Towards increasing lignocellulose to biofuel conversion by *Clostridium thermocellum*: co-culturing for increased hydrolysis and characterization of pyruvate phosphate dikinase for understanding atypical metabolism

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Abstract

Alternative low-carbon transportation fuels, such as biofuels, are needed to replace or supplement fossil fuels in order to lower global greenhouse gas emissions and combat climate change. Lignocellulosic biofuels have relatively low carbon emissions and are created using the non-food parts of crops and other plants, such as the leaves and stems, which are comprised mostly of a tough material called lignocellulose, composed of cellulose, hemicellulose, and lignin. One of the best lignocellulose degraders found in nature that is also capable of fermenting the released sugars to ethanol is *Clostridium thermocellum,* although improvements in both lignocellulose hydrolysis extent and ethanol yields are needed for commercial viability.

C. thermocellum, considered a cellulose-degrading specialist, was co-cultured with two different hemicellulose-specialists, *C. stercorarium* and *Thermoanaerobacter thermohydrosulfuricus*. The hypothesis was that the co-cultures might degrade more lignocellulose owing to the additional hydrolytic enzymes supplied by the partners and their ability to uptake the inhibitory hemicellulose sugars. All co-culture combinations were found to solubilize more wheat straw, among other lignocellulose materials, and produce more end-products, including ethanol, than *C. thermocellum* alone. These co-cultures were stable over multiple serial passages, on either wheat straw or pure cellulose, although some evidence of carbon competition was observed. The tri-culture was successfully used to screen the digestibility of various lignocellulose materials, revealing substantial difference between cattail harvested in different seasons. Cross-feeding of vital growth factors was observed between the various co-culture members in a defined medium.

The metabolism of *C. thermocellum* is atypical compared to many organisms, including the absence of a pyruvate kinase, and its substitution with both a malate shunt and a putative

pyruvate phosphate dikinase (PPDK), which may act to increase net ATP yields from glycolysis. The PPDK was cloned into *E. coli*, expressed, purified, and characterized, confirming its function as a PPDK for glycolysis and revealing strong activation by ammonium. The kinetic characterization of the PPDK will help inform future studies that measure and model levels of important intracellular metabolites, such as pyrophosphate and ammonium, to better understand the metabolism of *C. thermocellum* and allow further metabolic engineering to increase ethanol yields.

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Dedication

Dedicated to my late mother, who always believed in me and supported me.

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Abbreviations

ADP: adenosine diphosphate

AMP: adenosine monophosphate

ATP: adenosine triphosphate

CBM: carbohydrate binding module

CBP: consolidated bioprocessing

DNA: deoxyribonucleic acid

PEP: phosphoenolpyruvate

PPDK: pyruvate phosphate dikinase

PP_i: pyrophosphate

SDS-PAGE: sodium dodecyl sulfate – polyacrylamide gel electrophoresis

SSF: simultaneous saccharification and fermentation

qPCR: quantitative polymerase chain reaction

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Chapter 1: Literature Review

1.1 The case for biofuels

1.1.1 Climate change

Humankind has been releasing increasing amounts of carbon dioxide and other greenhouse gases into the atmosphere since the industrial revolution, with 37.1 gigatons of anthropogenic carbon dioxide being released in 2018, more than any other year on record [1]. This has resulted in an increase of the atmospheric carbon dioxide levels from around 280 ppm in pre-industrial times to over 400 ppm [2]. The higher concentration of carbon dioxide, among other greenhouse gases, absorbs more infrared radiation released from earth's surface that would otherwise have been lost to space, heating the earth and leading to climate change, and causes other negative effects such as ocean acidification. One obvious method to combat climate change is to reduce the amount of carbon dioxide we are emitting.

1.1.2 Decarbonizing the transport sector

The transportation sector is responsible for approximately 14% of global greenhouse gas emissions [3] and is an attractive target for decarbonization. The International Energy Agency has predicted that in order to limit global warming to 2 degrees Celsius above pre-industrial levels the share of transportation energy provided by biofuels will need to increase to approximately 26% by 2050, from under 3% in 2010 [4]. While electricity and hydrogen are predicted to supply most of the energy needed for light passenger vehicles, such as cars, much of the increased demand for biofuels will likely be driven by the aviation and ocean-shipping sectors [5]. This is because using electricity or hydrogen to power long-trip vehicles that have

significant weight and volume restrictions is technically challenging and will likely take significantly longer to implement [5].

1.2 Lignocellulosic biofuels

1.2.1 Second-generation biofuels

Biofuel is a broad term that encompasses fuels generated from recent biomass. They emit less net carbon dioxide compared to fossil fuels due to the fact that, although they emit carbon while being burned, the carbon is also taken up from the atmosphere to create the biomass in the first place, and thus biofuels are a preferable source of fuel. Much of the research on biofuels has centered around ethanol, which can act as a simple drop-in fuel to blend with gasoline in internal combustion engines or act as a complete replacement in modified engines [6]. Other potential biofuels include methanol, butanol, hydrogen, and methane gas [6]. Due to the fact that ethanol production in microbes is relatively easier to manipulate than some of the other, more desirable biofuels, such as butanol, it can act as a first milestone that the development of other biofuel-producing microbes can then build upon [7, 8].

Biofuels can be sub-divided based on the biomass used. First-generation biofuels use easy-to-utilize sugar or starch-containing plant-based materials, such as corn starch, as their feedstock [9] and comprise the vast majority of global biofuel production [10]. There exists a "food-versus-fuel" debate about the use of first-generation biofuels since they use crops which could otherwise go to feeding humans, with the argument being that using food crops for biofuel decreases the amount of food produced and thus increases the cost [11]. While first-generation biofuels do emit fewer carbon dioxide emissions overall than fossil fuels, using a life-cycle analysis, they still emit considerable amounts, over half of what fossil fuels produce, due to the significant farming needed to make the starchy feedstocks [12]. Second-generation, also known

as advanced or (ligno)cellulosic biofuels, as their name suggests, are based on lignocellulosic biomass, which can include a large variety of sources such as agricultural residues (e.g. straw and stover, the dried stems and leaves leftover from field crops), dedicated energy crops (e.g. miscanthus, switchgrass, and poplar), and forestry, industry, and municipal wastes (e.g. paper mill discards, sawdust, and paper coffee cups) [9]. These advanced biofuels have numerous advantages over first-generation technology, including using lower-cost feedstocks that can not be used for human consumption [11]. Advanced biofuels also create far fewer greenhouse gas emissions than first-generation biofuels, on a life-cycle basis, due to the decreased farming needed to acquire the same amount of feedstock [12]. Despite these benefits only around 0.5% of bioethanol in the U.S. is currently made using lignocellulosic biomass [13]. This is in large part due to the recalcitrant nature of lignocellulose itself, which is a complex substrate that can be quite difficult for any one microbe to completely degrade [14, 15], and thus usually requires expensive pre-treatment to increase its digestibility [16].

1.2.2 Lignocellulose – components and hydrolysis

Lignocellulose makes up plant cell walls and consists of three main structural polymers: cellulose, hemicellulose, and lignin (Fig. 1.1), which are discussed in more detail in the following sections. Briefly, cellulose is usually the most abundant polymer, making up between 35 and 50% of plant dry weight [17] and forms long, crystalline glucose homopolymers, imparting structural strength to plants [18, 19]. Hemicellulose, making up 20 to 35% of plant dry weight [17], is made up of heteropolymers of various sugars and other moieties that help strengthen the cell walls by forming cross-links with cellulose fibres and lignin [20]. Lignin makes up between 5 and 30% of plant dry weight [17], and forms a matrix along with

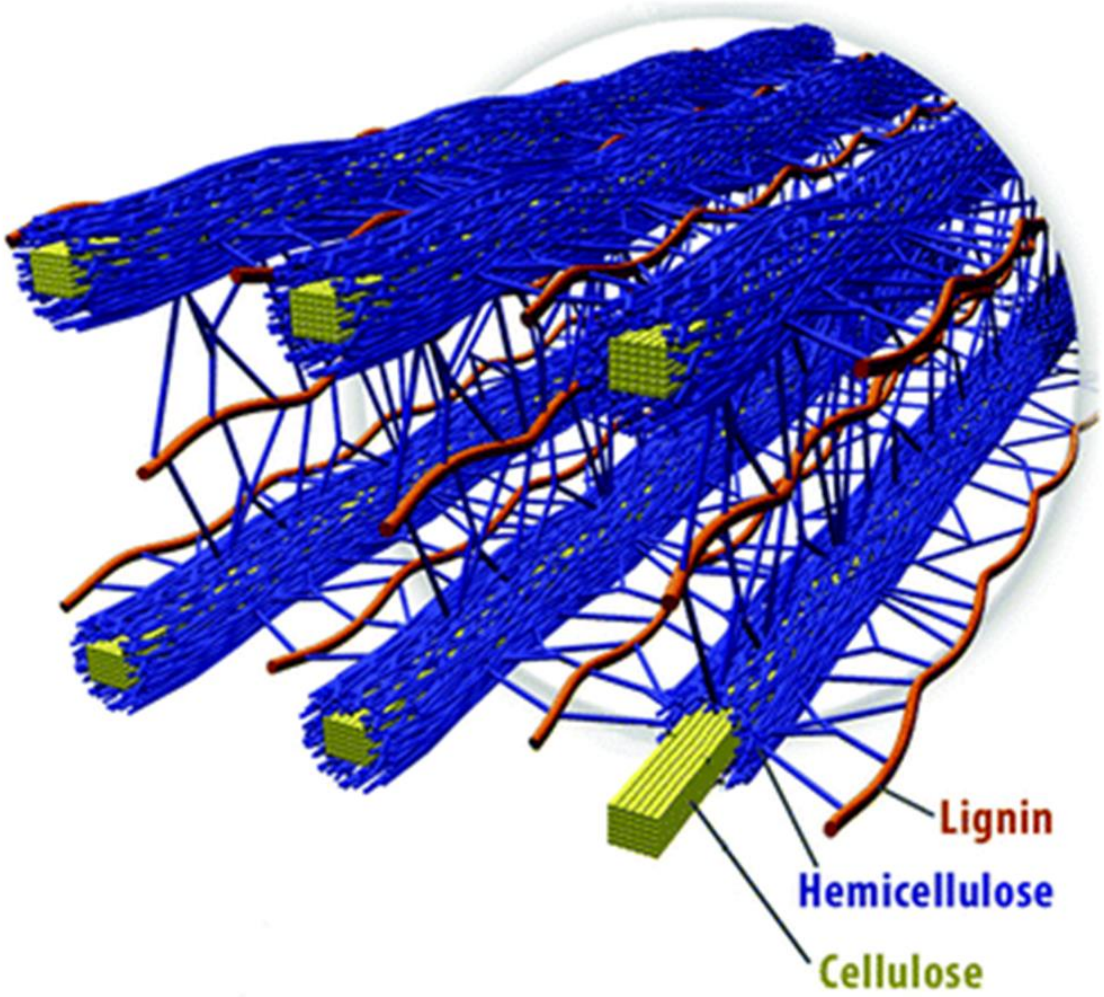

Fig. 1.1: Structure of lignocellulose. Adapted from [22] with permission.

hemicellulose that provides strength to the cell walls [18]. Due to its phenolic nature lignin is very resistant to biodegradation and helps to protect the plant against microbial pathogens [18, 21]. The linked structure of these and other components renders raw lignocellulose recalcitrant to microbial degradation.

1.2.2.1 Cellulose

Cellulose is a polysaccharide made up of glucose subunits with β-1,4 linkages, forming long, linear chains with no branching. The structure allows for many intra- and inter-molecular hydrogen bonds between chains, resulting in strong, tightly-packed, crystalline fibrils made up of approximately 30 individual parallel cellulose chains [17]. These elementary fibrils then aggregate together to form microfibrils, which themselves then aggregate to form cellulose fibres [17]. The crystalline structure of cellulose limits access to enzymes, inhibiting enzymatic digestion, although imperfections in the fibrils and amorphous regions with lower crystallinity exist, where digestion is enhanced [17, 23]. All cellulose found in nature is partially crystalline but the degree of crystallinity, sometimes referred to by a crystallinity index percentage, can vary depending on the source [24], and is partly indicative of the digestibility of the cellulose, with low crystallinity index substrates generally being easier to digest [24, 25]. Commercial celluloses can vary greatly in their crystallinity, from extremely crystalline (e.g. Avicel and Sigmacell), to moderately crystalline (e.g. Solka Floc), to very low crystallinity and highly water soluble cellulose derivatives (e.g. carboxymethylcellulose) [17]. The term “cellulolytic” must be taken with caution then, as some studies determine cellulolytic capabilities by using carboxymethylcellulose as the “cellulosic” substrate, but many organisms capable of degrading carboxymethylcellulose are incapable of degrading the microcrystalline cellulose found in nature [17, 26].

Enzymatic hydrolysis of cellulose can occur through various enzymes but is predominantly performed by glycoside hydrolases that can hydrolyze the β-1,4 bonds [17]. These cellulases include endoglucanases (EC 3.2.1.4) that cut at random sites along cellulose chains, producing new free ends and oligosaccharides of various length (cellodextrins), and exoglucanases (EC's 3.2.1.91 and 3.2.1.74) that cut only at the ends of the cellulose chains and produce either cellobiose (glucose β-1,4 disaccharide) or glucose, respectively [17]. Many cellulolytic organisms produce both endo- and exoglucanases, producing what is known as endo-exo synergy, whereby the endoglucanases are constantly producing new free ends for the exoglucanases to attack [17]. Exoglucanases act only at either the reducing or non-reducing end of the cellulose chains, not both, and another type of synergism (exo-exo) exists when both types of exoglucanases are present, such that both ends of the cellulose chains are attacked [17]. Some microbes that are unable to transport and utilize the cellodextrins themselves also produce and excrete β-glucosidases (EC 3.2.1.21) that cleave cellobiose and cellodextrins into glucose subunits [17]. A new group of cellulolytic enzymes was recently discovered, known as lytic polysaccharide monooxygenases (EC 1.14.99.54) which are produced by some aerobic microbes, that use molecular oxygen to oxidize and cleave cellulose chains [27].

1.2.2.2 Hemicellulose

Hemicellulose is a group of polysaccharides consisting of various pentose and hexose monomers but is based on a backbone made up of either xylose, mannose, glucose, or certain combinations of the three, connected with β-1,4 links [20]. This backbone can be highly branched with various links to different side-unit residues, including xylose, galactose, mannose, arabinose, fucose, rhamnose, glucuronic acid, acetic acid, and ferulic acid [20]. The exact composition of hemicellulose varies between cell type and between plants but commonalities

exist between various plant groups [20]. The branched and heterogeneous nature of hemicellulose prevents close packing of the polysaccharide chains together, and thus hemicellulose is more soluble and amorphous compared to cellulose and enzymatic hydrolysis is generally much quicker.

Enzymatic hydrolysis of hemicellulose occurs mainly through glycoside hydrolases and involves both endo- and exo-acting enzymes, similar to cellulose hydrolysis. Due to the wide variety of sugars present in hemicellulose, a large variety of enzymes is required for complete hydrolysis, with each enzyme having different sugar specificity [15]. Various non-glycoside hydrolases are also involved, including acetyl xylan esterases, ferulic acid esterases, and p-coumaric acid esterases [15]. Due to the diversity of hemicellulose structures and the accompanying diversity of enzymes required to depolymerize the hemicellulose, not every "hemicellulolytic" bacterium degrades all hemicellulose types efficiently. Each bacterium, due to their specific repertoire of hemicellulolytic enzymes, may depolymerize hemicellulose from certain plants or tissues faster and more effectively than others [15].

1.2.2.3 Lignin

Lignin's structure and enzymatic degradation is quite unlike that of cellulose and hemicellulose. Lignin is a highly complex and branched heteropolymer made up of phenyl propane subunits. Three different aromatic alcohol monomers exist: *p*-coumaryl, coniferyl, and sinapyl alcohol, which are linked by various carbon-carbon and aryl ether bonds [15]. Molecular oxygen is required for the enzymatic depolymerization of lignin, involving oxidative and hydrolyzation reactions and is performed by various peroxidases and laccases [15]. Lignin is thus quite recalcitrant to digestion and helps protect the plants from microbial attack [21].

1.2.2.4 Pectin

Pectin is another group of polysaccharides found in lignocellulose, cross-linking with hemicellulose and other components of the cell wall and lamella. Although it is only present in small amounts (around 0.1%) in the plant secondary walls, which makes up the majority of lignocellulose material [28], it can make up to 35% of the primary cell wall in some plants [29]. Pectin backbones consist of α-1,4-linked galacturonic acid residues, sometimes with alternating rhamnose, and can be highly acetylated and methylated, and can have complex side-chains with up to 12 different types of sugars [15]. Enzymatic hydrolysis involves pectin methyl esterases, polygalacturonases, and pectate lyases [15]. Although pectin makes up a small proportion of lignocellulose, its removal is still important for lignocellulose deconstruction [28], with many lignocellulolytic microbes also expressing pectinases [15].

1.2.2.5 Non-catalytic proteins involved in lignocellulose deconstruction

Microbes have evolved to employ a number of non-catalytic proteins and protein domains to aid in lignocellulose deconstruction. Carbohydrate-binding modules (or CBMs) are protein domains that bind strongly to various carbohydrates, including cellulose and hemicellulose. These CBMs are usually found in proteins that also contain catalytic domains, providing close access of the catalytic domains to the substrate of choice, thereby increasing hydrolysis [15]. Some research indicates that they might also play a role in altering cellulose structure and provide increased access to single cellulose chains [30]. Type IV pili may also play a role in lignocellulose digestion in some microbes by aiding in cellulose adhesion [31]. Some proteins related to solute-binding proteins have been found to be both highly expressed in the proteomes of cellulolytic organisms [32] and capable of binding to cellulose and other carbohydrates, although their exact role, if any, in lignocellulose deconstruction remains

uncertain [33]. Expansins, also known as swollenins, have no catalytic activity but act as plant cell wall-loosening proteins to increase access of catalytic enzymes to the lignocellulose substrate [34].

1.2.2.6 Lignocellulolytic enzyme systems

Three different main lignocellulolytic enzyme system variants have evolved over time. The first, and most basic system, involves free monofunctional enzymes secreted outside of the cells. This is predominantly found in aerobic fungi, but is also found in some bacteria [17]. These enzymes, which may or may not have CBMs, appear to be adequate for these aerobic fungi that can penetrate lignocellulose with their hyphal extensions, providing close access of their secreted enzymes to the substrate [17]. The second system, found mostly in hyperthermophilic anaerobic bacteria, utilizes secreted multi-functional enzymes, with single large enzymes containing two or more catalytic domains and one or more CBM domains [35]. The multiple catalytic domains often have different activities, creating a synergistic effect [35]. The third and final system, known as the complexed system, found in anaerobic bacteria, utilizes cellulosomes, which are modular protein structures that contain a non-catalytic, cell wall-bound scaffold protein (scaffoldin) backbone upon which multiple different cellulases, hemicellulases, and CBMs, are attached [17]. This system creates a high local concentration of hydrolytic enzymes near the substrate surface, creating a synergistic effect between the various cellulases and hemicellulases [17]. Cellulosomes also allow the attachment of cells to the surface of the lignocellulose substrates, creating a type of microbe-enzyme synergy that enhances cellulose degradation by live cells compared to dead cells or secreted enzyme systems [36, 37]. This microbe-enzyme synergy might be explained in part by the fact that the cells attached to the substrate are taking up the hydrolysis products quickly after release by the enzymes, thus

lowering the local concentration of hydrolysis products that could otherwise cause end-product inhibition of the hydrolytic enzymes [37, 38]. The evolution of these complexed systems might have arisen to facilitate close contact between the substrate and the anaerobic bacteria, which cannot penetrate the substrates as easily as the aerobic fungi, increasing access to solubilized sugars that other microbes are competing for, especially important under the low adenosine triphosphate (ATP)-yielding anaerobic conditions [17]. Although fungal and cellulosomal cellulases possess similar catalytic mechanisms, they display differential degradation effects on the substrate at a macro level. Whereas fungal cellulases tend to process the substrate by "sharpening" the ends of the substrate fibrils, the cellulosomes tend to splay the ends of the fibres, resulting in increased access to enzymatic attack [39]. This difference is highlighted by the synergism displayed between the two systems when combined for enzymatic hydrolysis of cellulose [39, 40].

1.3 Consolidated bioprocessing

There are a few different process configurations used for producing second-generation biofuels. The first step in all approaches is usually pretreatment, which involves cutting or milling the raw lignocellulosic substrate into smaller pieces, increasing the surface area for chemical and enzymatic reactions, and then subjecting the substrate to mechanical, thermal, biological, or chemical treatments, or a combination thereof, which either modifies the lignocellulose structure or simply removes the lignin or hemicellulose, increasing the enzymatic digestibility of the remaining residue [16]. The predominant approach, known as separate hydrolysis and fermentation, involves culturing a cellulolytic fungus, harvesting the secreted cellulases and hemicellulases, which can also be bought commercially, and then applying those hydrolytic enzymes to the pretreated substrate to saccharify the material into soluble monosaccharides under optimum conditions for those enzymes. A high ethanol-yielding microbe, usually yeast, is then added to ferment the glucose, from the hydrolyzed cellulose, into ethanol under optimum yeast conditions. Finally, another high ethanol-yielding, pentose-fermenting microbe is then added to ferment the pentoses from hemicellulose hydrolysis into ethanol under yet different conditions. This has been the standard for a number of years, due to the high yields possible, although the numerous steps required, especially the dedicated fungal cellulase production, create high production costs [41].

Another process configuration, simultaneous saccharification and fermentation (SSF), involves consolidating the enzymatic saccharification and glucose fermentation steps into a single process by utilizing conditions amenable to both the cellulolytic enzymes and the fermenting microbe. This has the significant advantage of keeping the glucose concentrations low, due to continual uptake by the microbe, and thus increases hydrolysis by decreasing the concentrations of the hydrolysis products that cause end-product inhibition of the

(hemi)cellulases [42]. A third configuration, known as simultaneous saccharification and cofermentation, then incorporates the pentose fermentation step into the saccharification and hexose-fermentation step as well, yet further consolidating the process and potentially lowering costs.

Consolidated bioprocessing, or CBP, consolidates all steps into a one-pot process, eliminating the need for a costly dedicated cellulase production step, while retaining the advantages of simultaneous saccharification and (co)fermentation, and presents the greatest promise for lowering the costs of lignocellulosic biofuel production to be competitive with fossil fuels [41]. There are a number of ways in which this can be done but the main method is by utilizing microbes capable of both producing their own (hemi)cellulases and then fermenting the solubilized oligo- and monosaccharides into ethanol [41].

Unfortunately, there has not yet been discovered, nor engineered, any microorganism with both sufficiently high lignocellulolytic degradation ability and high ethanol yield and titre to allow cost-competitive CBP bioethanol [7, 8, 43]. There are then two main approaches to developing an organism for CBP purposes. The first, known as the recombinant cellulolytic strategy, is to take a microbe that is already capable of high ethanol production and engineer it for lignocellulose deconstruction. The second, known as the native cellulolytic strategy, is to find a microbe with naturally high lignocellulose deconstruction capabilities and engineer it for high ethanol production. While there is some work being done on the former approach [43–45], the latter is generally considered more feasible [8, 46], due to the large variety of enzymes required for complete lignocellulose hydrolysis [43], as mentioned previously.

1.3.1 Candidate microorganisms for CBP

1.3.1.1 Recombinant cellulolytic candidates

The predominant microbe utilized for the recombinant cellulolytic strategy is *Saccharomyces cerevisiae* [47], but other yeast, such as *Kluyveromyces marxianus* [48] and bacteria, including *Escherichia coli* [49], *Zymomonas mobilis* [50], and *Geobacillus thermoglucosidasius* [51] have also been studied. Many of these organisms are well studied and have well-developed genetic systems available for manipulation. A common method is to express and secrete the endo- and exoglucanases from cellulolytic fungi in these hosts, which can degrade pure cellulose [52] but are inadequate for actual lignocellulosic substrates [49, 53]. More complex approaches include expressing cell membrane-attached cellulases and xylanases [54] and even expressing designer mini-cellulosomes on the cell walls of these high-ethanol hosts, which exhibit synergistic hydrolysis compared to individual secretion of the enzymes [55], although so far these mini-cellulosomes are purely designed for cellulose hydrolysis and have yet to start incorporating xylanases for complete lignocellulose utilization [47]. One of the disadvantages of *S. cerevisiae* is that it can not naturally utilize xylose [56], one of the main lignocellulosic sugars, and therefore xylose utilization has been engineered into *S. cerevisiae* through the addition of xylulose kinase and either: a xylose isomerase [57], or a xylose reductase and a xylitol dehydrogenase [58], although further engineering or adaptation is required for efficient xylose utilization [59].

1.3.1.2 Natively cellulolytic and ethanolgenic fungi

While cellulolytic fungi are well known for their ability to secrete high amounts of efficient cellulases [60], some cellulolytic fungi also possess the ability to produce various levels of ethanol, making them attractive candidates for CBP. Fungi from various genera have been

discovered that are both cellulolytic and ethanolgenic, including *Aspergillus*, *Rhizopus*, *Trichoderma*, *Fusarium*, *Monilla* [61], *Trametes* [62], *Orpinomyces* [63], *Phlebia*, *Flammulina*, *Neurospora*, and *Schizophyllum* [47]. These cellulolytic fungi tend to only produce and secrete high levels of cellulases and grow well on lignocellulose under aerobic conditions however [61], while ethanol production is predominantly an anaerobic process, used to oxidize NADH, which can not be oxidized through aerobic respiration in the absence of oxygen. This presents a problem to cellulosic ethanol production via fungi, as ethanol can not be produced in high amounts at the same time as the fungi are producing and secreting cellulases. This is partially overcome by altering culture conditions, the fungi are first grown under aerobic conditions, where they can increase in biomass and secrete hydrolytic enzymes, depolymerizing the lignocellulose, and then switched to microaerobic or anaerobic conditions, where the fungal cells then begin fermentation and convert the released sugars to ethanol [61, 64–66]. Some fungi have also been found to produce ethanol under fully aerobic conditions [62, 67], while strictly anaerobic, cellulolytic, ethanolgenic fungi have also been discovered [63].

1.3.1.3 Hyperthermophilic bacteria

The hyperthermophilic anaerobic bacteria, with a number of species from the *Caldicellulosiruptor* genus, represent another potential group of CBP candidates. These bacteria possess relatively high lignocellulose degradation abilities [68, 69], owing in part both due to their unique synergistic multi-functional enzymes [70] and the high temperatures they and their enzymes operate optimally at, around 65-78°C [46]. These hyperthermophilic anaerobic bacteria do not naturally produce high amounts of ethanol, but instead produce high amounts of hydrogen, and so have mainly been studied for their lignocellulosic hydrogen potential [71–73]. Certain *Caldicellulosiruptor* strains have been discovered that produce higher amounts of

ethanol, at least under certain conditions [74], while genetic engineering efforts have also managed to increase ethanol yields [75], although ethanol yields still remain far below commercially viable levels.

1.3.1.4 Mesophiles

Some of the most well-studied mesophilic, anaerobic, cellulolytic bacteria are *Acetivibrio cellulolyticus* (formerly *Clostridium cellulolyticum* [76]), *Clostridium cellulovorans*, and *Lachnoclostridium phytofermentans* (formerly *Clostridium phytofermentans* [77]) [44, 47]. Both *A. cellulolyticus* [78] and *C. cellulovorans* [79] utilize cellulosomes for lignocellulose deconstruction, as is usual for the anaerobic cellulolytic bacteria, while *L. phytofermentans* actually secretes mono-functional hydrolytic enzymes, similar to fungi [80]. *A. cellulolyticus* has been engineered for both higher ethanol production, via either addition of exogenous ethanol production enzymes [81] or deletion of competing pathways [82], and even butanol production [83]. *C. cellulovorans* has good lignocellulose degradation potential, able to grow on untreated substrates such as rice straw [84], but naturally produces mostly acetate and butyrate, with only trace amounts of ethanol [85]. Genetic engineering efforts have included adding alcohol dehydrogenases to pull flux from the acetyl-CoA and butyrl-CoA intermediates to simultaneously produce both ethanol and butanol [86, 87] or introduction of acetone-butanol-ethanol pathway genes to produce acetone, butanol, and ethanol [88]. *L. phytofermentans* secretes a large and various array of polysaccharide-hydrolyzing enzymes [89], allowing it to solubilize over 76% of pretreated corn stover polysaccharides [90], utilizes a wide array of lignocellulose sugars [89], and has a high yield of ethanol production (66% of theoretical maximum) [80].

Members of the Actinobacteria genus *Cellulomonas* are generally cellulolytic and some are also capable of producing ethanol [91, 92]. *Cellulomonas* species are either aerobic or facultatively anaerobic however, and can suffer the same issue as fungal CBP, with cellulase production only occurring aerobically while ethanol fermentation only occurs under anaerobic conditions [93]. Another candidate mesophile is *Ruminiclostridium cellobioparum* subsp. *termitidis* (formerly *Clostridium termitidis* [94]), originally isolated from the gut of a termite [95], which can hydrolyze and utilize a wide range of lignocellulosic substrates, including cellulose and xylan [96], and possesses an unusually large genome (~6.4 Mbp) compared to other Clostridia (~4.3 Mbp) [97], harboring one of the largest known collections of predicted glycosyl hydrolases and carbohydrate active enzymes [96], highlighting its potential role for lignocellulose deconstruction. The maximum ethanol yield obtained is only around 16% of theoretical maximum though, and the doubling on cellulose is relatively slow, around 19 hours [98].

1.3.1.5 Thermophiles

Thermophilic bacteria represent some of the most promising candidates for CBP [14, 46]. One of the, if not the most, promising candidate for CBP is *Acetivibrio thermocellus* (formerly *Clostridium thermocellum* [76]) [14, 99], which will be discussed in more detail later (See section 1.4). Other notable cellulolytic thermophilic CBP candidate bacteria include *Acetivibrio clariflavus* (formerly *Clostridium clariflavum* [76]), *Thermobifida fusca*, *Geobacillus* species, and *Thermoclostridium stercorarium* (formerly *Clostridium stercorarium* [94]). *A. clariflavus* is closely related to *C. thermocellum* [100], produces ethanol among other end-products [101], and utilizes a cellulosome for efficient cellulose and hemicellulose deconstruction [102]. *T. fusca* is an aerobic thermophilic actinomycete, very distantly related to the *Clostridium* and

Caldicellulosiruptor Firmicutes described so far [103], and uses a secreted fungi-like enzyme system for deconstructing lignocellulose [104]. Although *T. fusca* does not naturally produce any ethanol, it has been engineered to produce propanol, indicating its potential use for biofuel production [105]. While the oligosaccharide-utilizing *Geobacillus thermoglucosidasius* is relatively well-known for its CBP potential [47, 106], natively-cellulolytic, ethanolgenic species from the *Geobacillus* genus have also been discovered [107, 108]. While these natively-cellulolytic *Geobacillus* species have naturally low ethanol yields [108], the engineering of high-ethanol phenotypes in closely-related species [51] highlights their potential.

1.3.1.6 Clostridium stercorarium – *thermophilic cellulosic/hemicellulosic CBP candidate*

Clostridium stercorarium is a promising candidate for CBP due to its rare ability to degrade and ferment both microcrystalline cellulose and hemicellulose [109, 110]. *C. stercorarium* subsp. *stercorarium* is a strictly anaerobic, spore-forming, thermophilic (optimum growth temperature: 65°C), and rod-shaped bacteria with peritrichous flagella that was first isolated from a compost pile in 1983 [109]. Numerous studies indicate that *C. stercorarium* plays a highly active role in the degradation of plant biomass in anaerobic, thermophilic digesters, highlighting its potential for CBP [111–115]. In 2001 two other bacteria with different flagella arrangements and carbon source utilization , namely *Thermobacteroides leptospartum* [116] and *Clostridium thermolacticum* [117] were found to be closely related enough to *C. stercorarium* to be considered subspecies, necessitating the new name change of the type-species (strain DSMZ 8532) to *C. stercorarium* subsp. *stercorarium* [118], and in 2018 the genus of the three sub-species was reclassified to *Thermoclostridium* [94]. The rest of the thesis will only focus on *Thermoclostridium stercorarium* subsp. *stercorarium*, which, for the sake of simplicity, will simply be referred to by it's long-standing name, *Clostridium stercorarium*.

Unlike most other cellulolytic Clostridia, *C. stercorarium* utilizes a secreted free enzyme system, rather than cellulosomes, to degrade lignocellulose [110, 119–121]. Surprisingly, *C. stercorarium* is able to degrade microcrystalline cellulose with just two different cellulases: one endoglucanase [110, 119, 122–124], and one exoglucanase [110, 122, 125]. *C. stercorarium* also produces an extracellular β-glucosidase [110, 122], although it seems to prefer an energy-saving intracellular phosphorolytic system for metabolizing cellobiose [126]. This two-enzyme system is the simplest microbial system for depolymerizing crystalline cellulose discovered so far in nature [35], although its rate of crystalline cellulose hydrolysis is below that of cellulose-specialists, such as *C. thermocellum* [127, 128]. *C. stercorarium* does produce a highly active, extensive, and diverse array of hemicellulases, however, allowing it to depolymerize various types of hemicellulose in an efficient manner [110, 120, 121, 127, 129]. *C. stercorarium* is also able to utilize and grow well on a wide variety of lignocellulose sugars, including cellobiose, glucose, xylose, arabinose, galactose, mannose, and rhamnose [109]. Thus, due to its efficient hemicellulose hydrolysis and hemicellulose sugar utilization capabilities, *C. stercorarium* can be considered as more of a hemicellulose-specialist [127]. *C. stercorarium* produces a relatively high ratio of ethanol to acetate, as high as 2.5:1, depending on conditions, along with other end-products such as hydrogen, carbon dioxide, and small amounts of lactate [109, 129]. These features highlight the potential of *C. stercorarium* as a candidate for the CBP of lignocellulose, especially the hemicellulose fraction.

1.3.1.7 Thermophilic hemicellulolytic candidates – Thermoanaerobacter thermohydrosulfuricus *and others*

Non-cellulolytic microbes also have potential to act as important contributors to lignocellulosic biofuel production [47]. While members of the Clostridial genera

Thermoanaerobacter and *Thermoanaerobacterium* generally do not degrade cellulose (with a few exceptions [130]), they are known to secrete a variety of free hemicellulases that allow them to degrade and utilize hemicellulose fairly well [131–138]. Species from both genera are also naturally capable of producing high levels of ethanol [132, 133, 139–142]. Two species in particular, *Thermoanaerobacter ethanolicus* and *T. italicus*, have been found to naturally produce ethanol above 90% of the maximum theoretical limit [143, 144]. At least some species appear to be quite amenable to genetic engineering for higher ethanol yields, with knockouts of lactate and acetate production genes in *Thermoanaerobacterium saccharolyticum* causing ethanol yields to increase to near theoretical limits [145]. While some members of these genera seem to rely predominantly on bi-functional AdhE (aldehyde and ethanol dehydrogenase) enzymes for ethanol production [146, 147], others also require NADPH-dependent AdhA enzymes for their high ethanol yields [148, 149]. Some of these bacteria also possess naturally high ethanol tolerance, around 40 g/L [132], which is the value likely required for economic viability [150]. These hemicellulolytic thermophiles are thus promising candidates for the conversion of hemicellulose to biofuels via either monoculture, by converting the hemicellulose fraction of lignocellulose, or in co-culture with a cellulose degrader, allowing biofuel production from all lignocellulose sugars solubilized [144]. One hemicellulolytic *Thermoanaerobacter* strain that has been recently isolated and is of interest is *T. thermohydrosulfuricus* DSM 26960 (strain WC1) [151].

T. thermohydrosulfuricus DSM 26960 was initially isolated from a thermophilic wood compost pile using acid hydrolyzed hemicellulose, which is hemicellulose that has been hydrolyzed into various oligomers and monomers [152], as the enrichment substrate [151]. Numerous other very closely-related strains were also isolated along with DSM 26960, but DSM

26960 was chosen for further study for CBP applications based on its more extensive carbon source utilization (including most of the major lignocellulose sugars: cellobiose, glucose, xylose, mannose, and galactose), higher growth rate, and higher ethanol yield compared to the other isolated strains [151]. Genomic analysis of *T. thermohydrosulfuricus* DSM 26960 revealed the presence of an apparent suite of enzymes for hemicellulose deconstruction and utilization, in line with its ability to grow on xylan as a carbon source [135]. While *T. thermohydrosulfuricus* DSM 26960 only possesses a few predicted extracellular hemicellulases, including an endoxylanase, an acetyl-xylan esterase for deacetylating xylan, and a β-xylosidase, it contains a wide variety of carbohydrate transporters and intracellular hydrolytic enzymes active on cellulose and hemicellulose oligomers [135].

Carbon catabolite repression is a mechanism used by microorganisms in order to take up and use carbon sources sequentially, in order of preference, and to balance out metabolic energy demands [153, 154]. This carbon catabolite repression system has been found to be operative in some Clostridial species [155], in some cases preventing simultaneous usage of lignocellulose-derived sugars [156]. This is disadvantageous for CBP of lignocellulose as it would require longer incubation times in order to sequentially utilize all the sugars, as well as inhibiting the lignocellulose hydrolysis due to build-up of inhibitory products. While the genome of *T. thermohydrosulfuricus* DSM 26960 does encode carbon catabolite repression genes, proteomic analysis reveals that it does not express all of these genes in growth on cellobiose, xylose, or cellobiose plus xylose, and it has been experimentally shown to co-utilize a mixture of cellobiose and xylose simultaneously, an important advantage for a CBP candidate [157]. Although *T. thermohydrosulfuricus* DSM 26960 was originally found to produce mostly acetate and ethanol [151], the end-product profile shifted predominantly to lactate production under different growth

conditions, such as higher carbon loading and lower yeast extract, despite the high levels of enzymes for competing pathways such as acetate and ethanol production [157], and may be the result of the depletion of certain nutrient(s) in the medium. Thus, careful attention to growth conditions and/or metabolic engineering are needed to create high ethanol-yielding cultures of *T. thermohydrosulfuricus* DSM 26960 for CBP purposes.

1.4 *Clostridium thermocellum* – best candidate for CBP

Clostridium thermocellum is one of the best known and studied cellulolytic bacteria [15, 17, 18, 158–161]. *C. thermocellum* is a gram-positive, rod-shaped, spore-forming, strictly anaerobic, thermophilic (optimum temperature around 60°C) bacteria with lateral flagella [162]. It was first isolated from manure in 1926 [163] and further strains are frequently isolated from decaying biomass and soil [17, 164], some mesophilic environments [165], as well as thermophilic anaerobic digesters [166, 167]. Similarly to *C. stercorarium*, *C. thermocellum* has been reclassified numerous times in recent years, first to the *Ruminiclostridium* genus [77], then the *Hungateiclostridium* genus [94], and very recently to the genus *Acetivibrio*, as *A. thermocellus* [76]. For simplicity it will be referred to by its long-standing name, *Clostridium thermocellum,* for the remainder of the thesis.

Following the native cellulolytic strategy for lignocellulosic CBP, the first step would be to find the microorganism that can depolymerize the cellulose and hemicellulose fractions of lignocellulosic material to the greatest extent in the shortest amount of time. Assuming this organism is otherwise amenable to industrial processes the next steps would involve engineering the organism for better biofuel yields and titres. Initial comparisons between the secretomes of *C. thermocellum*, which contained cell-disassociated cellulosomes, and the fungal standard, *Trichoderma reesei*, found similar or slightly lower activity for *C. thermocellum* compared to *T. reesei* on various pure celluloses [168, 169]. When supplemented with exogenous β-glucosidase to reduce levels of inhibitory cellobiose, a much lower load of extracellular *C. thermocellum* cellulases was needed to solubilize an equivalent amount of ammonia-soaked rice straw compared to a *T. reesei* and commercial cellulase cocktail [170]. Microbe-enzyme synergy is an important aspect of the lignocellulose hydrolysis efficiency of *C. thermocellum* [37, 38], however, and actual CBP processes would utilize whole cells and not just extracellular protein

preps, so a more relevant comparison would test live cultures. In fact, *C. thermocellum* cultures has been found to solubilize significantly more substrate than fungal enzyme cocktails, on both untreated [171] and pretreated [172] lignocellulosic substrates. When compared against SSF technologies that utilize simultaneous fungal enzyme hydrolysis and yeast fermentation to keep levels of inhibitory hydrolysis products low, *C. thermocellum* is still able to solubilize a greater portion of switchgrass [14, 173], winter rye [174], and cellulose [37], as well as produce more total-end products on switchgrass [68].

Controlled comparisons between the various CBP candidates themselves are less common, unfortunately [14], though some do exist. *C. thermocellum* was found to be either equivalent to, or better, than either *Caldicellulosiruptor obsidiansis* [68] or *C. bescii* [68, 173], in terms of solubilization of switchgrass, depending on feedstock pretreatment conditions. A comparison between *C. thermocellum* and the closely-related *Clostridium clariflavum* found that *C. thermocellum* solubilized less untreated switchgrass than two different strains of *C. clariflavum* [102]. A comprehensive evaluation of numerous CBP candidates, including *C. clariflavum*, *C. bescii*, and a mixed consortium enriched from horse manure found that *C. thermocellum* solubilized the most glucan and xylan of untreated switchgrass [14]. These studies indicate that *C. thermocellum* is one of, if not the best, lignocellulose degraders among the currently known CBP candidates.

C, thermocellum also possesses other attributes that are amenable to industrial processes. It's anaerobic nature both prevents the need for oxygenation, which can be a costly and difficult process at larger scales [159, 160, 175], and is associated with it's relatively low cell yield, meaning more substrate is converted to end-product [159, 160]. The thermophilic nature also confers numerous advantages, including reduced chance of contamination, increased enzymatic

activity, reduced cooling cost of large-scale self-heating reactors, possibility of continuous distillation of volatile products such as ethanol, reduced viscosity, increased substrate solubility, and reduced gas solubility [159–161, 175–177]. Genetic tractability is an important attribute for industrial microorganisms, allowing directed mutations to alter target product yields [161]. Although transformation of *C. thermocellum* for genetic manipulations was initially quite difficult [161, 178] and strain dependant [179], requiring custom electroporation apparatus [179, 180] and cuvettes [179], later tests with various host methylation systems lead to increased transformation efficiencies, even with standard apparatus [181]. Combined with development of hypoxanthine-based counter selection tools that allow markerless deletions [182], this has led to an ease of genetic manipulation for *C. thermocellum*, highlighted by the numerous mutants created since then [8, 182–189]. The native carbon source utilization range for *C. thermocellum* is fairly small unfortunately, only able to grow on cellulose and cellulose hydrolysis products, such as cellobiose, but not glucose, and is unable to use any of the sugars found in hemicellulose, such as xylose, arabinose, mannose, or galactose, whether in their monomeric or oligomeric form [102, 162, 190, 191]. This is potentially problematic as these hemicellulose-derived monomers and oligomers have been found to inhibit both fungal cellulases [192–194] and hemicellulases [195–197], although they have not yet been confirmed to also inhibit hydrolytic enzymes from *C. thermocellum.* Some of the pentose sugars, xylose and arabinose, are known to significantly inhibit the growth and metabolism of *C. thermocellum* though, even in their oligomeric form, possibly via induction of a cell-signalling pentapeptide [198]. A strain of *C. thermocellum* has recently been engineered to utilize xylose however, providing one possible means of ameliorating this issue [199]. Although these attributes position *C. thermocellum* as a very strong candidate for CBP, it is still not commercially viable. The ideal candidate or process would be

able to both depolymerize minimally treated lignocellulose faster, and to a larger extent, and produce ethanol at higher yields.

1.4.1 Lignocellulose hydrolysis system of *C. thermocellum*

The remarkable lignocellulose hydrolysis ability of *C. thermocellum* is due in large part to the large multi-protein cellulosomes displayed on its cell surface [17, 111, 160]. The structure of the *C. thermocellum* cellulosome is shown in Fig. 1.2. Cell-surface SdbA proteins, anchored via S-layer homology domains, present cohesin II domains that interact specifically and strongly with dockerin II domains on the CipA scaffoldin backbone, anchoring the entire complex. The scaffoldin protein itself contains a family 3 CBM domain that binds strongly to crystalline cellulose, bringing and keeping the cellulosome complex into close proximity of its lignocellulose substrate [200], while also presenting nine separate cohesin I domains. These cohesin I domains then bind to dockerin I domains that are linked to enzymatic modules on any one of 72 various cellulosomal enzymes that *C. thermocellum* encodes for, including cellulases, hemicellulases, pectinases, chitinases, glycosidases, and esterases [201, 202]. The close proximity of the various depolymerizing enzymes to each other and to the cell creates a synergism that enables faster lignocellulose solubilization [202]. It is somewhat surprising that *C. thermocellum* produces enzymes to break down hemicellulose and pectin even though it is unable to use the resultant hydrolysis products, such xylose, as carbon sources [102, 190, 191]. It is thought that the purpose of these hemicellulases and pectinases is to remove the polysaccharides covering the favoured cellulose beneath [203]. While each individual cellulosome appears to be composed of nine randomly selected cellulosomal proteins, one for

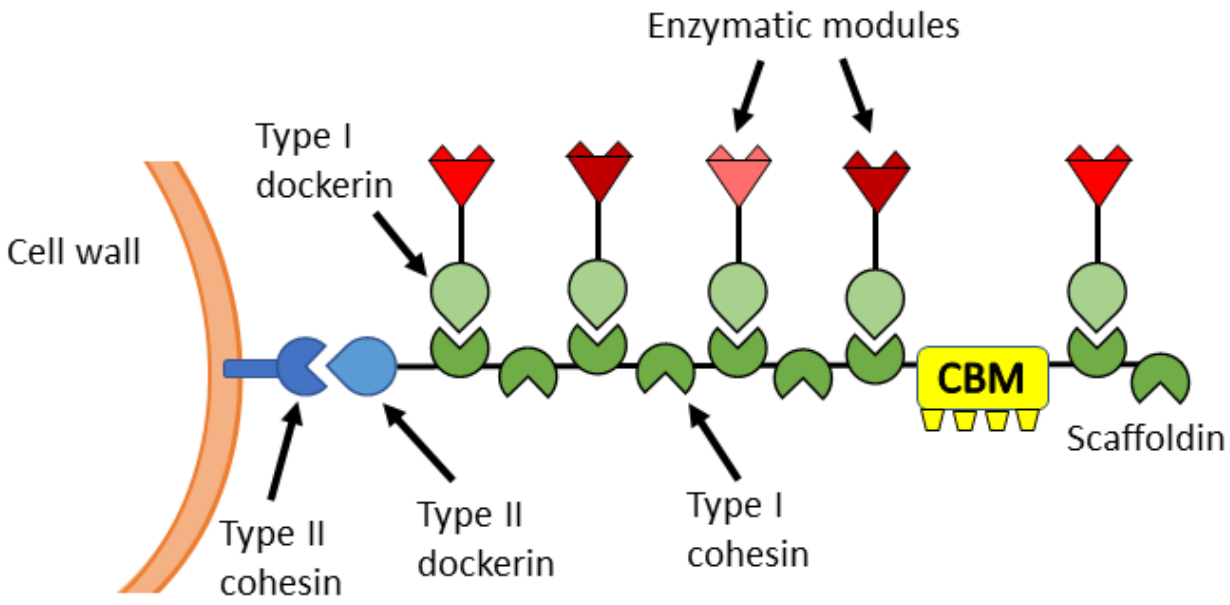

Fig. 1.2: Diagram of the *C. thermocellum* cellulosome. An S-layer homology domain-containing protein (dark blue) anchors the scaffoldin protein to the cell wall. The carbohydrate binding module (CBM) attaches the complex to the lignocellulose substrate while various cellulase and hemicellulase catalytic modules (red), anchored to the scaffoldin backbone via dockerin/cohesin interactions, hydrolyze the substrate into various oligo- and monosaccharides.

each cohesin/dockerin link [202], *C. thermocellum* does change overall expression levels of its cellulosomal proteins based on factors such as carbon source [204–207]. *C. thermocellum* is known to release cellulosomes from its cell surface during later stages of cell growth [208, 209] allowing for hydrolysis to continue. In addition to the canonical SdbA anchor protein + CipA scaffoldin protein cellulosome configuration described above, *C. thermocellum* also produces other structural proteins, albeit in lower abundance [204, 205]. These include the OlpA/C cell surface-anchored proteins that present a single cohesin I domain, allowing them to bind with a single dockerin I-linked cellulase/hemicellulase, and the OlpB and Orf2 cell surface-anchored proteins that present multiple cohesin II domains, allowing each to attach multiple scaffoldin proteins, forming what are known as polycellulosomes [160, 202]. *C. thermocellum* also secretes non-anchored Cthe_0736 proteins, simple multi-domain proteins that only contain 7 cohesin II domains, each binding a scaffoldin, forming free large polycellulosomes that diffuse away from the cells [202]. Most (>90%) of the cellulase activity of *C. thermocellum* is associated with the cellulosome however, as demonstrated in *cipA* mutants [210]. In addition to the cellulosome components *C. thermocellum* also secretes a number of non-cellulosomal lignocellulose hydrolysis enzymes that, apart from a cell surface-associated laminarinase, are secreted into the medium [211]. These include synergistically acting cellulases, as well as a putative xylanase, arabinofuranosidases, and chitinases [211].

1.4.2 Atypical central metabolism of *C. thermocellum*

In the standard model of glycolysis a net total of 2 ATP and 2 NADH are produced per glucose molecule [212], as shown in Fig. 1.3. In the upper “investment” phase, ATP is used to phosphorylate the substrate by both glucokinase and phosphofructokinase. Glyceraldehyde-3-

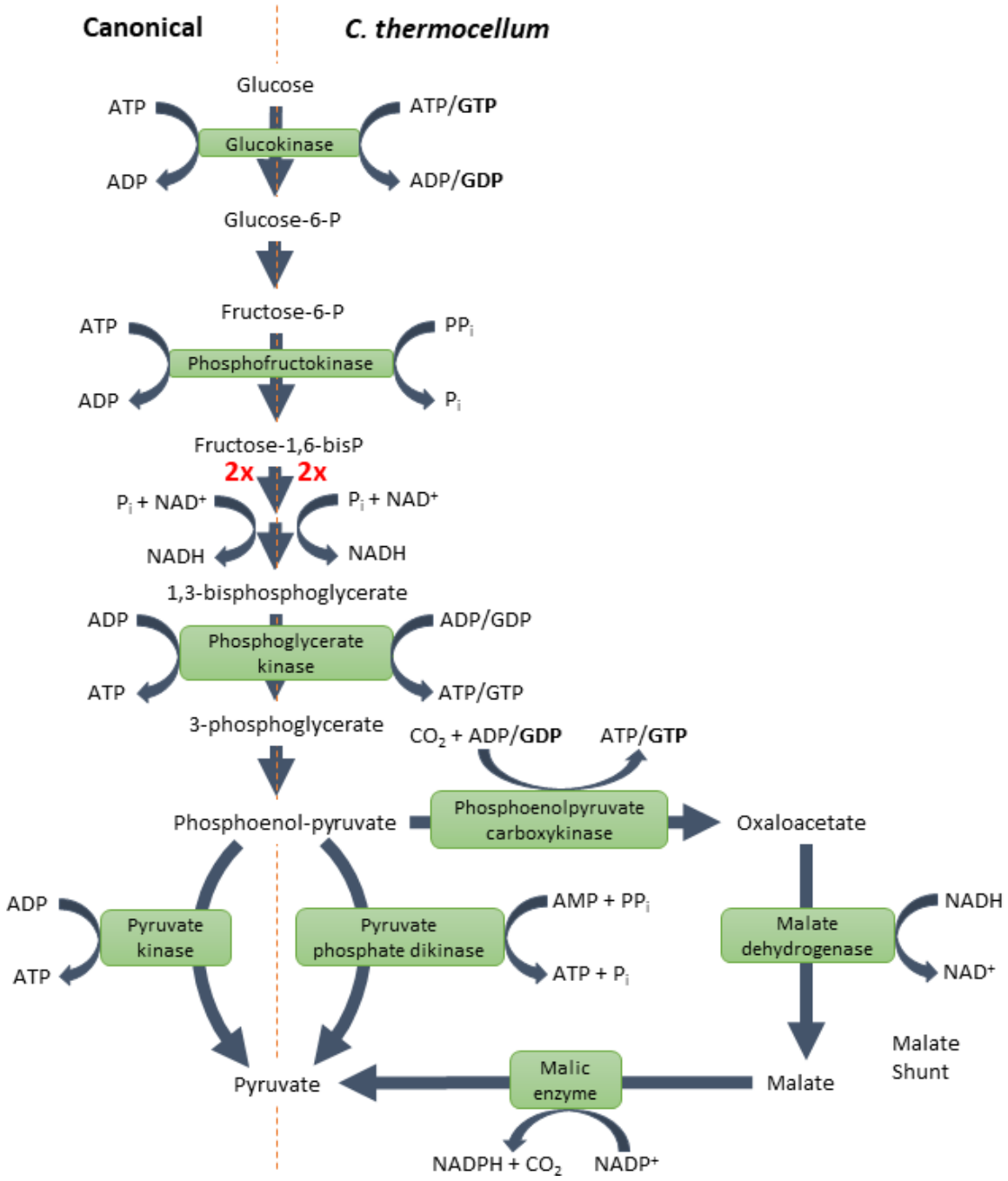

Fig. 1.3: Atypical glycolysis of *C. thermocellum*. Canonical cofactors and pathways are shown on the left while those for *C. thermocellum*, as determined by cell-free assays [213], protein characterization [214], and omics studies [215], are shown on the right. Bold words indicate greater cofactor preference. 2x represents two fluxes of bottom part of pathway for every one glucose. Although all reactions are drawn as unidirectional arrows some of these reactions exist near equilibrium and are reversible.

phosphate dehydrogenase then uses inorganic phosphate for phosphorylation of the substrate. Both phosphoglycerate kinase and pyruvate kinase then remove and utilize the substrate phosphates to convert adenosine diphosphate (ADP) to ATP, which is then used for many processes in cellular growth and maintenance [216]. Aerobic microbes can use the TCA cycle to further oxidize the carbon substrate and then use molecular oxygen to oxidize all the generated NADH, producing even more ATP. Anaerobes, such as *C. thermocellum*, must oxidize NADH (as well as reduced ferredoxin generated from the pyruvate ferredoxin oxidoreductase reaction) and regenerate NAD^+ (and oxidized ferredoxin) by ultimately either reducing the pyruvate leftover at the end of glycolysis, generating reduced end-products such as lactate and ethanol, or reducing protons, generating molecular hydrogen gas [217]. Reducing protons, thereby creating hydrogen gas, can be linked to the generation of proton motive force via membrane bound, proton-translocating hydrogenases (e.g. ferredoxin-dependent energy-conserving hydrogenase or Ech), freeing up the pyruvate to be used for other purposes, such as generating ATP by converting the pyruvate to acetate, ultimately increasing the net yield of ATP from glycolysis [217]. Many anaerobes, including *C. thermocellum*, possess mixed-acid fermentation, producing all of the mentioned end-products, and sometimes others, in various ratios [159, 217].

C. thermocellum has been found to use different cofactors and pathways in its atypical glycolysis (Fig. 1.3). One early study of glycolysis in *C. thermocellum*, using cell-free extracts, reported canonical ATP-dependent glucokinase, ATP-dependent phosphofructokinase, and pyruvate kinase activity [218], although the culture they were using may have been contaminated [213, 219]. Other studies, for instance, found relatively low, or no ATP-dependent glucokinase activity [219–221]. Later, a more comprehensive study found both ATP- and GTP-dependant glucokinase activity in cell-free extracts, although the GTP-dependent activity was 50-fold

higher [213]. This was later corroborated by purification and characterization of the *C. thermocellum* glucokinase, which was found to use both ATP and GTP, but had approximately 10-fold higher catalytic efficiency with the latter [222]. Genomic analysis revealed the presence of three putative phosphofructokinases in the genome, one of which was predicted to be ATP-dependent and another PP_i-dependent, with the last one remaining ambiguous [215, 222]. The PP_i-dependent phosphofructokinase was found to be expressed in much higher amounts than the others in a proteomic analysis [215], and cell-free extracts showed only PP_i-dependant activity [213]. This phosphofructokinase was later confirmed to only have activity with PP_i as its phosphate donor [222]. Phosphoglycerate kinase activity was found to be roughly equal in cell-free extracts with both ADP and GDP as cofactors [213] and the purified protein was also found to have fairly similar affinities for both ATP and GTP cofactors (working in the reverse direction) [222]. The pentose phosphate pathway is also missing the conventional aldolase and may be substituted with an alternate sedoheptulose bisphosphate pathway that utilizes a bifunctional fructose bisphosphate aldolase in conjunction with a bifunctional PP_i-phosphofructokinase [215, 223].

Genomic analysis also revealed the absence of any annotated pyruvate kinase [215, 224], which is strengthened by the lack of any pyruvate kinase activity in cell-free extracts [213], leading to the question of how *C. thermocellum* converts phosphoenolpyruvate (PEP) to pyruvate. There are several options for how this could work in *C. thermocellum* [213, 215]. PEP synthase can convert PEP to pyruvate but its expression in the proteome is quite low [215] and no activity has been observed in cell-free extracts [213]. PEP could also be converted to pyruvate though a pathway that first involves conversion to oxaloacetate through PEP carboxykinase, which is expressed highly [215] and whose activity has been observed in cell-free extract, with

10-fold greater activity with GDP than ADP as cofactor [213]. From this point oxaloacetate could be converted directly to pyruvate via oxaloacetate decarboxylase, which is expressed [215] although no activity for this enzyme has been observed [214, 225]. Another route, known as the malate shunt, involves converting oxaloacetate first to malate, via malate dehydrogenase, and then to pyruvate via malic enzyme. Both of the malate shunt enzymes are expressed highly in the proteome [215], their activities have been observed in cell-free extracts [213], both the enzymes have been purified and characterized [214], and genetic knockouts are lethal (unable to obtain any mutants using gene deletion methods) [225], making this a likely route. The malate shunt involves a transhydrogenation reaction, oxidizing NADH and forming NADPH [214]. This is interesting in light of the fact that the oxidative portion of the pentose phosphate pathway, normally used for generating NADPH needed for biosynthetic reactions [215], appears to be absent in *C. thermocellum* [215, 226], and so the malate shunt might work in its place, providing a sufficient supply of NADPH for anabolism or other purposes [215]. The last option for converting PEP to pyruvate is an enzyme known as pyruvate phosphate dikinase (PPDK), which concomitantly converts adenosine monophosphate (AMP) and pyrophosphate (PP_i) to ATP and P_i. PPDK is expressed highly in the proteome [215] but PPDK activity in cell-free extracts has been reported to be either absent [213] or relatively low [225]. However, knocking out PPDK has been shown to shift end-product profiles, increasing hydrogen production compared to wild-type [213, 225], and isotope-tracer analysis suggests that approximately 66% of the flux from PEP to pyruvate is mediated by PPDK [225].

The reasons behind this atypical metabolism are not fully known. The ancestral glucokinase might have been a polyphosphate-dependant glucokinase that evolved over time to utilize various nucleotide triphosphates, including ATP and GTP [212, 227, 228]. Utilization of

the guanosine phosphates in addition to the adenosine phosphates may confer metabolic flexibility to the cell by allowing utilization of multiple cofactors [212], and may aid in regulation, as both the glucokinase and phosphoglycerate kinase of *C. thermocellum* display substrate inhibition at higher cofactor concentrations [222]. PPi appears to act as an important regulator in the cell, as determined by its use in various glycolytic reactions [213, 225], its activation of glucokinase, and its inhibition of malic enzyme [214, 222].

Use of PP_i by phosphofructokinase and pyruvate phosphate dikinase may confer an energetic advantage to the cell by increasing net ATP yield from glycolysis. PP_i is typically considered a waste product, produced by various anabolic reactions, which is usually hydrolyzed to inorganic phosphate and heat by intracellular phosphatases (which *C. thermocellum* does not possess [212]) to keep the levels of PP_i low, creating thermodynamically favourable conditions for those anabolic reactions [212, 229, 230]. It is generally assumed that by utilizing PP_i, which would have been otherwise "wasted", instead of ATP as a phosphate donor, as in the case of phosphofructokinase, the net yield of ATP from glycolysis increases by one for each PP_i-dependant reaction, greatly benefiting the cell [231, 232]. The use of AMP and PP_i by PPDK is also predicted to increase the net ATP yield from glycolysis by one [8, 231]. This is assuming that anabolism is able to completely supply the PP_i needed for these reactions, however, and calculations have shown that anabolism would only supply approximately 20% of the PPi needed for a PP_i-dependent phosphofructokinase glycolysis, not even taking into account the PPDK [213]. This means that the cell requires other mechanisms to produce the other ≥80% of PP_i needed for glycolysis. Two main possibilities exist, either glycogen cycling or a membrane-bound proton-translocating pyrophosphatase. Glycogen cycling, which has been observed in other cellulolytic bacteria [233, 234], ends up converting ATP and P_i to ADP and PP_i, and since

adenylate kinase can convert 2 ADP to ATP and AMP this could still increase net ATP yield from PPi-dependant glycolytic reactions by 0.5 [213]. The ATP increase from using the proton-translocating pyrophosphatase is more difficult to calculate as the number of protons translocated per reaction varies between species, as does the number of protons translocated per membrane-bound ATP synthase reaction, which would presumably be used to generate the initial proton motive force needed for the membrane-bound pyrophosphatase. Based on conservative estimates though, the net ATP yield increase from this system would be around 0.5 as well [8]. Thus, a PPi-dependant glycolysis would provide some energetic advantages to a cell. This system does have disadvantages though, including lower overall thermodynamic driving forces for the PPi-dependant reactions, and thus glycolysis as a whole. This means that inhibition of glycolysis by end-product buildup is much more likely and that a higher concentration of enzyme would be needed to maintain glycolytic flux [232, 235].

Although quite different than the canonical metabolism of yeast or *E. coli* the variant metabolism seen in *C. thermocellum* might be more common than originally thought, at least among related species [212]. For instance, an analysis of bacteria from various phyla found the presence of both ATP- and PP_i-dependant phosphofructokinases in several genera, including *Ruminiclostridium*, *Caldicellulosiruptor*, *Thermotoga*, and *Thermoanaerobacter* [146]. Proteomic analysis revealed higher expression of the PPi-dependant variant in some instances [129]. The presence of PPDK was observed in an even greater diversity of genera, including *Thermoanaerobacterium* [229], *Pyrococcus*, *Thermococcus*, and *Bacillus*, although almost all of the species studied also encoded a pyruvate kinase [146]. *C, thermocellum*-type metabolism has been observed in very distantly related microbes, such as the microaerophilic, non-mitochondrial, human parasite single celled eukaryotes, *Entamoeba histolytica* [236] and

Giardia lamblia [237, 238], including both PPi-dependant and even GTP-dependant enzymes, which may have been horizontally transferred, suggesting the strong benefits this type of metabolism possesses in anaerobic, fermentative environments [239].

1.5 Strategies for improving consolidated bioprocessing processes

1.5.1 Pre-treatment and cotreatment

One of the approaches to improve the efficiency of CBP is to use various means to modify the lignocellulosic substrate itself, after it has been harvested and collected, to increase its digestibility and susceptibility to enzymatic digestion, collectively referred to as pretreatment. Pretreatments fall into a few different categories, including biological, physical, chemical, and physicochemical (also called thermochemical), the majority belonging to the latter [16]. Biological pretreatment typically involves cultivating the substrate with lignin-degrading fungi, such as white-rot fungi, to remove the protective lignin coating in order to improve access to the underlying cellulose and hemicellulose [240]. Many of these fungi also degrade and utilize some of the cellulose fraction for their own growth, however, reducing the amount available for conversion to biofuels, and the delignification process can take weeks [16]. Purely physical pretreatment is predominantly mechanical comminution of the material, via chipping, grinding, or milling, that both disrupts the plant cell wall architecture and reduces the particle size, increasing access for enzymatic attack, but physical pretreatment can also include irradiation [241]. Various chemicals can be applied to alter lignocellulosic structure, including dilute acid, which can hydrolyze the hemicellulose, or even the cellulose at higher concentrations, to its monomeric substituents, and alkali, which can alter and/or solubilize the lignin fraction, cause swelling of the biomass, and decrease cellulose crystallinity [16].

Physicochemical pretreatments combine chemical treatments with various physical treatments to extensively modify the lignocellulose substrate [16, 241]. Arguably the simplest physicochemical treatment, Liquid Hot Water treatment, involves incubating the substrate in water at high temperatures (160-230°C), at which point the acetyl and uronic acid moieties on hemicellulose are cleaved, which then go on to further hydrolyze the hemicellulose and some of

the lignin. Addition of exogenous acid, as in the case of Dilute Acid treatment, increases the severity of the treatment and reduces residence times. Steam-explosion involves treating the substrate to high temperature and high-pressure steam for a few minutes, causing similar autohydrolysis as seen in Liquid Hot Water treatment. Alkali can be added to this explosion process, as in the case of Ammonia Fibre Explosion treatment, to increase lignin solubilization and cause swelling of the material. Twin-Screw Extrusion is a unique treatment that involves shearing and extruding the material into smaller particles with increased surface area via two rotating screws, usually in conjunction with alkali treatment [242]. Another common pretreatment, termed Organosolv treatment, involves heating the substrate at high temperatures in organic solvents, usually an alcohol, with the option of an acidic catalyst, that causes extensive lignin removal and almost complete hemicellulose solubilization. Ionic liquids represent a newer approach that involves using liquids made up entirely of cations and anions to completely dissolve the cellulose, which can then be selectively recovered and utilized [16].

A common disadvantage among many physicochemical pretreatments is that they produce degradation by-products, such as furan derivatives and phenolic compounds, that can inhibit growth and metabolism of fermentative microbes [243]. These inhibitors can be washed away from solid residue but if the aqueous fraction from the pretreatment also contains valuable hemicellulose sugars, detoxification can be difficult and costly [16, 244]. This has lead to the development of a physical treatment, reminiscent of the continuous mechanical disruption that occurs in cattle rumen [245], that involves continuously ball-milling the substrate during the course of fermentation, termed ball-milling cotreatment [14, 173, 246, 247]. This type of substrate treatment is well suited to the anaerobic cellulolytic bacteria, such as *C. thermocellum*, that are much better able to withstand the mechanical forces generated by this technique, perhaps

due to their thick cell wall, compared to either yeast or *E. coli* [246, 247]. Ball-milling cotreatment nearly doubled the carbohydrate solubilization of switchgrass by *C. thermocellum* [14], allowing total carbohydrate solubilization extents of up to almost 90% on switchgrass, poplar, and corn stover [173, 246, 247].

1.5.2 Co-culturing

Complete lignocellulose hydrolysis in nature occurs via large and diverse microbial communities, with important interplay between the suites of enzymes each microbe secretes [15] and between the sugars and metabolic products swapped between species [248]. Selecting certain lignocellulolytic species from these communities and designing custom consortia can offset the weaknesses each single species possesses, including *C. thermocellum*, and various such co-cultures have been created and studied, displaying numerous advantages and benefits over monoculture of *C. thermocellum* alone. Under certain conditions, including low pH due to acetic acid production, and inhibition of growth from other metabolite buildups, *C. thermocellum* solubilizes more cellulose than it can utilize, resulting in residual soluble cellodextrins left unused in the medium [249, 250]. Co-culturing *C. thermocellum* with saccharolytic microbes that can utilize cellodextrins and possibly grow at lower pH values allows greater cellulose utilization and conversion to biofuels [182, 251–254]. Many of these saccharolytic microbes have greater substrate utilization ranges than *C. thermocellum*, allowing the utilization of a greater diversity of the sugars found in lignocellulose, further increasing substrate conversion [252, 255–258]. Utilization of both the soluble cellodextrins and hemicellulose-derived sugars has the potential to increase solubilization of lignocellulose due to the inhibitory effects these sugars can have on hydrolysis [192, 193, 195] and metabolism [198]. Co-culturing can also

allow the sugars released by *C. thermocellum* to be converted to other valuable end-products, including acetate [259] and butanol [260–262]. Methanogenic co-culture partners can convert the CO_2 and H_2 produced by *C. thermocellum* to methane [263, 264]. The lower hydrogen concentrations, due to continuous uptake, then causes a thermodynamic shift, making hydrogen production more thermodynamically feasible, resulting in an increase in acetate production by *C. thermocellum* [263, 264]. The use of facultatively aerobic organisms in co-cultures can even allow the strictly anaerobic *C. thermocellum* to degrade lignocellulose under aerobic conditions, presumably due to the aerobes using up the oxygen to respire the sugars released from lignocellulose hydrolysis [265–267].

C. thermocellum is known to secrete small amounts of various metabolite intermediates, including pyruvate, malate, valine, alanine, and proline, among others [250]. These secreted metabolites make up larger proportions of the total secreted end-products of *C. thermocellum* as carbon loading increases [268]. A naturally occurring co-culture of *C. thermocellum* and *T. thermohydrosulfuricus* was isolated from volcanic soil and both strains were found to secrete growth factors required by the other partner for growth, and even enhanced cellulose hydrolysis in the presence of yeast extract [269]. It was later revealed that the *C. thermocellum* strain secreted 4 vitamins and 1 amino acid while the *T. thermohydrosulfuricus* strain secreted 4 other vitamins [270]. It has been noted in the literature that *C. thermocellum* is difficult to isolate into pure culture due to formation of very stable co-cultures, and lab cultures frequently become contaminated [159, 213, 271], possibly due to this propensity for *C. thermocellum* to secrete these various other metabolites and form symbiotic relationships with other microbes in its environment. This characteristic is valuable to designer co-cultures as well, as *C. thermocellum* could secrete growth factors required by the partners for growth (and possibly vice-versa),

lowering medium costs by eliminating the need for exogenous growth factors [270] and possibly acting as a control mechanism to regulate the growth of the various members [248].

1.5.3 Metabolic engineering

The development of genetic tools to modify the genome of certain strains of *C. thermocellum* [182] has lead to a vast array of mutant strains aimed at increasing its potential as a CBP candidate. Genetic modifications have included allowing co-utilization of xylose and cellobiose [199], butanol production [272, 273], and increased resistance to pretreatment inhibitors [274]. Most modifications have aimed at increasing ethanol production though, with the majority of these studies deleting genes in pathways that produce other end-products, which would otherwise divert carbon or electrons away from ethanol production, including acetate [180, 182, 184, 187], lactate [182–184, 186, 187], malate shunt [183], hydrogen [188], formate [187, 189], and combinations thereof. Surprisingly, while many of these deletion mutants do indeed stop producing the targeted product, they do not produce a corresponding increase in ethanol [180, 189] and require laboratory evolution to significantly increase ethanol production, as well as increase the growth-rate, which decreases in many of the deletion mutants [182, 184, 275]. An important mutation commonly found in these adapted strains is a point mutation in *adhE*, which encodes a bifunctional aldehyde dehydrogenase/alcohol dehydrogenase that is responsible for most of the ethanol production in *C. thermocellum* [276], which then allows the AdhE to also utilize NADPH as a cofactor, rather than only NADH [186, 187, 275]. As a result of these engineering efforts, ethanol yields have reached up to 70% [187] and 75% [275] of the maximum theoretical yield. Engineering efforts to increase ethanol production have also included both the addition of exogenous genes, including pyruvate kinase [183], pyruvate

carboxylase and alcohol dehydrogenase from either *Zymomonas mobilis* [277] or various other sources [278, 279], glyceraldehyde 3-phosphate dehydrogenase from *Thermoanaerobacterium saccharolyticum* [280], pyruvate ferredoxin oxidoreductase from *T. saccharolyticum* [281], and *adhE's* from various species [282], and the overexpression of endogenous genes such as *rnf* (ion-translocating reduced ferredoxin:NAD^+ oxidoreductase) [283] and glyceraldehyde 3-phosphate dehydrogenase [280]. One major obstacle discovered through all these engineering efforts was that while ethanol yield could be improved significantly, this only occurred at low carbon loadings, and as carbon loadings increased the yield decreased and the ethanol titre was only around 9.5 g/l from 50 g/l cellobiose, requiring further adaptive evolution [275]. Metabolomics and modelling studies have revealed that growth and fermentation of *C. thermocellum* under high ethanol titres is hindered by metabolic bottlenecks in glycolysis, including glyceraldehyde 3-phosphate dehydrogenase and PPi-dependent reactions that exist near thermodynamic equilibrium and are easily inhibited by increases in product concentrations [275, 284]. Thus, to truly realize the ethanolgenic potential of *C. thermocellum*, further study and understanding of central metabolism is required.

1.6. Conclusion

The world has an imminent need to transition away from climate change-causing fossil fuels to more environmentally friendly alternatives, including lignocellulosic biofuels. The anaerobic thermophile, *Clostridium thermocellum*, is a promising microbial platform for a consolidated bioprocessing approach to producing ethanol from lignocellulosic biomass. This is in part due to the complex, multi-enzyme cellulosomes it utilizes to degrade lignocellulose, conferring *C. thermocellum* with some of the best lignocellulose hydrolysis abilities found in nature. Despite this, the recalcitrant structure of raw lignocellulose hinders hydrolysis by *C. thermocellum*, and requires expensive and harsh physiochemical pretreatments to allow for complete depolymerization in order to approach economic viability. While *C. thermocellum* naturally produces ethanol, the low yields show much room for improvement and efforts to metabolically engineer higher yields have been hindered by its atypical PP_i-dependant glycolysis. Therefore, efforts aimed at either increasing raw lignocellulose hydrolysis in *C. thermocellum*-based processes or deepening understanding of the atypical metabolism of *C. thermocellum* would increase its viability for use in economical lignocellulosic biofuel production.

1.7 Thesis objectives

This project was roughly split into two parts, with the overall goal of better understanding *C. thermocellum* and how it interacts in co-culture in the hopes of ultimately increasing its potential as a CBP candidate.

The first part dealt with investigating co-cultures of *C. thermocellum* with hemicellulose-utilizing partners grown on lignocellulose substrates. **1. The hypothesis was that the hemicellulose partners would increase hydrolysis rates of raw lignocellulose due to either the synergism of their hemicellulose degrading systems with the cellulose-specializing system of *C. thermocellum*, and/or their uptake of inhibitory hemicellulose-hydrolysis products.** The specific objectives were as follows:

Chapter 2

Investigate monocultures of *C. thermocellum*, *C. stercorarium*, and *T. thermohydrosulfuricus*, dual-cultures of all combinations, and a tri-culture of all three species when grown on carbon-limiting amounts of wheat straw. Monitor end-product profiles, lignocellulose depolymerization, and species-specific growth patterns via qPCR.

Chapter 3

Utilize superior hydrolytic properties of triculture to investigate hydrolysis of various untreated and pretreated lignocellulose substrates. Determine whether biomass feedstock characteristics such as lodging resistance or harvesting time affect hydrolysis by the triculture.

Chapter 4

Determine whether cross-feeding of any growth factors is present in any of the co-cultures, or whether any growth factors are present in the lignocellulose substrates itself, and determine whether the presence or absence of these factors affects lignocellulose hydrolysis.

The second part dealt with investigating the central metabolism of *C. thermocellum* by characterizing the PPDK. **2. The hypothesis was that the gene Cthe_1308 in *C. thermocellums* genome encodes a functional PPDK, with possible allosteric regulation, and that understanding the activity of the PPDK will allow deeper understanding of the regulation and control of the glycolysis of *C. thermocellum*.**

Chapter 5

Purify and characterize the PPDK of *C. thermocellum* in terms of kinetic parameters and allosteric regulation.

Chapter 2: Enhanced depolymerisation and utilization of raw lignocellulosic material by co-cultures of *Clostridium thermocellum* with hemicellulose-utilizing partners*

* Contributing authors: Froese A[1], Schellenberg J[2], Sparling R[3] (2019). Can J Microbiol 65:296–307. https://doi.org/10.1139/cjm-2018-0535. Contributions: [1]Experiment design, experimental work, first author; [2]research guidance; [3]research guidance, manuscript editing

2.1 Abstract:

Clostridium thermocellum is one of the most promising candidates for consolidated bioprocessing of low-cost lignocellulosic materials to biofuels but still shows poor performance in its ability to deconstruct untreated lignocellulosic substrates. One promising approach to increase *C. thermocellum*s rate of hydrolysis is to coculture this cellulose-specialist with partners that possess synergistic hydrolysis enzymes and metabolic capabilities. We have created cocultures of *C. thermocellum* with two hemicellulose-utilizers, *C. stercorarium* and *Thermoanaerobacter thermohydrosulfuricus*, which both secrete xylanolytic enzymes and are able to utilize the pentose oligo- and monosaccharides that inhibit *C. thermocellums* hydrolysis and metabolism. When grown on milled wheat straw the cocultures were able to solubilize up to 58% more of the total polysaccharides compared to the *C. thermocellum* monoculture control. Repeated passaging of the cocultures on wheat straw yielded stable populations with reduced *C. thermocellum* cell numbers, indicating competition for cellodextrins released from cellulose hydrolysis, although these stabilized cocultures were still able to outperform the monoculture controls. Repeated passaging on Avicel cellulose also yielded stable populations. Overall, the observed synergism suggests that coculturing *C. thermocellum* with other members is a viable option for increasing the rate and extent of untreated lignocellulose deconstruction by *C. thermocellum* for CBP purposes.

2.2 Introduction:

Biofuels, such as ethanol and hydrogen, are becoming an increasingly attractive alternative to petroleum-based transportation fuels [7]. Second-generation biofuels are generated from lignocellulosic substrates, such as wheat straw and other agricultural and industrial residues and, in contrast to first-generation biofuels, do not require any additional arable land to produce [7]. Consolidated bioprocessing (CBP) offers a potentially low-cost method of producing biofuels by utilizing microbes capable of both hydrolyzing lignocellulosic polymers and fermenting the released mono- or oligomeric sugars [17, 43, 285]. Raw lignocellulosic biomass is composed of three main polymers: cellulose, hemicellulose, and lignin. Cellulose is generally the most abundant polymer and its linear structure allows formation of rigid fibrils made up of many individual cellulose chains that are then encased in hemicellulose and lignin, resulting in a complex and recalcitrant substrate [19]. This had led to the practice of pretreating the material with various physical, chemical, or biological methods to disrupt and/or remove this matrix and increase the susceptibility of the underlying cellulose to enzymatic attack [241]. These pre-treatments can be costly however [241] and the ideal process would be one involving microbes capable of high performance on untreated or minimally pretreated substrates.

One of the most promising candidates for CBP is *Clostridium thermocellum*, an obligate anaerobic thermophile, capable of producing ethanol, that possesses cell wall-bound protein complexes known as cellulosomes that depolymerize crystalline cellulose relatively quickly [99, 160, 161]. *C. thermocellum* is also able to hydrolyze the hemicellulosic portions of lignocellulosic material, resulting in mainly xylooligosaccharides [102, 286–288], but it is unable to grow on these or xylose as a carbon source [102, 191]. This results in wasted potential carbon sources. As well, these xylo-oligomers have been found to have inhibitory effects on cellulases

[192–194, 289], hemicellulases [195–197], and growth of *C. thermocellum* [198], slowing down the rate-limiting lignocellulose hydrolysis step of CBP.

Coculturing *C. thermocellum* with a xylo-oligomer-utilizing microbe could therefore help alleviate this inhibition and ultimately increase the rate and/or extent of lignocellulose depolymerisation, especially of untreated substrates that still contain high amounts of hemicellulose. While other studies have observed higher cellulose utilization rates in *C. thermocellum* cocultures compared to *C. thermocellum* monocultures at high cellulose loading [251, 252, 254, 259] there have been very few studies examining cocultures grown in batch on lignocellulosics under carbon-limiting conditions [251], where pH inhibition and other growth-limiting factors that could be affecting *C. thermocellum* do not take place. Therefore, we decided to study cocultures of *C. thermocellum* and two xylanolytic thermophiles, namely *Clostridium stercorarium* DSM 8532 and *Thermoanaerobacter thermohydrosulfuricus* DSM 26960 (Table 2.1) grown on untreated lignocellulosic substrates under carbon-limited conditions (typically ≤ 2.2 g/l, [215]). This limitation also ensured that the carbon left behind was indeed inaccessible to the hydrolytic enzymes expressed by the organisms. The relative levels of different species in cocultures throughout growth is expected to provide insight into the interactions of cocultures, and therefore our studies included qPCR as a measurement of the different species cell concentrations.

Table 2.1: Characteristics of lignocellulolytic coculture members

Strain	Polysaccharide hydrolysis		Enzyme system		Pentose utilization	References
	Cellulose	Hemi-cellulose	Cellulosome	Free-floating extracellular hydrolases		
Clostridium thermocellum DSM 1237	Yes	Yes	Yes	Yes	No	[127, 160, 290]
Clostridium stercorarium DSM 8532	Yes[a]	Yes	No	Yes	Yes	[127]
Thermoanaerobacter thermohydrosulfuricus DSM 26960	No	Yes	No	Yes	Yes	[135, 151]

[a]Significantly less cellulolytic activity compared to *C. thermocellum*

2.3 Material and Methods:

2.3.1 Strains and media:

Lyophilized cultures of *C. thermocellum* DSM 1237 and *C. stercorarium* DSM 8532 were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) while *T. thermohydrosulfuricus* DSM 26960 was isolated from a previous study [151]. *C. thermocellum and C. stercorarium* have been recently renamed to *Acetivibrio thermocellus* [76] and *Thermoclostridium stercorarium* subsp. *stercorarium* [94], respectively, but their long-standing names are used here for clarity. Cultures were maintained by adding 5 mL (10% v/v) inoculum to 45 ml of modified ATCC 1191 medium containing 2 g/L of filter-sterilized cellobiose. All experiments were performed in modified ATCC 1191 medium, containing (per litre of milliQ water): KH_2PO_4, 1.5; Na_2HPO_4, 3.35; NH_4Cl, 0.5; $MgCl_2$, 0.18; yeast extract, 2.0; L-cysteine, 1.0, as reducing agent; and 1 ml 0.025% w/v resazurin solution as oxygen indicator. pH was set to 7.2 through addition of 5 M NaOH. Experiments were performed in Balch tubes sealed with butyl rubber septums and contained 10 mL liquid medium and 17 mL headspace filled with 100% N_2 gas. All tubes underwent 4 cycles of 1 min gassing with N_2 followed by 1 min vacuum before autoclaving for 20 min at 121°C.

2.3.2 Experimental conditions

Air-dried wheat straw was obtained as a gift from Dr. Belay Ayele (University of Manitoba, department of Plant Science), ground using a hammer mill and sieved through a 0.5 mm mesh. The wheat straw was sent to Feeds Innovation Institute of the University of Saskatchewan (Saskatoon, Saskatchewan, Canada), where compositional information was determined by AOAC methods 930.15, 973.18, and 2002.04 [291]. The composition is as follows (% dry weight): cellulose, 42.8; hemicellulose, 26.9; lignin, 10.8; crude protein, 3.4; ash,

6.5. Avicel was obtained from Sigma-Aldrich Canada (Oakville, ON). Substrates were added to the medium during its preparation and maintained in suspension via a magnetic stir bar prior to dispensing. Autoclaving the medium with wheat straw was found to have no effect on the digestibility of the wheat straw (Fig. S1). Final concentrations of substrates were either 3.6 g/L (time-course experiment) or 2 g/L (passaging experiments). Precultures were grown on either 2 g/L cellobiose overnight (*C. stercorarium* and *T. thermohydrosulfuricus* monocultures) or 2 g/L Avicel cellulose for 3 days (*C. thermocellum*-containing monocultures and cocultures). During the passaging experiments the cultures were passaged (10% inoculum) once a week (all cultures, wheat straw; non-*C. thermocellum* cultures, Avicel) or once every 3 days (*C. thermocellum*-containing cultures, Avicel). The last passage (#9 for wheat straw, #7 for Avicel) in both experiments contained no added substrate. All experiments were performed once in triplicate.

2.3.4 End-product analysis

Gaseous end-products (H_2 and CO_2) were measured with a Varian micro-GC (Agilent, Mississauga, USA). Liquid end-products (cellobiose, glucose, xylose, lactate, formate, acetate, and ethanol) were measured using a high-performance liquid chromatography system (Waters, Massachusetts, USA) with an HPX-87H column (Bio-Rad, California, USA) and a refractive index detector (Waters, Massachusetts, USA), at a flow of 0.6 mL/min of 5 mM H_2SO_4, at a temperature of 45°C. Xylooligomers were measured according to [292] with modifications. 110 µl of 5% (w/v) H_2SO_4 was added to 110 µl culture supernatant and autoclaved for 20 min at 121°C to hydrolyze the xylooligosaccharides, and the resulting monomers were measured using HPLC. Total carbon in end-products was calculated as TCEP (mM hex. eqv.) = [lactate]/2 + ([acetate] + [ethanol])/3 + ([formate] + [CO_2])/6. Percent conversion of total polysaccharides for

each end-product was calculated, based on hexose equivalents, as "% conversion" = [end-product] * (# carbon atoms in end-product)/6 * (1/[cellulose + hemicellulose available (mM hex. eqv.)]). The end-products formed from growth on yeast-extract, only significant for *Thermoanaerobacter thermohydrosulfuricus*, were accounted and corrected for in all calculations shown, excluding Supplementary Figs. A.2.3 and A.2.5.

2.3.5 DNA extraction and qPCR

For DNA extraction, the culture tube was mixed and 1 ml of sample was taken, followed by centrifuging at max rpm on a benchtop centrifuge for 5 min, and the resulting pellet, including substrate and cells, was used for DNA extraction with InstaGene Matrix according to manufacturer's protocol (Bio-Rad, California, USA). qPCR was performed with fluorescent probe-based technology targeting species-unique regions of the *cpn60* gene (Supplementary Table A.2.1) [293]. Primer and probe sets were designed using both SigOli [294] and Beacon Designer (Premier Biosoft, Palo Alto, CA, USA). Primers were purchased from Invitrogen (Waltham, MA, USA) and dual-labelled 5'-HEX/FAM 3'-Black Hole Quencher probes from LGC Biosearch Technologies (Petaluma, CA, USA). qPCR reactions were performed in duplicate and contained, per reaction (11 µL total volume): 5.5 µL iQ Multiplex Powermix (Bio-Rad), 2 x 0.33 µL of 25 µM forward and reverse primers, 0.22 µL of 10 µM probe, (optional) second set of primers and probes, 1 µL template DNA, and dH_2O to 11 µL. Thermo-cycling was performed in a CFX Connect Real-Time System (Bio-Rad) and cycling conditions were: 95°C for 3 min., and 40 cycles of 95°C for 10 s, 56°C for 10 s, and 72°C for 30 s. Standard series were prepared for each species by determining the number of genome copies per mL, using calculations based on both total DNA concentration, from DNA extractions of concentrated cells,

and on known values for genome sizes and composition for each species, and creating 10-fold serial dilutions in water. Efficiencies calculated for the standard series for single and mixed reactions were within 90% and 110%. Each primer and probe set was tested against the other species DNA to confirm specificity.

2.4 Results:

2.4.1 Growth measurements on wheat straw

To determine the effect of coculturing two or more of the lignocellulolytic species all monocultures and all possible coculture pairings were grown in medium with 3.6 g/L wheat straw over six days and samples taken every 24 hours for end-product and cell concentration analysis. The total carbon found in the end-products (TCEP), which is the sum of all major end-products produced (see methods) and represents a measure of the overall ability of the cultures to convert available polysaccharides into end-products, is shown in Fig. 2.1. The *C. thermocellum* monoculture produced around 2.3 mM TCEP, which represents around 15% of the total polysaccharides (cellulose + hemicellulose content), or 26% of the polysaccharides available to *C. thermocellum* (only cellulose), since *C. thermocellum* is unable to utilize any sugars that may result from depolymerisation of the hemicellulose fraction. Surprisingly, the *C. stercorarium* monoculture, which has relatively low cellulolytic activity [127] but can utilize the hemicellulose sugars, performed as well as *C. thermocellum*, producing roughly the same amount of total end-products in a similar time frame. The *T. thermohydrosulfuricus* monoculture performed poorly, only producing 0.5 mM TCEP, which represents just 3% conversion of total polysaccharides.

In general, all of the cocultures performed better than any of their monoculture constituents. The *C. thermocellum* + *C. stercorarium* coculture was the best performing dual-culture, producing up to 4.4 mM TCEP after six days, corresponding to 28% of total

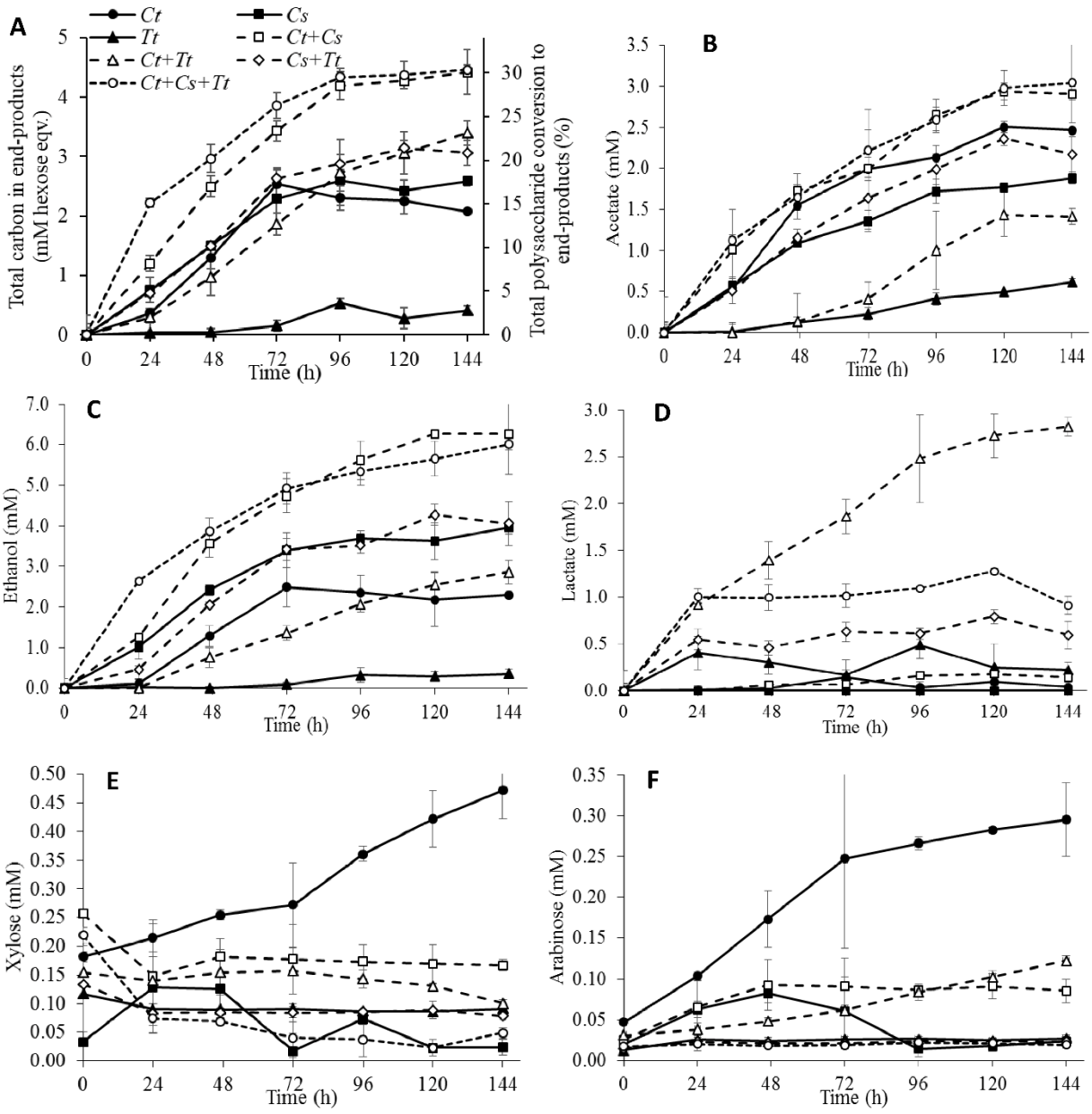

Fig. 2.1: Total carbon found in end-products (A), acetate (B), ethanol (C), lactate (D), xylose (E), and arabinose (F) production in all cultures grown on 3.6 g/l wheat straw. See Table 2.2 for species abbreviations.

polysaccharides conversion to end-products, representing almost a two-fold increase over either the *C. thermocellum* or *C. stercorarium* monocultures. In addition to greater extent of utilization, the *C. thermocellum* + *C. stercorarium* dual-culture also showed higher rates of end-product synthesis in the first two days of fermentation compared to the monocultures. The *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture also showed greater extent of conversion over the *C. thermocellum* mono-culture, with 22% total polysaccharide conversion, but had a slower rate during the first three days of fermentation. The *C. stercorarium* + *T. thermohydrosulfuricus* dual-culture showed only slight improvement in the extent of conversion over its monoculture counterparts, 19.5% total polysaccharide conversion compared to 16.5% in the *C. stercorarium* monoculture. The best performing culture of all was the *C. thermocellum* + *C. stercorarium* + *T. thermohydrosulfuricus* tri-culture, which had a similar total extent of conversion to the *C. thermocellum* + *C. stercorarium* dual-culture but had a much higher initial rate of conversion, especially in the first 24 hours.

While the acetate and ethanol production profiles (Fig. 2.1 B and C) have similar shapes among the cultures, with high initial production that gradually slows down, the lactate production (Fig. 2.1 D) varies significantly. While the *T. thermohydrosulfuricus* and *C. stercorarium* + *T. thermohydrosulfuricus* cultures produced small amounts of lactate, up to 0.8 mM, and mostly within in the first 24 hours, the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture produced substantially more lactate, around 2.8 mM over the entire experiment, suggesting that *T. thermohydrosulfuricus* utilizes a substantial portion of saccharides liberated by *C. thermocellum*. The tri-culture produced less lactate than the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture but more than the *C. stercorarium* + *T. thermohydrosulfuricus* dual-culture or the *T. thermohydrosulfuricus* monoculture, suggesting

significant competition between *T. thermohydrosulfuricus* and *C. stercorarium* for the sugars released by *C. thermocellum*. No accumulation of cellobiose or glucose was observed but small amounts of xylose (0.47 mM, Fig. 2.1 E) and arabinose (0.30 mM, Fig. 2.1 F) were found in the *C. thermocellum* monoculture supernatant.

The concentrations of the various end-products produced by each culture by the end of 144 hours is shown in Table 2.2. The *C. thermocellum* + *C. stercorarium* and *C. stercorarium* + *T. thermohydrosulfuricus* dual-cultures along with the *C. thermocellum* + *C. stercorarium* + *T. thermohydrosulfuricus* tri-culture all produced mostly ethanol and acetate, in a roughly 2:1 ratio, along with a small amount of lactate (2-6% of total end-product), which might indicate dominance of *C. stercorarium* in these cocultures due to the similarity of their end-product profiles. The *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture, on the other hand, produced relatively large amounts of lactate, amounting to 37% of the total end-products, which is a strong indicator for significant metabolism by *T. thermohydrosulfuricus*. End-point pH levels were equal to or greater than 6.57.

It should be noted that the results shown are corrected for end-products produced from growth on the yeast extract present in the medium. While both *C. thermocellum* and *C. stercorarium* only produce trace quantities of end-products from growth on yeast extract, *T. thermohydrosulfuricus* monocultures produce considerable amounts, up to 1.3 mM TCEP (Table 2.2). This can interfere with determining growth derived from actual depolymerisation of the lignocellulose and therefore all *T. thermohydrosulfuricus*-containing cultures were corrected for TCEP produced (1.3 mM hexose eqv.) by *T. thermohydrosulfuricus* from growth on the yeast extract present in the medium.

Table 2.2: Metabolite concentrations at the end of fermentation (144h) for all seven experimental cultures grown on 3.6 g/l wheat straw.

Culture	Metabolites produced, mM (% of total end-products[a])						TCEP[b] (mM hex. eqv.)
	Formate	Lactate	Acetate	Ethanol	CO_2	H_2	
Ct	0.38 (3)	0.04 (1)	2.47 (35)	2.30 (33)	3.97 (28)	4.47	2.33
Cs	0.11 (1)	0.00 (0)	1.88 (24)	3.96 (50)	3.98 (25)	3.45	2.63
Tt	0.00 (0)	0.22 (24)	0.61 (52)	0.33 (28)	0.45 (19)	0.90	0.39
Ct+Cs	0.07 (0)	0.14 (2)	2.91 (22)	6.27 (47)	7.67 (29)	5.73	4.42
Ct+Tt	0.00 (0)	2.82 (40)	1.40 (14)	2.83 (29)	4.06 (21)	4.04	3.30
Cs+Tt	0.00 (0)	0.59 (9)	2.16 (25)	4.03 (46)	4.68 (27)	3.93	2.94
Ct+Cs+Tt	0.00 (0)	0.91 (10)	3.03 (23)	5.99 (46)	6.49 (25)	5.03	4.34
Tt (uncorrected)[c]	0.11	0.97	1.32	1.11	2.43	2.25	1.72

[a]% of total end-products calculated on a mM carbon basis.

[b]TCEP: Total carbon found in end-products, calculated as described in methods.

[c]*Tt* (uncorrected) represents end-products resulting from growth on the yeast extract present in the medium; these values are accounted for in all other calculations.

Note: *Ct, Clostridium thermocellum*; *Cs, Clostridium stercorarium*; *Tt, Thermoanaerobacter thermohydrosulfuricus*

The cell concentration profiles over the course of fermentation for all cultures, based on qPCR measurements of genomic copies of *cpn60*, are shown in Fig. 2.2. *C. thermocellum* grew to lower maximum cell densities in the cocultures compared to monoculture, indicating competition for saccharides derived from the cellulosic portion. *C. thermocellum*'s growth profile was significantly different in the tri-culture compared to any of the other cultures, where it appeared to actually decrease in cell density over the first 24 hours while slow growth over four days was observed in the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture. Minimal or no growth of *T. thermohydrosulfuricus* was observed in the *C. stercorarium* + *T. thermohydrosulfuricus* dual-culture while immediate and substantial growth over the first day was observed in the tri-culture, also corroborated by the initial production of lactate in this culture (Fig. 2.1 D). Maximum cell concentrations for *T. thermohydrosulfuricus* were slightly higher in the *C. thermocellum*-containing cocultures compared to the *T. thermohydrosulfuricus* monoculture and the *C. stercorarium* + *T. thermohydrosulfuricus* coculture.

2.4.2 Multiple passages in medium with wheat straw

The initial experiments described above were performed using co-culture precultures that had been grown on Avicel cellulose. To investigate whether the apparent carbon source competition, indicated by the dominance of certain species over others in the cocultures, would eventually result in the complete disappearance of the less-competitive members, all the cultures were grown in medium containing 2 g/L wheat straw and sub-passaged once a week (10% inoculum) for 8 weeks. This time was selected because it appears to correspond to a time after substrate solubilization had mostly ceased (Fig. 2.1). A final ninth passage was performed in medium with no added wheat straw to confirm whether the different species, especially *T.*

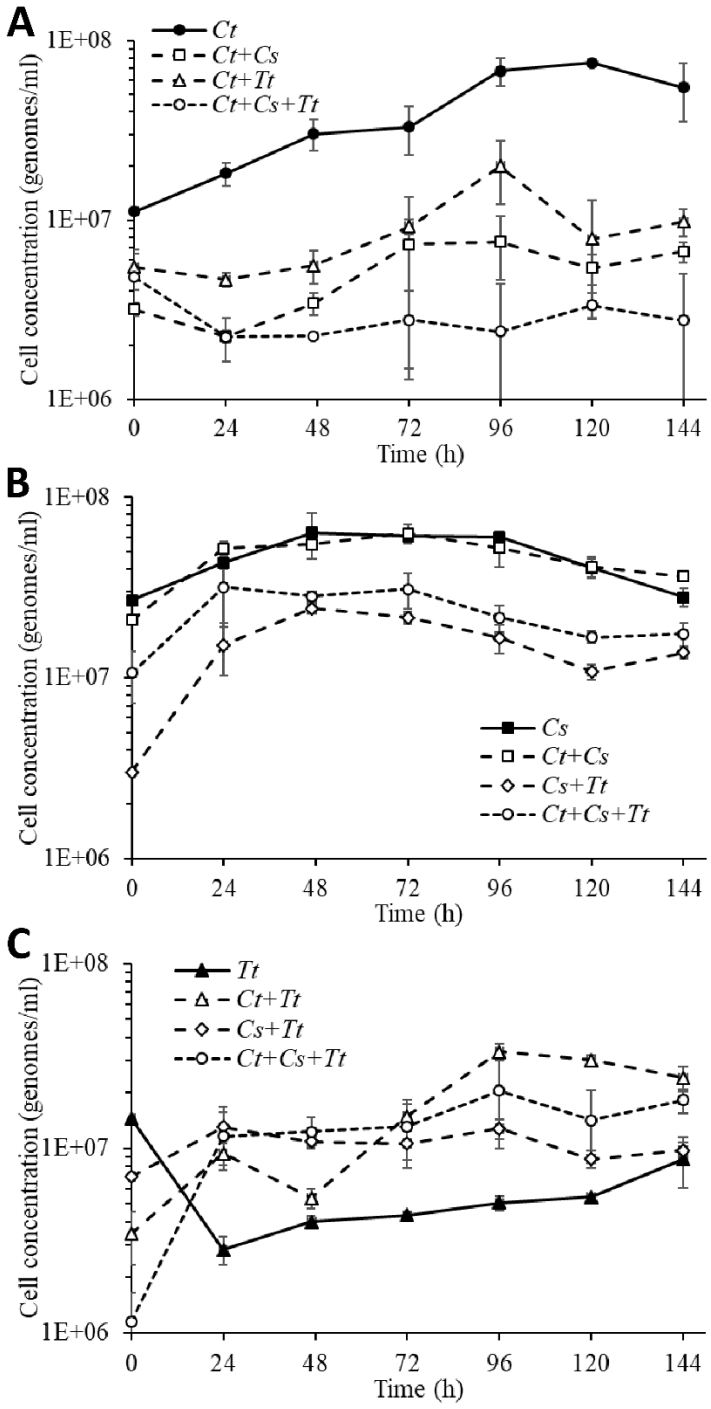

Fig. 2.2: Cell concentrations of *C. thermocellum* (A), *C. stercorarium* (B), and *T. thermohydrosulfuricus* (C) in mono- and different cocultures grown on 3.6 g/l wheat straw.

See Table 2.2 for species abbreviations.

thermohydrosulfuricus, were primarily utilizing the wheat straw or the yeast extract present in the medium. Although it is possible that some mutations could have occurred during this timeframe (approximately 27 generations in total), it is unlikely that significant changes occurred, with adaptive evolution experiments of *C. thermocellum* usually requiring many more generations [182, 275, 295].

The cell concentrations for each species at the end of the first, eighth, and last passages are shown in Fig. 2.3, although all coculture populations had stabilized by passage 5 (Supplementary Fig. A.2.2). The monoculture control populations all vary little throughout the passaging experiment. *C. thermocellum* cell concentrations stabilized at levels 0.19-fold, 0.40-fold, and 0.09-fold of the monoculture control numbers in the *C. thermocellum* + *C. stercorarium*, *C. thermocellum* + *T. thermohydrosulfuricus*, and *C. thermocellum* + *C. stercorarium* + *T. thermohydrosulfuricus* cocultures, respectively, but never washed out, indicating it remained an important component of these co-cultures. *C. stercorarium* levels remained fairly stable and the dominant species in every culture, and was especially high in the *C. thermocellum* + *C. stercorarium* dual-culture, reinforcing its role as a strong competitor for soluble sugars. Both *C. thermocellum* and *C. stercorarium* levels dropped approximately 10-fold between the eighth and ninth passages in all the cultures, corresponding to the 1 in 10 dilution from the inoculum and confirming that all growth was derived from wheat straw. The *T. thermohydrosulfuricus* levels were very similar between the eighth and ninth passages for the *T. thermohydrosulfuricus*, *C. stercorarium* + *T. thermohydrosulfuricus*, and *C. thermocellum* + *C. stercorarium* + *T. thermohydrosulfuricus* cultures, strongly suggesting that, after stabilizing, most or all of the *T. thermohydrosulfuricus* growth was derived from the yeast extract and not the wheat straw. *T. thermohydrosulfuricus* levels in the *C. thermocellum* + *T.*

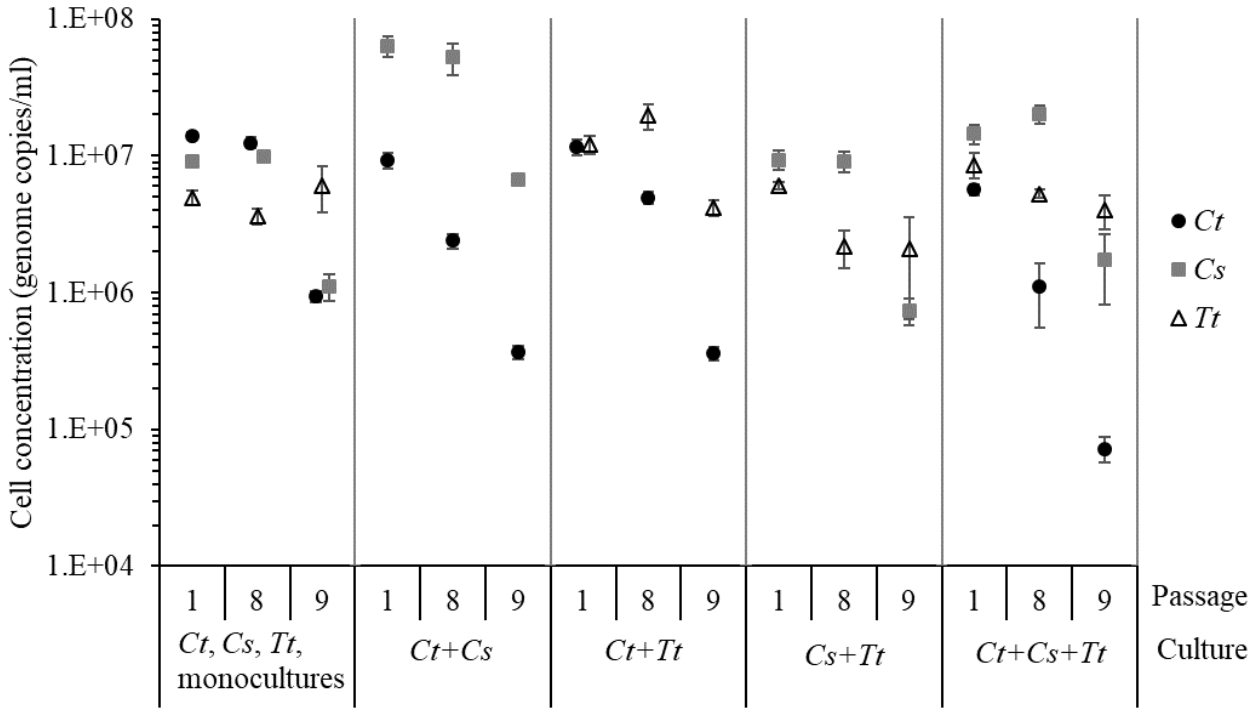

Fig. 2.3: Cell concentrations for each species in every culture at the end of passage 1, 8, and 9, when passaged once a week in medium containing 2 g/l wheat straw. The ninth passage contained no wheat straw. See Table 2.2 for species abbreviations.

thermohydrosulfuricus dual-culture, however, were significantly higher, indicating that *T. thermohydrosulfuricus* can utilize saccharides derived from hydrolysis by *C. thermocellum*, but not when competing with *C. stercorarium* for these same carbon sources.

The end-product concentrations at the end of each passage were measured to determine if the metabolite profiles varied over the multiple passages. The results from the first and eighth passage are presented in Fig. 2.4 in terms of how much of the total polysaccharides (cellulose + hemicellulose content) they represent. All values shown have had their ninth passage (yeast extract control) values subtracted from them; uncorrected values are available in Supplementary Fig. A.2.3. In contrast to the growth curve experiment, performed with 3.6 g/l wheat straw, in these experiments, using only 2 g/l wheat straw, *C. thermocellum* now produced more end-products (17.8% of total polysaccharides conversion) than *C. stercorarium* (13.5%), possibly due to lower amounts of the inhibitory pentose sugars released. There is no significant change in any of the monoculture's end-product profiles between passages, other than the elimination of the already low levels of lactate production in the *C. thermocellum* and *C. stercorarium* cultures. There is virtually no change in the *C. thermocellum* + *C. stercorarium* dual-culture and an increase in ethanol production in the *C. stercorarium* + *T. thermohydrosulfuricus* dual-culture between the first and last passages. The *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture shows the most changes, with a substantial 12-fold decrease in lactate, a small increase in acetate, and a significant increase in ethanol. A 33% drop in ethanol production was observed in the tri-culture. All the *C. thermocellum*-containing cocultures produced the most end-products within their first passage and then stabilized at a lower level of end-product production after a few passages, with the largest drop occurring in the tri-culture. This decrease in conversion rates

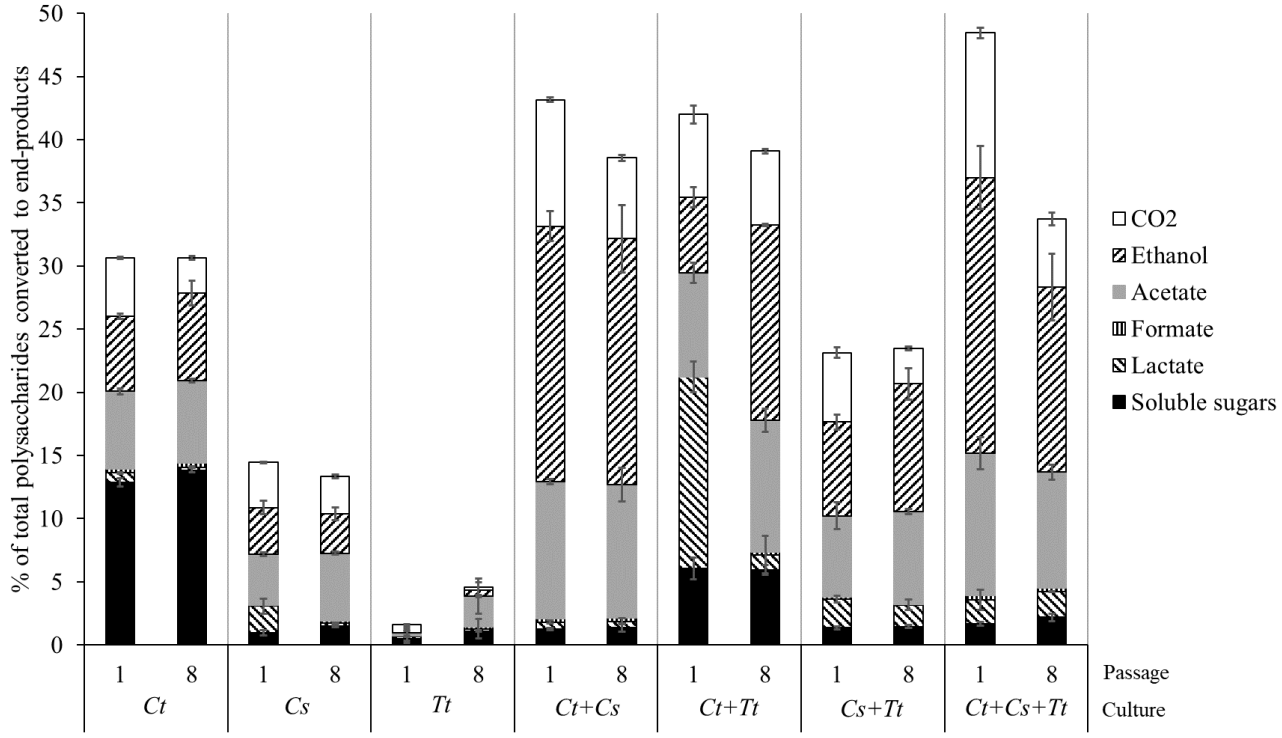

Fig. 2.4: Metabolite profiles for each culture at the end of passages 1 and 8 when passaged once a week in medium containing 2 g/l wheat straw. See Table 2.2 for species abbreviations.

is correlated with lower *C. thermocellum* cell concentration in the later passages (Fig. 2.3), confirming its role as the main driver of lignocellulose hydrolysis.

As *C. thermocellum* produces mainly xylose oligomers from the hydrolysis of hemicellulose [102, 287] we hydrolyzed all oligosaccharides in the supernatant to monomers (see methods) before measuring their concentration via HPLC. This is represented in Fig. 2.4 as "soluble sugars", which generally consisted of around 92% xylose and 8% arabinose monomers. This revealed that, in monoculture, *C. thermocellum* was able to hydrolyze approximately 37% of the available hemicellulose to soluble xylo-oligomers. The levels of xylo-oligomers were quite low in the other mono- and cocultures, presumably being taken up and used by both *C. stercorarium* and *T. thermohydrosulfuricus*, except in the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture, where approximately 17% of the available hemicellulose existed as soluble oligomers in the supernatant. This is somewhat surprising considering *T. thermohydrosulfuricus*'s ability to utilize xylan [135], hemicellulose-derived monomeric sugars [151], and xylose simultaneously with cellobiose [157]. Measuring total polysaccharide solubilization by summing the carbon found in end-products and soluble oligosaccharides (Fig. 2.4) reveals that *C. thermocellum* is able to solubilize much more of the total polysaccharides, around 30%, compared to *C. stercorarium* (13.4%) or *T. thermohydrosulfuricus* (4.6%). After stabilizing, the *C. thermocellum* + *C. stercorarium* (38.5% total polysaccharide conversion), *C. thermocellum* + *T. thermohydrosulfuricus* (39.1%), and *C. thermocellum* + *C. stercorarium* + *T. thermohydrosulfuricus* (33.7%) cocultures were still able to outperform the *C. thermocellum* monoculture, even with significantly lower *C. thermocellum* cell concentrations (Fig. 2.3), suggesting that the *C. thermocellum*-containing coculture members are acting synergistically to

degrade and solubilize more of the untreated lignocellulosic substrate than *C. thermocellum* by itself.

2.4.3 Multiple passages in medium with Avicel cellulose

To further investigate the stability of the cocultures and test whether growth on a single carbon source would increase competition and lead to washout or a significant decrease in any member we passaged all the cultures in medium containing 2 g/l Avicel cellulose as the only carbon source for six weeks and measured the cell concentrations for each species at the end of the first and sixth passage (Fig. 2.5), at which point they were relatively stable (Supplementary Fig. A.2.4). It is important to note that Avicel can be contaminated with small amounts of other sugars, including around 2 % xylose [296], which could contribute slightly to the growth of *C. stercorarium* and *T. thermohydrosulfuricus*. This experiment also included a final passage in medium with no added carbon source to control for growth from yeast extract, as was done in the previous experiment. In general, *C. thermocellum* grew to higher cell concentrations, roughly 40-fold, than when grown on 2 g/l wheat straw (Fig. 2.3), and cell concentrations were similar in all passages and all cultures, apparently growing unhampered in any of the cocultures. *C. stercorarium* alone grew to similar densities compared to growth on wheat straw, but was able to grow to higher numbers, roughly 15-fold, in the *C. thermocellum*-containing cocultures, in agreement with the strong competitiveness shown in the previous experiment. The cell concentrations of *T. thermohydrosulfuricus* in monoculture are not significantly higher than the passage 7 control, in agreement with the fact that it cannot grow on cellulose [135, 151]. *T. thermohydrosulfuricus* though, similar to *C. stercorarium*, was able to grow to higher densities, >5-fold, when cocultured with *C. thermocellum*. Overall the cocultures were relatively stable and

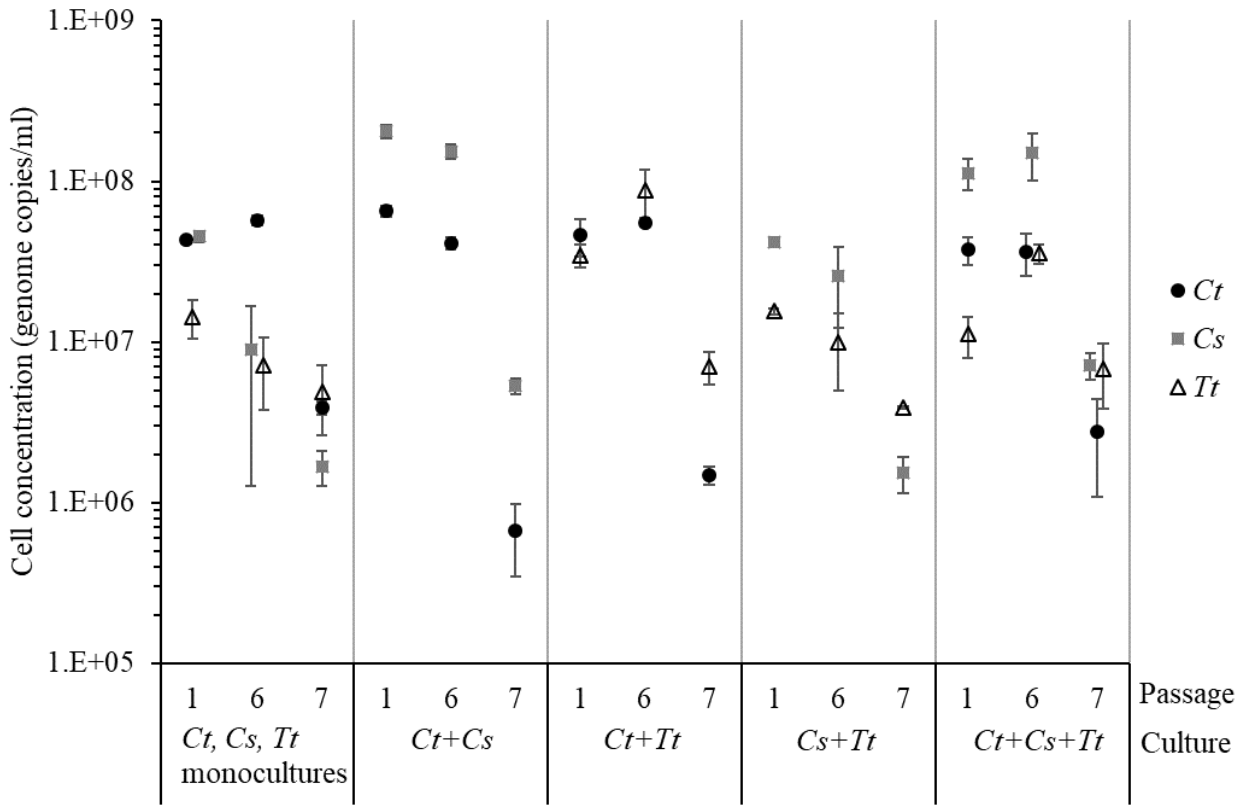

Fig. 2.5: Cell concentrations for each species in each culture at the end of passages 1, 6, and 7 when passaged once a week in medium containing 2 g/l Avicel. The seventh passage contained no Avicel. See Table 2.2 for species abbreviations.

all the members were successfully able to compete for a substantial portion of the saccharides released, which was mainly hydrolyzed by *C. thermocellum*.

End-product concentrations at the end of the passages were also measured and corrected for the last passage yeast extract control values (Figs. 2.6 and Supplementary Fig. A.2.5). The end-product profiles for most of the cultures were fairly stable. Exceptions were the *C. stercorarium* monoculture, which was only producing very small amounts of end-products by the last few passages, and the *C. stercorarium* + *T. thermohydrosulfuricus* dual-culture, which also showed decreased end-product levels but surprisingly was still producing more than the *C. stercorarium* monoculture. Lactate production was especially high in the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture, which also had the highest cell concentrations of *T. thermohydrosulfuricus* (Fig. 2.5), while the tri-culture had the highest ethanol:acetate ratio, around 3.4:1. *C. thermocellum* produced fewer end-products (Fig. 2.6) and less biomass (Fig. 2.5) than the *C. thermocellum* + *C. stercorarium* and *C. thermocellum* + *C. stercorarium* + *T. thermohydrosulfuricus* cocultures, although equivalent amounts of cellulose were consumed based upon visual inspection for overt cellulose particles, indicating an incomplete carbon recovery for *C. thermocellum*. *C. thermocellum* has been shown to secrete considerable amounts of amino acids under certain conditions [250, 268], which may be occurring here undetected.

2.5 Discussion:

Although *C. thermocellum* is one the best known anaerobic lignocellulose degraders [99, 161] and generally performs better than current fungal enzyme technologies [14, 174], solubilization rates of untreated lignocellulosic material remain below economic viability [14, 102, 297]. This was also observed in the present study, where it was only able to solubilize and convert approximately 26% of the cellulosic portion of the substrate (17% of total

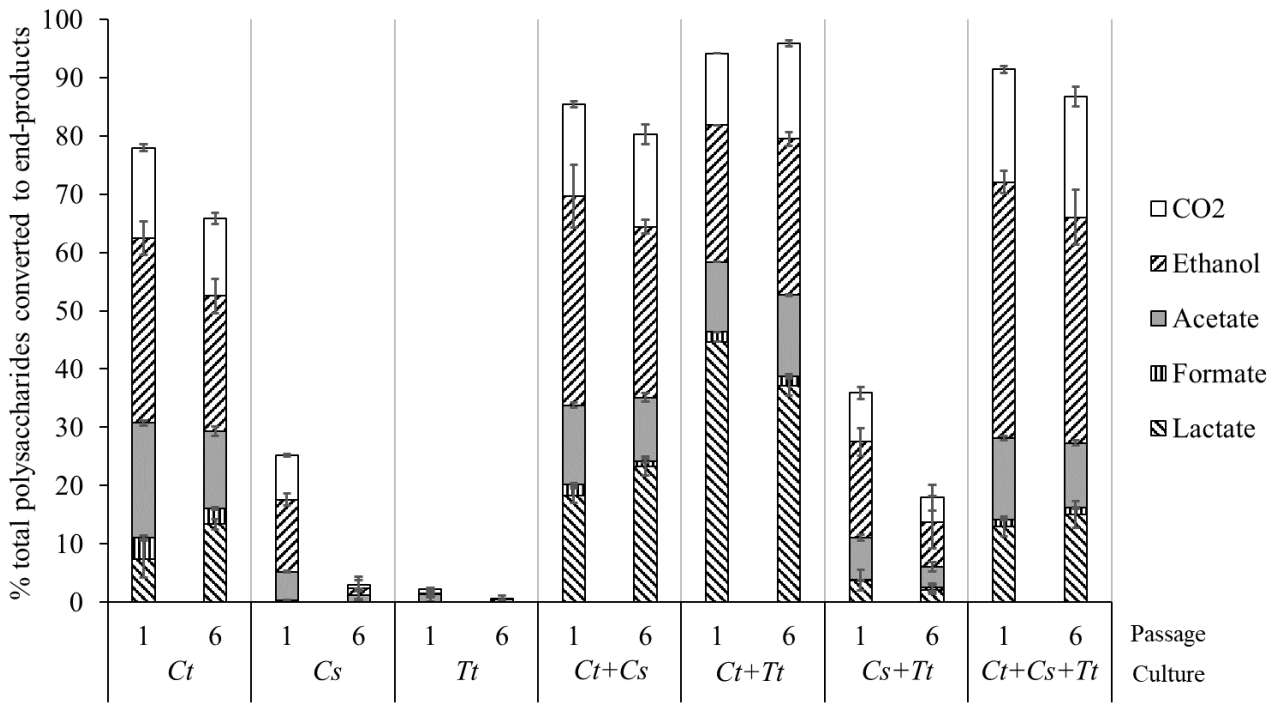

Fig. 2.6: Metabolite profiles for each culture at the end of passages 1 and 6 when grown on 2 g/l Avicel cellulose. See Table 2.2 for species abbreviations.

polysaccharide) to end-products and 37% of the hemicellulosic portion to soluble oligo- or monosaccharides, with 31% total polysaccharide solubilization of milled wheat straw after a week-long incubation. Unlike *C. thermocellum*, which is considered more of a cellulose-specialist [127], *C. stercorarium* is considered as more of a hemicellulose-specialist as it can grow very well on hemicellulose, and only secretes a few cellulases that allow it to grow relatively slowly on crystalline cellulose [127]. Few studies have looked at the performance of *C. stercorarium* strains on actual lignocellulosic material. However, those that have found modest performance comparable to *C. thermocellum*, at least in terms of total end-product production [111, 128, 298], which we also found, with *C. stercorarium* converting 13.4% of total polysaccharides to end-products. As *C. stercorarium* is able to breakdown and ferment both cellulose and hemicellulose, a separate analysis for the cellulose and hemicellulose breakdown is not available as it is for *C. thermocellum*. Although *T. thermohydrosulfuricus* does not possess any cellulases it does secrete xylanases and can grow on xylan derived from beechwood [135]. In our study however, *T. thermohydrosulfuricus* performed poorly in monoculture on lignocellulosic substrates and was only able to convert 4.6% of total polysaccharides (13.8% of hemicellulosic portion) to end-products.

A significant increase in the amount of substrate solubilized and converted to end-products (and sugars) was observed in all the *C. thermocellum*-containing cocultures compared to the *C. thermocellum* monoculture (Fig. 2.1 A and 2.4), even when the cell numbers of *C. thermocellum* in the cocultures were substantially lower than the monoculture control (Fig. 2.2 and 2.3). One possible reason for this could be that the hydrolytic enzymes secreted by *C. stercorarium* and *T. thermohydrosulfuricus* are acting in a synergistic manner with the cellulosomes and secreted enzymes of *C. thermocellum*, allowing faster depolymerisation, and to

a higher extent, of the cellulose and hemicellulose matrix. An additional reason could be that *C. stercorarium* and *T. thermohydrosulfuricus* are utilizing the pentose oligo- and monosaccharides that result from the breakdown of hemicellulose and inhibit *C. thermocellum*'s hydrolysis [194, 196] and growth [198]. Previous studies have observed increases in cellulose consumption in cocultures of *C. thermocellum* with another saccharolytic partner [251, 254] but these were performed under high carbon loadings where *C. thermocellum*'s growth has ceased before all the hydrolysis products are consumed. Numerous studies have found increases in total end-product production in cocultures of *C. thermocellum* with various partners when grown on actual lignocellulosic substrates [251, 252, 255] but total sugars, including xylo-oligomers, were not measured and thus total substrate solubilization data are not readily available. This study shows an actual increase in the total amount of substrate polysaccharides solubilized, as measured by total end-products and soluble sugars, unrelated to cellulose breakdown products, by coculturing with either *C. stercorarium* and/or *T. thermohydrosulfuricus.*

The relatively slower growth observed for *C. thermocellum*, compared to either *C. stercorarium* or *T. thermohydrosulfuricus*, on wheat straw is not surprising as cellulose, the only portion of the material that can be utilized by *C. thermocellum*, is depolymerized at a slower rate compared to hemicellulose [43]. The lower levels of *C. thermocellum* in the cocultures grown on wheat straw, compared to the monoculture control, and the higher levels of *C. stercorarium* and *T. thermohydrosulfuricus* in the *C. thermocellum*-containing cocultures grown on cellulose, compared to their respective monocultures, strongly suggest that significant competition for cellodextrins exists between *C. thermocellum* and the other components of the co-cultures. The levels of *C. thermocellum* in the cellulose-grown cocultures were similar compared to the monoculture however, which would not be expected with cellodextrin competition, and remains

unexplained. This competition appears to be negatively affecting overall hydrolysis of the lignocellulosic substrate however, as lower total solubilization is observed in the cocultures in the later passages (Fig. 2.3 and 2.4) that have reduced *C. thermocellum* levels. This suggests that *C. thermocellum* contributes the most to the substrate solubilization and that the ideal coculture partner would be one that does not compete with *C. thermocellum* for cellodextrins. Even though *C. thermocellum* releases cellulose hydrolysis products into the medium where *C. stercorarium* and *T. thermohydrosulfuricus*, both not known to physically attach to cellulose, can take them up, the concentration of *C. thermocellum* never diminishes to the point that it washes out of the cocultures. This indicates that *C. thermocellum* is still able to take-up enough of the released cellodextrins, most likely due to its proximity to the released products, to survive and reproduce.

We have developed cocultures, containing *C. thermocellum* and one or two xylanolytic partners, which were shown to solubilize and utilize more of the total polysaccharides present in the untreated lignocellulosic substrate than the *C. thermocellum* monoculture. This enhancement was observed even when levels of *C. thermocellum* were reduced, indicating significant synergism between the coculture members with respect to their ability to solubilize the cellulose and hemicellulose fractions. The reduction in *C. thermocellum* levels occurred after passaging the co-cultures numerous times and allowing the populations time to find their own equilibrium. There has been at least one other report in the literature that shows a slight decrease in the amount of cellulose degraded by an *C. thermocellum*-containing coculture when passaged multiple times [254]. Thus, optimizing co-culture CBP processes for maximal lignocellulose degradation might involve using freshly prepared and mixed co-cultures with defined cell numbers rather than using an inoculum from a previous batch run. This study shows that

cocultures represent a possible avenue to help increase the rate-limiting hydrolysis of lignocellulosic material by *C. thermocellum* for consolidated bioprocessing.

Chapter 3: Digestibility of wheat and cattail biomass using a coculture of thermophilic anaerobes for consolidated bioprocessing*

* Contributing authors: Froese AG[1], Nguyen T-N[2], Ayele BT[3], Sparling R[3] (2020). BioEnergy Res 13:325–333. https://doi.org/10.1007/s12155-020-10103-0. Contributions: [1]Experiment design, experimental work, first author; [2]Feedstock growth, harvesting, and processing; [3]experiment design, research guidance, manuscript editing

3.1 Abstract:

A consolidated bioprocessing (CBP) approach utilizing a synergistic coculture of three lignocellulolytic bacteria (*Clostridium thermocellum, C. stercorarium,* and *Thermoanaerobacter thermohydrosulfuricus*) was compared to the monoculture controls using a variety of different lignocellulosic substrates. In addition to increasing the average ethanol:acetate:lactate ratio from 49:45:6 in the *C. thermocellum* mono-culture to 66:26:7 in the triculture, the amount of total polysaccharides utilized in the triculture relative to the monocultures was increased in all untreated and pretreated substrates tested, with up to 3-fold increases observed. Under relatively low carbon loads of 2 g/l, the highest ethanol titre achieved was 0.298 g/l, corresponding to a yield of 0.149 g ethanol/g substrate. The coculture was then used to screen the digestibility of different cattail (*Typha spp.*) species harvested in different seasons as well as wheat straw samples from different cultivars that vary in their resistance to lodging. Cattail plants harvested in July, contained less cellulose and lignin and were up to 80% more digestible than those harvested in September. Although moderate differences in wheat straw digestibility were observed, this variation did not significantly correlate with differences in lodging resistance or cellulose, hemicellulose, and lignin contents. Our study suggests the importance of screening feedstocks using CBP approaches in order to determine the attributes that affect digestibility with CBP.

3.2 Introduction

Second-generation biofuels are derived from lignocellulosic feedstocks and can provide a greener alternative to petroleum-based fuels while utilizing various inexpensive or "waste" biomass from different sources [7, 12]. Flax and hemp are both multi-use plants, grown for their seeds and fibre, and the straw left over from processing of these plants represents possible feedstocks for these biofuels [299, 300]. Wheat straw is an abundant low-cost agricultural residue, with over 850 Tg (850 million tons) produced worldwide annually, some of which is otherwise burned on the field and wasted [301]. It has comparable overall lignocellulosic composition to other crop residues [43] and shows potential for use as a feedstock in biofuel production [301]. There are many different commercial wheat cultivars available and these cultivars can show considerable variation in the degree to which their straws are able to be depolymerized by lignocellulolytic enzymes into their fermentable constituent sugars [302–304].

Cattails (*Typha spp.*) are perennial, prolific, highly adaptable, fast-growing plants that grow preferentially in wetlands and marginal crop lands [305], furthermore, they are arising as promising phytoremediation candidates since they are reported to accumulate unwanted compounds such as heavy metals [306] and excess nitrogen and phosphorous [305]. Harvesting, which can occur while the plants are still green and growing [307], can permanently remove those compounds from the ecosystem and the biomass collected can potentially be used as a lignocellulosic feedstock for bioethanol production [308]. If the cattail is harvested primarily for heavy metal phytoremediation purposes, however, the high concentrations of heavy metals may interfere with bacterial metabolism and this problem would need to be addressed. It is currently unknown to what extent the degradability of the biomass of cattail plants is affected by variation in species or harvesting time.

The most common methods to produce lignocellulosic ethanol involve costly pretreatments of the substrates with physical and/or chemical methods, hydrolyzing the substrate with commercially available fungal cellulases and hemicellulases, followed by fermentation of the released sugars by high ethanol-yielding yeast or *Escherichia coli* [7, 309]. An alternative approach, known as consolidated bioprocessing (CBP), consolidates the (hemi)cellulase enzyme production, substrate saccharification, and sugar fermentation steps into a single process or reactor, offering a simpler and potentially more cost-effective method of producing lignocellulosic biofuels [17]. CBP utilizes either monocultures of microbes capable of both producing their own (hemi)cellulases and fermenting the saccharides to ethanol or other biofuels, or cocultures where the depolymerization and fermentation tasks might be split between the various members [17]. A major advantage of CBP is the higher degree of lignocellulose solubilization observed with the use of CBP enzymes and microbes compared to fungal enzyme cocktails [14, 170, 174, 310]. Other advantages of CBP include the cost-savings realized from the use of only a single vessel and the absence of dedicated cellulase production, which help place CBP as a promising lower-cost alternative to, and logical next step of, current fungal enzyme and yeast technologies [7, 17, 43].

The two main approaches to CBP involve starting with either bacteria [14, 311] or fungi [62, 312] that naturally have high lignocellulose deconstruction abilities and at least some ethanol production or starting with bacteria [49, 50] or yeast [313, 314] that naturally have high ethanol yields and engineering them for lignocellulose deconstruction (Table 3.1). The cellulosomes of certain cellulolytic, anaerobic bacteria, including some of the most promising CBP candidates, deconstruct lignocellulose in a different manner than fungal cellulase systems [39, 315] and thus are affected differently by different lignocellulosic biomass characteristics

Table 3.1: Consolidated bioprocessing reports on various untreated and pretreated lignocellulosic substrates

Substrate	Pretreatment	Substrate load (g/L)	Organism(s)	Polysaccharide utilization (%)	Ethanol yield (g/g substrate)	Ethanol titre (g/L)	Ref.
Cattail	-	2	*Ct+Cs+Tt*[a]	24.9	0.056	0.11	This study
Hemp	-	2	*Ct+Cs+Tt*	25.8	0.087	0.17	This study
Wheat straw	-	2	*Ct+Cs+Tt*	37.2	0.119	0.24	This study
Wheat straw	-	10	mixed culture TMC7	44.2	-	-	[316]
Wheat straw	-	6.67	mixed culture XDC-2, aerobic	25.0	-	-	[317]
Wheat straw	-	4.7	*Thermoanaerobacterium thermosaccharolyticum* M18	59.2	0.025	0.12	[130]
Wheat straw	-	10	mixed culture TC-5, anaerobic	35.3	-	-	[318]
Wheat straw + bran (10:1)	-	110	*Fusarium oxysporum* 11C	-	0.080	8.80	[312]
Switchgrass (mid-season)	-	14.3	*C. thermocellum* DSM 1313	64.6	0.018	0.26	[14]
Switchgrass (mid-season)	-	14.3	mixed culture DC3, anaerobic	58.0	0.043	0.61	[14]
Switchgrass (mid-season)	-	14.3	*Clostridium clariflavum* DSM 19732	49.4	0.003	0.04	[14]
Switchgrass (mid-season)	-	14.3	*Clostridium cellulolyticum* H10	45.9	0.015	0.21	[14]
Switchgrass (mid-season)	-	14.3	*Caldicellulosiruptor bescii* DSM 6725	26.6	0.000	0.00	[14]
Rice straw	-	20	*Trametes versicolor* KT9427	-	0.240	4.80	[62]
Switchgrass	-	20	*C. bescii* JWCB049, engineered for ethanol production	-	0.004	0.07	[75]
Switchgrass (senescent)	-	13.2	*C. thermocellum* DSM 1313	41.0	-	-	[14]
Switchgrass (senescent)	Ball-milling cotreatment	13.2	*C. thermocellum* DSM 1313	68.0	-	-	[14]

Cattail	Twin-screw extrusion	2	*Ct+Cs+Tt*	30.2	0.091	0.18	This study
Flax straw	Twin-screw extrusion	2	*Ct+Cs+Tt*	59.5	0.147	0.29	This study
Wheat straw	Dilute acid	17.5 cellulose equivalent	*Trichoderma reesei* Rut C30 + *Saccharomyces cerevisiae* VTT C-79095 + *Scheffersomyces stipitis* VTT C-07806T	-	-	9.80	[319]
Switchgrass	Dilute acid	15	*C. thermocellum* DSM 1313	-	0.032	0.48	[185]
Switchgrass	Dilute acid	15	*C. thermocellum* M1570, engineered for high ethanol yield	-	0.080	1.20	[185]
Corn stover	Dilute acid	100	*Saccharomyces cerevisiae* 590.E1, engineered for cellulase production	-	0.205	20.51	[53]
Corn husk	Dilute alkaline	20	*S. cerevisiae* YI13_cel3A[cel7A], engineered for cellulase production	-	0.163	3.38	[320]
Rice straw	Compressed liquid hot water	10	*Zymomonas mobilis* pGEX-4T-3 BI 120-2, engineered for cellulase and xylanase production	-	0.265	2.65	[50]
Giant reed	Dilute acid	50	*E. coli* MS04, engineered for cellulase and xylanase production	-	0.152	7.60	[49]

[a]*Ct*: *Clostridium thermocellum* DSM 1237; *Cs*: *Clostridium stercorarium* DSM 8532; *Tt*: *Thermoanaerobacter thermohydrosulfuricus* DSM 26960

[14]. It is therefore helpful to screen lignocellulosic biomass samples with cellulosomal CBP technologies, as opposed to conventional fungal cellulase and yeast-based methods, in order to elucidate the most important biomass characteristics.

A recent study has developed a coculture for CBP consisting of *Clostridium thermocellum*, one of the best known candidates for CBP [14, 99] (Table 3.1), and two hemicellulose-utilizing thermophiles, and this coculture was capable of outperforming *C. thermocellum* alone and could solubilize a substantial portion of untreated lignocellulosic material [321]. In this study, firstly, the performance of this newly developed tri-culture was evaluated relative to the constituent member controls on multiple lignocellulosic substrates. The tri-culture was then used to screen the digestibility of cattail biomass from plants harvested in different seasons, and of straw from different wheat cultivars that had a broad range of lodging quality.

3.3 Materials and Methods

3.3.1 Plant material

Cattail biomass of two species, *Typha angustifolia* and *Typha latifolia*, were harvested from Libau area, Manitoba, Canada, in both September 26, 2013 (represents later stages of plant development) and July 17, 2014 (represents earlier stage of development). Six hexaploid wheat cultivars with a broad range of lodging resistance, namely 5602 HR, AC Domain, AC Intrepid, Harvest, Kane and McKenzie were used for this study. Plants were grown in 1-gallon plastic pots (one seed per pot) containing Super Mix supplied with 18 g of fertilizers (ACER®nt 13-12-12 consisting of 13 % N, 12 % P_2O_5, 12 % K_2O, and micro elements) in a greenhouse until maturity as described in [322]. The straw samples of cv. Glenn were obtained from the Ian N. Morrison Research Farm, Department of Plant Science, University of Manitoba. Hemp biomass samples

were obtained from the Emerson Hemp Company, Emerson, Manitoba. Other cattail biomass samples of unknown species or harvesting time were a gift from the International Institute for Sustainable Development in Winnipeg, Manitoba, and collected from the Nettlie Marsh area of Lake Winnipeg, Manitoba. Cattail and flax straw biomass that had been pretreated via twin-screw extrusion (200rpm, 100°C, 5% NaOH) was a gift from Dr. Simon Barnabé, Université du Québec à Trois-Rivières, Department of Chemistry, Biology, and Physics. All biomass samples were air-dried at room temperature for at least a week, cut into smaller pieces with a coffee grinder, and passed through a 0.5 mm sieve. Biomass samples were sent to Feeds Innovation Institute of the University of Saskatchewan (Saskatoon, Saskatchewan, Canada), where their lignocellulosic composition was determined by AOAC methods 930.15, 973.18, and 2002.04 [291] (Table 3.2).

3.3.2 Bacterial strains and media:

Strains and media used in this study are as described in [321]. Lyophilized cultures of *C. thermocellum* DSM 1237 and *Clostridium stercorarium* DSM 8532 were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) while *Thermoanaerobacter thermohydrosulfuricus* DSM 26960 was isolated from a previous study [151]. The first 2 strains have recently been reclassified to the genus *Hungateiclostridium* and *Thermoclostridium* respectively [94], however for the sake of clarity their longstanding names have been maintained throughout the text. Cultures were routinely passaged in modified ATCC 1191 medium containing 2 g/L of cellobiose. All experiments were performed in modified ATCC 1191 medium, containing (per litre of milliQ water): KH_2PO_4, 1.5 g; Na_2HPO_4, 3.35 g;

Table 3.2: Composition of the lignocellulosic substrates used for comparing cultures

	Hemp	Cattail	TSE[a] Cattail	Wheat straw	TSE[a] Flax straw	Avicel cellulose
Cellulose (%)	57.7	36.7	37.3	42.8	32.0	100
Hemicellulose (%)	17.8	16.9	27.2	26.9	19.2	0
Lignin (%)	16.8	12.5	12.3	10.8	7.4	0

[a]TSE: twin-screw extrusion pretreated

NH_4Cl, 0.5 g; $MgCl_2$, 0.18 g; yeast extract, 2.0 g; L-cysteine, 1.0 g, as reducing agent; and 1 mL 0.025% w/v resazurin solution as oxygen indicator; pH was set to 7.2 through addition of 5 M NaOH. Lignocellulosic substrates were added before autoclaving to a final concentration of 2 g/L. Experiments were performed in Balch tubes sealed with butyl rubber septa and contained 10 mL liquid medium and 17 mL headspace filled with 100% N_2 gas. All tubes underwent 4 cycles of 1 min. gassing with N_2 followed by 1 min. vacuum before autoclaving for 20 min. at 121°C.

3.3.3 Culture experimental conditions

Precultures were inoculated with 10% (v/v, monocultures), 5/5% (v/v, dual-cultures), or 3.3/3.3/3.3% (v/v, triculture) inocula of overnight cultures from each respective culture member grown on 2 g/l cellobiose. Precultures were then grown on either 2 g/L cellobiose overnight (*C. stercorarium* and *T. thermohydrosulfuricus* monocultures) or 2 g/L Avicel cellulose for 3 days (*C. thermocellum*-containing mono and cocultures). The O.D.'s of the cultures prior to inoculation were approximately: *C. thermocellum*, 0.45; *C. stercorarium*, 0.54; *T. thermohydrosulfuricus*, 0.66; *C. thermocellum + C. stercorarium*, 0.53; *C. thermocellum + T. thermohydrosulfuricus*, 0.45; *C. stercorarium + T. thermohydrosulfuricus*, 0.68; *C. thermocellum + C. stercorarium, + T. thermohydrosulfuricus*, 0.59. Samples were inoculated using a 10% (v/v) inoculum of the preculture into tubes containing 2 g/l substrate, incubated for 4 days at 60°C, and then harvested. All experiments were done once or twice in triplicate.

3.3.4 End-product analysis

Analysis of the end products was performed as described in [321]. Gaseous end-products (H_2 and CO_2) were measured with a Varian micro-GC (Agilent, Mississauga, USA). Liquid end-

products (cellobiose, glucose, xylose, lactate, formate, acetate, and ethanol) were measured using a high-performance liquid chromatography system (Waters, Massachusetts, USA) with an HPX-87H column (Bio-Rad, California, USA) and a refractive index detector (Waters, Massachusetts, USA), at a flow of 0.6 mL/min of 5 mM H_2SO_4 and temperature of 45°C. Total carbon in end-products (TCEP) was calculated as TCEP (mM hex. eqv.) = [lactate]/2 + ([acetate] + [ethanol])/3 + ([formate] + [CO_2])/6. Percent conversion of total polysaccharides for each end-product was calculated, based on hexose equivalents, as "% conversion" = [end-product] * (# carbon atoms in end-product)/6 * (1/[cellulose + hemicellulose available (mM hex. eqv.)]).

3.4 Results

Previous research has showed that dual- and tri-cultures of *C. thermocellum* with either *C. stercorarium* and/or *T. thermohydrosulfuricus* were able to solubilize and convert a greater portion of wheat straw to end-products than the *C. thermocellum* monoculture [321]. To determine whether the ability of the co-cultures to convert more substrate to end-products was substrate-specific or if it was a general characteristic for all lignocellulosic substrates we tested the mono- and cocultures on a variety of substrates and measured total end-products (Tables 3.3 and 3.4). In general, *C. thermocellum* performed the best out of the three different mono-cultures tested, producing the highest total amount of end-products, while *T. thermohydrosulfuricus* generally produced the least end-products by a substantial margin.

The *C. thermocellum*-containing co-cultures, especially the *C. thermocellum* + *C. stercorarium* dual culture and the tri culture, produced more total end-products than the *C. thermocellum* mono-culture on all of the substrates tested (Table 3.3). The array of substrates tested included multiple untreated and twin-screw extrusion pre-treated substrates, suggesting

Table 3.3: Amount of substrate converted to fermentation end-products by all cultures, in percent relative to *C. thermocellum* monoculture, on 2 g/L of various untreated (only milled) and pretreated lignocellulosic substrates.

Culture	Amount of substrate converted to end-products (% relative to *Ct*)						Ethanol: Acetate: Lactate ratio[b]
	Cattail	TSE[a] Cattail	Wheat straw	Hemp	TSE[a] Flax straw	Avicel cellulose	
Ct[c]	100	100	100	100	100	100	49 : 45 : 6
Cs[d]	64	71	58	90	129	16	56 : 42 : 2
Tt[e]	85	41	28	46	25	4	25 : 17 : 58
Ct+Cs	174	164	170	300	233	115	64 : 33 : 4
Ct+Tt	154	123	154	198	177	123	39 : 27 : 34
Cs+Tt	88	30	93	92	154	14	54 : 36 : 11
Ct+Cs+Tt	165	161	184	267	223	126	66 : 26 : 7
	Total polysaccharides converted to fermentation end-products (%)						
Ct	15	19	20	10	27	72	

[a]TSE: twin-screw extrusion pretreated

[b]Average ethanol:acetate:lactate end-product profile ratio (mM basis) across all substrates (see Table 3.4 for substrate-specific ratios)

[c]*Ct*: *Clostridium thermocellum*

[d]*Cs*: *Clostridium stercorarium*

[e]*Tt*: *Thermoanaerobacter thermohydrosulfuricus*

The data represent means of 3 independent biological replicates

Table 3.4: Ethanol:acetate:lactate end-product ratios of cultures grown on 2 g/l various untreated (only milled) and pretreated lignocellulosic substrates

Culture	Cattail	TSE[a] Cattail	Wheat straw	Hemp	TSE[a] Flax straw	Avicel cellulose
Ct[b]	45 : 51 : 3	59 : 35 : 6	50 : 49 : 1	46 : 54 : 1	50 : 46 : 3	41 : 34 : 25
Cs[c]	45 : 54 : 1	49 : 44 : 7	60 : 38 : 1	40 : 59 : 1	71 : 29 : 1	71 : 28 : 1
Tt[d]	30 : 26 : 44	14 : 24 : 63	13 : 45 : 43	43 : 8 : 49	10 : 0 : 90	20 : 45 : 35
Ct+Cs	58 : 41 : 1	64 : 33 : 3	67 : 32 : 1	57 : 42 : 1	74 : 25 : 0	61 : 22 : 17
Ct+Tt	40 : 33 : 27	42 : 24 : 33	36 : 29 : 35	42 : 31 : 27	36 : 25 : 39	37 : 18 : 45
Cs+Tt	26 : 42 : 32	64 : 25 : 11	52 : 48 : 0	39 : 49 : 12	75 : 21 : 4	60 : 24 : 16
Ct+Cs+Tt	58 : 31 : 11	70 : 25 : 5	71 : 27 : 3	66 : 33 : 1	75 : 24 : 1	58 : 19 : 23

[a]TSE: twin-screw extrusion pretreated

[b]*Ct*: *Clostridium thermocellum*

[c]*Cs*: *Clostridium stercorarium*

[d]*Tt*: *Thermoanaerobacter thermohydrosulfuricus*

that the significant positive effect of co-culturing *C. thermocellum* with a hemicellulose-utilizing partner is a general phenomenon that occurs with many lignocellulosic substrates. The magnitude of the effect did vary between substrates and co-culture partners however, ranging from a 1.23-fold increase in amount of substrate converted with a *T. thermohydrosulfuricus* partner on twin-screw extruded cattail to a 3-fold increase with a *C. stercorarium* partner on hemp. The large increase in substrate conversion using hemp biomass is surprising as hemp contained significantly more cellulose, the main carbon source for *C. thermocellum*, and less hemicellulose, which is utilized only by *C. stercorarium* and *T. thermohydrosulfuricus*, than the other substrates (Table 3.2).

The tri-culture was then used to screen the digestibility of four cattail samples consisting of two different species, both harvested in two different harvest seasons, September (later in growing season, before wintering) and July (earlier in growing season) by measuring total end-product concentrations after four days of incubation (Table 3.5). Although little difference in digestibility was observed between the two cattail species, there was a substantial difference in both biomass lignocellulosic composition and amount of polysaccharides converted to end-products between the plants harvested at different times. The cattail plants harvested in July, contained, on average, 42 mg g^{-1} biomass (dry weight) less cellulose, 22 mg g^{-1} biomass less lignin, but 34 mg g^{-1} biomass more hemicellulose. The plants harvested in July were much more digestible by the tri-culture, with a 63% increase in the fraction of total polysaccharides converted to end-products, on average, compared to the plants harvested in September. This harvesting time effect was similar between the two different species.

A similar screen was applied to wheat straw from six different commercial wheat cultivars grown in a greenhouse under identical environmental conditions (Table 3.6). The

Table 3.5: Compositional information and amount of total polysaccharides converted to end-products by tri-culture of two different cattail species (*Typha spp.*), each species harvested at two different times.

Cattail Species	Harvest date	Composition (%)			End-product carbon (mM hex. eqv.)	Total polysaccharides utilized (%)[a]
		CE	HC	LI		
T. angustifolia	July 17	33.5	27.7	9.7	2.01	26.2 ± 0.9
	Sept. 26	38.4	25.3	10.6	1.15	14.5 ± 1.8
T. latifolia	July 17	33.8	27.9	8.9	1.98	25.5 ± 1.6
	Sept. 26	37.3	23.5	12.3	1.34	17.6 ± 0.8

[a]mean ± standard deviation

CE: cellulose; HC: hemicellulose; LI: lignin.

The data represent means of 3 independent biological replicates

Table 3.6: Compositional information and amount of total polysaccharides converted to end-products by tri-culture of various wheat straw varieties.

	Composition (%)					Resistance to lodging[a]	End-product carbon (mM hex. eqv.)	Total polysaccharides converted (%)[b]
Variety	CE	HC	LI	PR	Ash			
McKenzie	40.5	25.1	6.2	6.2	10.2	Fair	2.22	27.0 ± 1.4
5602HR	33.6	21.8	5.9	9.9	15.3	Fair	2.14	30.9 ± 4.6
AC Intrepid	40.9	26.4	6.5	5.8	11.4	Good	2.36	28.0 ± 3.5
Kane	40.6	25.6	6.7	6.4	10.3	Good	2.64	31.8 ± 3.5
AC Domain	39.6	24.4	6.7	8.2	10.1	Very good	2.22	27.7 ± 3.3
Harvest	39.4	25.0	6.4	7.4	12.1	Very good	2.31	28.6 ± 2.4
Glenn (field[c])	42.8	26.9	10.8	3.4	6.5	Very good	2.40	27.4 ± 3.9

[a][323]

[b]mean ± standard deviation

[c]Plants of cv. Glenn were grown under field conditions and included as a control; the other cultivars were grown under greenhouse condition

CE: cellulose; HC: hemicellulose; LI: lignin; PR: protein.

The data represent means of 6 independent biological replicates between two separate experiments (3 replicates per experiment)

amount of total polysaccharides converted to end-products varied slightly between the cultivars, ranging from 27.0% to 31.8%. While the wheat cultivars used in the study differ in their lodging resistance quality [323], which represent the ability of a plant to stand up straight and resist bending over, the composition of their straw biomass varied little, except 5602HR, which had lower cellulose and hemicellulose and higher ash contents than the other cultivars (Table 3.6). No statistically significant correlation was measured between either the lodging resistance quality or the straw lignocellulosic composition and the amount of carbon converted.

3.5 Discussion:

The fact that the cocultures, notably the *C. thermocellum* + *C. stercorarium* dual-culture and the tri-culture, were consistently able to convert a larger fraction of all the various substrates tested to end-products (Table 3.3) confirms that this effect occurs due to general characteristics of lignocellulosic materials and is not particular to wheat straw or non-pretreated material. This is expected as many lignocellulosic substrates contain significant amounts of hemicellulose, which *C. thermocellum* can hydrolyze but not utilize for growth [102, 287], leaving the hemicellulose saccharides in the medium for the co-culture partners to utilize and convert to end-products. The consistent increase in total end-products in the co-cultures confirms that the *C. thermocellum*-containing co-cultures are a robust and reliable system for increasing polysaccharide-to-end-product conversion for lignocellulosic substrates. While the final ethanol yields were relatively low, ranging from 0.013 g ethanol/g substrate (monocultures) to 0.149 g ethanol/g substrate (co-cultures on flax straw), significant improvements could be made by utilizing high-ethanol strains of *C. thermocellum* [275, 278, 279] and co-treatment of the lignocellulosic substrates [14] (Table 3.1). The ethanol titre could be improved by using high substrate loadings, which were kept intentionally low in this study to prevent any confounding

factors that occur in batch at higher substrate loadings from interfering with analysis of substrate degradation characteristics.

Only a small number of studies exist that have looked at the lignocellulosic feedstock potential for cattail biomass [308, 324, 325]. Studies on other plants have observed significant changes in cell wall composition throughout growth, with general increases in the cellulose and lignin content of the leaves and stems over time [326–329]. Studies have also observed general decreases in % digestibility of different potential biomass crops as they mature, as determined either by enzymatic digestion [14, 299, 330], biochemical methane potential [329], or CBP [14, 174]. The present study reveals that these same trends occur in cattails, with increased cellulose and lignin content and reduced digestibility with increasing crop maturity, and indicate that optimal harvest times might occur before full maturity of the plant. The total biomass, nitrogen, and phosphorous levels in the above ground portion of cattail plants have been found to peak around August or September and then plateau or decline thereafter [331, 332]. Thus, the greater digestibility that occurs earlier in the growing season must be balanced against the biomass yields as well as stem and leaf nitrogen and phosphorous levels that might peak slightly later to ensure maximum biofuel yields and phytoremediation potential [332].

The amount of total polysaccharides in wheat straw converted to end-products by the tri-culture in this study is comparable to what other authors have found using cellulase plus hemicellulase preparations on untreated wheat straw (32.2%) [333]. The relative standard deviation of the total polysaccharide conversion for the group of wheat straw substrates was 6.4%, similar to what other studies have found using enzymatic hydrolysis on wheat straw [302, 304]. The present study failed to observe any significant correlation between either lodging quality or biomass lignocellulosic composition and digestibility for this set of cultivars

(Supplementary Fig. A.3.1), suggesting that lodging quality alone is not a strong indicator of digestibility of wheat straw. The lack of any significant correlation could possibly be due to a low number of cultivars tested or due to difference in growth condition, that is, between the greenhouse conditions under which the plant materials used for this study were grown and the field conditions under which lodging quality was assessed [323]. Previous studies, though, have found significant positive correlations between fraction of total sugars released by enzymatic hydrolysis and lignin content, and negative correlations with cellulose content [304]. These relationships were reversed when correlated with absolute total amount of sugars released however [303, 304], suggesting that higher cellulose content is associated with a disproportionate increase in difficult-to-access cellulose [304]. It should be noted though that these studies employed commercial fungal enzyme preparations, in contrast to the CBP approach employed in this study, and cellulosomes are known to have different mechanisms of action [39, 315] and are not always influenced equally by the same biomass characteristics [14].

3.6 Conclusion:

Overall these results indicate that synergistic cocultures, as a potential CBP process, can be used to reliably screen a wide range of plant species and cultivars for digestibility as lignocellulosic feedstock samples. Harvest time, and to a lesser extent cultivar selection, can significantly affect digestibility of these feedstocks. The underlying substrate properties responsible for these differences remain elusive however, as simple compositional differences appear to have only a small effect. These growth stage-dependant properties may be especially important for plants such as cattail and other grass species that are currently used and harvested in phytoremediation scenarios [305, 307, 332], where the harvested material is expected to have a dual purpose: nutrient removal and biomass production for biofuel generation.

Chapter 4: The role of cross-feeding and wheat straw extractives in enhancing growth of co-cultures of *Clostridium thermocellum* with other lignocellulolytic partners*

* Contributing authors: Froese AG[1], Sparling R[2]. 2021. Bioprocess and Biosystems Engineering (In-press) https://doi.org/10.1007/s00449-020-02490-7. Contributions: [1]experiment design, experimental work, first author; [2]research guidance, manuscript editing

4.1 Abstract

Co-cultures consisting of three thermophilic and lignocellulolytic bacteria, namely C*lostridium thermocellum*, *C. stercorarium*, and *Thermoanaerobacter thermohydrosulfuricus*, have previously been found to degrade lignocellulosic material in a synergistic manner. These species were tested for their ability to cross-feed nutrients between one another, and each species was found to secrete vital growth factors required by at least one other species for growth in a defined minimal medium. Growth factors also appeared to be present in water-soluble extractives obtained from wheat straw, which also allowed growth in a defined minimal medium. Cell enumeration during growth on wheat straw in this medium revealed different growth profiles of the members that varied between the co-cultures. End-product profiles also varied substantially between the cultures, with significantly higher ethanol production in all co-cultures compared to the mono-cultures. Understanding interactions between co-culture members, and the additional nutrients provided by lignocellulosic substrates, will aid us in consolidated bioprocessing design.

4.2 Introduction

The production and use of second-generation biofuels, generated using lignocellulosic feedstocks, is a promising approach to reduce global use of fossil fuels and greenhouse gas emissions [7]. Consolidated bioprocessing (CBP) approaches to produce second-generation biofuels utilize microbes that can simultaneously depolymerize the lignocellulosic polysaccharides into shorter-chain sugars and convert those sugars into biofuels such as ethanol [41]. However, no single organism has yet been discovered that possesses all of the qualities needed for an economically viable CBP process [43, 44]. Co-culturing selected microbial species together allows complementation of different traits and can have numerous benefits, including increased hydrolysis rates [251, 254, 321], enhanced sugar utilization [251, 321], end-product profile shifts [253, 263, 334], and aerotolerance [265–267]. Cross-feeding has also been observed in co-cultures, whereby one or more members will secrete nutrients, such as vitamins and amino acids, that can either enable or enhance the growth of the other members [253, 269, 270]. This cross-feeding can allow for potential control over the co-culture and the population ratios by modulating growth of certain members [248] and can also decrease the number of nutrients that need to be added to the medium, further reducing the overall cost of the process [270].

Previous reports from our lab have described initial tests of co-cultures consisting of three different lignocellulose utilizers: *Clostridium thermocellum* DSM 1237, *C. stercorarium* DSM 8532, and *Thermoanaerobacter thermohydrosulfuricus* DSM 26960, using qPCR techniques to monitor the growth of the separate components of the defined consortium [321, 335]. *C. thermocellum* is considered more of a cellulose-specialist due to the fact that although its cell-wall bound protein complexes (cellulosomes) efficiently hydrolyse cellulose and hemicellulose [99, 160], it cannot utilize any pentose sugars from the breakdown of

hemicellulose [102, 191]. *C. stercorarium* and *T. thermohydrosulfuricus* both secrete hydrolytic enzymes that can degrade xylans from hemicellulose, but have relatively low or no activity on cellulose, respectively [119, 127, 135, 151]. However, both species can utilize the pentose sugars from hemicellulose that can inhibit both the cellulases [193, 194, 289] and the growth of *C. thermocellum* [198]. When either *C. stercorarium* or *T. thermohydrosulfuricus* are co-cultured with *C. thermocellum* on lignocellulosic substrates the lignocellulose hydrolysis rates and the extent of substrate solubilization are increased compared to *C. thermocellum* mono-culture controls [321, 335].

The current study was undertaken to understand the interactions between the co-culture members, specifically whether any nutrient cross-feeding was occurring, and how this might be related to, or impact, growth on lignocellulosic substrates. Cross-feeding of growth-promoting compounds, possibly amino acids, between the co-culture members was observed in a defined medium. The water-soluble extractives from wheat straw were also found to contain certain growth-promoting compounds that allowed the growth of *C. stercorarium* and *T. thermohydrosulfuricus* in the same defined medium.

4.3 Materials and methods

4.3.1 Strains and media

Lyophilized cultures of *C. thermocellum* DSM 1237 and *C. stercorarium* DSM 8532 were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany). The strains have recently been reclassified to the genera *Acetivibrio* [76] and *Thermoclostridium* [94], respectively, however for the sake of clarity, their longstanding names have been maintained throughout the text. *T. thermohydrosulfuricus* WC1 (DSM 26960) was isolated in a previous study [151]. All experiments were performed in a

modified form of the ATCC 1191 medium [336]. The sodium sulfide was replaced with 2 g/L cysteine as a reducing agent. The yeast extract was omitted and the concentrations of all vitamins were ten times higher than the amount in ATCC 1191, to match other frequently used minimal media [257, 337]. The pH was set to 7.2 through the addition of 5 M NaOH. Experiments were performed in either Balch tubes containing 10 mL liquid medium or serum bottles containing 50 mL liquid medium, sealed with butyl rubber septums and the headspace filled with 100% N_2 gas. All other aspects of medium preparation were as performed in [336]. Cultures were maintained, prior to the experiments, via passaging, using a 10% (v/v) inoculum, in a different modified form of 1191 medium [321], with no added vitamins, but with 2 g/L yeast extract and 2 g/L cellobiose as the carbon source.

4.3.2 Wheat straw and extractives

Air-dried wheat (cv. Glenn) straw was provided as gift from Dr. Belay Ayele (University of Manitoba, Department of Plant Science, Faculty of Agriculture), ground using a hammer mill and sieved through a 0.5 mm mesh. The compositional analysis of the wheat straw was performed by the Feeds Innovation Institute of the University of Saskatchewan (Saskatoon, Saskatchewan, Canada) via AOAC methods 930.15, 973.18, and 2002.04 [291]. The composition was determined to be (% dry weight): cellulose, 42.8; hemicellulose, 26.9; lignin, 10.8; crude protein, 3.4; ash, 6.5. Wheat straw extractives-containing medium was obtained by preparing medium containing 2 g/L wheat straw, autoclaving, filtering the medium through a 0.22 µm filter, dispensing the filtrate into new tubes, re-gassing/degassing, re-autoclaving, and adding filter-sterilized cellobiose to a final concentration of 2 g/L prior to inoculation. Extractive-free wheat straw was obtained by incubating a bottle of distilled water containing 50

g/L wheat straw at 90°C for 30 minutes, filtering, and repeating with new water for 14 cycles in total, then performing another 14 cycles with 95% ethanol at 60°C, at which point no more colour was evident in either wash, and air-drying the material.

4.3.3 Experimental conditions

The pre-cultures for all of the experiments were grown in modified ATCC 1191 medium containing 2 g/L yeast extract but no added vitamins. All experiments were performed in medium containing 2 g/L carbon source (cellobiose or wheat straw). For the passaging experiments, all the passage 1 cultures were inoculated using the same total volume (1 mL, 10% (v/v) of total liquid volume), but the co-cultures inoculum was split into equal volumes of the different members. The pre-cultures were grown on 2 g/L cellobiose in non-modified ATCC 1191 medium overnight. For all of the passaging experiments the cultures were passaged, using a 10% inoculum, either every 7 days or, only for the experiments using cellobiose, once full growth was achieved. The growth curves using wheat straw were performed in 50 mL bottles with cultures (mono-cultures and co-cultures) that had been passaged on wheat straw three times. Three bottles were inoculated for each culture and 1 mL samples were taken aseptically at each time-point after briefly inverting and mixing the bottle to obtain a homogenous sample. All experiments were conducted in triplicate unless otherwise stated.

4.3.4 End-product analysis

Gaseous end-products (H_2 and CO_2) were measured using a Varian Micro-GC (Agilent, Mississauga, USA). Liquid end-products (cellobiose, glucose, xylose, arabinose, lactate, formate, acetate, and ethanol) were measured by HPLC using an HPX-87H column (Bio-Rad, California,

USA) and a refractive index detector (Waters, Massachusetts, USA) on a Waters HPLC system with a flow rate of 0.8 mL/min of 5 mM H_2SO_4. Oligosaccharides were measured by acid hydrolysis (depolymerizing to monosaccharides) and HPLC as in [321]. Total carbon in end-products (TCEP) was calculated as TCEP (mM hex. eqv.) = [lactate]/2 + ([acetate] + [ethanol])/3 + ([formate] + [CO_2])/6. Percent conversion of total polysaccharides for each end-product was calculated, based on hexose equivalents, as "% conversion" = [end-product] * (# carbon atoms in end-product)/6 * (1/[cellulose + hemicellulose available (mM hex. eqv.)]).

4.3.5 Post-fermentation substrate residual composition

The composition of the material left-over after fermentation was measured according to [338] with modifications. Briefly, the substrate particles were left to settle by gravity, the supernatant was removed, the substrate was washed with distilled water, left to settle again, the wash water decanted, and the substrate was then dried at 60°C. 300 µL of 72% H_2SO_4 was added to each tube, containing ≤ 20 mg of substrate, and incubated at room temperature for one hour with frequent stirring. 8.4 mL of distilled water was added to dilute the acid concentration to 4% and the solution was autoclaved at 121°C for 20 minutes. 3.5 mL of 20% ammonium bicarbonate was then added to neutralize the pH. The samples were centrifuged at max rpm in a table-top centrifuge. The supernatant containing the monosaccharides was analyzed via HPLC as described above. Uninoculated medium tubes were used as controls.

4.3.6 DNA and qPCR for cell enumeration

Genomic DNA was extracted on sample pellets containing substrate and cells with InstaGene Matrix (Bio-Rad, USA) according to manufacturer's protocol. qPCR targeting the *cpn-60* gene for cell enumeration [293] was performed as described in [321].

4.3.7 Statistical analysis

Statistical analysis was performed in Microsoft Office Excel using the T.TEST function.

4.4 Results

4.4.1 Cross-feeding in defined medium

To investigate any possible cross-feeding, the mono-cultures and all co-culture pairings were inoculated into a defined medium that did not contain any amino acids (except cysteine, as reducing agent) or complex nutrient sources, with cellobiose as a carbon source. All cultures were passaged once a week, or sooner if full growth was observed, for several passages and *cpn-60* based qPCR was used to determine species-specific cell numbers (Fig. 4.1). *C. thermocellum* was the only mono-culture able to consistently grow to high cell densities, indicating no nutrient auxotrophies in this defined medium. Both *C. stercorarium* and *T. thermohydrosulfuricus* populations decreased approximately 10-fold between each passage initially, indicating that the cells were dormant, with no significant reproduction or lysis. Eventually *C. stercorarium* cell numbers stabilized around 10^4 cells/ml while *T. thermohydrosulfuricus* stabilized around 10^5 cells/ml, indicating that both species were able to grow in this medium but were limited to considerably lower maximum cell numbers, possibly due to trace amounts of growth factor still present. When either *C. stercorarium* or *T. thermohydrosulfuricus* were co-cultured with *C. thermocellum* however, they were able to consistently grow to much higher cell concentrations,

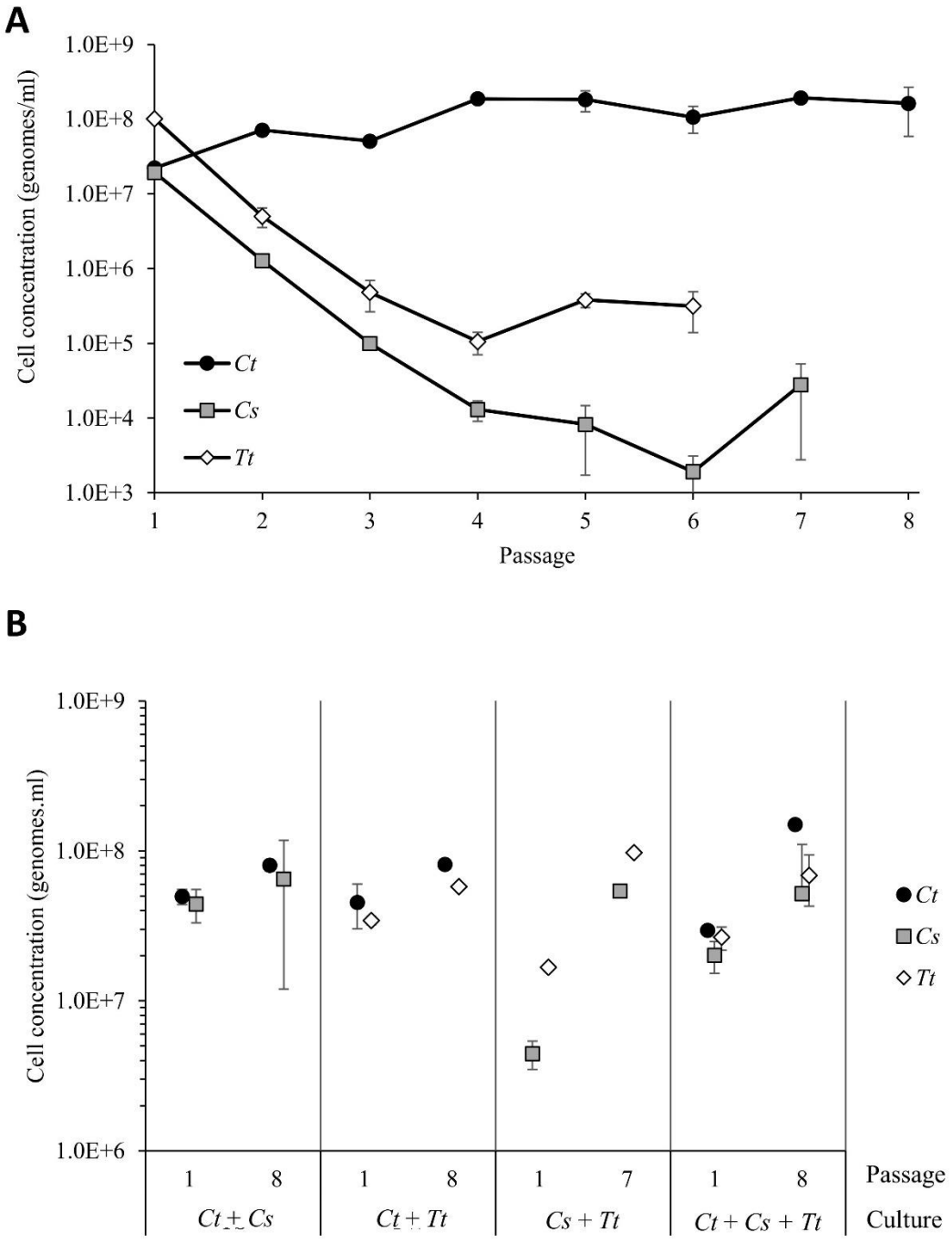

Fig. 4.1: Cell concentrations for each species in the mono-cultures at the end of each passage (a), and in the co-cultures at the end of the first and last passages (b), when grown in defined medium with 2 g/l cellobiose and passaged once a week. *Ct: C. thermocellum; Cs: C. stercorarium; Tt: T. thermohydrosulfuricus*

between 10^7 and 10^8 cells/ml (Fig. 4.1 and Supplementary Fig. A.4.1), suggesting that *C. thermocellum* was secreting nutrients needed by *C. stercorarium* and *T. thermohydrosulfuricus* for efficient growth. This effect was also observed in the tri-culture, with all three different members growing to high cell numbers together. Cross-feeding was also apparent in the *C. stercorarium* + *T. thermohydrosulfuricus* co-culture where, after several passages of adaptation, both *C. stercorarium* and *T. thermohydrosulfuricus* were able to grow to the high cell numbers seen in the other co-cultures. End-product profiles were also measured at the end of each passage (Supplementary Fig. A.4.2), revealing, in general, relatively stable end-product profiles for each culture.

4.4.2 Passaging in defined medium with wheat straw

The mono- and co-cultures were then passaged in defined medium containing wheat straw as the carbon source to determine how effective the cultures would be in deconstructing lignocellulose in the absence of yeast extract, considering that *T. thermohydrosulfuricus* has previously somewhat obfuscated inferences into its contribution to co-cultures due to its growth on yeast extract [321]. The end-product profiles, including all soluble sugars, for the averages over all the passages are shown in Fig. 4.2 (see Supplementary Fig. A.4.3 for data from each separate passage) and compared with data from previous studies that used medium containing yeast extract and that has been corrected for end-products produced from yeast extract. The end-product profiles generally change little between the first and last passages, other than an increase in lactate and decrease in acetate and ethanol in the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture, indicating the overall stability of the co-cultures. Other than a small amount of arabinose (≤0.25 mM), the vast majority of the soluble saccharides, after depolymerization into

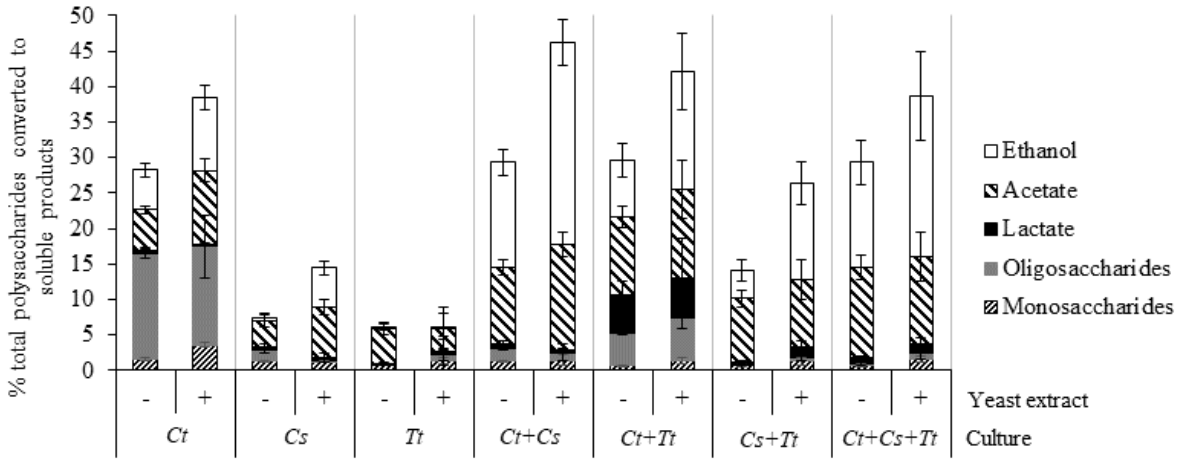

Fig. 4.2: Percent of total polysaccharide converted to various soluble products for each culture when grown on 2 g/l wheat straw in minimal medium, and when grown on 2 g/l wheat straw in simplified ATCC 1191 medium containing yeast extract. Cultures were passaged once every 7 days for 6 weeks and the data shown are averages for all passages. Error bars represent standard deviation for each separate product. Data for yeast extract-containing medium controls taken from [321]. *Ct: C. thermocellum; Cs: C. stercorarium; Tt: T. thermohydrosulfuricus*

monosaccharides, travelled under a single peak on the HPLC with a retention time shared with xylose, galactose, and mannose, making the three monosaccharides indistinguishable. Most of the sugars in this peak are presumed to be xylose based on the relatively higher content of xylose in wheat straw hemicellulose [339]. The sum of the end-products and soluble saccharides released allows an indirect measurement of how much of the substrate was depolymerized and solubilized by each culture. *C. thermocellum* produced significantly less acetate and ethanol in the absence of yeast extract, while releasing slightly more soluble sugars, resulting in an overall 27% relative decrease in the total amount of substrate solubilized in the absence of yeast extract. *C. stercorarium* also solubilized less substrate overall in the absence of yeast extract and exhibited a significant shift in the acetate to ethanol ratio, from 1.34 with yeast extract to 10.2 without. An increase in the acetate to ethanol ratio was not observed with *C. thermocellum* but was also observed in the *T. thermohydrosulfuricus* mono-culture and in all the co-cultures. All the co-cultures exhibited a decrease in the amount of substrate solubilized in the absence of yeast extract, ranging from 24% to 47% relative decreases, such that the *C. thermocellum*-containing co-cultures solubilized only marginally more substrate (3.8% to 4.7% relative increases) than the *C. thermocellum* mono-culture. Surprisingly, the *T. thermohydrosulfuricus* mono-culture behaved the same in the presence or absence of yeast extract.

The cell concentrations at the end of the first and sixth passage were measured to determine any population shifts (Fig. 4.3 and Supplementary Fig. A.4.4). Most of the cultures were fairly stable between the passages but some differences existed when compared to growth in medium containing yeast extract. *T. thermohydrosulfuricus* tended to grow to higher numbers, in all of the cultures, in the presence of yeast extract, which it can use as a carbon source. The other notable difference was the *C. thermocellum* + *C. stercorarium* dual-culture, in which *C.*

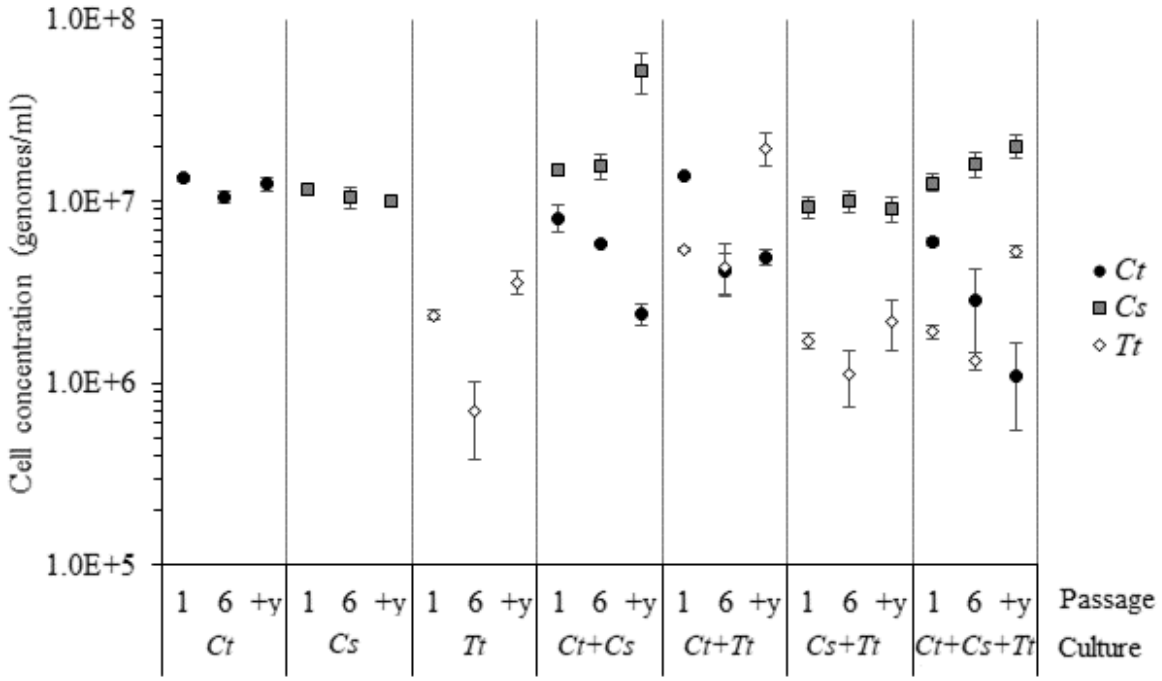

Fig. 4.3: Cell concentration for each species in each culture at the end of passages 1 and 6 when grown in minimal medium with 2 g/l wheat straw and when grown in medium containing yeast extract and 2 g/l wheat straw (+y). Data for +y controls taken from [321]. *Ct: C. thermocellum; Cs: C. stercorarium; Tt: T. thermohydrosulfuricus*

stercorarium grew to higher densities and *C. thermocellum* to lower densities in the presence of yeast extract, leading to an even lower *C. thermocellum* to *C. stercorarium* ratio. Indeed, the *C. thermocellum* to either *C. stercorarium* or *T. thermohydrosulfuricus* cell number ratio is much higher in all co-cultures when grown in defined medium with no yeast extract (Supplementary Fig. A.4.5), likely due to the reliance *C. stercorarium* and *T. thermohydrosulfuricus* now have on the growth factors secreted by *C. thermocellum*.

4.4.3 Growth measurements in defined medium with wheat straw

The cultures were grown in defined medium with wheat straw and monitored every few hours to determine their growth characteristics. Quantification of cell numbers (Fig. 4.4) revealed that *C. thermocellum* tended to grow slowly over 3 or 4 days, sometimes after a lag of a few hours, and generally reached similar maximum cell numbers in all the cultures, other than in the *C. thermocellum* + *C. stercorarium* co-cultures, where there was no lag and *C. thermocellum* reached higher cell numbers. *C. stercorarium* tended to grow more quickly in the first 24 hours and then more slowly over the rest of the fermentation period in all of the cultures. The co-cultures exhibited higher maximum *C. stercorarium* cell numbers, especially the tri-culture. *T. thermohydrosulfuricus* grew identically in all cultures in the first 12-18 hours, with quick growth to similar cell numbers, but deviated after that, gradually declining in the mono-culture, slowly growing in the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture, and stabilizing at a constant level in the other co-cultures.

Soluble sugars gradually increased in the *C. thermocellum* mono-culture over time (Fig. 4.5A), as expected, since it cannot utilize any pentose sugars. Most of the other cultures saw little change in the levels of soluble sugars over time but concentrations never dropped below 0.22

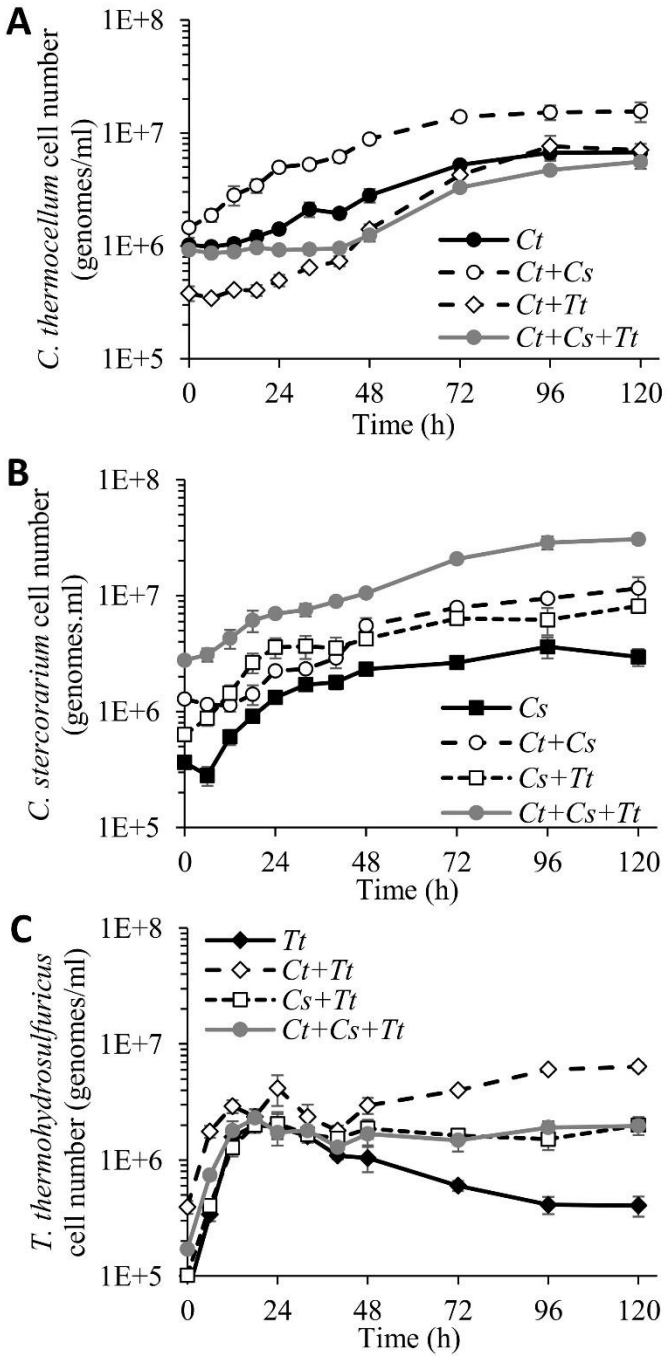

Fig. 4.4: Cell concentrations for *C. thermocellum* (a), *C. stercorarium* (b), and *T. thermohydrosulfuricus* (c) in each culture when grown in minimal medium with 2 g/l wheat straw. *Ct: C. thermocellum; Cs: C. stercorarium; Tt: T. thermohydrosulfuricus*

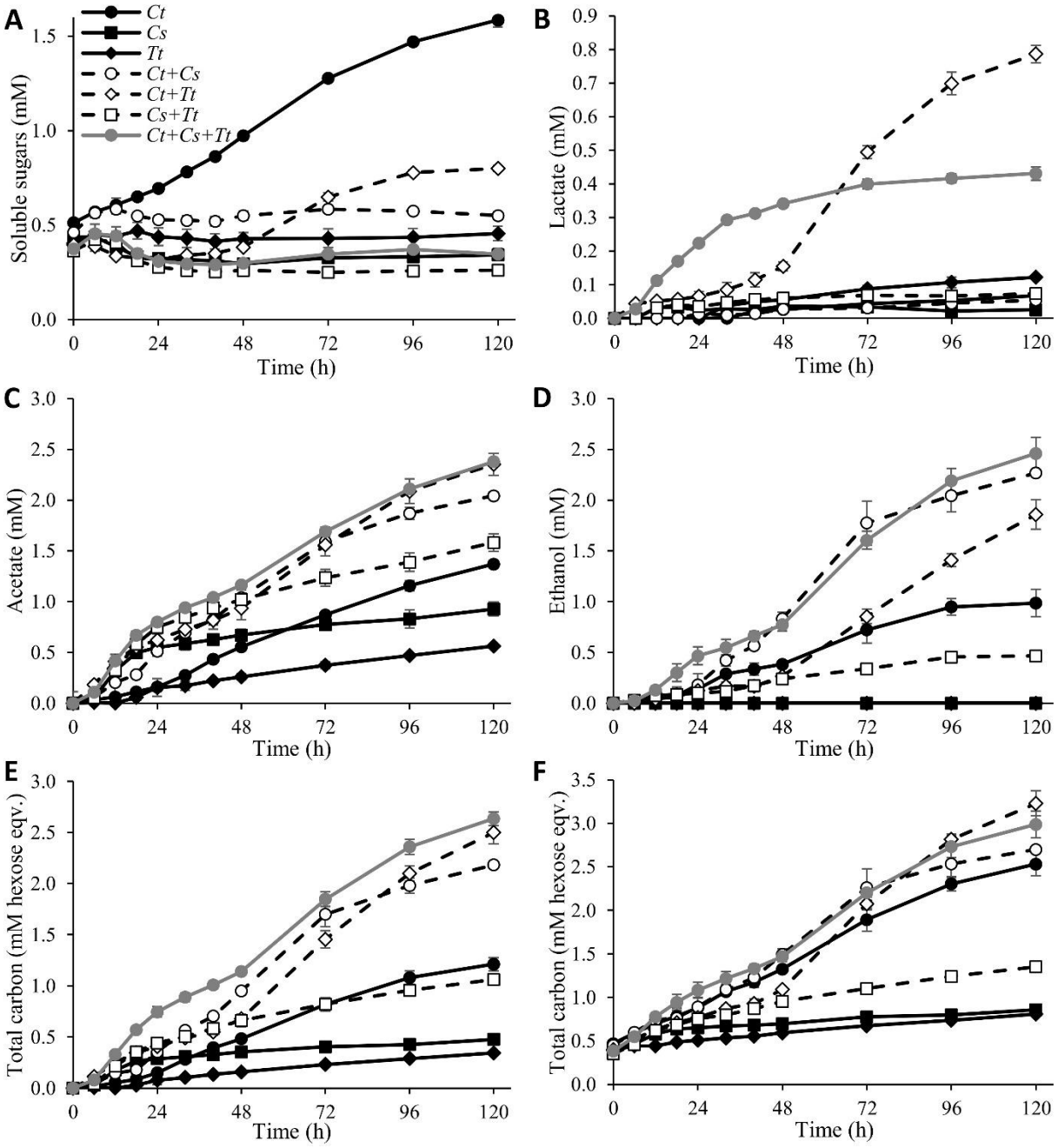

Fig. 4.5: Total soluble mono- and oligomeric sugars (a), lactate (b), acetate (c), ethanol (d), total carbon in end-products (e), and total carbon in end-products plus soluble sugars (f) concentrations over 5 days for each culture when grown in defined medium with 2 g/l wheat straw. *Ct: C. thermocellum; Cs: C. stercorarium; Tt: T. thermohydrosulfuricus*

mM xylose/mannose/galactose for any of the cultures. This is somewhat surprising given that both *C. stercorarium* and *T. thermohydrosulfuricus* can utilize xylose, mannose, and galactose [109, 151]. Significant lactate production was only observed in the *C. thermocellum + T. thermohydrosulfuricus* and *C. thermocellum + C. stercorarium + T. thermohydrosulfuricus* cultures (Fig. 4.5B). Most of the lactate in the *C. thermocellum + T. thermohydrosulfuricus* dual-culture was produced after 40h, which correlates well with the *T. thermohydrosulfuricus* growth curve (Fig 4.4C). Up until 40 hours *T. thermohydrosulfuricus* appears to grow identically in all cultures, but after 40 hours it only increases in cell number in the *C. thermocellum + T. thermohydrosulfuricus* co-culture (Fig. 4.4C). It is interesting to note that although the cell numbers for *T. thermohydrosulfuricus* in the *C. stercorarium + T. thermohydrosulfuricus* and *C. thermocellum + C. stercorarium + T. thermohydrosulfuricus* cultures mirror each other very well (Fig. 4.4C) lactate is only produced in the tri-culture.

Other than the *C. thermocellum* and *T. thermohydrosulfuricus* mono-cultures the rate of acetate production for the other cultures was generally highest in the first 24 hours after which point it slowed down (Fig. 4.5C). In contrast, the ethanol production tended to lag for a few hours, and in some cases maximal production rates were observed between 48 and 72 hours (Fig. 4.5D). However, neither *C. stercorarium* nor *T. thermohydrosulfuricus* produced any detectable levels of ethanol in their respective mono-cultures. Overall the co-cultures were able to produce end-products faster than any of the mono-cultures, especially during the first 30 hours, and produced more over-all (Fig. 4.5E). With the soluble sugars factored in (Fig. 4.5F), *C. thermocellum* was superior to the other mono-cultures and to the *C. stercorarium + T. thermohydrosulfuricus* co-culture in terms of substrate solubilization while the other co-cultures were able to solubilize up to 34% more material than the *C. thermocellum* mono-culture.

4.4.4 Post-fermentation substrate compositional analysis

To confirm that summing end-products and soluble sugars was a reliable measurement of the total amount of substrate solubilized by the cultures, the substrate residue left after fermentation underwent compositional analysis. This also allowed us to directly determine the amount of cellulose and hemicellulose solubilized by the cultures (Fig. 4.6). In general, the total amount of substrate solubilized as determined by post-fermentation compositional analysis tended to be slightly higher (ranging from 0.86-fold to 2.04-fold higher) than the estimations of substrate solubilization as determined by the sum of the end-products and soluble sugars. Part of this discrepancy could be due to cell biomass, which was not measured directly. Small amounts of cellulose loss were apparent in the *T. thermohydrosulfuricus* mono-culture, which should not be able to degrade cellulose, and might represent a systematic error that could also partially explain the above-mentioned discrepancy. The analysis confirmed that *C. stercorarium*, *T. thermohydrosulfuricus*, and their co-culture, hydrolyzed far less cellulose and hemicellulose than the *C. thermocellum*-containing cultures, as was previously observed [321]. The total amount of substrate solubilized as determined by this technique was more similar between the *C. thermocellum* mono-culture and the *C. thermocellum*-containing co-cultures although a slight increase, around 10%, was still observed in the *C. thermocellum* + *T. thermohydrosulfuricus* and *C. thermocellum* + *C. stercorarium* + *T. thermohydrosulfuricus* co-cultures.

4.4.5 Fulfillment of nutrient auxotrophies by wheat straw extractives

Observations of higher cell numbers of *C. stercorarium* and *T. thermohydrosulfuricus* in defined medium with wheat straw (Fig. 4.3) compared to defined medium with cellobiose (Fig.

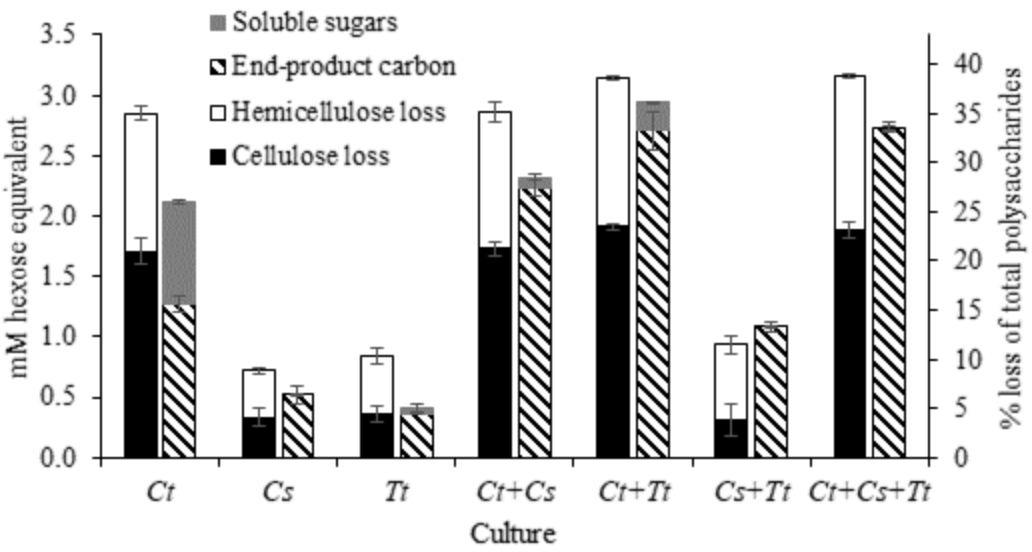

Fig. 4.6: Amount of carbon solubilized, as determined by post-fermentation compositional analysis, and carbon found in end-products and soluble sugars for all cultures grown on 2 g/l wheat straw in defined medium for 5 days. *Ct: C. thermocellum; Cs: C. stercorarium; Tt: T. thermohydrosulfuricus*

4.1A) led to the hypothesis that water-soluble extractives from wheat straw contained nutrients that allowed *C. stercorarium* and *T. thermohydrosulfuricus* to grow to higher densities. To confirm this the various mono-cultures were passaged in medium containing extractives obtained from wheat straw. Briefly, this was accomplished by preparing medium containing wheat straw, filtering off the wheat straw particles, re-degassing and re-autoclaving the medium that now contained the wheat straw extractives, and then adding cellobiose as a carbon source (see methods for full details). Results from the fourth passage showed that while *C. thermocellum* utilized all the cellobiose regardless of the presence of the wheat straw extractives, *C. stercorarium* and *T. thermohydrosulfuricus* were able to utilize 55% and 100% of the cellobiose in the presence of the extractives, respectively, compared to negligible amounts in the controls that did not contain any wheat straw extractives (Supplementary Fig. A.4.6). This indicates that the wheat straw extractives do contain nutrients that allow the auxotrophic *C. stercorarium* and *T. thermohydrosulfuricus* to grow and utilize cellobiose in the defined medium used, although in the case of *C. stercorarium* utilization is still below that seen in the presence of yeast extract.

4.4.6 Degradation of extractive-free wheat straw

To determine whether the extractives obtained from wheat straw affected the ability of the different cultures to degrade wheat straw the cultures were grown and passaged in defined medium with extractive-free wheat straw. The total amount of end-products produced was lower for all cultures on the extractive-free wheat straw (Fig. 4.7) in a statistically significant manner, at least by the third passage, indicating that the cultures abilities to utilize the substrate was reduced in the absence of the extractives. The amount of growth and end-products observed in the *C. stercorarium* and *T. thermohydrosulfuricus* mono-cultures grown on the extractive-free

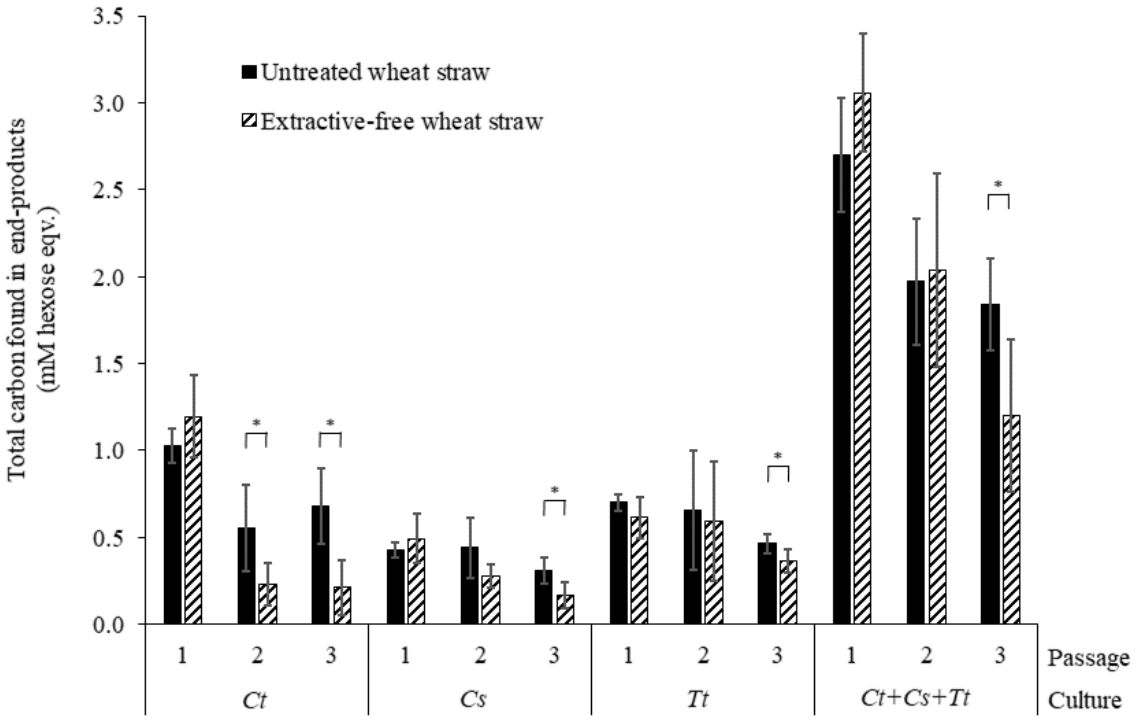

Fig. 4.7: Total carbon found in end-products for each monoculture and the tri-culture at the end of each passage when grown in defined medium containing 2 g/l of either untreated wheat straw or extractive-free wheat straw. *: P-value ≤0.05. *Ct*: *C. thermocellum*; *Cs*: *C. stercorarium*; *Tt*: *T. thermohydrosulfuricus*. Error bars represent standard deviation from biological replicates.

wheat straw were surprisingly high given the low amount of growth observed in defined medium with cellobiose (Fig. 4.1A). This could imply that the washing steps are not removing all of the nutrients present in the wheat straw. The tri-culture still produced significantly more end-products than any of the mono-cultures, with or without extractives.

4.5 Discussion

A previous cross-feeding co-culture consisting of *C. thermocellum* and *T. thermohydrosulfuricus* strains was isolated from nature, and both species were found to secrete vitamins and/or amino acids required by the other member for full growth [269, 270]. Cross-feeding of unidentified growth factors has also been observed in at least one other environmentally-isolated cellulolytic co-culture [340, 341]. The multiple occurrences of cross-feeding observed in the present study is consistent with the fact that these organisms are expected to live in nature in complex communities, even though each of the members of our current tri-culture was isolated from a different environment [109, 151, 163]. The growth factors being cross-fed are likely amino acids considering that the medium used contains abundant vitamins and that *C. thermocellum* is known to secrete amino acids [184, 250, 268].

Although the additional growth factors provided by co-culturing and wheat straw extractives were able to sustain growth of the auxotrophic members, the total substrate solubilization levels on wheat straw were, in general, considerably lower than in yeast extract-containing medium, indicating that the growth factors are not in sufficient quantity to allow for full and optimal growth of the cultures. Thus, although *C. stercorarium* and *T. thermohydrosulfuricus* were able to grow in the *C. thermocellum*-containing co-cultures, presumably utilizing the pentose oligosaccharides, they were able to only slightly increase

substrate solubilization. The extremely low ethanol production observed in the *C. stercorarium* and *T. thermohydrosulfuricus* mono-cultures, possibly due to the low amounts of polysaccharides that these two species were able to depolymerize into utilizable sugars, has not been observed before [109, 151, 321]. In contrast, the *C. stercorarium* + *T. thermohydrosulfuricus* dual-culture produced substantial amounts of ethanol, highlighting the significant effects of co-culturing. None the less, the higher cell number ratios of *C. thermocellum* to either *C. stercorarium* or *T. thermohydrosulfuricus* in medium without yeast extract, when cross-feeding appears to be necessary, suggests culturing these co-cultures in medium lacking certain growth factors could be a tool for controlling and optimizing the co-culture populations [248]. This could be especially important when the main lignocellulose solubilizing organism is inhibited or competes with the partners, as observed in Chapter 2.

Different growth profiles were observed for each species when grown alone on wheat straw compared to when they were grown with various co-culture partners, such as the different lag periods for *C. thermocellum* that varied between 0 and 40 hours and that indicate substantial differences in the growing conditions. The nearly identical growth of *T. thermohydrosulfuricus* in the first 18 hours in all cultures suggests that *T. thermohydrosulfuricus* utilizes some carbon source, possibly some form of soluble oligomeric sugar, that the other co-culture partners are unable to use. The divergence in *T. thermohydrosulfuricus* growth after 32 hours of incubation also indicate different conditions and correlates with more oligosaccharides that would be available in the co-cultures, especially the *C. thermocellum* + *T. thermohydrosulfuricus* dual-culture where no competition for released oligosaccharides with *C. stercorarium* would exist.

Water-soluble extractives obtained from raw lignocellulosic biomass are known to contain proteins and other compounds [342]. These extractives have not received attention as to

their ability to alleviate nutrient auxotrophies and thereby reduce medium costs. The complementation of auxotrophies for *C. stercorarium* and *T. thermohydrosulfuricus* with wheat straw extractives shown here indicates that this might be a promising avenue for further research. The auxotrophy alleviation provided by the extractives could also possibly partially explain why the cultures had a reduced ability to degrade and utilize extractive-free wheat straw. At least one study has shown that certain compounds found in water-soluble wheat straw extractives, possibly proteins, can work synergistically with fungal cellulase preparations to increase hydrolysis [343]. The potential lack of these synergistic compounds in the extractive-free substrate could also explain the reduced hydrolysis, especially in the case of *C. thermocellum*, which does not require the extractives to fulfill nutrient auxotrophies.

Cross-feeding of vital nutrients, potentially amino acids, was observed between *C. thermocellum* and two other lignocellulolytic species that allowed the formation of stable co-cultures in defined medium. Alongside other benefits, such as increased hydrolysis rates, cross-feeding in co-cultures provides an opportunity to help reduce medium costs. Understanding how these different species grow and utilize wheat straw, independently or in co-cultures, will allow us to reduce the need for the addition of growth supplements, such as yeast extract, and further optimize CBP processes.

Chapter 5: Characterization of the pyruvate phosphate dikinase from *C. thermocellum**

* Contributing authors: Froese AG[1], Taillefer M[2], Donald L[3], Sparling R[4]. Contributions: [1]Experiment design, experiment work, first author; [2]experiment design, cloning; [3]mass spectrometry; [4]research guidance, manuscript editing

5.1 Abstract

Clostridium thermocellum is one of the most promising candidates for a consolidated bioprocessing approach of converting lignocellulosic substrates to biofuels such as ethanol. The ethanol yield of wild-type *C. thermocellum* is fairly low however, but it has been revealed that variations in glycolysis can significantly impact ethanol production. Glycolysis in *C. thermocellum* is different than canonical systems in multiple ways, including the absence of a pyruvate kinase for converting phosphoenolpyruvate to pyruvate. Two likely alternative routes exist in *C. thermocellum*: a malate shunt, and a pyruvate phosphate dikinase (PPDK) that utilizes pyrophosphate and possibly increases net ATP yields from glycolysis. Previous studies have found low or absent PPDK activity in cell-free extracts despite relatively high protein expression and high predicted flux from modelling studies. The PPDK from *C. thermocellum* was therefore characterized to gain further insights into the metabolism of *C. thermocellum*. The maximum velocity in the catabolic direction, 27 U/mg, was relatively high compared to other characterized PPDKs. Phylogenetic analysis grouped the *C. thermocellum* PPDK with others predicted to go in the catabolic direction, and together with relatively low PP_i and high P_i K_m values, this suggests a glycolytic direction for *C. thermocellums* PPDK. Ammonium was a strong activator, with a K_a of 1.2 mM and a maximum 5-fold activity increase, suggesting strong regulation of this glycolytic node by ammonium. This work furthers our understanding of phosphoenolpyruvate to pyruvate metabolism in *C. thermocellum* and can inform future metabolic studies ultimately aimed at optimizing *C. thermocellum* as a lignocellulosic biofuel platform organism.

5.2 Introduction

Clostridium thermocellum, an anaerobic, thermophilic Firmicute, is one of the most promising candidates for a consolidated bioprocessing approach to producing biofuels using lignocellulosic feedstocks [14, 99]. The ethanol yield of wild-type *C. thermocellum* remains far below the desired level of around 90% [150] due to the production of other end-products such as acetate, lactate, formate, and hydrogen [215, 336]. Efforts to increase ethanol yield in *C. thermocellum* by knocking out genes involved in the production of these other end-products have been moderately successful, at least at low carbon loadings [182, 184, 187]. These mutant strains have significantly lower growth rates and at higher carbon loadings the ethanol yields decrease significantly [182, 187, 275]. In fact, even wild-type *C. thermocellum* displays altered metabolism at high carbon loadings, secreting end-products that never appear at lower carbon loadings, including malate, pyruvate, amino acids, soluble glucans, and various alcohols [250, 268]. Metabolomics and modelling studies have revealed that the thermodynamic driving force for glycolysis in *C. thermocellum* is much lower than that in other organisms, leading to strong inhibition by build-up of end-products under high carbon conditions [235, 280, 344]. This low thermodynamic driving force is due in large part to a few key glycolytic reactions that are atypical compared to other microorganisms [213, 235].

While *C. thermocellum* possesses non-canonical phosphoglycerate kinase and glucokinase enzymes that can utilize guanosine phosphate cofactors in addition to the usual adenosine phosphate cofactors, with either similar or even greater affinity, respectively [213, 222], these cofactor variations do not greatly affect the reaction thermodynamics. *C. thermocellum* also possesses at least one non-canonical phosphofructokinase that utilizes PP_i as a phosphate donor in place of ATP [213, 222, 223] in a highly reversible reaction [235, 284]. In place of pyruvate kinase, which *C. thermocellum* does not possess [213, 215], in contrast to most

other organisms [345, 346], it possesses two other possible pathways for converting phosphoenolpyruvate (PEP) to pyruvate [225]. One pathway, known as the malate shunt, involves PEP carboxykinase, malate dehydrogenase, and malic enzyme. The PEP carboxykinase produces GTP [213] and the rest of the pathway involves a transhydrogenation, with electrons transferred from NADH to $NADP^+$, and is inhibited by PP_i and activated by NH_4^+ [214]. The other option for converting PEP to pyruvate in *C. thermocellum* is a pyruvate phosphate dikinase (PPDK), which concomitantly converts AMP and PP_i to ATP and P_i. The use of PP_i as a phosphate donor by both PP_i-dependant phosphofructokinase and PPDK is thought to confer an energetic advantage to the cell [8, 213], since ATP is saved and PP_i, produced from various anabolic reactions, is otherwise typically wastefully hydrolyzed to heat and P_i by soluble pyrophosphatases in most other organisms [230, 231]. Anabolism does not provide all the PPi required though, and additional PPi must be produced via other mechanisms, such as glycogen cycling or membrane-bound proton-translocating pyrophosphatases, and although these mechanisms ultimately require ATP to produce PPi, each ATP produces more than one PPi, meaning there is still a net increase in the glycolytic ATP yield [8].

The relative flux of PEP to pyruvate conversion through either the PPDK or the malate shunt can thus impact the net ATP yield from glycolysis and how much NADPH is produced, which is especially important since *C. thermocellum* does not have an operative oxidative portion of the pentose phosphate pathway to produce NADPH for anabolic reactions [214, 215, 223]. There are conflicting reports on whether the PPDK is active in exponentially-growing *C. thermocellum* however [213, 225]. Therefore, we cloned the Cthe_1308 gene from *C. thermocellum*, encoding the PPDK, and expressed, purified, and characterized the PPDK in terms of kinetic parameters and regulation.

5.3 Materials and Methods

5.3.1 Strains and reagent

E. coli DH5α was used as the host for plasmid screening. *E. coli* T7 Shuffle (partial genotype: *lacZ::T7 gene1 λatt*::pNEB3-r1-*cDsbC*) (Life Technologies Corp.) was used as the expression strain for recombinant protein expression. PCR purification was done using QIAquick PCR Purification Kit (Qiagen Inc.). All restriction enzymes used were purchased from New England Biolabs. Plasmids were extracted using Qiagen Plasmid Mini Kit (Qiagen Inc.). Recombinant proteins were purified using HiTrap^{TM} chelating column (GE Healthcare Bio-Sciences Corp.). All other reagents used were purchased from either Sigma-Aldrich or Fisher Scientific.

5.3.2 Plasmid preparation

Plasmids were designed using the corresponding gene sequence for the PPDK (Cthe_1308) from Integrated Microbial Genomics [347]. The plasmids were synthesized by GeneArt (Life Technologies Corp.) using a pRSET-A backbone to form the recombinant plasmid pAHCT1308. The recombinant plasmid was transformed and confirmed by restriction digest and sequencing in *E. coli* DH5α. The confirmed plasmids were transformed into *E. coli* T7 Shuffle (New England Biolabs) for recombinant protein expression.

5.3.3 Overexpression and recombinant protein isolation

E. coli T7 Shuffle containing the plasmid pAHCT1308 was cultured overnight in TB medium containing ampicillin (100 µg/mL) at 30°C. Overnight cultures were re-inoculated into fresh TB medium containing ampicillin (100 µg/mL) and grown aerobically at 30°C until an

OD600 of 0.5-0.7. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM. The cultures were grown for an additional 12 hours at 30°C. Cells were harvested by centrifugation and resuspended in buffer containing 20 mM NaH_2PO_4 (pH 7.4), 0.5 M NaCl, and 20 mM imidazole. Cells were lysed at 37°C after 15 minutes incubation with 1 mg/mL lysozyme, 1% Triton X-100, 5 μg/mL DNase, 5 μg/mL RNase, and 5 mM $MgCl_2$ [348]. Cell lysates were centrifuged at 10 000 RPM for 30 minutes at 4°C in a Fiberlite F13-14x50cy rotor. Supernatants were filtered through a 0.22 μm filter prior to being loaded on a Ni^{2+} HiTrap™ metal affinity column and purified following the manufacturer's instructions (GE Healthcare Bio-Sciences Corp.) on an AKTA prime liquid chromatography system (GE Healthcare Bio-Sciences Corp.). The recombinant His_6-tagged protein was eluted using a stepwise imidazole gradient. Imidazole and NaCl were removed from the recombinant protein samples using HiTrap™ Desalting Column eluting with 20 mM NaH_2PO_4 (pH 7.4). Enzyme purity was verified by SDS-PAGE with a 12% resolving gel and a 5% stacking gel. Visualization of the recombinant proteins was done by staining the gels with Coomassie Brilliant Blue R-250. Protein concentration was measured on a Qubit Fluorometer (Thermo Fisher Scientific) using manufacturers' protocol. Protein size and sequence was also verified by mass spectrometry. Briefly, purified proteins were dialyzed into 50 mM NH_4CO_3 and then diluted to approximately 10 μM in a 50% methanol, 1% acetic acid solution. Analysis was performed via electrospray on a time-of-flight instrument built in the Department of Physics and Astronomy at the University of Manitoba [349]. Protein identity was also verified via mass spectrometry of peptide digests. Briefly, 1 mg/mL protein solutions were digested with trypsin and then mixed with an equal volume of DHB matrix (160 mg/mL 2,5-dihydroxybenzoic acid in 3:1 acetonitrile:water, 2%

formic acid solution). Analysis was performed via MALDI on a Bruker Ultra FLEX ToF-ToF instrument [350].

5.3.4 Enzyme assays

All enzyme activities were measured in a 300 µl well with a total reaction volume of 200 µl at 50°C. PPDK activity in the forward pyruvate-forming direction was measured in a standard coupled reaction containing 100 mM MOPS (pH 7.4), 10 mM DTT, 2.5 mM $MgCl_2$, 20 mM NH_4Cl, 0.4 mM PP_i, 2 mM phosphoenolpyruvate, 2 mM AMP, 0.2 mM NADH, and 10 U/mL lactate dehydrogenase, similar to [225]. Phosphoenolpyruvate, PP_i, AMP, and NH_4Cl concentrations were varied. When testing for pyruvate inhibition the reaction solution substituted 2 mM glucose, 0.2 mM $NADP^+$, 10 U/ml hexokinase, and 10 U/mL glucose-6-phosphate dehydrogenase in place of NADH and lactate dehydrogenase, similar to [351]. The assays were started by addition of PP_i. PPDK activity in the reverse phosphoenolpyruvate-forming direction was measured in a standard reaction containing 100 mM MOPS (pH 7.4), 10 mM DTT, 5 mM $MgCl_2$, 20 mM NH_4Cl, 2 mM P_i, 2 mM pyruvate, 2 mM ATP, 0.2 mM NADH, 4 mM Na_2CO_3, 10 U/mL phosphoenolpyruvate carboxylase, and 10 U/mL malate dehydrogenase, similar to [352]. Pyruvate, P_i, ATP, and NH_4Cl concentrations were varied. The assay was started by addition of P_i. The lactate dehydrogenase and phosphoenolpyruvate carboxylase enzymes purchased from Sigma as ammonium sulfate suspensions were desalted using HiTrap™ desalting column into 20 mM MOPS buffer to ensure no carry-over of ammonium in the assays. Changes in cofactor (NAD(P)H) concentration were monitored at 340 nm using either a BioTek Synergy 4 or a Molecular Devices SpectraMax iD5 plate reader.

5.3.5 Kinetic properties

The kinetic properties for the recombinant PPDK were determined by varying substrate or cofactor concentrations while keeping the concentration of all other constituents at saturating levels at 50°C and pH 7.0. Inhibition assays were performed using the standard reaction conditions above, but with varying inhibitor concentration at 50°C. All kinetic parameters were determined by fitting the data to the Michaelis-Menten equation using Sigma-Plot (Systat Software Inc.).

5.3.6 Phylogenetic analysis

The PPDK amino acid sequences from all species were obtained from the UniProt database. Sequences were aligned using ClustalW in MEGA X. The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model [353]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. Evolutionary analyses was conducted in MEGA X [354].

5.4 Results

The monomeric molecular mass of the cloned and purified His-tagged PPDK was approximately 100 kDa, corresponding to the predicted molecular mass of the PPDK protein containing the 6xHis Tag, and the protein sequence was confirmed as that encoded by the Cthe_1308 gene, as determined by SDS-PAGE (Supplementary Fig. A.5.1) and mass spectrometry (Supplementary Fig. A.5.2). PPDK activity was found to be specific for AMP with no activity detected with GMP. While the K_m values for PEP and pyruvate were similar, the K_m

value for AMP was 34-fold lower than for ATP, and the K_m value for PP_i was 143-fold lower than for P_i (Table 5.1). The maximum velocity rate in the forward direction, 27 U/mg, was almost double that of the reverse direction, 15 U/mg. The activity in both the forward and reverse directions was found to be significantly increased in the presence of NH_4^+, with 5.0-fold and 2.9-fold increases, respectively, in the presence of 20 mM NH_4^+ (Fig. 5.1). This monovalent cation activation could only be partially substituted by K^+, with 69% activity relative to NH_4^+ at 20 mM, and not at all by Na^+. No significant substrate inhibition was observed in either direction, but the forward reaction was found to be inhibited by the product pyruvate in a competitive manner with phosphoenolpyruvate, with 57% inhibition at 2 mM pyruvate under standard conditions. Other inhibitory compounds were also identified: 3-phosphoglycerate (16% inhibition at 2 mM), acetyl-CoA (29% inhibition at 1 mM), and alpha-ketoglutarate (27% inhibition at 2 mM). The enzyme required Mg^{2+} for activity and this requirement could not be substituted by other divalent cations such as Co^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+}. Some divalent cations were found to completely inhibit the reaction at a concentration of 2 mM: Ca^{2+}, Cu^{2+}, and Mn^{2+}. The pH optimum for the forward reaction was 6.8 while the optimum for the reverse reaction was slightly higher, around pH 7.3 (Fig. 5.2). The PPDK from *C. thermocellum* clustered strongly with the closely related *C. symbiosum* and weakly with other primarily glycolytic PPDKs from eukaryotic parasites (i.e. *Trypanosoma* species and *Giardia intestinalis*) during phylogenetic analysis (Fig 5.3). The PPDKs from plants all clustered strongly together and weakly with other gluconeogenic bacteria, while the bottom clade contained both gluconeogenic and glycolytic sub-clusters.

Table 5.1: Kinetic values of PPDK from *C. thermocellum* and other organisms

Organism	Predicted physiological direction	Specific activity (U/mg)		K_m values (µM)						Ref.
		Glycolytic direction	Gluconeogenic direction	AMP	PP_i	PEP	ATP	P_i	pyruvate	
Triticum aestivum (common wheat)	gluconeogenic	-	0.2	-	-	-	36	430	25	[355]
Zea mays (maize)	gluconeogenic	1.49	1.61	<10	40	140	15	1500	250	[352]
Sugarcane	gluconeogenic	-	-	<4	40	110	90	500	110	[356]
Microbispora rosea	gluconeogenic	9.92	-	5	38	280	200	-	130	[357]
Propionibacterium freudenreichii subsp. *shermanii*	gluconeogenic	2.1	-	1.5	-	-	4	1000	100	[358]
Komagataeibacter xylinus	gluconeogenic	-	0.88	1.6	60	100	400	800	200	[359]
Acetobacter aceti	gluconeogenic	5.8	1.8	6	62	130	200	830	27	[360]
Giardia intestinalis	glycolytic	17	-	1.3	29	33	32	1800	75	[361]
Trypanosoma brucei	glycolytic	2.5	-	7.5	50	40	600	500	300	[362]
Trypanosoma cruzi	glycolytic	0.9	-	17	70	320	-	-	-	[363]
Entamoeba histolytica	glycolytic	8.1	1.5	2	91	30	284	-	305	[364]
Thermoproteus tenax	glycolytic	2.5	1.1	20	80	500	-	3500	800	[365]
Clostridium symbiosum	glycolytic	35	-	15	80	100	50	800	100	[358]
Clostridium thermocellum	glycolytic	27	15	4	27	202	137	3864	115	This study

-: information not available

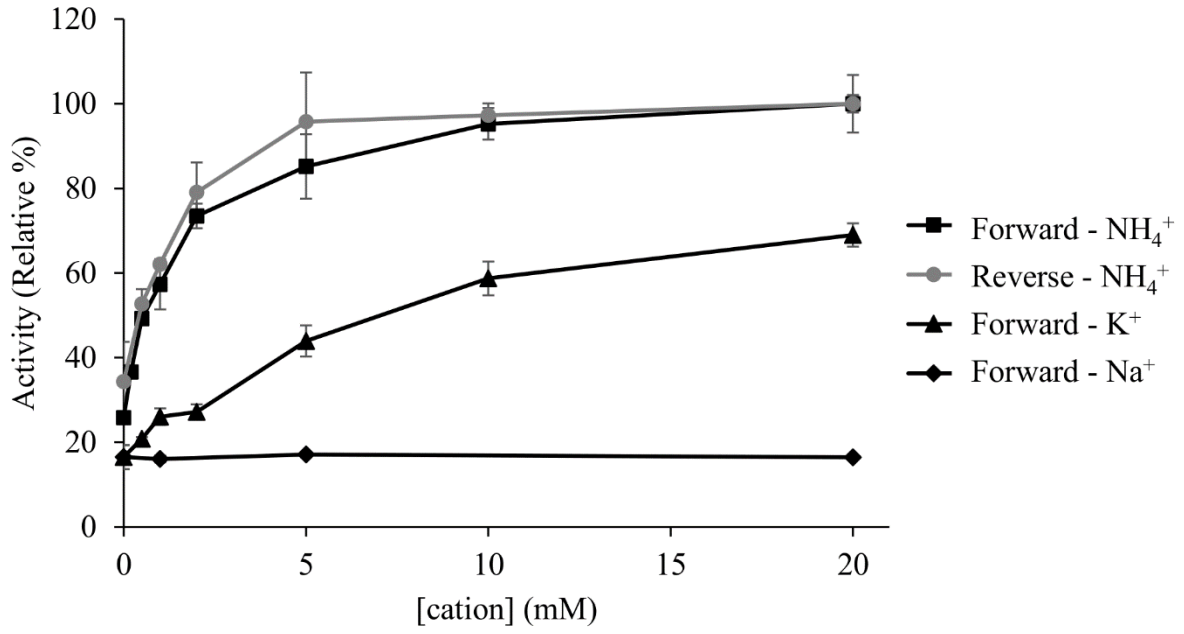

Fig. 5.1: Effect of ammonium and other monovalent cations on the activity of the recombinant PPDK in both directions.

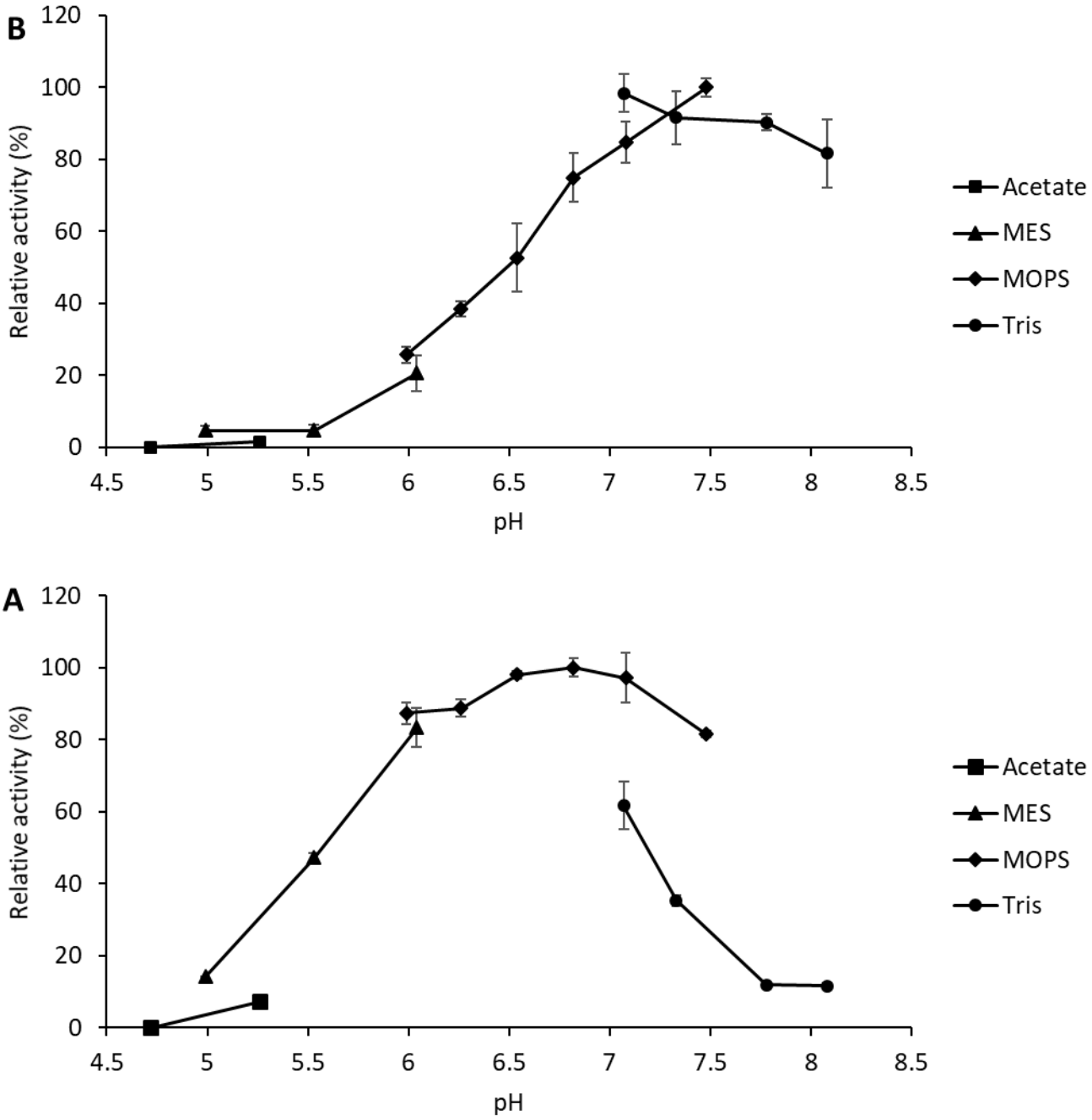

Fig 5.2: Effect of pH on recombinant PPDK activity in the forward (A) and reverse (B) directions in specified buffers

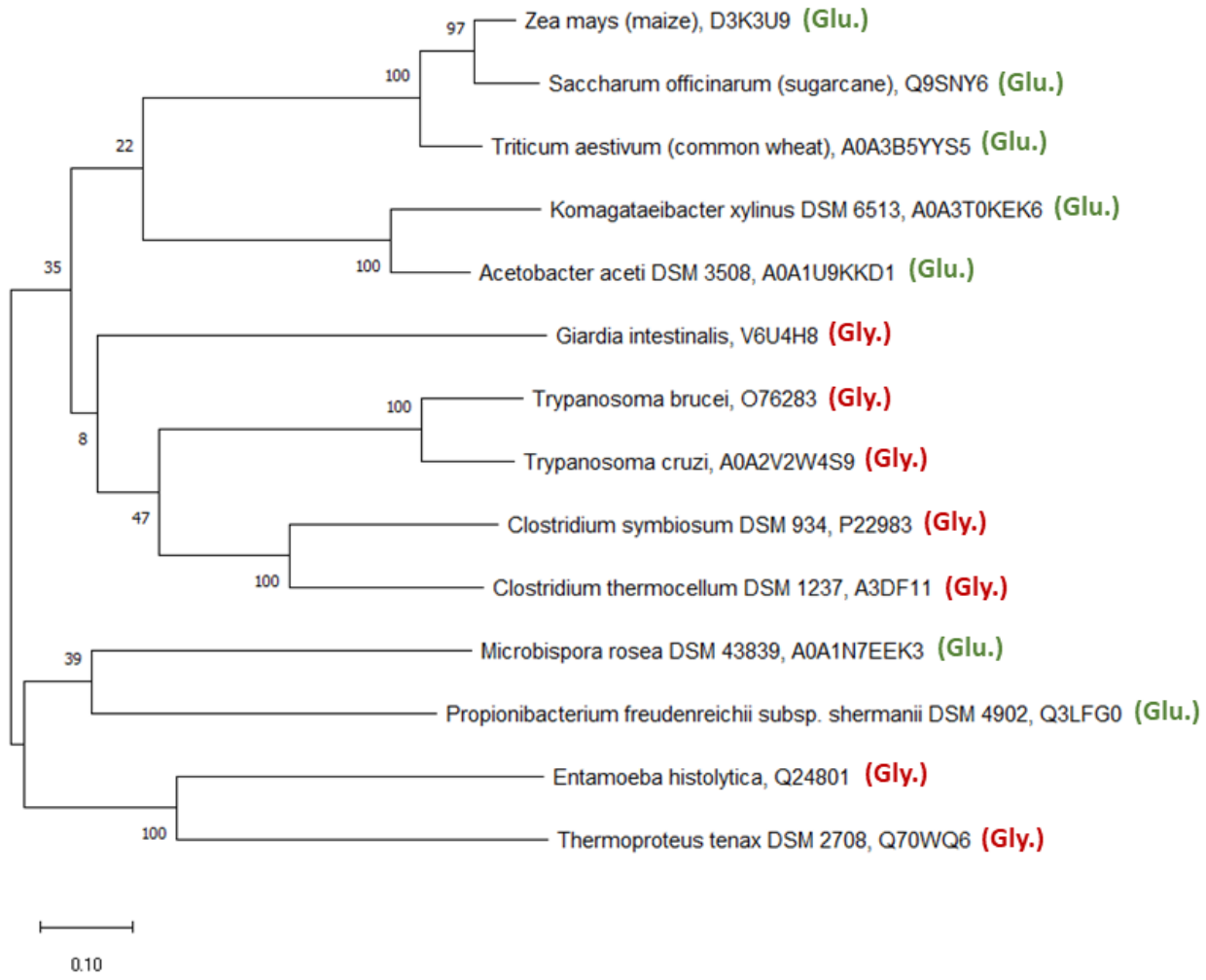

Fig. 5.3: Phylogenetic analysis of biochemically characterized PPDKs from various species using Maximum Likelihood method. The tree with the highest log likelihood (-18662.44) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. UniProt accession numbers for each protein are indicated. Predicted main physiological direction of PPDK is also indicated: **(Glu.)**, gluconeogenic; **(Gly.)**, glycolytic

5.5 Discussion:

The Cthe_1308 gene was confirmed to encode a functional PPDK. Most of the kinetic parameters fall within the range of values observed from previously characterized PPDKs (Table 5.1). The specific activity of the *C. thermocellum* PPDK is quite high though compared to most other PPDKs, and approaches the 35 U/mg activity of the most active PPDK tested to date [358]. The K_m values for PP_i and P_i are exceptional as well, being the lowest and highest values, respectively, ever recorded for those substrates [361, 365]. The activation of PPDK by ammonium may help to explain why certain previous reports failed to detect any measurable PPDK activity in cell-free extracts [213] while more recent reports have detected activity under high ammonium conditions [225]. This monovalent cation activation effect has been observed before for other PPDKs [351, 363, 366], and is thought to aid in the enolization step of the reaction [367], but this cation activation effect does not appear to occur for all PPDKs [365, 368]. It is interesting to note that both of the pathways for converting PEP to pyruvate in *C. thermocellum*, PPDK and the malate shunt, are activated significantly by ammonium [214, 369], suggesting its internal concentration could be an important regulator of metabolism.

The PPDK reaction, which is essentially reversible [365], is known to operate in the anabolic direction in some plants [370], and certain bacteria that undergo gluconeogenesis [371, 372], and operates in the catabolic direction in certain eukaryotic parasites [364], anaerobic bacteria [351, 373], and archaea [365]. While there does not appear to be any grouping of gluconeogenic [352, 357, 359, 360] vs glycolytic [358, 361, 366, 374] PPDKs based on K_m values (Table 5.1), there does appear to be some grouping based on amino acid sequence (Fig. 5.3). Thus, a glycolytic role for the PPDK in *C. thermocellum* is suggested by its phylogenetic grouping, the higher activity in the catabolic direction, unusually wide disparity between PP_i and P_i K_m values, and high expression during glycolytic growth [215], as well as the general lack of

need for gluconeogenesis. This suggests that the PPDK could account for a substantial amount of the flux from PEP to pyruvate. This has been recently demonstrated in exponentially growing *C. thermocellum* via isotope labelling and metabolic modelling, which found that approximately 66% of the flux from PEP to pyruvate proceeded through PPDK, with the rest through the malate shunt [225]. This portion of flux could change throughout growth and under different conditions, however, due to possible changes in the concentrations of certain metabolites, such as PP_i and ammonium, that strongly regulate this node [214]. For instance, the concentration of PP_i in the closely related *Caldicellulosiruptor saccharolyticus*, which also possesses a PP_i-dependant phosphofructokinase and a PPDK, changes significantly between exponential and stationary phase [373]. PP_i was also found to vary significantly throughout growth in two other Clostridia, *Clostridium pasteurianum* and *Clostridium thermoaceticum,* in contrast to *E. coli*, in which it remained fairly constant [375]. Attempts were made to measure the concentration of PP_i in *C. thermocellum* in this study but were unsuccessful.

C. thermocellum possesses many atypical aspects of its central metabolism, including two non-pyruvate kinase routes for converting PEP to pyruvate (Fig. 1.3). Now that both the malate shunt (excluding the PEP carboxykinase) and the PPDK have been characterized, it allows further studies, notably metabolomics, to determine the flux between the two routes in various conditions and further understand the role of both routes in the metabolism of *C. thermocellum* and how it might affect production of various end-products, such as ethanol for CBP purposes.

Chapter 6: Conclusions and future directions

6.1 Thesis conclusions

The ever-increasing effects of climate change demonstrate a significant need to transition away from fossil fuels to "greener" alternatives, including lignocellulosic biofuels. Consolidated bioprocessing represents a promising lower-cost approach to producing these advanced biofuels by hydrolyzing the substrate and fermenting the sugars simultaneously in one vessel under constant conditions. *C. thermocellum* is one of the most promising candidates for this process due to its efficient cellulosomes that allow relatively high lignocellulose degradation rates. However, the production of ethanol, as transportation fuel, from lignocellulose by *C. thermocellum* is not currently cost-competitive with fossil fuels. This is, in large part, due to incomplete hydrolysis of raw lignocellulose, necessitating expensive and harsh feedstock pretreatment, as well as low ethanol yields and titres [7].

The overarching aim of this thesis was to further the possibilities of realizing commercial lignocellulosic ethanol production by *C. thermocellum*. This was split into looking at ways in which lignocellulose hydrolysis might be improved and characterizing the glycolytic metabolism of *C. thermocellum* that can impact ethanol production and cell yields. Co-cultures of *C. thermocellum* with hemicellulose-utilizing partners were investigated for their potential to: increase hydrolysis rates and extents of raw lignocellulosic substrates, screen digestibility of various feedstocks, and cross-feed nutrients between each other. The pyruvate phosphate dikinase of *C. thermocellum* was then characterized to further determine its role in glycolytic metabolism. Specifically, the conclusions for each objective are as follows.

6.1.1 Objective 1: Characterize lignocellulolytic potential and growth profiles of co-cultures

In efforts to increase the hydrolysis rate and extent of raw or minimally treated lignocellulose material, *C. thermocellum* was co-cultured with two different bacteria that could each utilize hemicellulose, or at least xylan, on their own, and produce ethanol. Since *C. thermocellum* is unable to utilize the hemicellulose sugars, leading to build-up of the sugars that can inhibit hydrolysis and growth, the hypothesis was that co-culturing *C. thermocellum* with these hemicellulose-utilizers would lead to a decrease in the inhibitory sugar concentration and therefore increase the overall solubilization of the lignocellulose. The additional free cellulolytic and hemicellulolytic enzymes secreted by the partners might also contribute to lignocellulose hydrolysis.

As predicted, *C. thermocellum* was able to hydrolyze a portion of the hemicellulose fraction of the raw wheat straw substrate, leading to a build-up of both monomers and soluble oligomers in the medium (Chapter 2). The levels of hemicellulose-derived sugars were much lower in co-cultures, indicating uptake and conversion to end-products, which were much higher in the co-cultures as well. When estimating total substrate solubilization by summing end-products and soluble sugars it was discovered that the co-cultures of *C. thermocellum* with either *C. stercorarium* and/or *T. thermohydrosulfuricus* solubilized significantly more total polysaccharides, up to 58% more, than the *C. thermocellum* monoculture. This solubilization difference almost completely vanished after serial passaging however, and stabilized co-cultures solubilized either the same amount or only slightly more than the *C. thermocellum* mono-culture control (Fig. 6.1). Some competition between the co-culture members appeared evident as shown by the decrease in *C. thermocellum* cell numbers during serial passaging in the co-cultures (Fig. 6.2), which is possibly what lead to the decreased solubilization in serially passaged cultures.

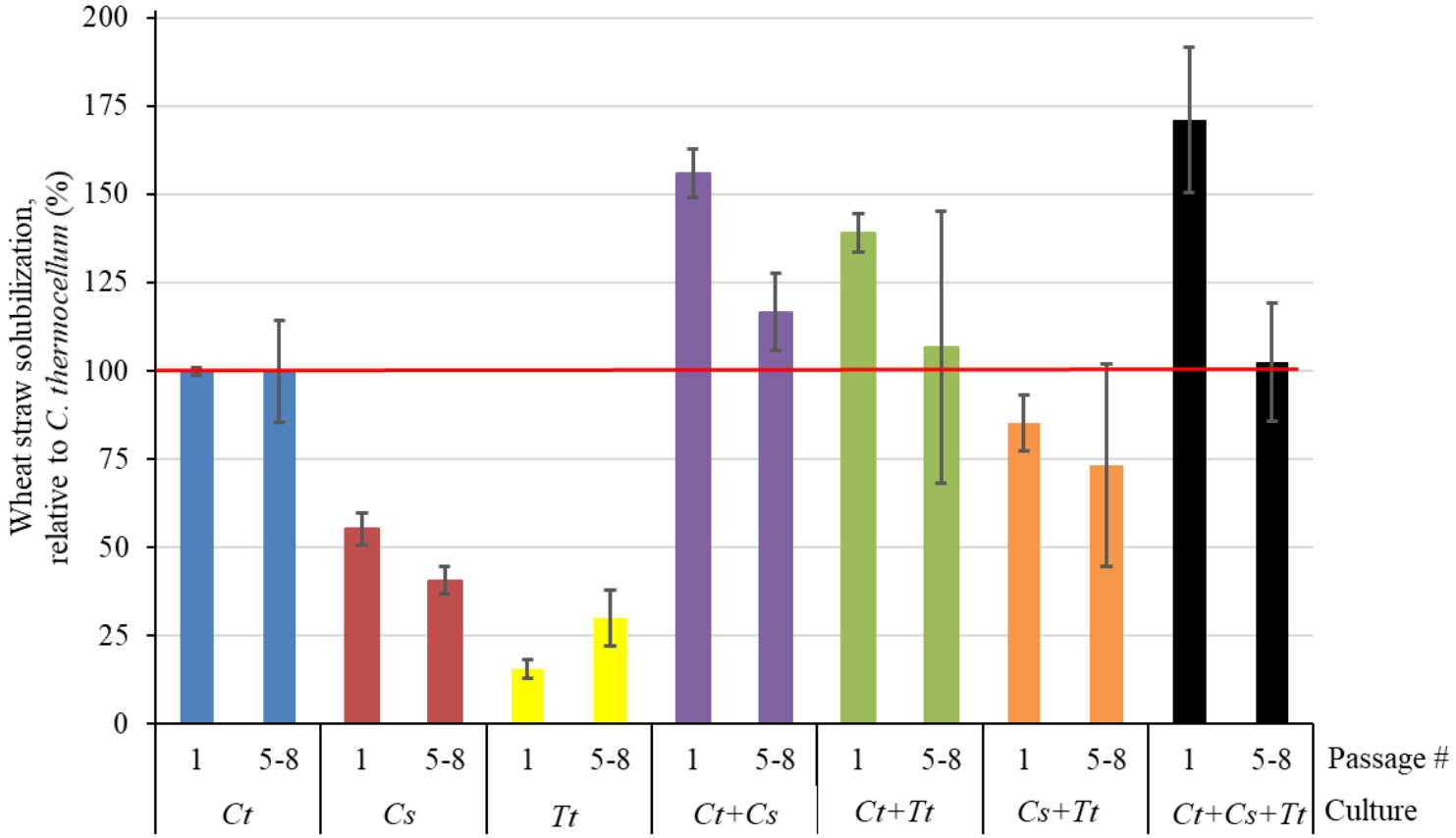

Fig. 6.1: Wheat straw solubilization of *C. thermocellum*-containing co-cultures, compared to the *C. thermocellum* mono-culture control, were significantly higher in the first passage but similar in the later passages. Solubilization was determined by summing end-products and soluble sugars released from hydrolysis. Data from Chapter 2. Data shown are for passage 1 and the averages from passage 5 to 8.

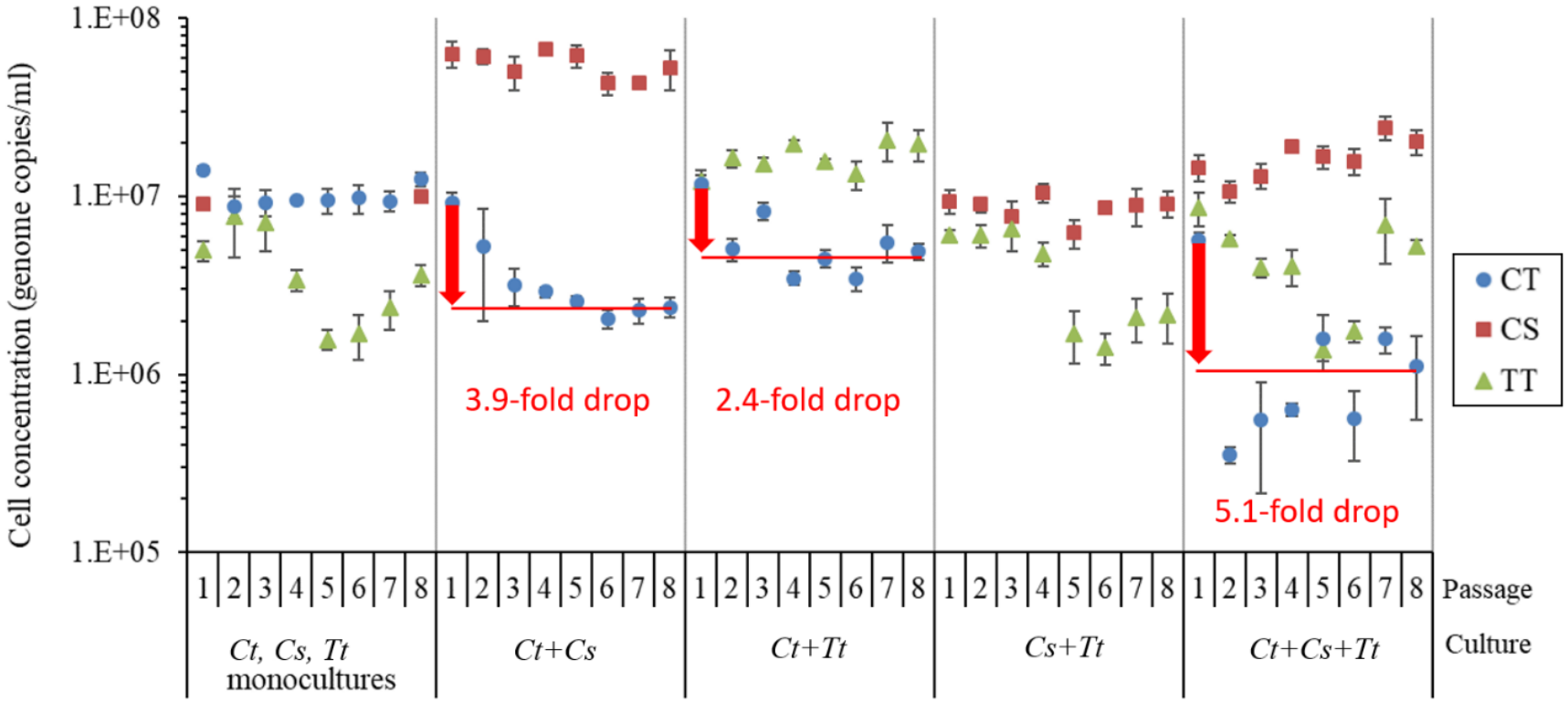

Fig. 6.2: *C. thermocellum* cell numbers drop significantly in the co-cultures during serial passaging, compared to the stable mono-culture control. *C. thermocellum* cell number decrease values shown are from the first passage to the average of passages 5 to 8. Data from Chapter 2. *Ct*: *C. thermocellum*, *Cs*: *C. stercorarium*, *Tt*: *T. thermohydrosulfuricus*

Despite lower *C. thermocellum* cell numbers in the later passages of the co-cultures, the solubilization of these passages were still similar to *C. thermocellum* control levels, indicating continued hydrolysis synergy. The reason for this synergy, whether inhibitory-sugar removal, or additional hydrolysis enzyme suites, was not resolved.

6.1.2 Objective 2: Screen digestibility of various wheat straw and cattail feedstock samples with tri-culture

Screening the digestibility of various lignocellulose feedstock samples, whether different cultivars or different samples of a single cultivar grown or harvested under various conditions, is important in order to elucidate the properties that impact digestibility. Most screening in the literature is done via commercial enzyme and yeast technologies, due to its amenability to high throughput assays. Cellulosome-utilizing CBP candidates such as *C. thermocellum* degrade lignocellulose in a different manner than fungal enzymes however, and thus, if CBP technologies are needed to fully realize economic viability of lignocellulosic ethanol production, then it is important to screen lignocellulose via those same CBP technologies in order to determine the biomass properties that affect digestibility by CBP and that might not affect digestibility by fungal enzymes in the same manner or to the same degree. Wheat is an important food crop and cattail has high bioremediation potential, and thus their lignocellulosic portions are potential feedstocks for CBP purposes. Therefore, we validated the synergy of the co-cultures on various lignocellulose feedstocks and then screened various wheat straw cultivars and cattails harvested under different conditions.

The co-cultures produced significantly more end-products than the *C. thermocellum* monoculture on all substrates tested, although the percent increase varied significantly depending

on treatment and feedstock source (Chapter 3). The tri-culture was successfully used to screen various feedstocks. While cattail digestibility did not vary considerably between the two species tested, it did vary drastically between harvesting seasons, with much greater digestibility observed when the plants were harvested during the growing season, in July. The wheat straw samples also showed little variation between cultivars and this variation was not correlated with either lodging resistance or lignin, cellulose, or hemicellulose composition.

6.1.3 Objective 3: Determine cross-feeding potential of co-cultures and nutrient potential of wheat straw

Symbiotic relationships are widely found throughout nature, including environmental microbial communities. Various compounds are secreted by certain members and taken up by others, interchanging throughout the community, including carbon sources, amino acids, and vitamins [248]. Cross-feeding can be beneficial for industrial microbial processes, allowing the fulfilment of auxotrophies present in one or more of the microbes, which prevents the need for adding such exogenous growth factors, thereby lowering medium costs. Cross-feeding can also control community population ratios by constraining the growth of certain members to the growth of others [248]. Lignocellulose feedstocks are typically only characterized in terms of digestibility of their lignocellulose structure and the other compounds they might contain, including proteins and other metabolites, are often overlooked. Therefore, we endeavored to determine 1) whether any cross-feeding was occurring in our designed co-cultures and 2) determine whether the presence of other compounds in wheat straw could affect the CBP process.

Cross-feeding of growth factors was observed from *C. thermocellum* to both *C. stercorarium* and *T. thermohydrosulfuricus*, and between *C. stercorarium* and *T. thermohydrosulfuricus* themselves, that allowed growth in a defined medium (Chapter 4, Fig. 4.1). These cross-fed growth factors did not fully compensate for a lack of yeast extract, however, observed by reduced solubilization of wheat straw in defined medium. Growth profiles of the various members, especially *T. thermohydrosulfuricus*, varied significantly between the co-cultures, indicating significant reliance on the other members for providing carbon sources and/or other growth factors. Indeed, the cell number ratios of *C. thermocellum* to either *C. stercorarium* or *T. thermohydrosulfuricus* were much higher when the co-cultures were grown in medium without yeast extract (Supplementary Fig A.4.5), in which cross-feeding is apparently needed, indicating the potential of cross-feeding for controlling population ratios. Post-fermentation substrate analysis confirmed that the co-cultures were solubilizing more cellulose and hemicellulose than the *C. thermocellum* control. Growth factors were revealed to be present in wheat straw extractives, enhancing the growth and hydrolysis of the members, especially *C. stercorarium*, in defined medium.

6.1.4 Objective 4: Characterize PPDK of *C. thermocellum*

Efforts to metabolically engineer *C. thermocellum* for higher ethanol yields have been moderately successful, but high ethanol titres at high carbon loadings have been difficult to achieve due to ethanol easily inhibiting the atypical glycolysis that has lower thermodynamic driving force than other microorganisms [235]. The central metabolism of *C. thermocellum* has numerous aspects that differ from the canonical system from model organisms such as *E. coli*, including two possible alternate routes for converting phosphoenolpyruvate to pyruvate in lieu of

a pyruvate kinase: the malate shunt and a pyruvate phosphate dikinase. A possible reason for the use of the PPDK instead of the usual pyruvate kinase may be to increase the net yield of ATP from glycolysis, as the PPDK uses PP_i, a by-product of anabolic reactions that is typically otherwise wastefully hydrolyzed using a soluble pyrophosphatase (e.g. *E. coli* [230]). While isotope-tracing and modelling estimate that approximately 66% of the flux from PEP to pyruvate occurs through PPDK, with the balance through the malate shunt [225], some studies have failed to find any PPDK activity in cell-free extracts [213]. Therefore, we cloned the Cthe_1308 gene, putatively encoding the PPDK, and expressed, purified, and characterized PPDK.

The Cthe_1308 gene was confirmed to encode a functional PPDK (Chapter 5). The enzyme was strongly activated by ammonium, possibly explaining why some previous reports failed to detect any activity. The kinetic parameters were similar to other characterized PPDKs, other than a few unusual values, such as the high activity, low PP_i Km, and high P_i Km values. The catabolic direction-favouring K_m values, high activity, and high expression in proteome during glycolytic growth support the hypothesis that PPDK plays an important role in converting PEP to pyruvate in *C. thermocellum*. The ammonium activation of both PPDK and the malate shunt indicate that ammonium and PP_i may be important regulators of *C. thermocellum's* metabolism, especially the PEP to pyruvate node (Fig. 6.3).

6.2 Future work

Although lignocellulose hydrolysis synergism was observed in the co-cultures, the exact reason remains unclear. One of the important next steps in the project would be to determine whether the enhanced hydrolysis effect observed in the co-cultures is due to either the additional lignocellulolytic enzymes provided by *C. stercorarium* or *T. thermohydrosulfuricus*, or the uptake of inhibitory hemicellulosic mono- and oligosaccharides, or perhaps both. One way in

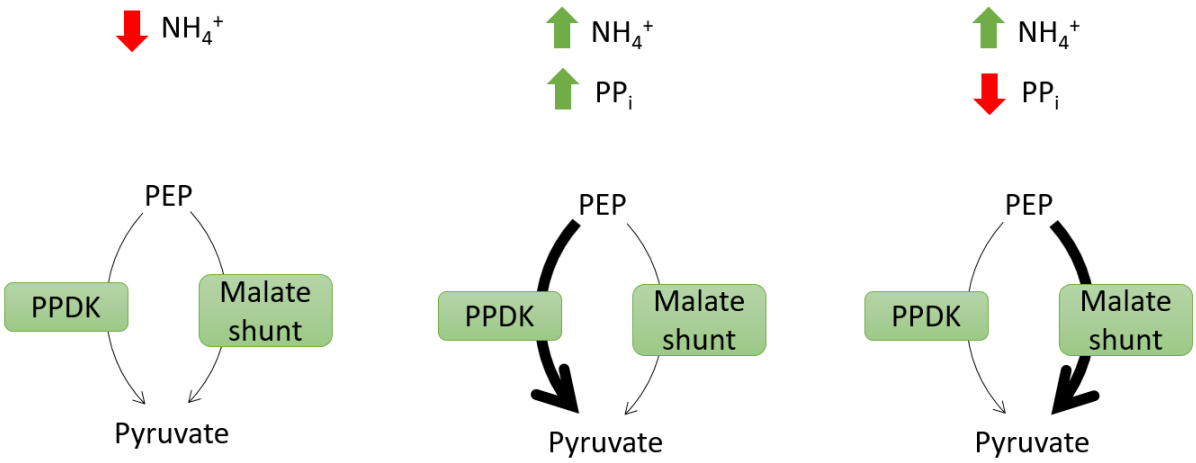

Fig. 6.3: Model of regulation for phosphoenolpyruvate (PEP) to pyruvate metabolism in *C. thermocellum*. Larger arrow indicates higher flux. Under low ammonium conditions (left panel) flux would be low through either pathway since both need ammonium for activation. Under high ammonium and high PP_i (middle panel) the PPDK would be favoured due to inhibition of the malate shunt, but when PP_i is low (right panel) the malate shunt would be favoured due to no inhibition of the malate shunt and no available PP_i substrate for the PPDK.

which this could be achieved would be to collect and concentrate the supernatants of *C. stercorarium* and *T. thermohydrosulfuricus* monocultures, containing their respective secreted enzyme suites, add it to *C. thermocellum* monocultures grown on lignocellulosic substrates, and determine whether any increase in solubilization is achieved. Another method, perhaps in complementation to the above, would be to co-culture *C. thermocellum* with a different partner that is able to up-take hemicellulosic mono- and oligosaccharides but does not secrete any hydrolytic enzymes of its own, and compare that with the already characterized co-cultures.

One potential issue with the co-cultures identified was the moderate cellodextrin competition observed between *C. thermocellum* and its partners (Chapter 2). This appeared to limit the growth of *C. thermocellum*, which might explain the decreased solubilization observed in the serially passaged cultures. It would be interesting to determine whether lignocellulose hydrolysis could be further increased by eliminating this cellodextrin competition, either by selecting a hemicellulose-utilizer that is incapable of utilizing glucose and cellodextrins, or by knocking out needed glucose and cellodextrin transporters in either *C. stercorarium* or *T. thermohydrosulfuricus*.

While *C. thermocellum* cells are known to attach to the surface of cellulose, forming monolayer biofilms [376], and *C. stercorarium* appears to attach to cellulose via multidomain S-layer homology domain-containing proteins [377], it is unknown whether *T. thermohydrosulfuricus* cells attach to the surface of cellulose. It would be valuable to know whether any of these species attach to hemicellulose as well, and if the attachment to raw lignocellulose materials changes depending on the source. This could be accomplished via either microscopy of lignocellulose cultures or even bacterial quantification of pelleted substrate versus the supernatant. Determining whether any potential attachment is altered in co-cultures would

also be of interest, considering the possible formation of layered biofilms, which could have significant effects on lignocellulose hydrolysis [248].

While cross-feeding of growth factors was observed in this work (Chapter 4), the exact components were not resolved. This would be another interesting direction for future research, part of which could be accomplished through bioinformatic analysis. It would also be interesting to measure the levels of these growth factors in wheat straw extractives, and perhaps other lignocellulose substrates as well.

The exact role of PPDK in the metabolism of *C. thermocellum* is still unknown, considering some studies estimate that approximately 66% of flux from PEP to pyruvate is estimated to proceed through PPDK [225] but yet deletions of PPDK appear to have minimal phenotypic effects [213, 225]. The next step in understanding the central metabolism of *C. thermocellum* and the role of the PPDK would be to accurately determine the concentration of important metabolites and regulators, such as PP_i, ammonium, and ATP, and whether they change throughout growth or under various conditions, such as growth on cellulose or wheat straw, when glycolytic flux is lower. This would be especially informative when combined with metabolic modelling to create and test models of the central metabolism of *C. thermocellum* and how it would react to various genetic perturbations or different conditions. This work would greatly benefit from being able to compare wild-type strains against PPDK knockout strains already created in other labs in other strains of *C. thermocellum* (DSM 1313 for the most part).

6.3 Future perspectives

Although hydrogen and electricity are predicted to supply the majority of transportation energy needs for light passenger vehicles, busses, and trains by 2075 there still exists need for biofuel in the interim for light passenger vehicles and into the foreseeable future for

transportation modes that are difficult to otherwise fuel, such as air and ocean travel [5]. Although ethanol is not an ideal biofuel, it is a good starting point and proving grounds for lignocellulosic biofuels before more advanced biofuels can be engineered [7, 8]. CBP promises to be a great approach to producing lignocellulosic biofuels by lowering costs through the implementation of single vessels in which hydrolytic enzyme production, hydrolysis, and fermentation all take place simultaneously, both reducing complexity and facilitating synergism between hydrolysis and fermentation. Despite this promising concept, no organism has been found to possess all of the required attributes required for CBP, including high lignocellulose hydrolysis and high ethanol yield [7]. One of the most promising candidates for CBP, *C. thermocellum*, still requires pretreatment in order to completely hydrolyze lignocellulose materials [14] and ethanol yields and tires remain below ideal values, despite numerous and moderately effective genetic engineering attempts [187, 275, 378]. Advances will be needed on both the lignocellulose hydrolysis and ethanol yield/titre fronts in order to realize commercially viable ethanol production from *C. thermocellum*.

One recent technique that shows promise in increasing lignocellulose hydrolysis by *C. thermocellum* and similar lignocellulolytic bacteria is ball milling co-treatment [14, 173, 247], which can increase total polysaccharide solubilization by up to 480% on especially recalcitrant substrates, such as poplar [246]. Ball milling has historically been considered too energy-intensive for CBP purposes [379–381], but that had been considering it as a pre-treatment, not as co-treatment, in which the slurry conditions might lower energy requirements enough for feasibility [246, 247], but this requires further research. Another promising approach to increase hydrolysis is to supplement *C. thermocellum* with synergistic hydrolytic enzymes, possibly via co-culturing. Although the increases in hydrolysis observed in this thesis were modest, possibly

impeded by cellodextrin competition, this work indicates the approach has potential, supported by previous synergism observed between *C. thermocellums* cellulosome system and secreted enzyme systems [382].

Hemicellulose-derived sugar inhibition remains a barrier to complete lignocellulose hydrolysis by *C. thermocellum*, especially at higher carbon loadings that would occur in industrial-scale processes [198]. One method to ameliorate this issue is by genetically engineering *C. thermocellum* to utilize pentose and pentose oligosaccharides simultaneously with cellodextrins, which has been accomplished, although the recombinant strain was unable to degrade and utilize cellulose and xylan simultaneously, a serious problem that indicates unknown regulatory mechanisms that would need to be discovered and resolved [199]. The other way to ameliorate this issue is through co-culturing with pentose-utilizing partners, as described throughout this work. Additional metabolic engineering may be required for each additional partner incorporated in order to achieve high ethanol yields though, but higher ethanol yields have been much simpler to engineer in certain hemicellulose-utilizing partners than *C. thermocellum* [8].

The highest ethanol-yielding strains of *C. thermocellum* created so far, through competitive pathway knockouts and adaptive evolution, have yielded, on high industrially-relevant carbon loads, around 50% [378] and 75% [275] of the maximum ethanol yield from 100 g/l and 60 g/l cellulose, respectively, falling below the 90% target value mark [150]. Part of this inability to achieve higher ethanol yields from high carbon loadings appears to be the PP_i-dependent metabolism of *C. thermocellum* and other cellulolytic anaerobes [232] that allow them to benefit from increased ATP yields from glycolysis but have the drawback of resulting in thermodynamic inhibition of glycolysis at relatively low ethanol titres [235]. Greater

understanding of this atypical metabolism and its implications in *C. thermocellum* growth and ethanol yield will be needed in order further engineer this species to attain the required levels of ethanol yields needed at higher carbon loadings to fully realize the promise of CBP for lignocellulosic biofuel production.

Appendix A.1: Supplemental information for Chapter 1

Table A.1.1: Name changes of bacterial species used in text

"Older" or common name	Current name	Reference
Clostridium cellulolyticum	*Acetivibrio cellulolyticus*	[76]
Clostridium phytofermentans	*Lachnoclostridium phytofermentans*	[77]
Clostridium termitidis	*Ruminiclostridium cellobioparum* subsp. *termitidis*	[94]
Clostridium thermocellum	*Acetivibrio thermocellus*	[76]
Clostridium clariflavum	*Acetivibrio clariflavus*	[76]
Clostridium stercorarium	*Thermoclostridium stercorarium* subsp. *stercorarium*	[94]

Appendix A.2: Supplemental information for Chapter 2

Table A.2.1: Primer and probe sequences used for *cpn60* based qPCR for cell enumeration

Primer/probe	Sequence (5' - 3')	Target (locus tag)	Amplicon length (bp)
CT5-F	TCC TCA TTA CAG ACA AGA A	*Clostridium thermocellum* DSM 1237 *cpn60* (Cthe_2892)	148
CT5-R	CCT CTT AAT TTG TTT ACA AGC		
CT5-probe*	CCT CAA CAT CCT CAG CAA TGA TAA CC		
CS5-F	TCC TGA TTA CTG ACA AGA A	*Clostridium stercorarium* DSM 8532 *cpn60* (Clst_2558)	149
CS5-R	TCC TCT CAG TTT GTT CAG		
CS5-probe*	TGC CAA TGC TTC GCC TTC AA		
TT5-F	ACC ACT CTT AGA ACA AAT AG	*Thermoanaerobacter thermohydrosulfuricus* DSM 26960 *cpn60* (TthWC1_2665)	125
TT5-R	CAG CTA CAC ATG TAA ATG TA		
TT5-probe*	ATG TTG CCA ATG CTT CTC CTT CTA C		

* 5'-(FAM or HEX); 3'-Black Hole Quencher

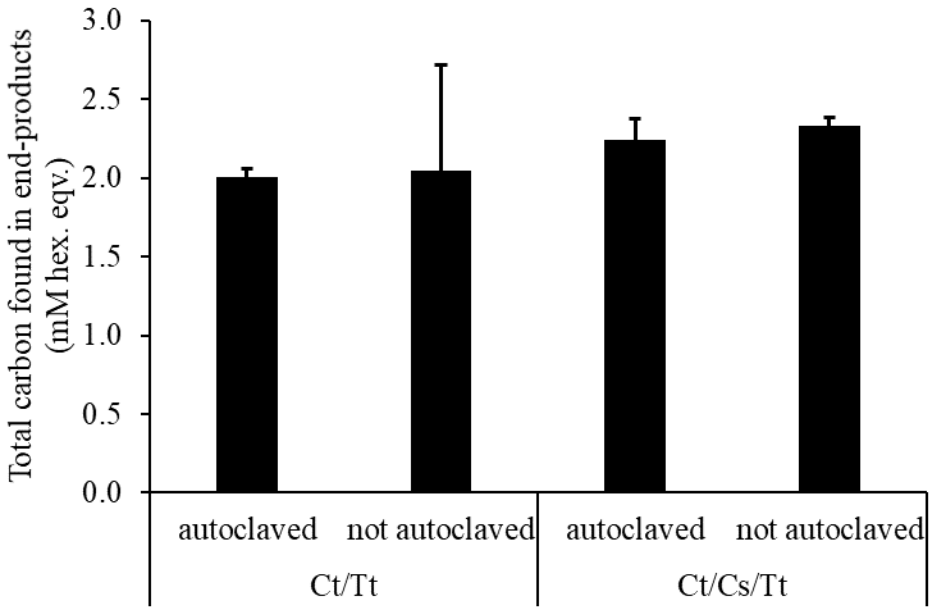

Fig. A.2.1: Total carbon found in end-products for Ct/Tt dual-culture and Ct/Cs/Tt tri-culture grown on autoclaved or non-autoclaved wheat straw (2 g/l). Autoclaving had no statistically significant impact on total lignocellulose conversion. Ct: *C. thermocellum*, Tt: *T. thermohydrosulfuricus*, Cs: *C. stercorarium*

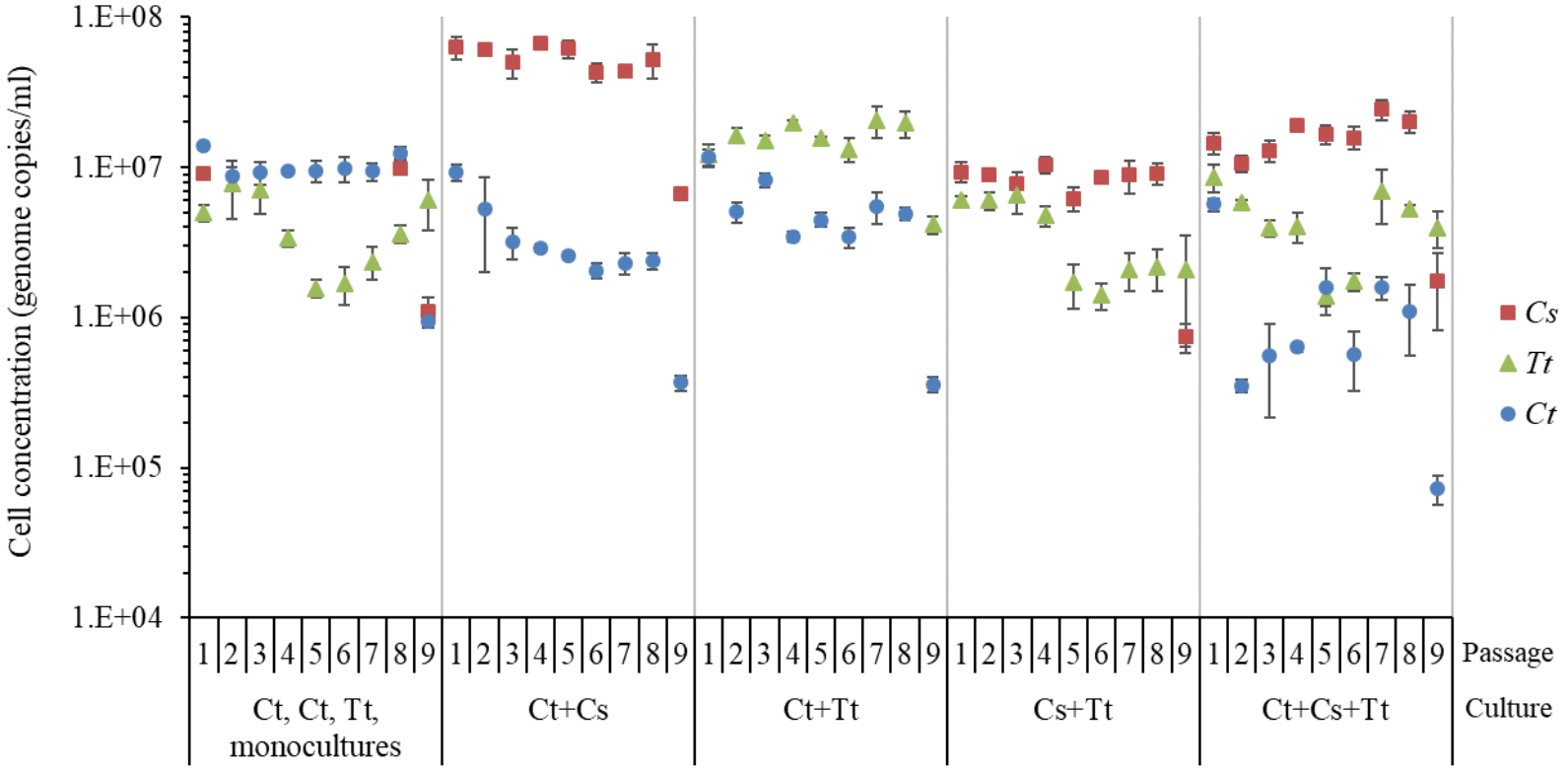

Fig. A.2.2: Cell concentrations for each species in every culture at the end of each passage when passaged once a week in medium containing 2 g/l wheat straw. Ninth passage contained no wheat straw in order to determine growth arising from yeast extract alone. Ct: *C. thermocellum*, Tt: *T. thermohydrosulfuricus*, Cs: *C. stercorarium*

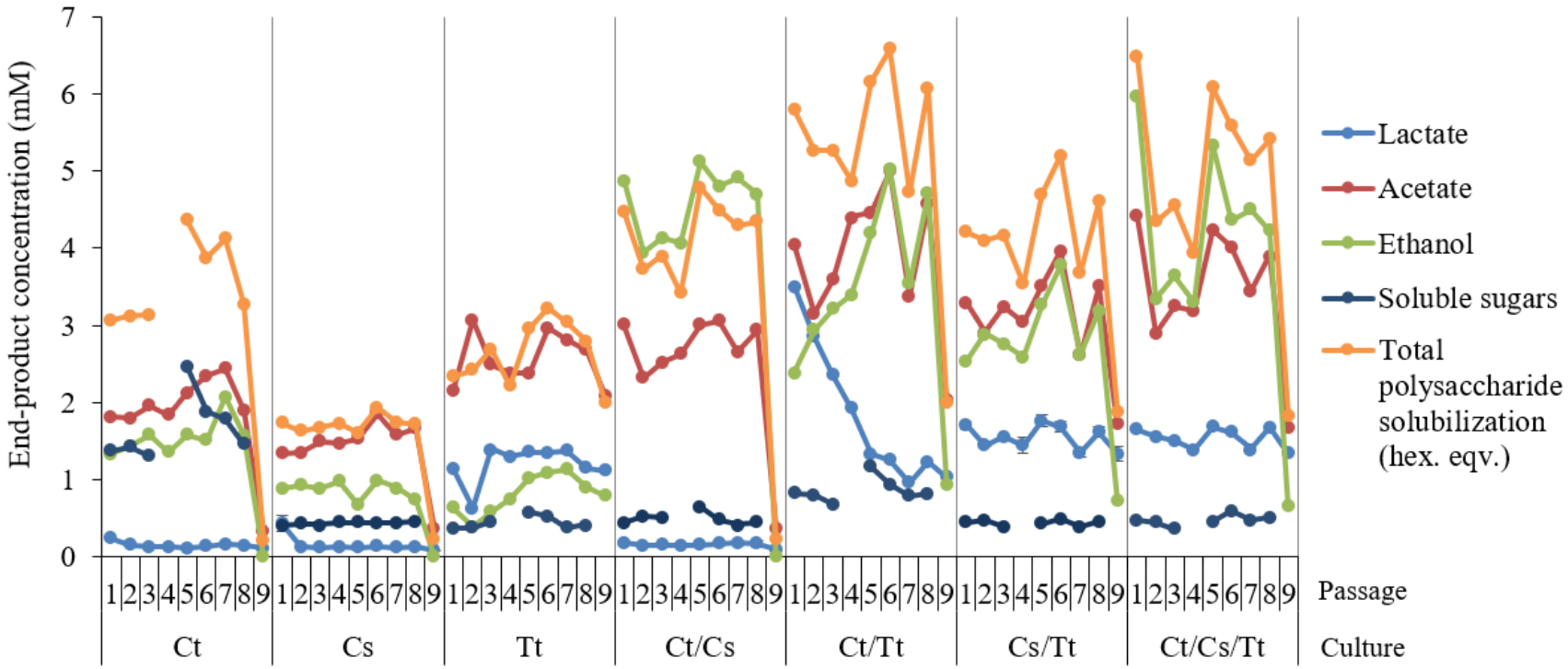

Fig. A.2.3: Metabolite profiles for each culture at the end of every passage (7 days) when passaged once a week in medium containing 2 g/l wheat straw. Ninth passage contained no wheat straw. Values are not corrected for growth on yeast extract (ninth passage). Ct: *C. thermocellum*, Tt: *T. thermohydrosulfuricus*, Cs: *C. stercorarium*

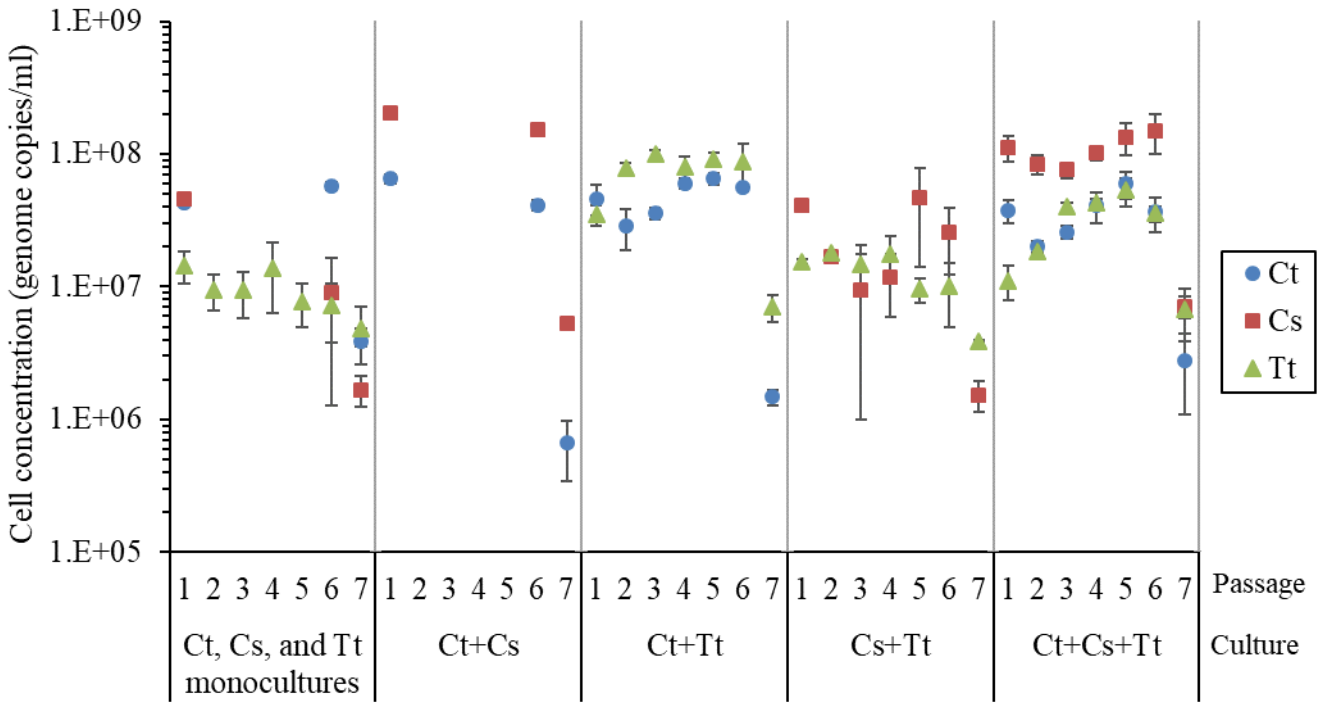

Fig. A.2.4: Cell concentrations for each species in every culture at the end of each passage when passaged once a week in medium containing 2 g/l Avicel. The seventh passage contained no Avicel in order to determine growth arising from yeast extract alone. Ct: *C. thermocellum*, Tt: *T. thermohydrosulfuricus*, Cs: *C. stercorarium*

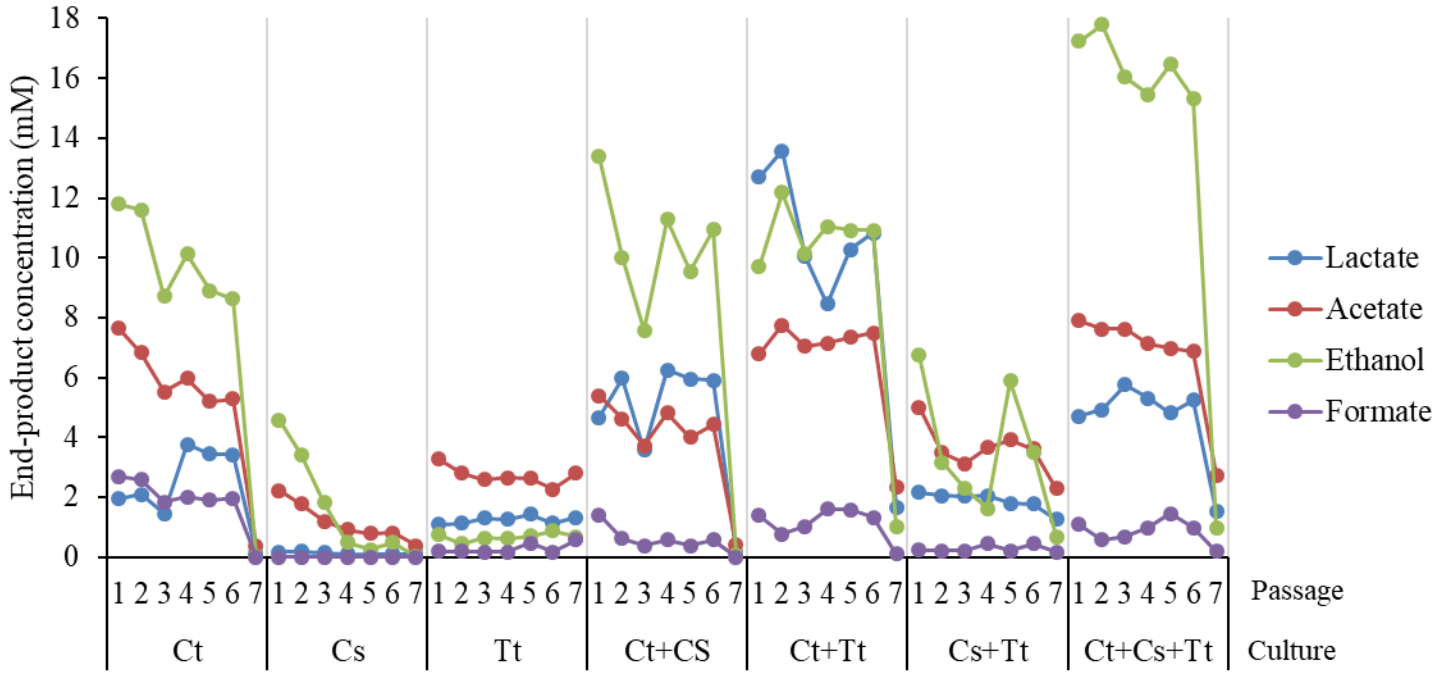

Fig. A.2.5: Metabolite profiles for each culture at the end of every passage when passaged once a week in medium containing 2 g/l Avicel. The seventh passage contained no Avicel. Values are not corrected for growth on yeast extract (seventh passage). Ct: *C. thermocellum*, Tt: *T. thermohydrosulfuricus*, Cs: *C. stercorarium*

Appendix A.3: Supplemental information for Chapter 3

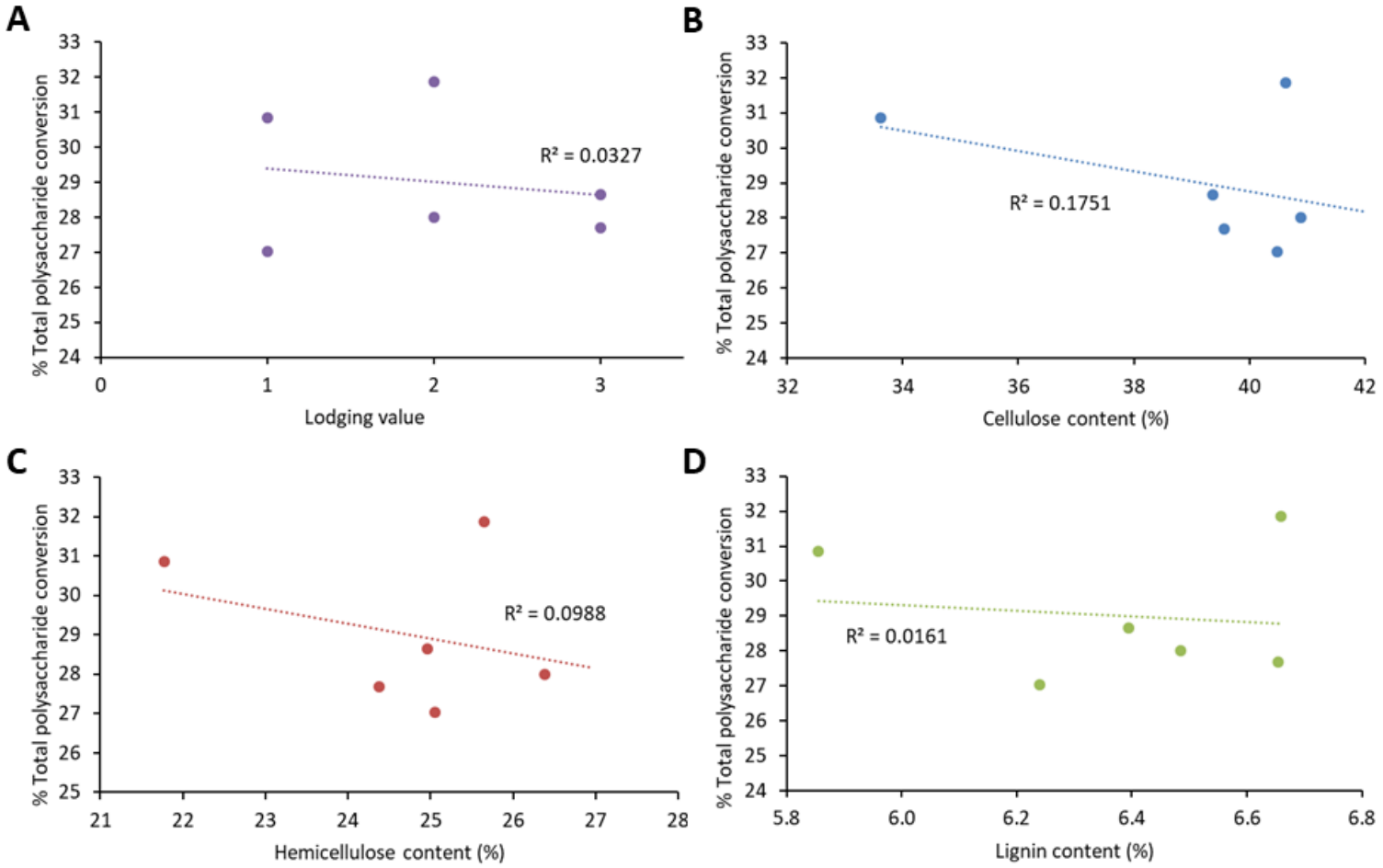

Fig. A.3.1: No significant correlation between total polysaccharide conversion of wheat straw by tri-culture and lodging value (A), cellulose content (B), hemicellulose content (C), or lignin content (D) of wheat straw from various cultivars. Lodging values for (A): 1, fair; 2, good; 3, very good

Appendix A.4: Supplemental information for Chapter 4

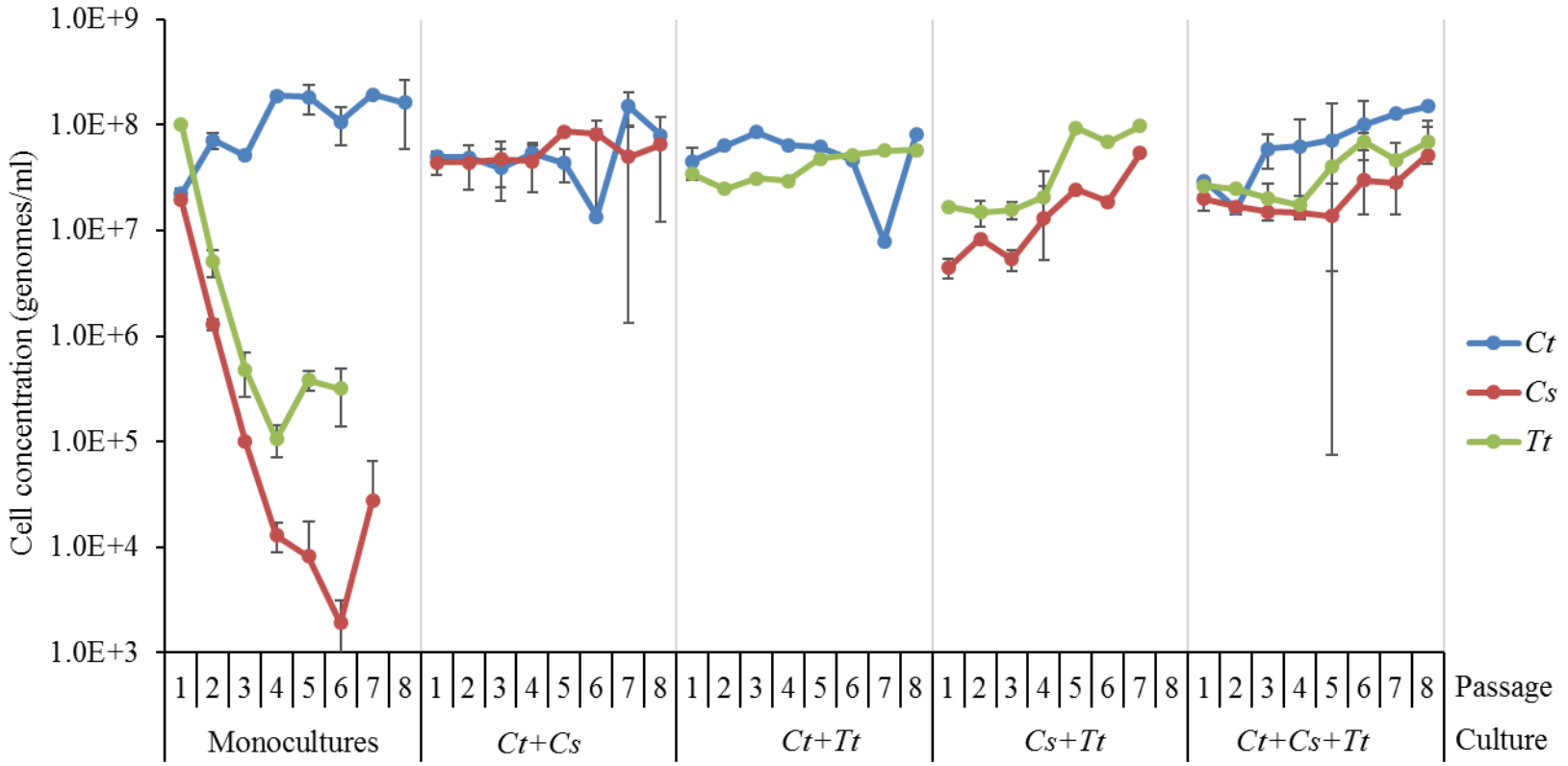

Fig. A.4.1: Cell concentrations of *C. thermocellum* (*Ct*), *C. stercorarium* (*Cs*), and *T. thermohydrosulfuricus* (*Tt*) in each mono- and co-culture when passaged in defined medium with 2 g/L cellobiose. Significantly higher cell numbers of *C. stercorarium* and *T. thermohydrosulfuricus* in co-cultures suggests presence of growth factor cross-feeding. See methods for culturing details.

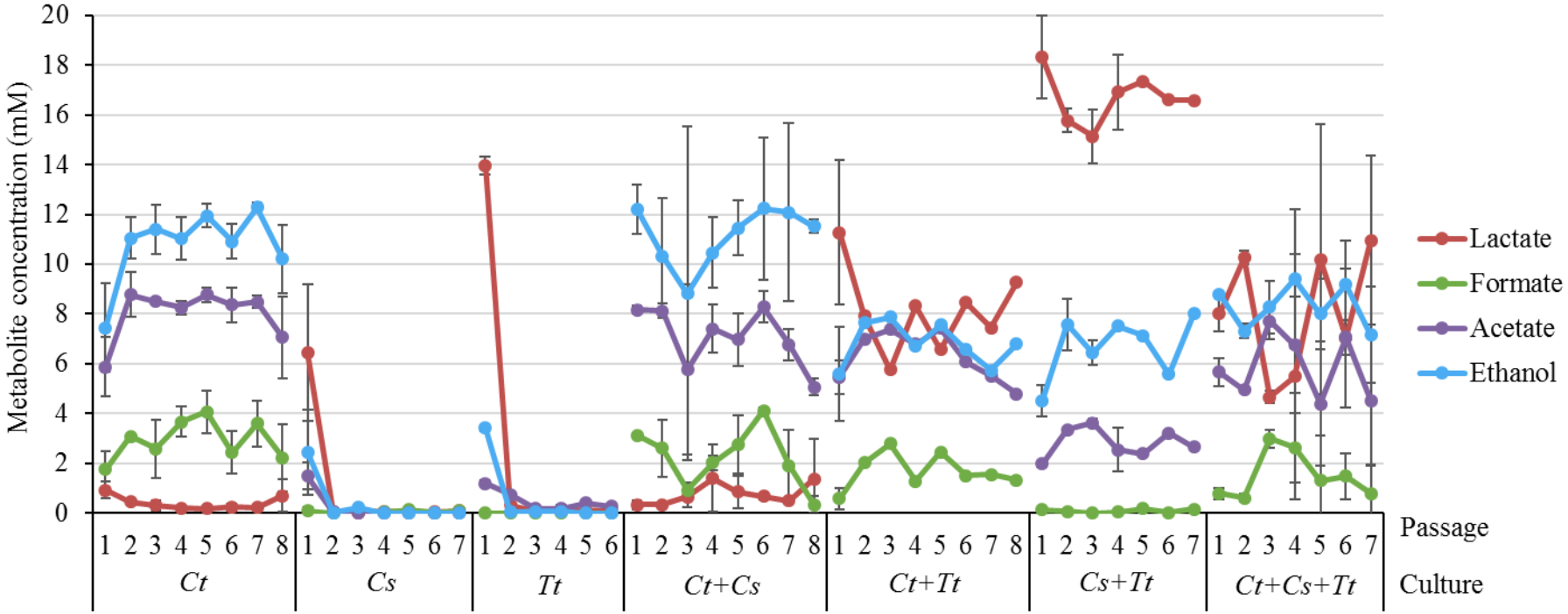

Fig. A.4.2: End-product concentrations at the end of each passage for all cultures passaged in defined medium with 2 g/L cellobiose. The metabolites produced in the first passage of the *C. stercorarium* and *T. thermohydrosulfuricus* cultures are likely due to carry-over of yeast extract nutrients in the pre-culture medium. *Ct*: *C. thermocellum*; *Cs*: *C. stercorarium*; *Tt*: *T. thermohydrosulfuricus*

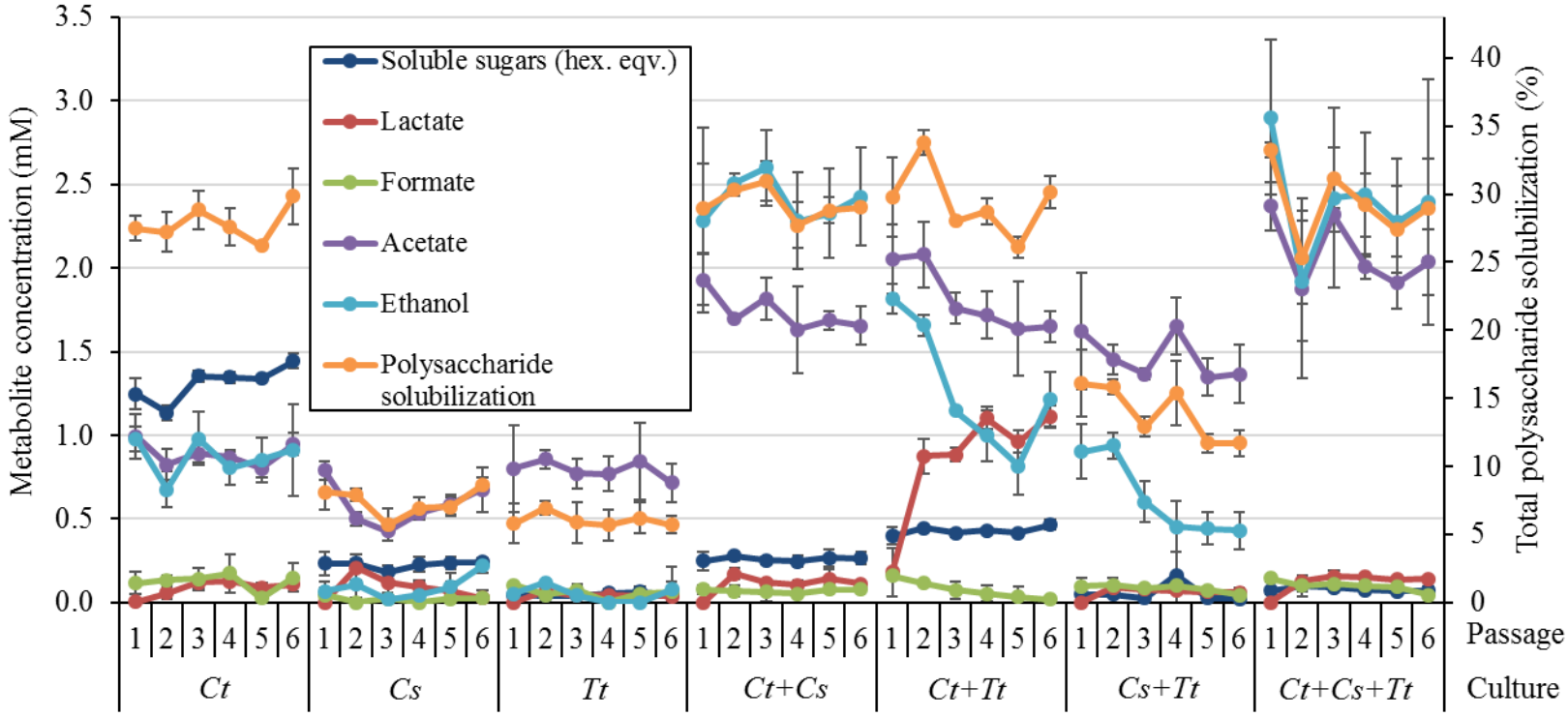

Fig. A.4.3: End-product concentrations and total polysaccharide solubilization for each culture at the end of each passage when passaged in defined medium with 2 g/L wheat straw once a week for six weeks. *Ct*: *C. thermocellum*; *Cs*: *C. stercorarium*; *Tt*: *T. thermohydrosulfuricus*

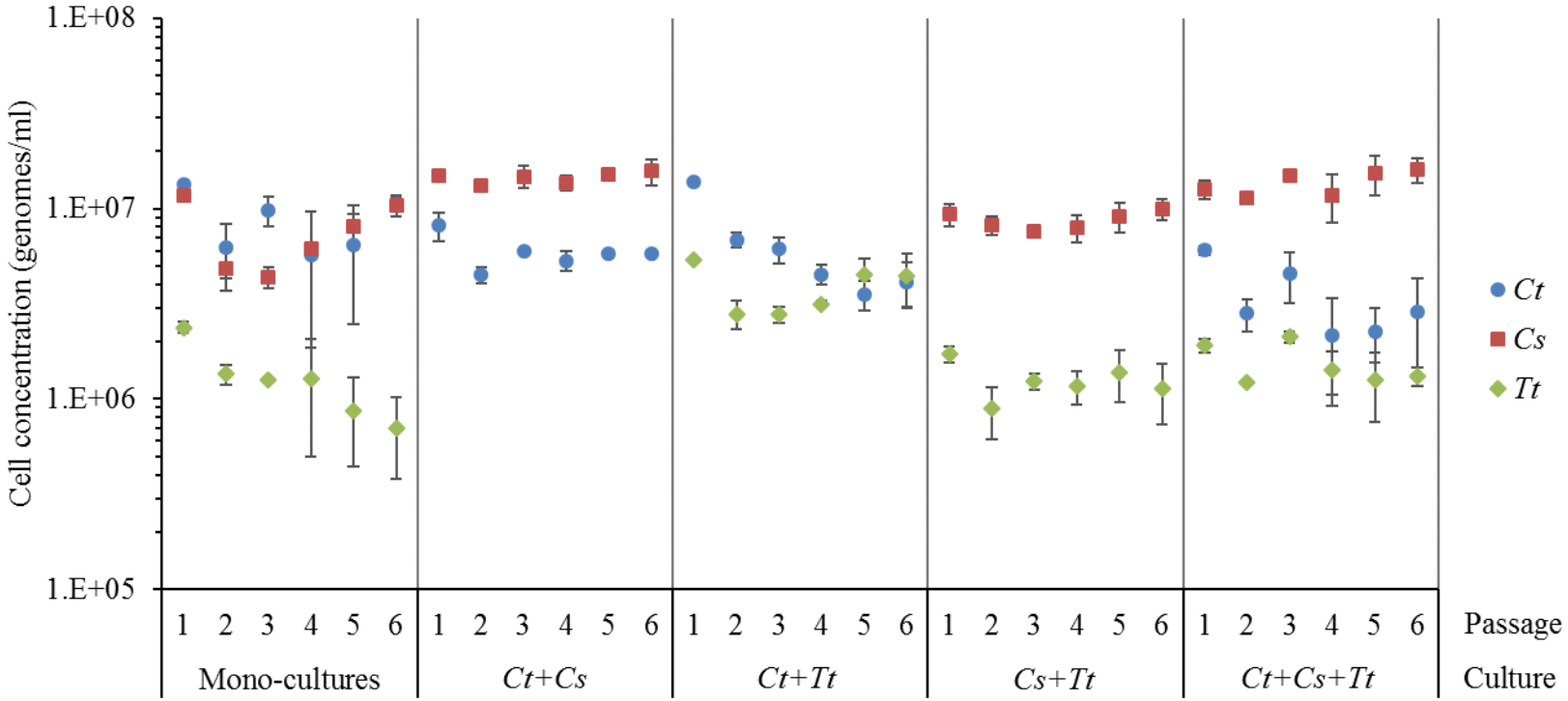

Fig. A.4.4: Cell concentrations for each species for each culture at the end of each passage when passaged in defined medium with 2 g/L wheat straw once a week for six weeks. *Ct*: *C. thermocellum*; *Cs*: *C. stercorarium*; *Tt*: *T. thermohydrosulfuricus*

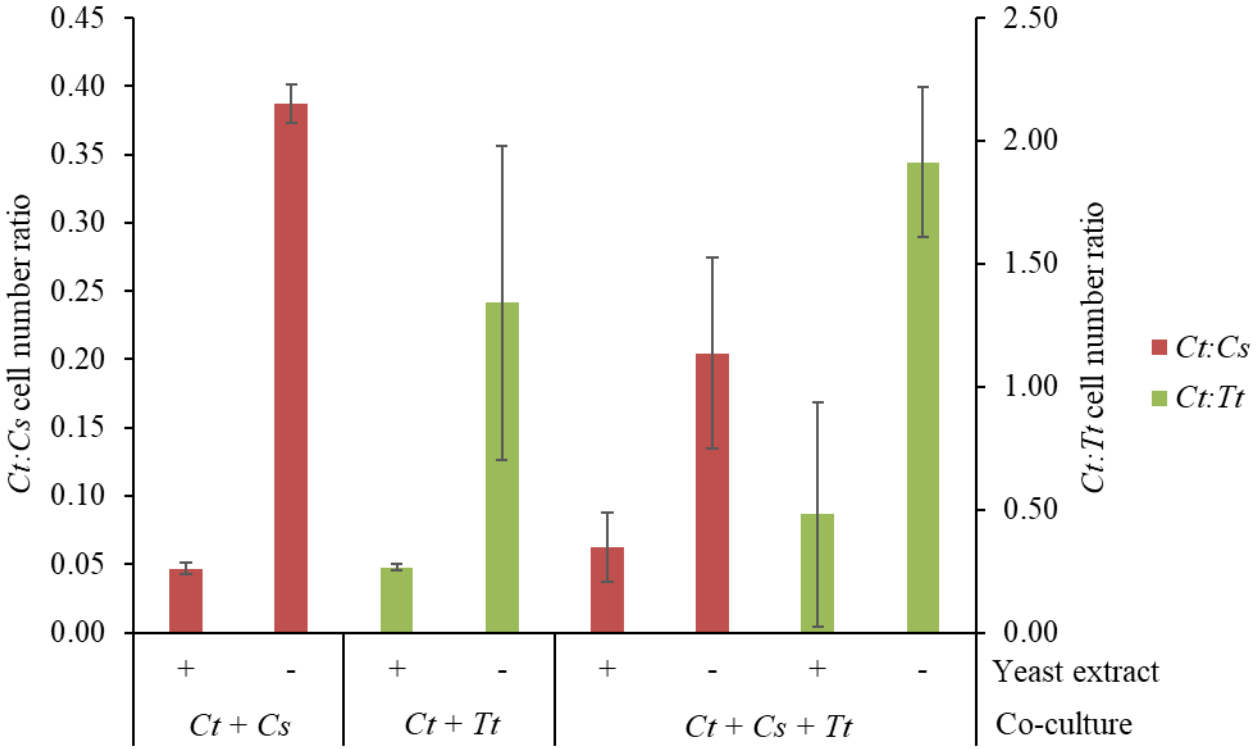

Fig. A.4.5: Cell number ratio of *C. Thermocellum* to either *C. stercorarium* or *T. thermohydrosulfuricus* in co-cultures when grown in either complex medium with yeast extract (+), or in defined medium without yeast extract (-). The higher ratios in medium without yeast extract suggests reliance on cross-fed nutrients from *C. thermocellum* to the other partners in this medium is altering the population dynamics. *Ct*: *C. thermocellum*, *Cs*: *C. stercorarium*, *Tt*: *T. thermohydrosulfuricus*. Data shown are averages for passages 3 to 6 from each serially passaged experiment.

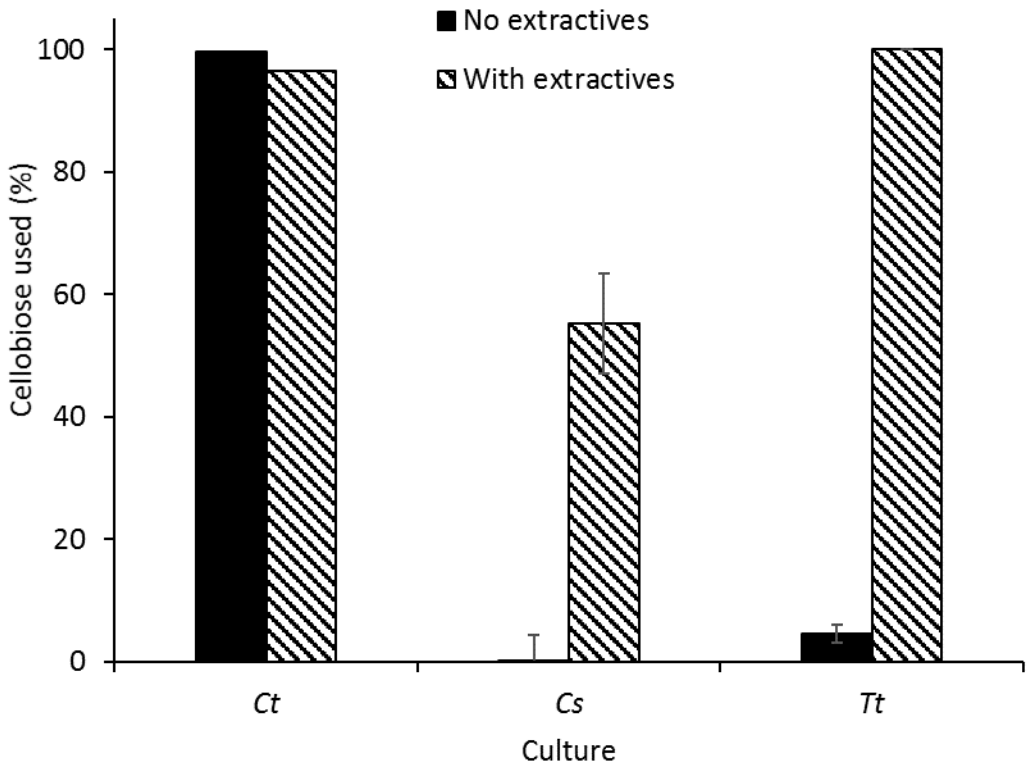

Fig. A.4.6: Amount of cellobiose utilized by each mono-culture at the end of the third passage when grown in defined medium with 2 g/l cellobiose and with or without wheat straw extractives. Utilization of cellobiose only in medium with extractives suggests the presence of growth-factors. *Ct*: *C. thermocellum*; *Cs*: *C. stercorarium*; *Tt*: *T. thermohydrosulfuricus*

Appendix A.5: Supplemental information for Chapter 5

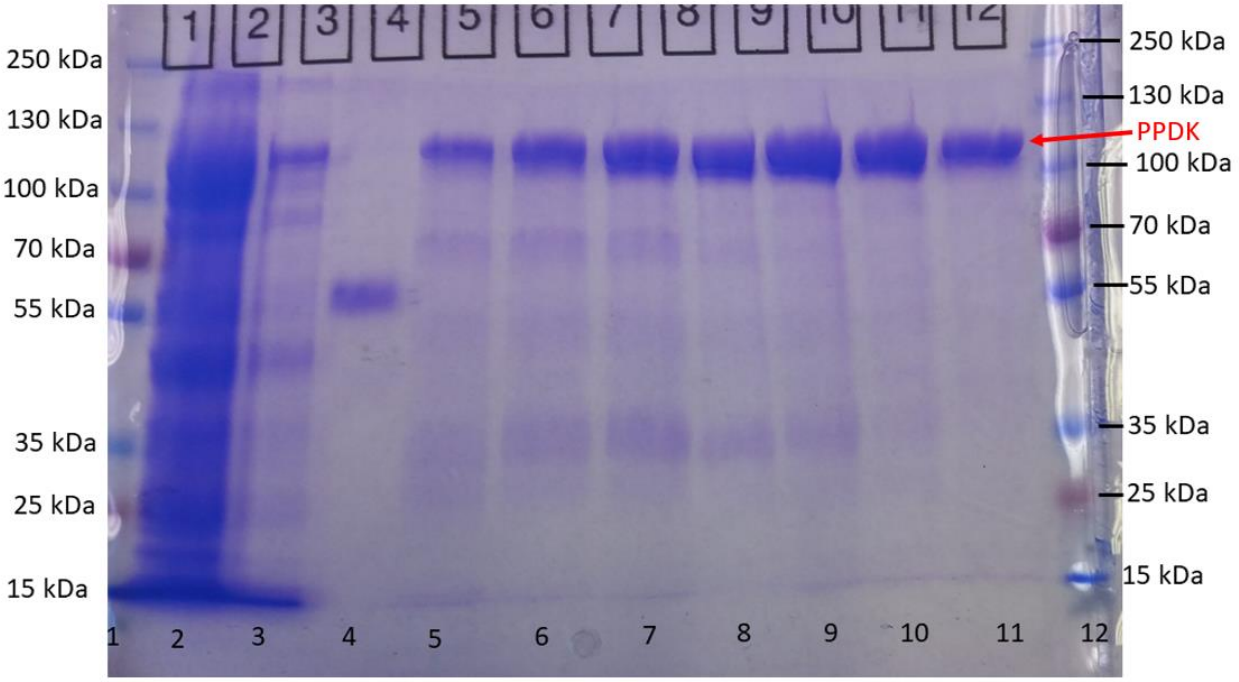

Fig. A.5.1: PPDK expression, purification, and size confirmation with SDS-PAGE. Lanes: **1**, PageRuler plus protein ladder; **2**, crude protein lysate from *E. coli* expressing the PPDK; **3**, 1:10 dilution of crude protein lysate; **4**, 100 µg/ml bovine serum albumin control; **5-11**, fractions #12-18 from AKTA prime column purification; **12**, PageRuler plus protein ladder. Expected size of PPDK is 103 kDa

Cthe_1308 pyruvate phosphate dikinase, Expected size: 103037 Da, Observed size: 103341 Da

0001 MRGSHHHHHHGMASMTGGQQMGRDLYDDKDRWGSELEMAKYVYLFSEGNASMRDLLGGKG
0061 ANLAEMTSLGLPVPRGFTITTEACTRYYQDGKVIAKEIEDEIYRTMEKLEEIVGKKFGDP
0121 SNPFLVSVRSGARVSMPGMMDTILNLGLNDEVVVGLAKLTNNERFAYDSYRRFIQMFSDV
0181 VMEVEKSKFEAILDAVKEENNCENDCDLSAENLKEVVRRYKELFKKEKGFDFPQDPKTQL
0241 MEAVKAVFRSWENPRAIVYRRLNDIPGDWGTAVNVQEMVYGNMGNDSGTGVAFTRNPATG
0301 EKKLYGEFLMNAQGEDVVAGIRTPQSIDQLKEVMPDVYNQFVEIAEKLERHYRDMQDMEF
0361 TIERGKLFMLQTRNGKRTAAAALKIAVDLVNEGMVTKEEAILKVDPKQLDTLLHPNFEPS
0421 ALKNAKPIAKGLPASPGAATGKIYFRAEDAVEATKNGEKDIILVRLETSPEDIEGMHVSK
0481 GILTGRGGMTSHAAVVARGMGTCCVAGCSEIRINEEEKYFVDKNGKKYVEGDWISLDGST
0541 GNVYGEKLPTVEPEMTGDFATLMQWADEIRTLKIRTNADTPADAIQARKFGAEGIGLCRT
0601 EHMFFDSDRIPAMREMIVARTEEQRRKALDKLLPMQRKDFEELFTAMEGYPVTIRFLDPP
0661 LHEFLPQEDEDIEALAKEMGITFDELKAIVTGLHEFNPMMGHRGCRLAVTYPEIAEMQTR
0721 AVIEAAINVSRKNIKVVPEIMIPLVGDVKELKYVKDVVVRTANELIEKSGVKIEYKVGTM
0781 IEIPRAAITADEIAKEAEFFSFGTNDLTQMTFGFSRDDAGKFLEEYYNKKIYEFDPFAKL
0841 DQDGVGKLVEMAAKLGRQTRPDIKLGICGEHGGDPSSIEFCHQIGLNYVSCSPFRVPIAR
0901 LAAAQARVNEIKGTKDLGQK

Fig. A.5.2: PPDK protein sequence confirmation via mass spectrometry. Red sequence represents probable match with ions seen in the MALDI spectra of tryptic digests. Observed mass is that of the most abundant species with error about 20 Da.

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