

5.2 Conclusion

As stated in Chapter 1, there is a growing trend towards increased consolidation in bioprocessing for production of biocommodities, and further development of the integrated biorefinery depends on processing of lignocellulosic biomass in the most efficient manner. The way to low cost biorefining can only be acheived through research and development that will elucidate the fundamental knowledge of biomass structure, pretreament chemistries, digestibility trends, and fermentability. In this work we have moved from a “black box” approach to pretreatment to seeking a greater understanding of the factors that influence bioconversion of pretreated biomass, such as innate cellulose content and the structural components of the cellulose polymers.

In Chapter 2, we showed that the yields of fermentation of different fractions of the same feedstock primarily depend upon innate cellulose contents of the feedstock and the ability of the consolidated bioprocessing microbe to access the cellulose chains within the biomass. In Chapter

3, we showed that following pretreatment structural changes to cellulose within the substrate affects subsequent digestibility or yields of direct microbial conversion. In the end (Chapter 4) we demonstrated that bioaugmentation with cellulosome-associated enzymes derived from a cellulolytic bacterium can be used to enhance end-product yields during microbial conversion, and that a very low dosage of exogenous glycosyl hydrolases was able improve the rate of end-product accumulation. After conducting numerous fermentability test and feedstock analyses, a novel parameter predictive of the extent of direct microbial conversion of any given substrate to end-products was identified as the "quotient of accessibility" (QA). The lower the QA the more accessible the cellulose in the feedstock will be to CBP microbes for direct microbial conversion to biofuels and co-products (Supplementary Table 1).

5.3 Future Prospects

Clostridium thermocellum and other cellulolytic *Clostridium* species are emerging as a utility candidates for CBP of lignocellulosic biomass (Akinosho et al., 2014; Hasunuma and Kondo, 2012; Xu et al., 2009). More research is required to established a minimum change in % CAC that results in significant change in digestibility and its application in a consolidated bioprocesses that are feedstock agnostic. The hydrolytic ability of the multi-enzyme complex of cellulolytic bacteria is the main reason behind why they are able to grow rapidly on cellulosic material (Demain et al., 2005; Desvaux, 2005; Fu and Holtzapple, 2009; Lynd et al., 2002). The isolation and use of these complex enzymes system for industrial purposes will establish new frontiers for biomass hydrolysis to sugars. It is evident from this research work that synergies between this complex cellulase system and cellular fermentation should be investigated to establish more efficient bioprocessing steps with a greater output. With more strategies for the isolation and purification of more active cellulosomes under investigation (Bayer and Lamed,

1992; Bayer et al., 1994; St Brice et al., 2014). and greater tools for metabolic engineering of clostridial species, more work on enzyme and cellulosome synergies with the hydrolytic complexes of live cultures needs to be investigated to established effective CBP systems. More research in understanding the properties of pretreated lignocellulosic feedstocks to make them digestible is also required so that industrial-scale consolidated bioprocesses that are feedstock agnostic can be developed.

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Appendix

Supplementary Table 1. All substrates composition of all test substrates showing the quotient of accessibility, (% DM = percent dry / percent cellulose).

		Results on a "Dry Matter Basis"								
	Sample ID:	**Purified Wood**	**Purified Hemp**	**Hemp Hurds**	**Hemp Fibre**	**Purified Flax**	**Flax Shive**	**Cattails**	**Purified Rice Straw**	**Beverage Cup Cellulose**
	Method Used									
Dry matter (%)	AOAC 930.15	95.33	94.94	93.55	93.50	95.43	90.97	91.64	90.02	95.62
Crude protein (%)	AOAC 984.13	0.37	0.87	1.05	2.01	0.53	2.01	7.23	2.79	0.27
Lignin (%)	ANKOM 08/05	2.13	7.42	16.81	3.31	14.37	22.71	8.14	10.88	5.19
ADF (%)	ANKOM Method 5: 08-16-06	93.40	86.36	74.50	81.82	91.40	72.87	45.60	49.49	91.47
NDF (%)	ANKOM Method 6: 08-16-06	95.57	91.30	92.31	88.44	97.30	91.06	66.54	64.79	98.01
ash (%)	AOAC 942.05	0.10	4.31	1.03	2.10	0.54	1.90	8.15	15.55	0.50
Soluble crude protein (%)	Roe et al., 1990	0.20	<0.05	0.10	0.36	<0.05	0.47	-	0.60	0.08
Cellulose (%)	cellulose = ADF - lignin	91.27	78.94	57.69	78.51	77.03	50.16	36.46	38.61	86.28
Hemicellulose (%)	hemicellulose = NDF - ADF	2.17	4.94	17.81	6.62	5.90	18.19	20.94	15.30	6.54
Quotient of Accessibility = (%DM/%C)		*1.04*	*1.20*	*1.62*	*1.19*	*1.24*	*1.81*	*2.45*	*2.33*	*1.11*