Fed-Batch Fermentation of *Clostridium thermocellum* ATCC 27405 with High Cellulose Concentrations for the Production of Biofuels

by

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Abstract

Consolidated bioprocessing is a one-step process that allows the direct microbial conversion of cellulosic substrates to ethanol and hydrogen. The fermentation was initially performed in batch cultures, in a pH and temperature controlled reactor using *Clostridium thermocellum* ATCC 27405. With an objective of increasing the production of ethanol and hydrogen, various types of fed-batch fermentations were investigated: variable volume (VV) fed-batch, fixed volume (FV) fed-batch, and semi-continuous fermentation. Semi-continuous processes were carried out at low (10-15 g/L) and high (20-25 g/L) cellulose concentrations. The maximum ethanol production obtained in batch, VV, FV, semi-continuous with low concentrations and high concentrations were 554 mmol, 336 mmol, 477 mmol, 695 mmol and 741 mmol respectively. In the same order, the total hydrogen production was 288 mmol, 364 mmol, 231 mmol, 434 mmol, and 387 mmol. Overall, the semi-continuous fermentation showed more promise in terms of large-scale deployment compared to batch, VV, and FV fed-batch.

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Chapter 1

Literature Review

1.1 Introduction

1.1.1 The need for renewable energy and its global situation

Increasing problems related to greenhouse gases and climate changes have spurred tremendous interest to seek renewable, non-fossil energy sources. Alternative energy sources such as solar, hydro, wind and biomass have been embraced globally as solutions for the environmental issues and problems associated with the oligopoly in the world's fossil fuel supply (Herzog et al. 2001).

Biomass energy is the energy harvested from the sun through photosynthesis and is stored in all living and recently living organisms. This energy can be derived from organic material originating from plants, trees and crops (Mabee 2006). It is abundantly available and is available at a low cost. The total global production of wood by terrestrial plants alone is 1.3×10^{10} metric tons (dry weight basis) of wood which if converted correspond to 7×10^{9} metric tons of coal. If this total energy could be harnessed it could suffice two-thirds of the global energy requirement (Demain et al. 2005). This bioenergy can be harnessed through combustion, gasification or as liquid or gaseous biofuels (Herzog et al. 2001).

Brazil, France, Germany and the United States are the current global leaders in the biofuel industry. In comparison, the Canadian biofuel industry experienced a slow initial growth in spite of the large volume of biomass generated by its agriculture industry annually. However, due to generous subsidies, the Canadian biofuel production capacity has increased rapidly since 2005. Between 2006 and 2008, approximately C$860 million to C$1.02 billion was invested in the biofuel industry, with an average of C$300 million per annum (Laan et al. 2009).

As of end 2013, Canada has made a total investment of C$2.69 billion in the development of new infrastructure in the biofuel sector. This includes 26 operating biofuel plants across Canada with the capacity to produce approximately 2.52 billion litres of biofuel annually (CRFA 2013). Despite these developments, the Canadian biofuel sector does not make a significant contribution at a global scale. In the year 2010, the total Canadian biofuel production of 2 billion litres represented only 2% of the global production and only 4% of the domestic production of the United States (CRFA 2010). Hence, the Canadian biofuel industry has a long way to go to reach its full potential.

As opposed to fossil fuels, the net emission of carbon dioxide (CO_2) in the combustion of ethanol is almost zero since the CO_2 emitted during combustion is absorbed back in to new biomass through photosynthesis (Lave et al. 2000). Nevertheless, this concept of carbon neutrality has been widely debated as the current agricultural practices still require fossil fuels to power transport or harvesting machines causing a net emission of CO_2 (Potters et al. 2010). However, as an added benefit, the combustion of biofuels eliminates the emission of pollutants such as carbon monoxide, nitrogen oxides, and volatile organic compounds (Wingren et al. 2003).

Studies show that complete replacement of petroleum fuels with biofuels in the automotive industry alone could reduce the total CO_2 emissions by 60-90% (Brown et al. 1998). Nevertheless, the combustion of pure ethanol can result in the emission of aldehydes, predominantly acetaldehyde, almost two to four times higher than during the combustion of gasoline (Carere et al. 2008).

Hydrogen (H_2) gas produced from biological sources (biohydrogen) is another source of bio-renewable energy, which has received widespread attention due to its environment

friendliness. Upon combustion H_2 produces no CO_2 and the only end-product formed is water. Hydrogen fuel cells have the ability to directly convert chemical energy to electrical energy without any combustion required. Theoretically it has been proven that the efficiency of this conversion can be as high as 90% (Carere et al. 2008). Demonstrations of electronic devices and even electric automobiles driven by hydrogen fuel cells have practically proven the efficiency of hydrogen as a fuel (Dunn 2002). Although the H_2 fuel cell only produces water, the biological production of H_2 through dark fermentation results in the generation of significant amounts of CO_2 (Carere et al. 2008). However, as recently produced organic material is used for the fermentation, CO_2 emitted during fermentation can be considered carbon neutral.

1.1.2 Biofuels of first generation, second generation and beyond

First generation biofuels mainly include ethanol produced from sugar or starch based plant matter and biodiesel produced from vegetable oil. These fuels are commonly used in Brazil, North America, and Europe. Once blended with petroleum fuels, bioethanol and biodiesel are compatible with current engines without any modifications (Mabee 2006). The major disadvantage of first generation fuels is that the feedstocks used to make them are derived from food crops, creating competition between the use of these crops, as well as the land used to grow them, for food/feed versus fuel (Srinivasan 2009). In addition, despite use of large acreages of arable land for bioethanol production, the volume of fuel produced is insufficient to meet the full demand for transportation fuel (Hannon et al. 2010). In response to these problems, strategies for second generation biofuels have been developed. Second (2^{nd}) generation biofuels use feedstocks that are much more diverse and abundantly available. Where 1^{st} generation biofuels are produced from sugar and starch, which constitutes a major component of cereal crops, 2^{nd} generation biofuels are produced from lignocellulose, a complex polymer matrix that creates the structural

components of all plants and trees (Hamilton 1987). Lignocellulosic feedstocks from agriculture and other sources, such as municipal and industrial wastes, are abundantly available at about 180 million tons per year (Demain et al. 2005).

1.2 Lignocellulosic biomass

Lignocellulosic biomass mainly constitutes of lignin, cellulose and hemicellulose. The composition of each of these may vary from one type of biomass to the other. Table 1.1 shows the chemical composition of commonly found biomass (Kumar et al. 2009). Cellulose is the principle component of lignocellulosic biomass. It's a homopolysaccharide composed of repeating β-D-glucopyranose units (Agbor et al 2011). The susceptibility for hydrolytic processes of cellulose will differ from species to species, depending on parameters such as degree of polymerization, crystallinity and surface structure (Yang et al. 2011). Cellulose can be divided as crystalline and amorphous depending on the orderliness of the microfibrills formed by the cellulose chains (Agbor et al. 2011). Generally, a larger portion of cellulose is more crystalline whereas a smaller percentage shows a more amorphous structure. These amorphous portions of the cellulose, with its unorganized cellulose chains, are shown to be more susceptible to enzymatic degradation (Kumar et al. 2009).

Hemicellulose is a heterogeneous polymer containing pentoses (b-D-xylose, a-L-arabinose), hexoses (b-D-mannose, b-D-glucose, a-D-galactose) and/or uronic acids (a-D-glucuronic, a-D-4-O-methylgalacturonic and a-D-galacturonic acids). (Gírio et al. 2010). Hemicellulose increases the structural integrity of the cellulose– hemicellulose–lignin matrix by serving as a connection between the lignin and the cellulose fibers (Laureano-Perez et al. 2005).

Table 1.1 Cellulose, Hemicellulose and Lignin contents in common agricultural residues and wastes.

Lignocellulosic Material	**Cellulose %**	**Hemicellulose %**	**Lignin %**
Hard wood Stems	40-55	24-40	18-25
Soft wood stems	45-50	25-35	25-35
Corn cobs	45	35	15
Paper	85-99	0	0-15
Wheat Straw	30	50	15
Waste paper from chemical pulps	60-70	10-20	5-10
Switch Grass	45	31.4	12

Lignin is the third major constituent of lignocellulosic biomass and it is a complex polymer matrix containing phenyl propane (p-coumaryl, coniferyl, and sinapyl alcohol). Lignin serves as glue that binds the other components together to form a more rigid and impermeable structure (Howard 2003). The impermeable barrier formed by lignin provides resistance against microbial degradation and oxidative stress on the lignocellulosic biomass (Joshi et al. 2011). The amorphous structure, water insolubility and optical inactivity of lignin are some of the key characteristics responsible for its recalcitrant nature (Feldman 1983).

The lignin-hemicellulose-pectin matrix provides an extremely recalcitrant seal around cellulose (Feldman 1983). In order to overcome its recalcitrant nature, this matrix structure should be altered in macroscopic and microscopic levels, facilitating cellulase enzymes to access the cellulose to obtain a higher yield of soluble sugars. This alteration process is called pretreatment of lignocelluloses (Mosier 2005). The pretreatment processes may involve physical, chemical and/or biological treatments. This is the most vital and rate determining step in converting lignocellulose to soluble sugars (Agbor et al. 2011). After pretreatment, cellulose and hemicellulose is hydrolysed to generate soluble hexose and pentose sugars (saccharification) followed by subsequent fermentation and distillation steps (Varga et al. 2004; Wingren et al. 2003).

1.3 Hydrolysis and Fermentation of Lignocellulosic Biomass

For the hydrolysis of cellulose into soluble sugars that can be fermented by microbes, a variety of enzymatic activities are required. Enzymatic hydrolysis of cellulose is slower and more complicated when compared to the degradation of other natural polysaccharide such as starch or hemicellulose. It is implemented by two different cellulase systems: a non-complex cellulase system and a complex cellulase system called the “cellulosome” (Lynd et al. 2002,

Poole 1995). Both systems incorporate the enzyme activities of endoglucanase (EG) and exoglucanases, including cellobiohydrolase (CBH), cellodextrinase, and β-glucosidase (BGL). Endoglucanase randomly acts upon the amorphous regions of cellulose chains and produces reducing and non-reducing ends for CBH. Cellobiohydrolase then acts on the reducing or non-reducing ends of crystalline cellulose to produce cellobiose. Hence, the efficient conversion of cellulose to soluble cellobiose and cellooligosaccharides is due to the endo–exo synergism of EG and CBH. In the final step of hydrolysis, the BGL acts upon the cellooligosaccharides to subsequently produce glucose (Joshi et al. 2011; Yang et al. 2011).

The process of converting pretreated lignocellulosic biomass to ethanol can take different process pathways. Essentially there are four possible process routes for this conversion. They are, separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SScF) and consolidated bioprocessing (Agbor et al. 2011). Figure 1.1 summarizes the process routes of each fermentation method (Lynd et al. 2002).

1.3.1 Separate Hydrolysis and Fermentation

Separate hydrolysis and fermentation (SHF) is a two-step process where a cocktail of exogenous cellulase enzymes would hydrolyze the cellulose in to glucose in the first step and the glucose is fermented in to ethanol in the latter step. Separate hydrolysis and fermentation allows each step to be carried out at its optimum temperature, which results in higher reaction rates. One of the main drawbacks of SHF is that high glucose concentrations cause severe inhibition on the cellulase activity during hydrolysis. Therefore, lower cellulose concentrations and higher enzyme loadings are required to achieve acceptable ethanol yields (Geddes et al. 2011; Ohgren et al. 2006; Um 2004).

1.3.2 Simultaneous Saccharification and Fermentation

In Simultaneous Saccharification and Fermentation (SSF) process, the enzymatic saccharification and fermentation steps are performed in the presence of fermentative microorganisms in a single step. The first step of the SSF process is the filtration of the pretreated biomass to separate the liquid from the solid. As a large portion of the hemicellulose is hydrolysed during the steam pretreatment, this liquid fraction will contain mostly pentose sugars (Erdei et al. 2012). The liquid stream will then be neutralized through the addition of lime and then a separate fermentation process will be carried out for the xylose fermentation. The solid stream generated from the post pretreatment is hydrolyzed and fermented simultaneously using exogenous cellulase enzymes and yeast (Ohgren et al. 2006; Stenberg et al. 2000; Zhang et al. 2010). This technique makes the overall process more economically feasible through the elimination of a separate hydrolysis step. As both saccharification and fermentation occur simultaneously, there is no build-up of glucose or cellobiose in the system to inhibit the cellulase activity. Thus, SSF enables the operation at higher cellulose concentrations resulting in higher ethanol yields without the requirement of high cellulase loadings (Joshi et al. 2011; Ohgren et al. 2006). One of the disadvantages of using SSF process is the requirement of a lower operating temperature of approximately 37 to 38°C. This affects the reaction rates and overall ethanol production rate (Joshi et al. 2011).

1.3.3 Simultaneous Saccharification and co-fermentation (SScF)

In Simultaneous Saccharification and co-Fermentation (SScF), the pretreated lignocellulosic biomass is not separated through filtration as in SSF. Instead, both liquid and solid fractions are neutralized and exposed to various enzymes and microorganism that have the ability to convert both cellulose and hemicellulose in the soluble sugars and ferment the sugars to

ethanol. The microorganisms used for this process are genetically engineered species that combine the robustness and high yields of *Saccharomyces cerevisia* or *Escherichia coli* with the ability to ferment xylose. Examples for genetically modified industrial strains include, *S. cerevisiae* TMB3400 (Wahlbom et al. 2003), *E. coli* KO11 (Ohta et al. 1990), and *Zymomonas mobilis* AX101 (Su et al. 2012). This process is very economically desirable when the biomass contains a high content of xylose.

1.3.4 Consolidated Bio-processing Processing

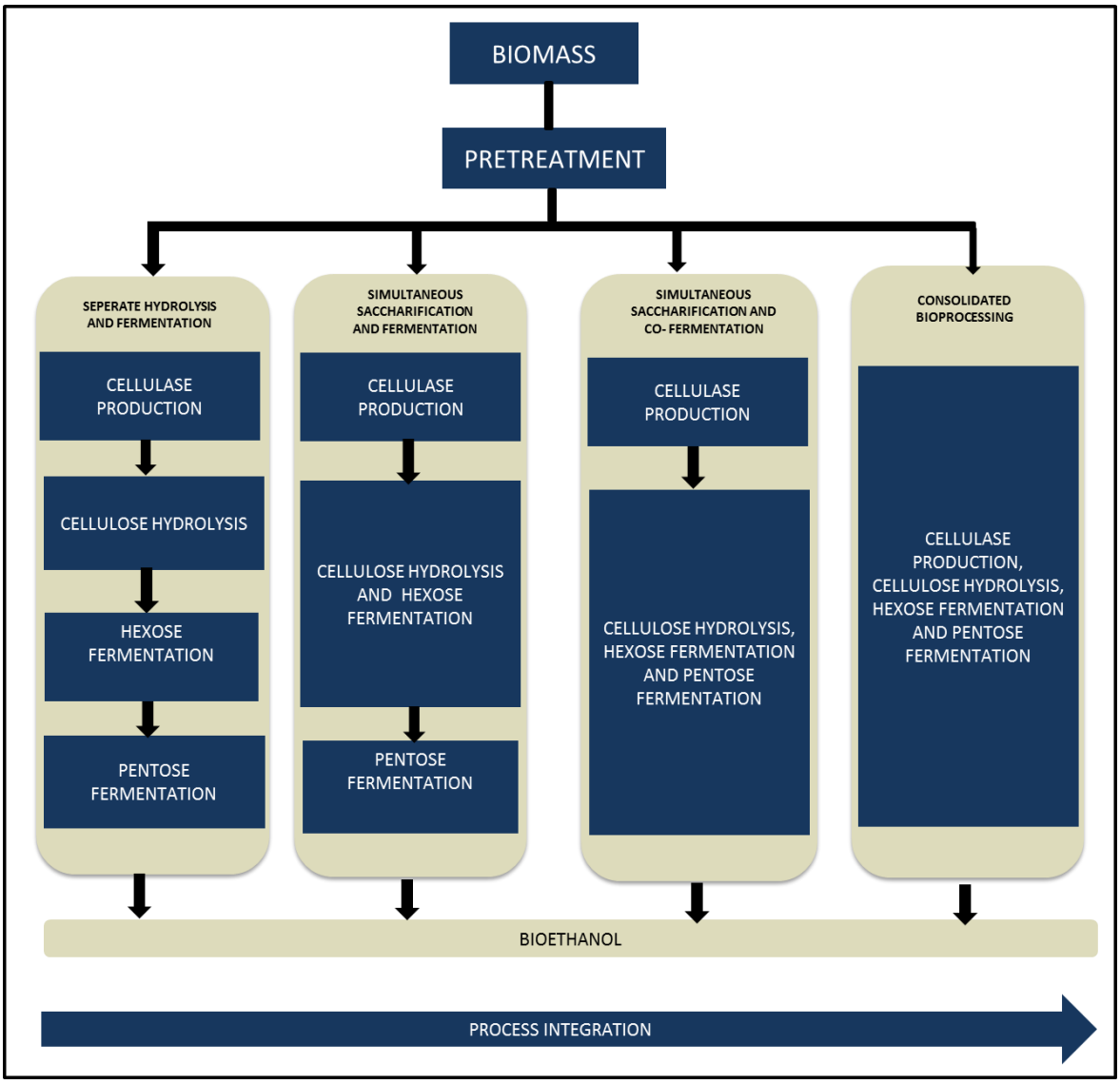
Consolidated bio-processing (CBP) is a relatively novel process strategy that holds immense potential in terms of long term, commercial scale deployment. It is a single step process in which cellulase production, cellulose and hemicellulose hydrolysis and fermentation takes place in one reactor by one microbial consortium (Demain et al. 2005; Lynd et al. 2005). Consolidated bio-processing is also considered a "third generation" biofuel production and can be used to produce H_2 and ethanol (Levin et al. 2009).

The most beneficial feature of CBP compared to other conversion processes is the elimination of the cellulase production step and thereby removing the operating and capital costs associated with dedicated enzyme production (Olson et al. 2012). Also, it gives the benefit of lower energy inputs than SSF or SScF based processes (Levin et al. 2009). An economic feasibility study done by Lynd et al. (2005) showed that the total cost of ethanol production from SScF is 6.1 CAD¢/L (including the cost of cellulase production) and the projected cost for CBP was only 1.4 CAD¢/L proving that CBP is a far more economically viable option.

1.4 *Clostridium thermocellum*

Clostridium thermocellum can be considered as a model organism for CBP and is extensively studied in the field of consolidated bioprocessing (Demain et al. 2005).

Figure 1.1 Lignocellulose to ethanol conversion process configurations (Lynd et al. 2002).



C. thermocellum is a gram-positive, anaerobic and thermophilic bacterium that has the ability to degrade cellulose and produce valuable end-products such as ethanol and H_2 (Xu et al. 2010). *C. thermocellum* is commonly found in nature in decomposing organic materials.

Common places where *C. thermocellum* was found naturally were in municipal/agricultural wastes, sewage digestion sludge, soil, cotton bales, river mud, and hot springs (Ulbrik 1991). *C. thermocellum* was initially isolated by Viljoen et al. (1926) from manure and later described by McBee (1948). Detailed studies of the *C. thermocellum* type strain (ATCC 27405, equivalent to DSMZ 1237) began in Massachusetts Institute of Technology in 1970s. Since then much research has been done on *C. thermocellum* to increase the ethanol yield, improving the tolerance to ethanol and modifying metabolic pathways to eliminate less valuable by products (Biswas et al. 2014; Herrero et al. 1980; Tailliez et al. 1989; Xu et al. 2010).

1.4.1 End-Products of *C.thermocellum*

During anaerobic fermentation of cellulosic substrates, *C. thermocellum* has the ability to produce H_2 as well as ethanol. Acetate, formate, lactate and CO_2 are the other major end-products of cellulose fermentation of *C. thermocellum* (Islam et al. 2006). Batch culture studies show that while other end products are synthesised during the exponential phase, lactate and formate synthesis occur only when the culture reaches stationary phase (Islam et al. 2006; Levin et al. 2006; Sparling et al. 2006). As the pH in these cultures were not controlled, the pH was dropped to a ~ 6.3 as the cultures reached stationary phase.

The end-products synthesis of *C.thermocellum* can be shifted by the addition of exogenous end-products or by increasing the hydrostatic pressure in the reactor. Rydzak et al. (2011) identified that increased concentrations of end-products resulted in slower growth and reduced cell biomass. Also, it was observed that exogenous ethanol and lactate resulted in higher H_2 and

acetate yields while exogenous hydrogen (H_2), acetate, and lactate resulted in higher ethanol yields. Multiple other studies using different strains of *C.thermocellum* including SS21, SS22, AS39, JW20, and YS have shown the increase in ethanol yield in the presence of exogenous hydrogen (Bothun et al. 2004; Lamed et al. 1980; Rani et al. 1997). Bothun et al. (2004) showed that during continuous fermentation, elevated hydrostatic pressures from 7.0 MPa to 17.3 MPa can increase the ethanol/acetate ratio by more than 100 fold when compared to fermentation carried out at atmospheric pressure.

The high rate of cellulose degradation of *C. thermocellum* makes it a good candidate for the production of H_2 from cellulosic biomass. As high temperatures reduce the solubility of gases (Henry's law), the high optimal growth temperature of *C.thermocellum* enables more efficient removal of gaseous-end products such as H_2 and CO_2 (Demain et al. 2005). In the studies done with the *C. thermocellum* strain of ATCC 27405, the highest production of H_2 reported is 2.32 moles of H_2/mole glucose equivalent utilized when fermented in lab scale batch cultures using delignified wood fibres as substrate (Levin et al. 2006). This value is still considerably lower than the theoretical maximum of 4 of H_2/mole glucose equivalent.

1.4.2 Properties of *C. thermocellum*

C. thermocellum grows in complete anaerobiosis and in the thermophilic temperature range. The optimum temperature for growth is 60-64 °C and the optimum pH ranges from 6.1 to 7.5 (Freier et al. 1988). The cells have a straight or slightly curved rod shape and often occur singly or in pairs. The spores are slightly oval and terminal (Freier et al. 1988; Ulbirk 1991). *C. thermocellum* has a relatively slow growth rate. When grown on cellulose, the shortest doubling time reported is 7 hours. The doubling time on cellobiose is 2.5 hours (Maugeri et al. 1988).

One of the main advantages of using *C. thermocellum* is that its optimal growth temperature of 60 °C prevents the growth of many other contaminating microorganisms. This makes the sterilization requirements relatively less stringent compared to cultures maintained at lower temperatures (Levin et al. 2006). Thermophilic temperatures enable the elimination of a sterilization step for the incoming biomass while carrying out pure-culture fermentation. Also, its thermophilic nature reduces the post-heating cost and facilitates ethanol removal and recovery (Demain et al. 2005).

1.4.3 Effect of agitation

Several studies have been carried out to show the effect of agitation on the end-product synthesis of *C. thermocellum* (Bothun et al. 2004; Freier et al 1988; Lamed et al.1988). The studies of Lamed et al. (1988) showed that agitation facilitates H_2 transfer in to the headspace and thereby reducing the dissolved H_2 in the media. This in turn will relieve the inhibition of acetate formation. As a result, higher agitation resulted in decreased ethanol to acetate ratios. The studies by Freier et al. (1988) show that no significant cell detachment occurred for an agitation rate of up to 125 strokes per minute.

1.4.4 Cellulosome

Cellulosome is a multi-component cellulolytic exocellular complex of proteins found in many thermophilic and mesophilic cellulose-degraders in nature including *C. thermocellum* that mediates cellulose binding and degradation (Demain et al 2005; Xu et al. 2010). In addition to the enzymatic activity described in Chapter 1 (Section 1.3), the cellulosome also plays a major role in physically adhering the microorganism to the cellulose structure. The cellulosome system gives the cellulolytic *Clostridium* species the following advantages: 1) due to the specific and direct attachment to the substrates, any other non-cellulosome utilizing cellulolytic bacteria must

compete with the cellulosome for the binding sites in the cellulose; and 2) by reducing the distance between the cellulose and the cell, oligo-cellodextrins cannot diffuse away from the cell in to the medium.

When cellulose or cellobiose is used as the substrate, a water insoluble substance called yellow affinity substance (YAS) is produced (Ljungdahl et al. 1983). It's clearly visible on cellulose when compared its appearance on cellobiose (Kristjansson 1991). Its formation precedes cellulase appearance. It attaches itself to the cellulose fibers and forms a bridge between cellulose and the cellulolytic system that facilitates the hydrolysis process. YAS is a carotenoid-like compound and its exact chemical structure is not yet known (Ljungdahl et al. 1983). Recent studies revealed that supplementation of exogenous beta-glucosidase can significantly enhance the synthesis of YAS (Morag 2011).

1.4.5 End-product inhibition

Unlike other alcohol-producing microbes, such as yeasts, *C. thermocellum* is severely inhibited at relatively low ethanol concentrations (Herrero et al. 1980). The work of Kundu et al. (1983) and Wang et al. (1983), show that at ethanol concentrations of 5 g/L, approximately 15% of the cells are inhibited, and 50% of cells are inhibited at an ethanol concentration of 12 g/L. Therefore, in order to achieve higher ethanol concentrations, strains with higher ethanol tolerance levels have been developed (Herrero et al. 1980; Tailliez et al. 1989; Xu et al. 2010).

1.5 Fermentation in high substrate concentrations

An important factor in the process economics of biofuel production is the concentration of the substrate used in the reactor. In theory, increasing the substrate concentration should result in increased synthesis of end-products of interest. Multiple studies with *C. thermocellum* have shown that increasing cellulose concentration results in increased ethanol and hydrogen

production (volumetric production = mol end-product/L culture). Nevertheless, while high cellulose concentrations result in higher ethanol and hydrogen concentrations, the molar yields for ethanol and hydrogen (mols product/mol hexose equivalent substrate) are lower (Holwerda et al. 2014; Islam et al. 2006; Jiang et al. 2013). Conversely, low substrate concentrations give lower ethanol concentrations but higher molar yields. Therefore, it is important to optimize the substrate concentrations in fermentation processes to achieve the best balance of volumetric production and molar yield for the desired end-product.

The product concentration has a significant impact on capital costs, due to the requirement of smaller sizes of equipment (distillation column, tanks etc.) and lower operating costs that result from reductions in heating, cooling, and mixing power requirements. High concentrations in the product streams will also reduce the cost of residual disposal because of less water used (Mohagheghi et al. 1992; Wingren et al. 2003). Several techno-economic feasibility studies have shown that the ethanol concentration in the input stream of the distillation should be above 4% (w/w) for an economically viable process (Jørgensen et al. 2007; Lynd 1996). Assuming an average cellulose content of 60% and an ethanol conversion yield of 0.5 g/g, to maintain 4% w/w of ethanol will require 15% (w/w) of dry matter (Jørgensen et al. 2007; Wingren et al. 2003). In spite of this requirement, high solid concentrations cause the rheology of the fermentation media to change and thereby pose numerous operational difficulties such as insufficient uniform mixing and limited heat and mass transfer (Jørgensen et al. 2007; Mohagheghi et al. 1992)

1.6 Fed-Batch Fermentation

A fed-batch fermentation is a process where the substrate and/or nutrients are added continuously or intermittently after the initial inoculation (Babu et al. 2014). Fed-batch fermentations have gained popularity in many industries, as they combine the benefits of both

batch and continuous operation, while eliminating a number of other disadvantages. The main advantage of fed-batch fermentations is that through the intermittent feeding strategy of fed-batch fermentations, substrate inhibition and catabolite repression encountered in batch fermentations can be eliminated (Wang 2014). Other advantages include the requirement for less initial biomass and reduced risk of contamination and mutation compared to continuous reactor runs. A disadvantage of fed-batch operation is the requirement of substantial operator skill and additional control instruments for the feeding (Babu et al. 2014).

The development of the feeding strategy plays a pivotal role in the efficiency of any fed-batch fermentation process. The types of feeding strategies involved in fed-batch fermentation are: 1) Nutrient feeding according to a predetermined feeding profile based on previous data or growth rate calculations; 2) Nutrient feeding according to nutrient uptake; and 3) Nutrient feeding according to product formation (Lee et al. 1999). The objective of all these strategies is to ensure that the culture is not overfed or underfed at any given time. In fact, an ideal feeding strategy should optimize the environment conditions such that the growth or production phase of the desired end- product is maintained for a longer time. Theoretically it can be shown that when the specific growth rate of the micro-organism follows a "Monod-type" profile, a fed-batch culture will attain a quasi-steady-state where the cell and substrate concentrations remain constant while the total volume of the media increases (Pirt 1974).

1.7 Semi-Continuous fermentation

A semi-continuous fermentation is a type of fed-batch fermentation also referred to as 'repeated fed-batch fermentation'. A semi-continuous system is differentiated from a regular fed-batch culture (as described in Section 1.6) by the removal of a portion of the media and the addition of an equivalent amount of fresh media to replenish the nutrients in the fermentation

reaction. The residual broth acts as an inoculation for the second cycle. A repeated fed-batch culture can be operated indefinitely, as it is not limited by the reactor volume unlike in regular fed-batch fermentation (Jiang et al. 2012).

Semi-continuous fermentations are especially favoured when one or more of the end-products inhibit the growth of the organism. Once the media reaches a certain threshold concentration of the inhibitory end-product, a portion of the media is removed and replenished with fresh media making concentration more suitable for the growth or performance of the organism (Bauer et al. 2005). Another advantage of a semi-continuous system is that due to its long operation times, non-productive downtime associated with filling, heating, sterilization etc. can be reduced compared to batch and variable volume fed-batch operation. Several studies have shown the advantage of fed-batch fermentation over batch fermentation for the production of cellulosic ethanol and H_2 using the different process routes of SHF (Gupta el al. 2012), SSF (Fan et al. 2003; Rudolph et al. 2005; Zhang et al. 2010), SScF (Moreno et al. 2003), and CBP (Jiang et al. 2013; Wang et al. 1983) .

One of the common challenges in batch fermentation of cellulosic material is that due to its insoluble nature, higher concentrations of cellulosic materials will pose difficulties in mixing and mass transfer. Through intermittent feeding done in fed-batch fermentation a higher amount of cellulose can be hydrolyzed while maintaining lower cellulose concentrations in the media at all times. Semi-continuous fermentation also addresses the issue of ethanol inhibition observed in high cellulose concentrations. By periodic removal of the spent media, the ethanol concentration in the media is maintained at below inhibitory levels.

1.8 Research scope and Approach

The objective of the research is to investigate the performance of *C. thermocellum* ATCC 27405 for the direct conversion of cellulose in to ethanol and H_2 under high cellulose concentrations using fed-batch fermentation techniques. The baseline for this study was the results obtained in batch fermentation of *C. thermocellum* using an optimized media and a cellulose concentration of 25g/L. Throughout the chapters, this initial batch fermentation experiments will be referred to as the "Batch fermentation".

The following improvements were expected from augmenting the batch process to a fed-batch process while keeping other process parameters constant.

- Achieve higher product concentrations and yields for both H_2 and ethanol production. As explained in Chapter 1, Section 1.5, when increasing the cellulose concentration, there exists a trade-off between the concentrations of ethanol and hydrogen and the substrate specific yield. Therefore, through this research, the optimum conditions can be recognized;
- Attain longer operation times in order to avoid the down time associated with batch fermentation; and
- Elucidate the end-product synthesis profiles of *C. thermocellum* under different feeding strategies with high cellulose loadings.

By achieving these objectives, we will gain insight to the suitability of *C. thermocellum* for the direct microbial conversion of cellulose to ethanol and H_2 for more industrially relevant applications.

Chapter 2

General Materials and Methods

This chapter will explain the general materials and methods that were employed in all feeding strategies. Materials and methods that are specific to different feeding strategies will be described in the respective chapters.

2.1 Microorganism and Growth Media

Clostridium thermocellum DSM 1237 obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) was used throughout this study. *C. thermocellum* DSM 1237 was cultured in modified MJ medium (Johnson et al. 1981) that had been optimized for carbon excess conditions, as described by Islam et al. (2009). The medium contained (per liter) 1.5 g KH_2PO_4, 2.9 g K_2HPO_4, 10.0 g MOPS, 150 mg $CaCl_2.2H_2O$, 1 g $MgCl_2$.6H2O (g), 1.25 mg $FeSO_4.6H_2O$, 2.1 g urea, 1.0 mg resazurin and 3.0 g sodium citrate, 1 g yeast extract, 20 mg biotin, 200 mg pyridoxamine-HCl, 40 mg p-aminobenzoic acid and 20 mg cyanocobalamin.

Medium used in bioreactor studies had the same composition with the exception of 1.75 g per litre (/L) of $MgCl_2.6H_2O$ and 2 g/L yeast extract. The pH of the media was increased to 7 by the addition of KOH pellets. Urea and vitamin solutions were prepared in 100 times concentrated stock solutions and added to the media using a filter syringe (0.22 μm pore size) after autoclaving. The cellulose concentrations used in culture maintenance stocks and reactor inoculums were 5g/L and 10g/L, respectively. The cellulose concentrations in the reactor runs were varied in each feeding strategy and are explained in the respective chapters.

C. thermocellum was sub-cultured twice and aliquots were stored as glycerol stocks at -80 °C to be used as a master stock only in the case that working stocks became contaminated.

Dormant cultures were maintained at 4 °C and revived by passaging at least two times before each experiment.

2.2 Inoculum preparation

All inoculations were performed by transferring 10% (V/V) aliquots of a culture in mid-exponential phase to fresh medium. For instance, for a bioreactor with a 3 L working volume, the culture would be revived in a 120 mL serum bottle with a working volume of 30 mL and then inoculated in to a 1 L serum bottle with 300 mL working volume before the reactor is inoculated. Previous studies had determined that hydrogen (H_2) gas production follows closely cell mass production (Islam et al., 2006; 2009). As growth of *C. thermocellum* on an insoluble substrate, like cellulose, cannot be measured by optical density, H_2 gas concentration was used as an indicator of cell mass increase in the exponential phase. The culture gas production was measured every eight hours to ensure that the culture had attained mid-exponential phase prior to inoculation. The H_2 gas concentration at mid-exponential phase was used as a reference point for all subsequent experiments to eliminate disparity in inoculum cell density among replicate reactor runs. The anaerobic conditions of the serum bottles were achieved by adding 1 g/L of L-Cysteine hydrochloride and gassing and degassing with nitrogen gas prior to autoclaving (Daniels et al. 1986).

2.3 Experimental design for bioreactor studies

All bioreactor runs were conducted in a 7 L autoclavable vessel (Applikon Biotechnology), where the pH, mixing, and temperature were maintained via a programmable control tower (Applikon EZ-Control). The pH was maintained with the automatic addition of 5M KOH. Mixing was performed using a marine impeller and a Rushton impeller. The mixing rate was maintained at 100 rpm and was increased to 200 rpm for two minutes prior to sampling only.

Pure nitrogen gas (N_2) was filtered through a 0.2 μm filter (Milipore) and was continuously sparged at 10mL/min using an L-type sparger. The outlet port of the reactor directed headspace gases through a cold-water condenser to limit the ethanol evaporation (Figure 2.1).

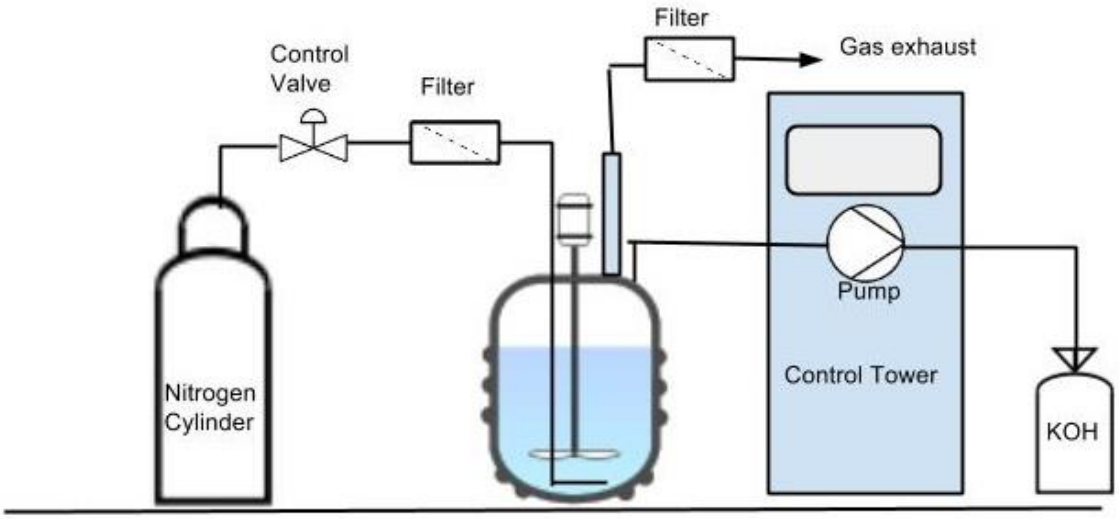
After autoclaving, 1g/L of L-Cysteine hydrochloride was added to the reactor by filtering the solution through a 0.2 μm filter and a higher sparging rate of 50-55 mL/min was maintained to remove any dissolved oxygen from the medium. The medium was considered completely oxygen free when the colour changed from pink to clear. After the media was confirmed to be anaerobic, the inoculum was added through a feeding port. Stirring was maintained at 40 rpm for the first 8 hours for a gentle transition of the inoculum in to the reactor media.

2.4 Analytical techniques

The composition and concentrations of gases in the headspace were measured using Gas Chromatography (SRI Instruments, Model 8610C or Agilent 7890A GC System). The aqueous phase H_2 and CO_2 concentrations were quantified by calculating their solubility in water by using Henry's law (Sander 1999). For CO_2, the bicarbonate concentration was calculated using the Handerson-Hasselbach equation (pH = pKoverall + log10) (Darrett and Drisham 1995). The equations used for the quantification of total gas end-products are shown in the Appendix I.

At each time point, approximately 10 mL samples were extracted using the sample port. From each 10 mL sample, 2 mL were transferred to microcentrifuge tubes and centrifuged for 10 minutes at 4000 g in a Sorval legend micro 21 (Thermo Scientific) centrifuge. The supernatants and pellets were separated and stored separately at -20 °C for liquid product analyses and protein assays respectively.

Figure 2.1 Schematic of the bioreactor configuration.



The remaining 8 mL of each time point sample was stored at -20 °C for the measurement of residual cellulose. Aqueous phase end-product concentrations were quantified using High Performance Liquid Chromatography (HPLC).

Concentrations of organic acids (acetate, lactate, formate), simple sugars (glucose, xylose, cellobiose), and ethanol were measured using an Aminex HPX 87H (300 x 7.8 mm) ion exclusion column (Bio-Rad) fitted with Cation-H, micro-guard cartridge (40 x 4.6 mm) and a refractive index detector (Dionex ICS- 3000, Thermo Fisher Scientific, Waltham, MA, USA; or Waters Breeze 2, Waters, Milford, MA)

The protein concentration was quantified using a modified Bradford method (Bradford 1976) after processing and extracting as described previously (Sparling et al. 2006). The protein samples were loaded in to 96 well plates and the absorbance was measured at a wavelength of 595 nm using a microplate reader (Biotek Synergy 2, Hamilton Robotics). Cell dry weight (cdw) was estimated based on the protein concentration as detailed in Islam et al. (2009).

The residual cellulose concentration was measured by vacuum filtering the sample using pre-weighed Whatman Grade 1 qualitative filter paper with an 11 μm pore size. The filter paper was dried in an oven at 80 °C for 24 hours and then weighed again. The residual cellulose was calculated by subtracting the weight of the paper before filtering the sample and the weight of cell dry weight from the weight of the paper after filtering the sample.

The metal ion concentrations of the samples were measured by CCD simultaneous Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES; Model: VISTA MPX; Varian Inc.). All samples were diluted 10 times with distilled water prior to the analysis to adjust the concentrations to the maximum measurable levels.

Chapter 3

Comparison of fed-batch fermentation with batch fermentation

3.1 Introduction

For a fermentation to be cost effective, it is important to develop a strategy that produces the desired products with high productivity and yield. Fed-batch cultivation is generally employed to improve the cost effectiveness of batch fermentation by feeding one or more nutrients intermittently. Ideally a fed-batch culture should reach a quasi-steady state where cell and substrate concentrations are constant, while the volume increases (Kalogerakis 1979). The most crucial step in fed-batch cultivation is the development of a suitable feeding strategy that ensures that the culture is not overfed or underfed at any given time (Lee et al. 1999).

As detailed in Chapter 1 (Section 1.6), there are several feeding strategies that are commonly employed in bioprocesses. This chapter summarises the work done on the development of two feeding strategies and compares their performance with batch cultivation. The two feeding strategies developed were:

1) Variable volume fed-batch fermentation; and
2) Fixed volume fed-batch fermentation.

The variable volume fed-batch fermentation involves a predetermined feeding strategy based on the results obtained from the batch fermentation. In the fixed volume fed-batch fermentation, the feeding profile was based according to the substrate uptake.

3.2 Materials and methods

The media composition, reactor set-up and inoculation, and end-product analysis in both feeding strategies are as described in Chapter 2, Sections 2.1, 2.2, and 2.3 respectively.

3.2.1 Variable volume fed-batch fermentation

In this feeding strategy, the culture was started as a batch fermentation and was fed more nutrients and cellulose once it reached mid-exponential phase in order to maintain active growth over a longer period of time. The time taken to reach mid- exponential phase was estimated from the cell growth profiles obtained from the batch culture studies. The batch cultures reached mid-exponential phase at approximately 24 hours post inoculation (h pi). At this time, 50% of the initial cellulose added was consumed. Based on this result, a hypothesis was developed that, the culture will consume 50% of the existing cellulose concentration every 24 hours (h). This hypothesis led to the development of the feeding strategy shown in Table 3.1.

The reactor was started with a working volume of 2 L, and approximately every 24 hours, 1 L of media containing the cellulose concentrations shown in Table 3.1 was added. The media and cellulose were gassed-degassed and autoclaved in 1 L bottles (Bellco Glass Inc.). The added media in the bottles were clear in colour indicating that it was completely reduced prior to addition. The nitrogen-sparging rate was increased from 10 mL/min to 25 mL/min for 20 minutes after each feeding to remove any oxygen that may enter the system during the feeding.

3.2.2 Fixed volume fed-batch fermentation

In this feeding strategy, the reactor run was started with a working volume of 4 L and a cellulose concentration of 25 g/L. Cellulose was then fed according to the uptake to maintain the concentration at 25 g/L which was found to be the optimum concentration in batch fermentation studies. No other nutrients were replenished during the run. The cellulose was autoclaved and fed in to the reactor sterilely using a sealed funnel. The cellulose hydrolysis was quantified by measuring the residual cellulose concentration.

Table 3.1 Feeding strategy for the variable volume fed-batch fermentation.

	Initial	**Feeding 1**	**Feeding 2**	**Feeding 3**
Time (Hours)	0	25	48	73
Volume of media added (L)	2	1	1	1
Feed stream [cellulose] (g/L)	25	50	62.5	72
Amount of cellulose added (g)	50	50	62.5	72

A modified method which combined the saccharification technique detailed in Updergraph (1969) and the phenol sulphuric method detailed in Dubois (1951) was used to measure the residual cellulose concentration. For the saccharification of the cellulose, 1 mL of each sample was spun down at a speed of 14000 x g for 5 minutes in a micro centrifuge (Sorval Legend Micro 21, Thermo Scientific). The supernatant was discarded, the pellet vortexed and 67% sulphuric acid was added to the pellet. After the sample was completely dissolved, 0.2 mL of the sample was combined with 0.2 mL of phenol and 1 mL sulphuric acid. After a dilution of 5 times of this mixture with distilled water, the optical density was measured using a spectrophotometer (Biomate 3, Thermo Scientific) at a wavelength of 520 nm. Along with all samples, standards ranging from 5 g/L to 30 g/L were processed identically in order to get a standard curve. All standard curves had an R^2 value above 0.97. Both feeding strategies were performed in duplicates and the average results are shown in the tables and graphs.

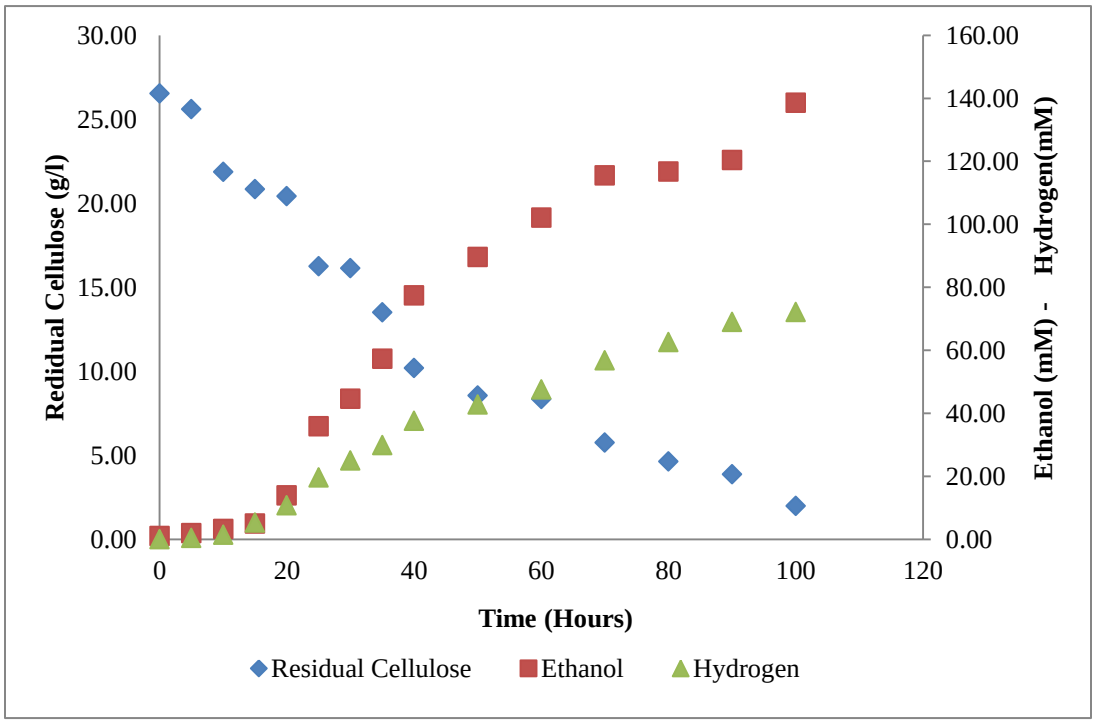
In all figures where the end-product concentrations are presented in mM or mmoles/L corresponds to mmoles end-product produced per one litre of culture.

3.3 Results and Discussion

3.3.1 Batch fermentation and development of the fed-batch fermentation strategy

The results obtained from the batch fermentation studies were used as a base line in the development of the fed-batch fermentation strategies. This section will explain the rationale behind developing each strategy. As visualized in Figure 3.1, synthesis of ethanol and hydrogen (H_2) continued to increase over the 100 h of the fermentation reaction. The rates of synthesis of these products, however, differ. After an initial lag- phase of approximately 20 h, ethanol concentration increased rapidly until 40 h pi.

Figure 3.1 Ethanol, hydrogen, and residual cellulose concentrations in *C. thermocellum* batch fermentation cultures.



Ethanol production continues at a slower rate between 40 and 72 h pi, and appeared to plateau between 72 and 96 h pi. Hydrogen synthesis also increased, after the initial lag-phase, at a moderate rate between 20 and 48 h pi, and at a slower rate between 48 and 100 h pi. At 100 h pi, almost all the cellulose was consumed. The residual cellulose concentration at 100 h pi was only 0.98 g/L. The slower rate of ethanol and H_2 synthesis in the later stages of the fermentation reactions could be due to end-product inhibition, depletion of cellulose, depletion of some other essential nutrient(s), or a combination of these factors.

3.3.2 Variable Volume Fed-batch fermentation cultures

The variable volume fed-batch fermentation was designed to eliminate all three reasons that may result in reduced growth and end-product synthesis rates. Cellulose was supplemented continuously to ensure that there was a sufficient concentration of substrate present at all times. Fresh medium with more nutrients was added periodically to ensure that there is no depletion of essential nutrients. Also, the volume increase in the reactor would dilute the end-product concentrations, therefore minimizing the effect of end-product inhibition. Despite the expected improvements, the performance of the variable volume fed-batch fermentation was not as high as expected. As shown in Figure 3.2, the gas production rate was significantly lower in the variable volume fed-batch fermentation when compared to the batch fermentation culture. It was also observed that after the first cellulose and media addition at 24 h pi, the gas production rate decreased sharply in the variable volume fed-batch fermentation compared to the batch fermentation culture. This could be due to the poor reduction in the added media. Despite pre-reducing the media prior to addition, during the feeding, some oxygen may enter the system making the overall media poorly reduced. Although the fermentation reaction lasted for 230 h, the final concentration of H_2 achieved by the variable volume fed-batch fermentation culture was

similar to that of the batch fermentation culture. A similar trend was observed for all the aqueous phase end-products, ethanol, acetate, and formate with the exception of lactate (Figures 3.3 and 3.4).

The ethanol production rate in the variable volume fed-batch fermentation culture was significantly lower than that observed in the batch fermentation culture. The total ethanol production in the variable volume fed-batch fermentation culture was less than half of the ethanol concentration observed in the batch fermentation culture. At the end of the fermentation reaction (100 h), the ethanol: acetate (E: A) ratio of the batch fermentation culture was 3.37 while the E: A ratio in the variable volume fed-batch fermentation was only 0.96.

There was a sharp rise in the lactate concentration during the late log-phase in the variable volume fed-batch fermentation. This is a common trend reported by other studies conducted with high cellulose concentrations (Islam et al. 2009; Tailliez et al. 1989). The aqueous phase end-product profiles indicated that under the high cellulose concentrations in our experiments, more carbon flowed towards the synthesis of lactate and away from the pathways that synthesize the end-products of interest, ethanol and H_2.

The cell protein concentration at the stationary phase is approximately 400mg/L in both variable volume fed-batch fermentation and batch fermentation (Figure 3.5). Nevertheless, the variable volume reached this concentration at 70 hours while the batch fermentation required only 25 hours. The slow initial growth rate of the variable volume fed-batch fermentation culture may be due to the smaller working volume at the initial phase.

Since the working volume of the variable volume fed-batch fermentation culture changed with time, the total cellulose consumed was used as a parameter to compare the uptake of

Figure 3.2 Gaseous end-products (H_2 and CO_2) in the *C. thermocellum* variable volume fed-batch and batch fermentation cultures. The arrows indicate the points of media addition at 25h, 48h and 73h respectively.

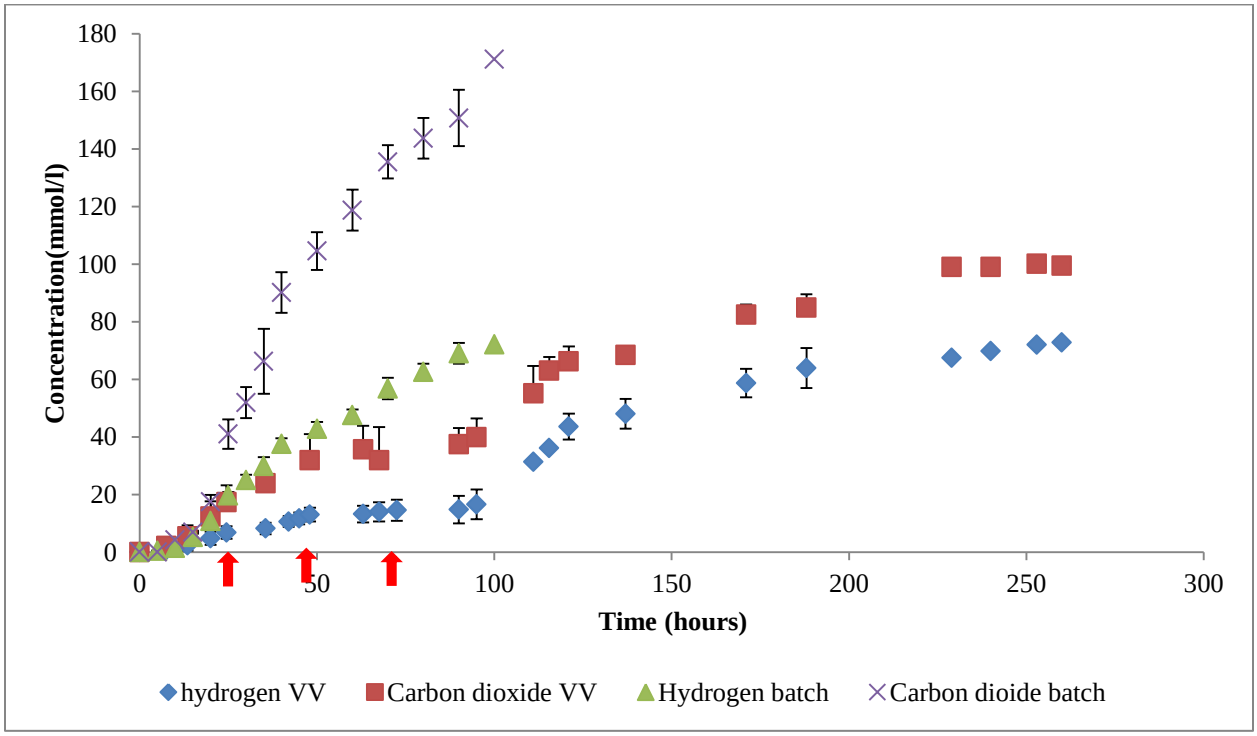


Figure 3.3 Ethanol and acetate concentrations in the *C. thermocellum* variable volume fed-batch and batch fermentation cultures. The arrows indicate the points of media addition at 25h, 48h and 73h respectively.

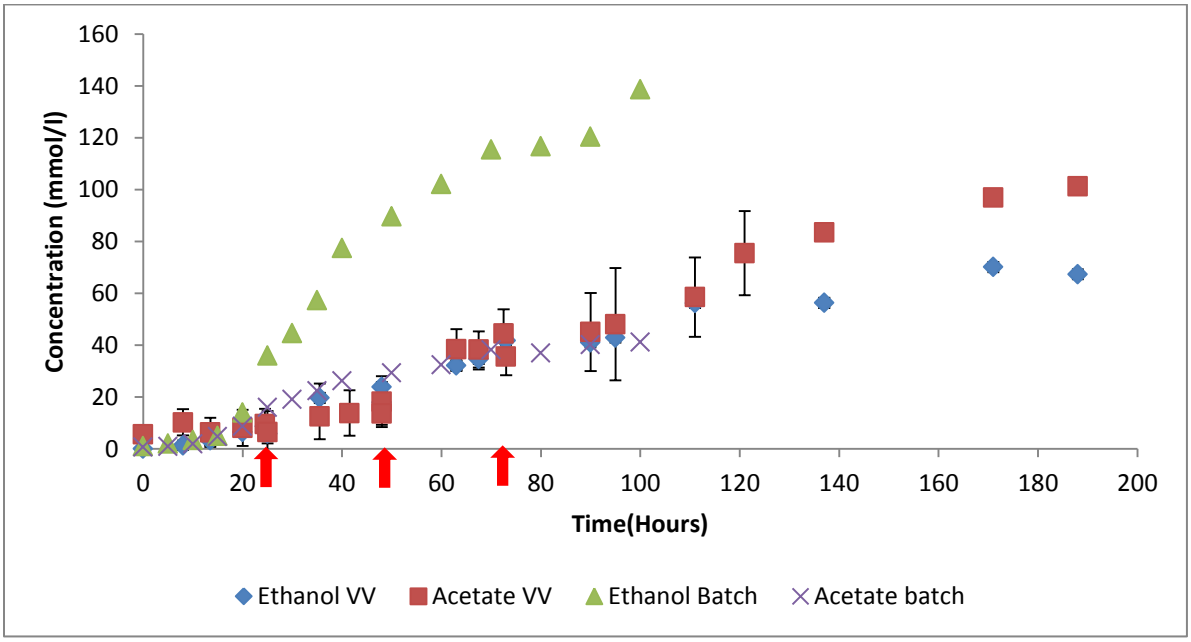


Figure 3.4 Lactate and formate concentrations in the *C. thermocellum* variable volume fed-batch fermentation and batch fermentation cultures. The arrows indicate the points of media addition at 25h, 48h and 73h respectively.

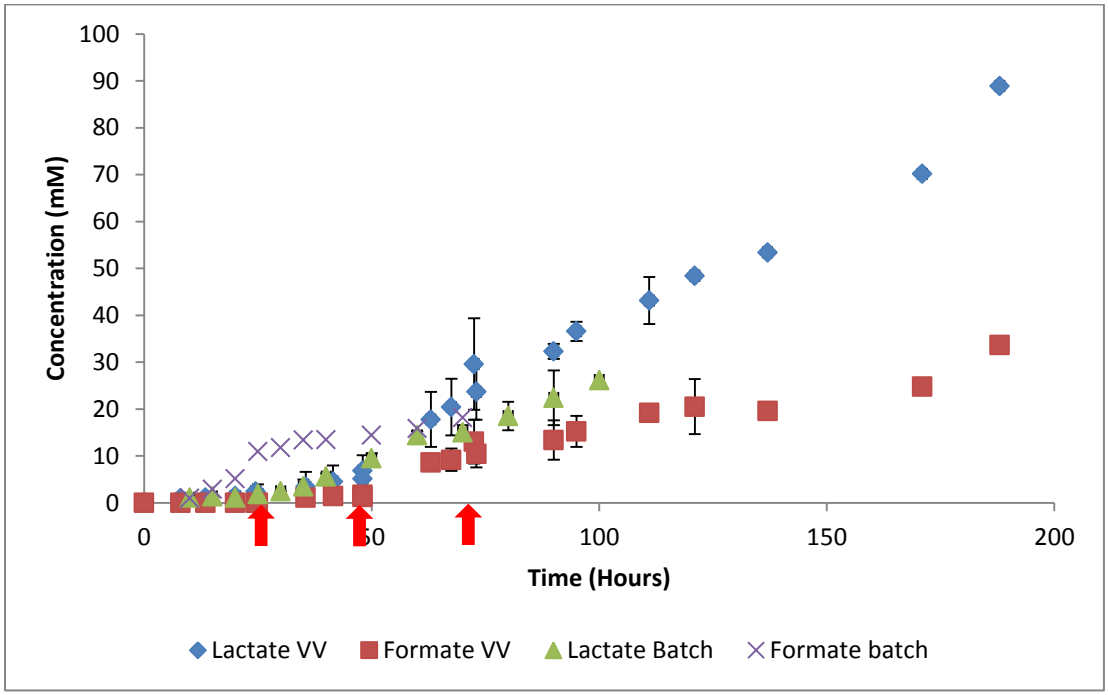
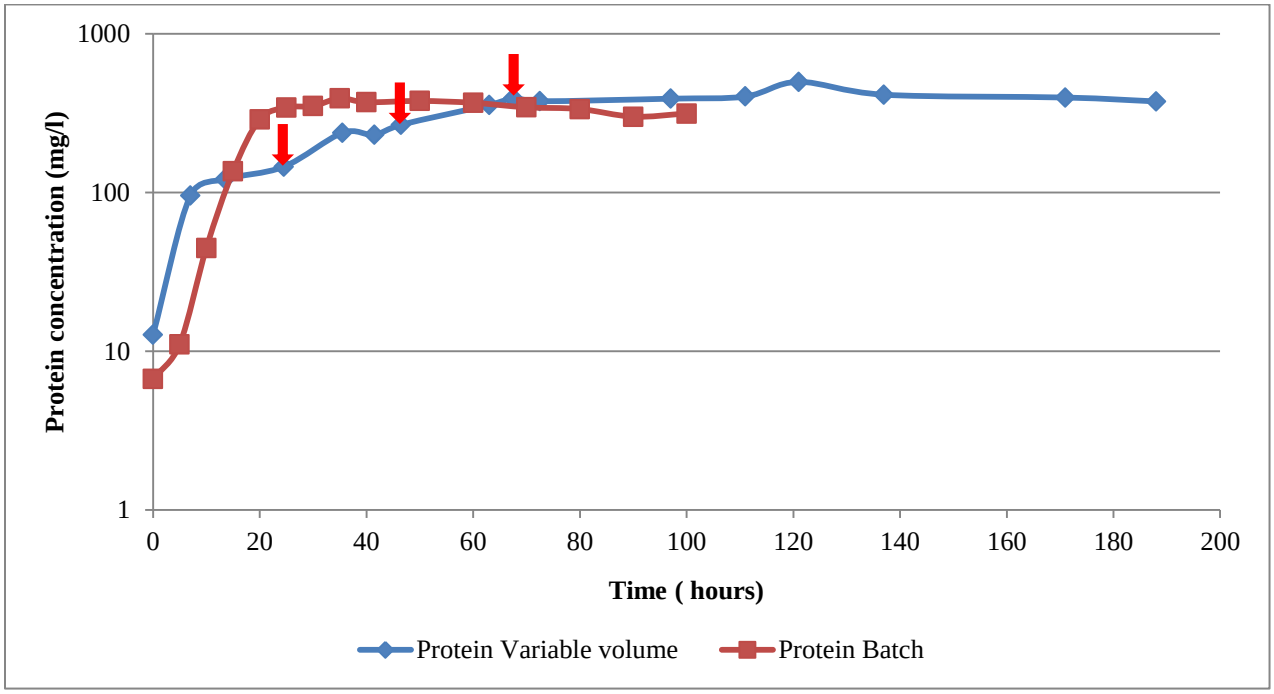


Figure 3.5 Total *C. thermocellum* protein concentrations extracted from the variable volume fed-batch fermentation and batch fermentation cultures. The arrows indicate the points of media addition at 25h, 48h and 73h respectively.



cellulose by *C. thermocellum* in the variable volume fed-batch fermentation versus the batch fermentation culture (Figure 3.6). Consistent with the end-product profiles, there was a slower rate of cellulose consumption in the variable volume fed-batch fermentation culture (Figure 3.6). However, the rate of cellulose consumption in the batch fermentation culture decreased after 50 hours, while the rate of cellulose consumption in the variable volume fed-batch fermentation culture increased, such that the total of amount of cellulose consumed by 100 h pi was similar in both fermentation cultures. In the end, a greater amount of cellulose was consumed by *C. thermocellum* in the variable volume fed-batch fermentation than in the batch fermentation culture, but this is because the duration of the variable volume fed-batch fermentation was much longer and a higher amount of cellulose was added throughout the fermentation when compared to the batch fermentation cultures.

Although the cellulose consumption rate was not constant in either the variable volume fed-batch fermentation or the batch fermentation culture (Figure 3.6), the variation in substrate consumption was more pronounced in the variable volume fed-batch fermentation culture .The amount of cellulose consumed after each addition of substrate decreased in variable volume fed-batch fermentation culture, such that the cellulose concentration increased with each successive addition, ultimately reaching concentrations greater than 35 g/L (Figure 3.7). However, the increased amounts of cellulose in the culture contributed to neither increased cell mass production, nor end-product yields. In fact, the increased amount of substrate appeared to have an adverse effect on cell growth. Figure 3.8 shows the change in the distribution of end-products in between each feeding in the variable volume fed-batch fermentation. Despite the increased cellulose consumption rate in variable volume fed-batch fermentation observed after ~50 hours (Figure 3.6), a significant amount of the carbon was directed towards the production of lactate.

Figure 3.6 Cellulose consumption rate by *C. thermocellum* in the variable volume fed-batch and batch fermentation cultures.

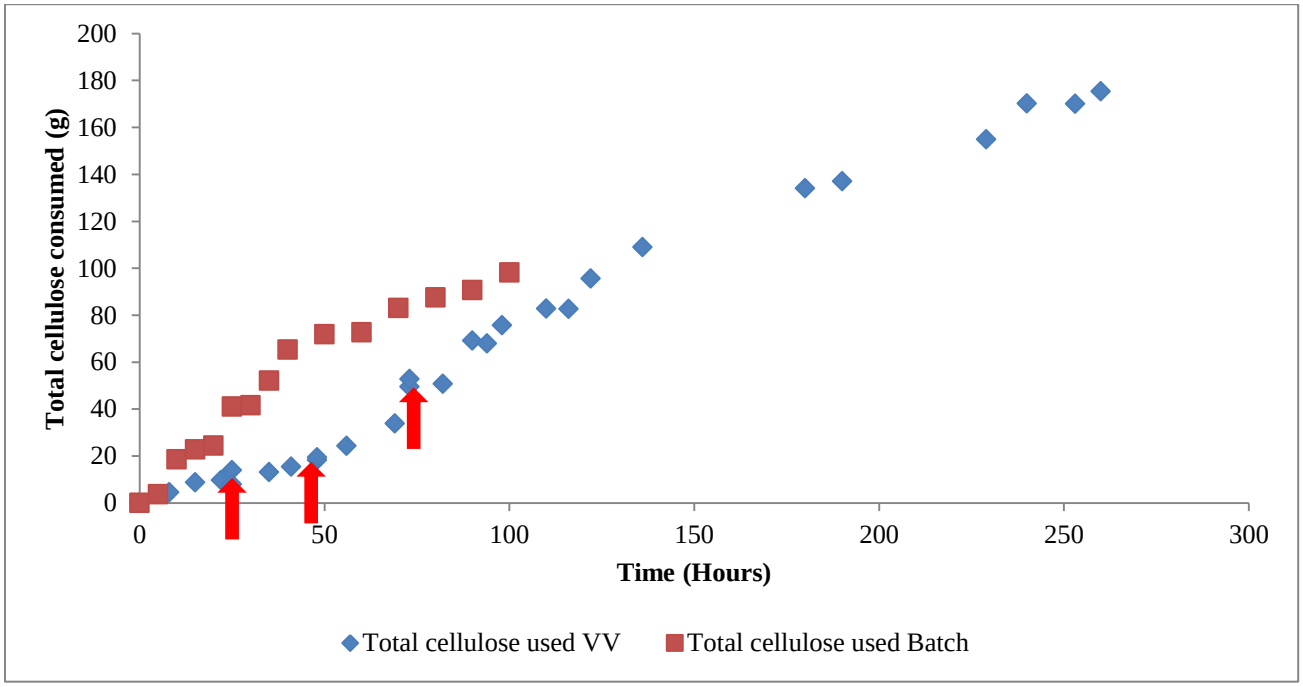


Figure 3.7 Residual cellulose concentrations in the variable volume fed-batch fermentation culture.

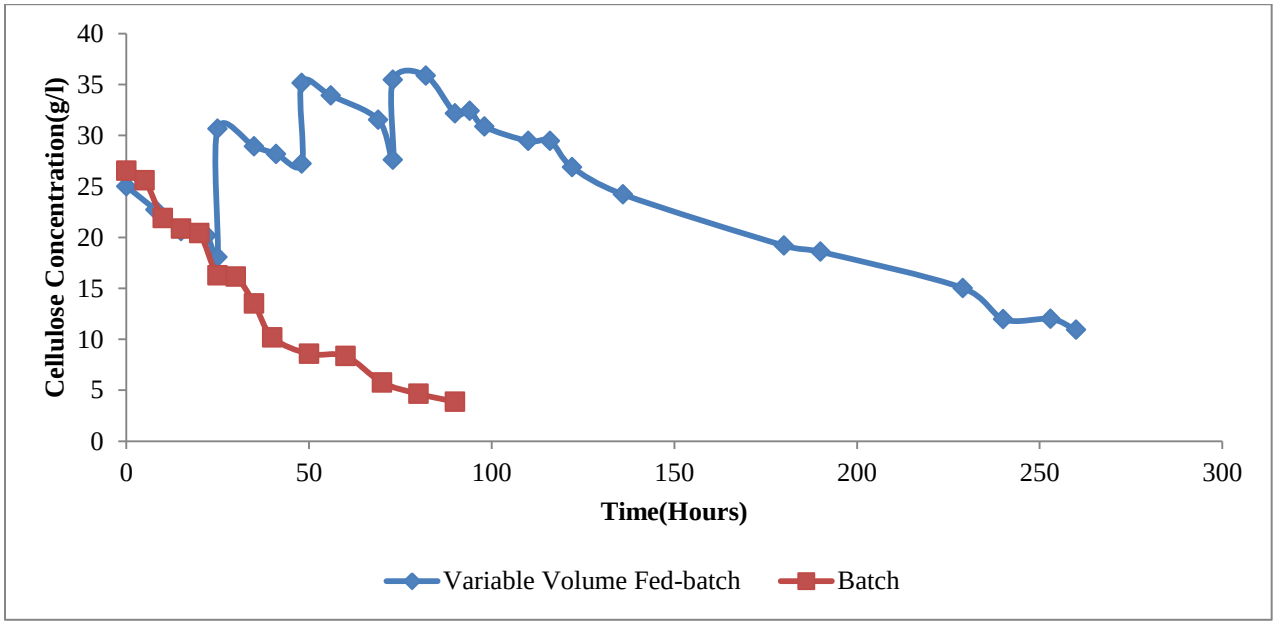
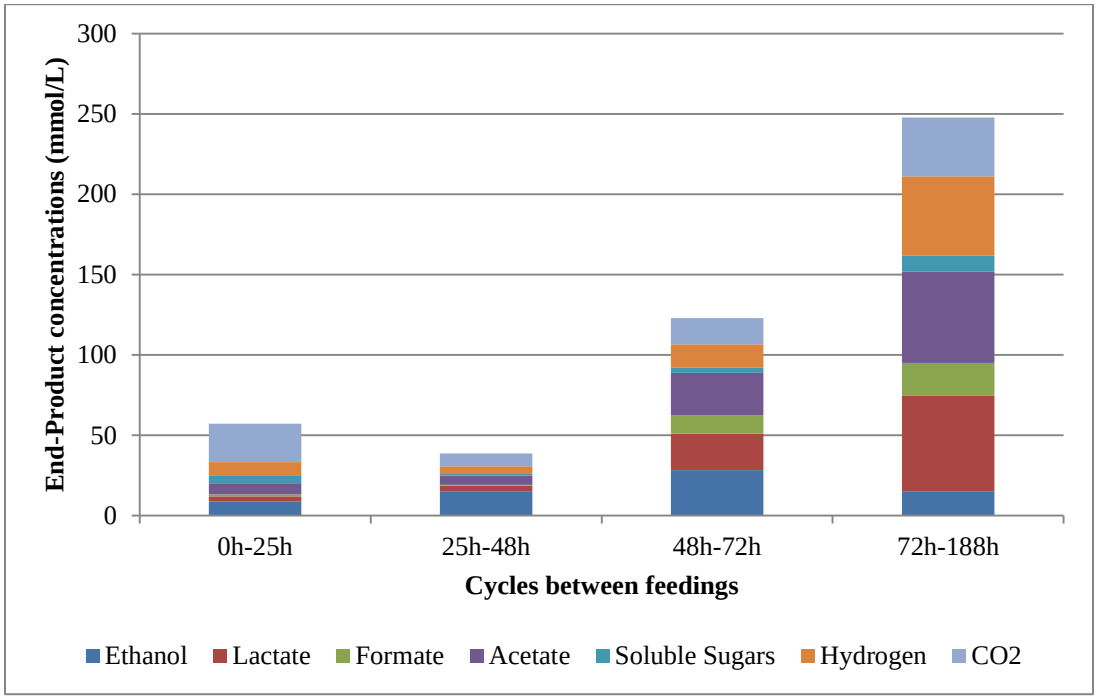


Figure 3.8 The end-product distribution between each feeding in the variable volume fed-batch fermentation



As shown in Figure 3.8, in cycles 2 and 3, due to the increased lactate production, the production of other end-products were severely compromised. Figure 3.8 also shows that cycle 2 (within hours 25-48) showed the minimum cellulose consumption. This could be due to poor reduction in the fresh media added as this addition had the highest fermented media to fresh media ratio (2:3).

Overall, the variable volume fed-batch shows that increased cellulose concentration resulted in poorer production of the desired end products. While the reason for this adverse effect is not clear, it has been demonstrated that fermentation media with higher concentrations of solids tend to have different rheological properties, which could negatively affect proper mixing within the culture and this effect mass transfer of nutrients (Jiang et al. 2012). At concentrations greater than 25 g/L, when a stirring rate gentle enough for the microorganisms was employed, a compact bed of cellulose formed at the bottom of the reactor which made it difficult for the bacteria to access a major portion of the cellulose. Also accumulation of cellulose at the bottom can lead to poorer mass transfer that results in higher concentrations of dissolved gases and other liquid end-products within the bed of cellulose (Islam et al. 2009). Accumulation of acidic end-products can result in lower pH values within the cellulose bed when compared to the liquid above the bed. Although other studies (Holwerda et al. 2014), used higher stirring speeds (300 rpm) in cellulose fermentations with *C. thermocellum*, the current work used a stirring rate of 100 rpm, because higher rates of stirring resulted in foaming on the culture surface. The tendency to create foam in a bioreactor depends on the level of mechanical stress created by the stirring system, as well as on the protein content in the medium, which will stabilize the foam (Routledge 2012).

Another reason for the poor performance of the variable volume fed-batch fermentation culture could be that with each addition of fresh media, the media composition varied from the optimum composition that was used in the batch experiments. The intermittent addition of fresh media could result in certain components that were not consumed to at a high rate to be present in excess in the media. This is a common problem in fed-batch fermentation, as it is difficult to know the uptake rate of all nutrients. In order to avoid this, Jiang et al. (2012) increased the amounts of KH_2PO_4, NH_4Cl and yeast extract added to a cyclic fed-batch fermentation proportionally to the increasing cellulose concentration.

3.3.3 Fixed Volume Fed-batch Fermentation

The fixed volume fed-batch fermentation strategy was designed to maintain the cellulose concentration at the optimum concentration. The main objective of this strategy was to eliminate the high cellulose concentrations seen in variable volume fed-batch fermentation culture. In order to keep the number of variables to a minimum, the volume of the culture was maintained and no other nutrients were added during the fermentation. As explained in the materials and methods (Chapter 3, Sections 3.2.2), the residual cellulose concentrations were measured intermittently and cellulose was replenished to maintain the concentration at 25 g/L. As shown in Figure 3.9, cellulose was added at 43 and 72 h pi. At 43 h pi, 30 g of cellulose was added and at 72 h pi 40 g of cellulose was added. After replenishing the cellulose at each time, the total cellulose concentrations were 21.9 ± 1.7 g/L and 20.1 ± 0.8 g/L, respectively. The discrepancies in these values from the planned 25 g/L are due to errors of the methods of cellulose measurement. Also, some of the cellulose became attached to the reactor walls while feeding, thus reducing the total amount of cellulose in the fermentation culture medium. Although in total 170 g of cellulose was added intermittently, it is important to note that the total amount of

cellulose consumed was only 103 ± 8.05 g. This value is similar to the total amount of cellulose consumed in the batch experiments, which was a total of 100 g/L.

Concentrations of gaseous (H_2 and CO_2) end-products synthesized in the fixed volume fed-batch fermentation culture were also similar to those of the batch fermentation experiments (Figure 3.10). Carbon dioxide was synthesized in much greater amounts than H_2 in both the fixed volume fed-batch fermentation and batch fermentation cultures. However, the production rate of all end-products with the exception of lactate and formate appeared to decrease in the fixed volume fed-batch fermentation at approximately 48 h pi, despite the presence of excess cellulose. The lactate concentrations in the fixed volume fed-batch fermentation followed a similar profile as lactate production in the batch fermentation culture, until the first addition of cellulose. After that a steep increase in the lactate concentrations was observed until the end of the fermentation (Figure 3.12). This shows that similar to variable volume fed-batch, the increased cellulose concentrations stimulated the synthesis of lactate.

At 100 h pi, ethanol concentrations in the fixed volume fed-batch fermentation and batch fermentation were 5.49 g/L and 6.38 g/L, respectively (Figure 3.11). It is well known that the viability of *C. thermocellum* cells is compromised by ethanol at these concentrations (Kundu et al. 1983 ;Wang et al. 1983). The final acetic acid concentration of the fixed volume fed-batch fermentation and batch fermentation cultures were 1.68 g/L and 2.42 g/L, respectively (Figure 3.11). According to Kundu et al. (1983), these concentrations are too low to cause a significant inhibitory effect on cell growth. Therefore, it can be concluded that the accumulation of ethanol was a contributing factor to the plateauing of the end-products. Nevertheless, the concentrations were not high enough for it to be the sole contributing factor.

Figure 3.9 Residual cellulose concentration in the fixed volume fed-batch fermentation culture.

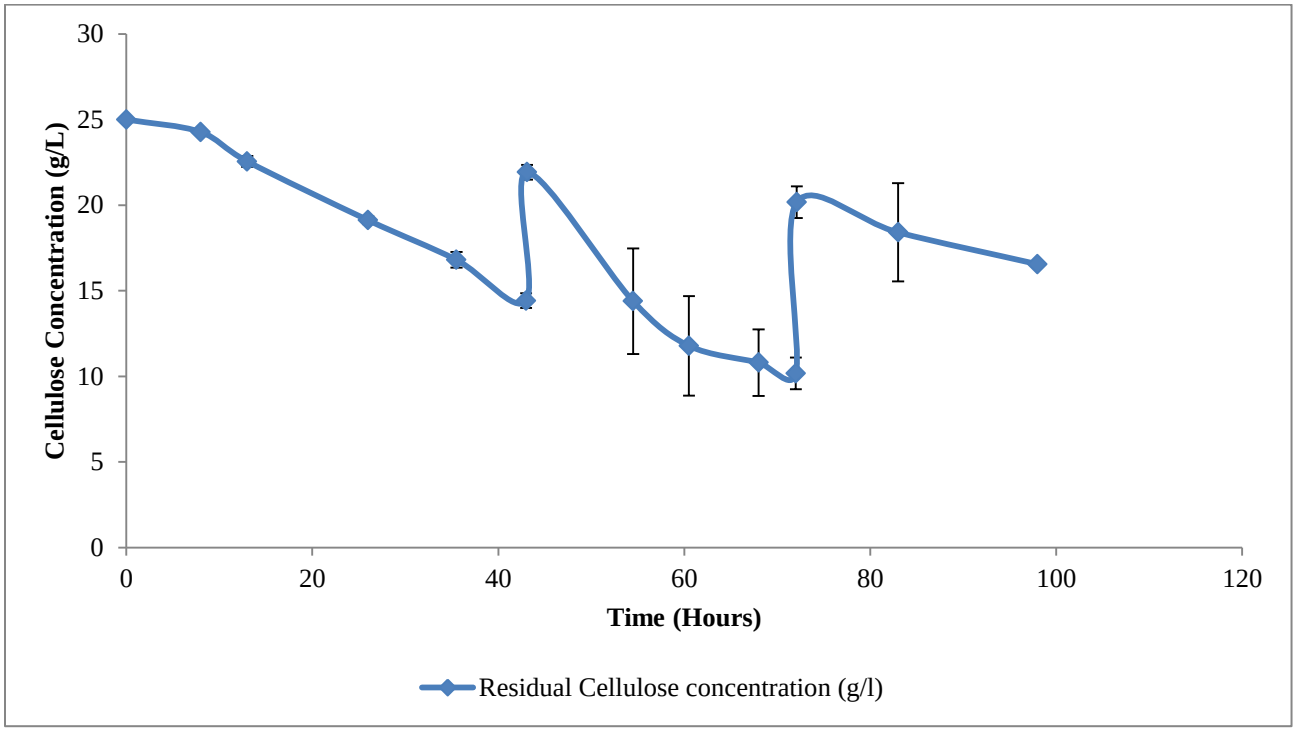


Figure 3.10 Gaseous (H_2 and CO_2) end-product profiles of *C. thermocellum* in the fixed volume fed-batch and batch fermentation cultures. The arrows indicate the time points where cellulose was added to the reactor (43 hours and 72 hours).

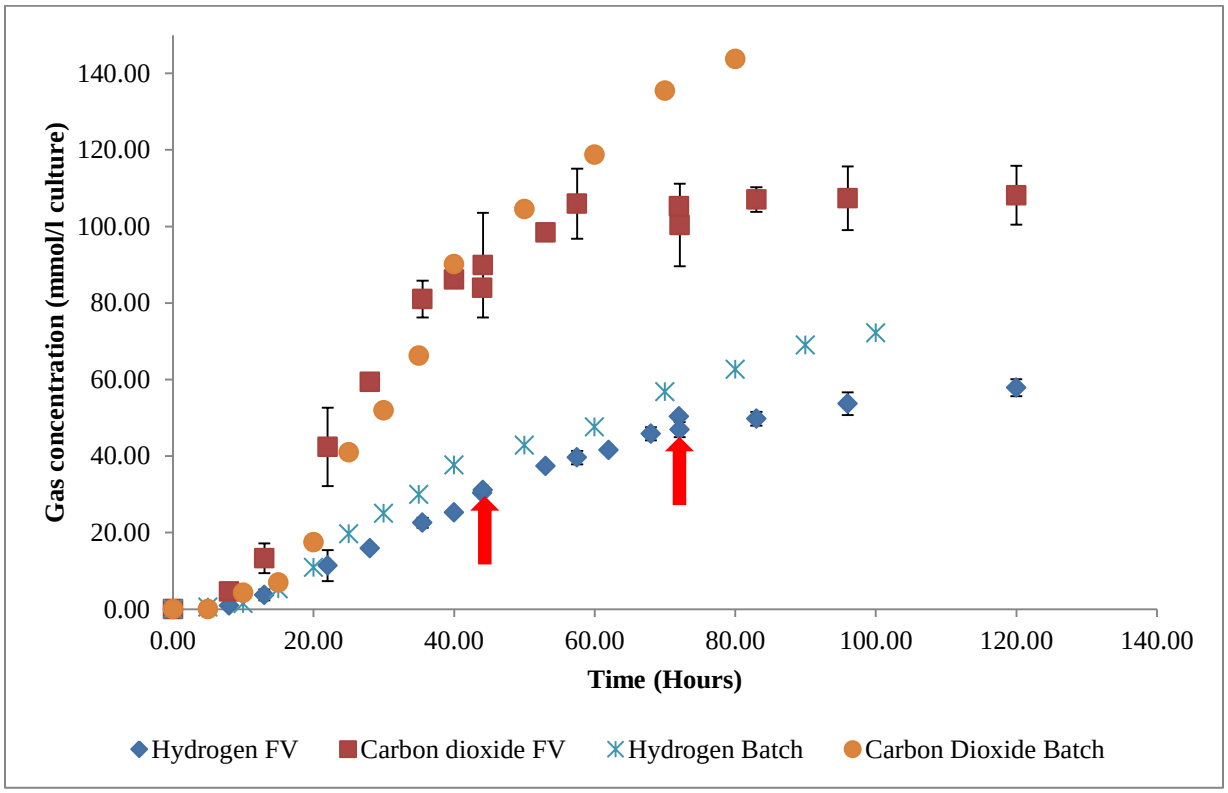


Figure 3.11 Ethanol and acetate concentrations in the fixed volume fed-batch fermentation and batch fermentation cultures. The arrows indicate the time points where cellulose was added to the reactor (43 hours and 72 hours).

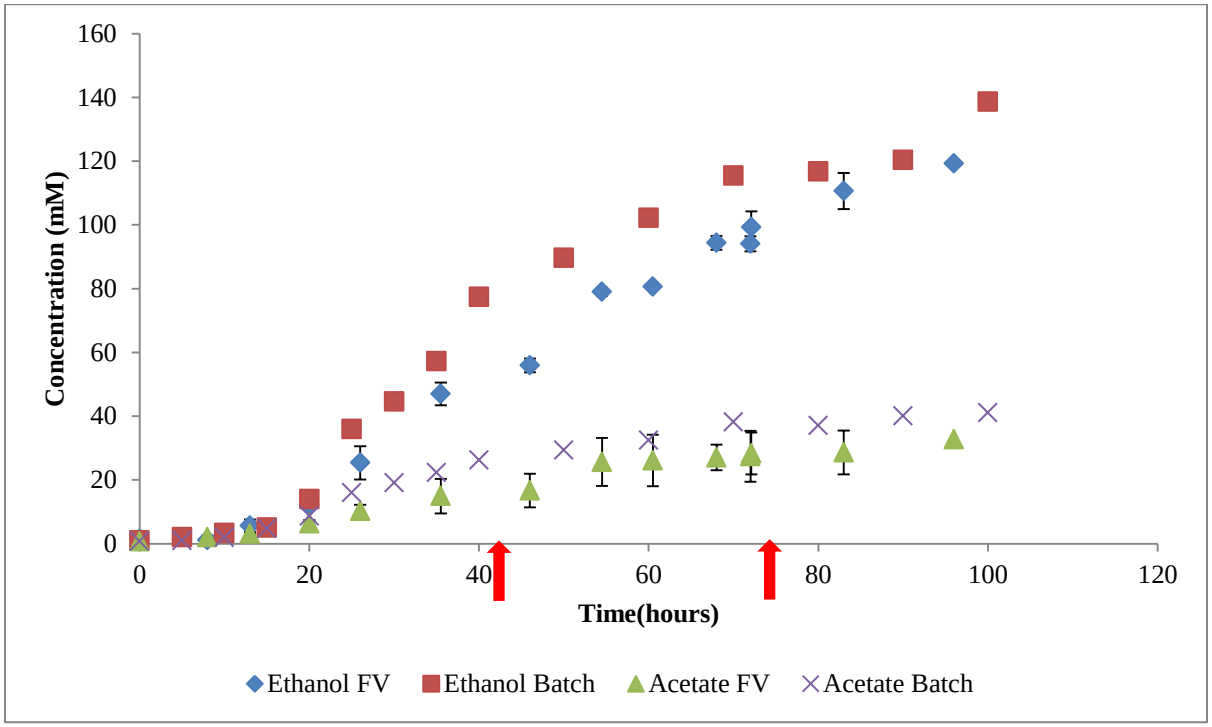
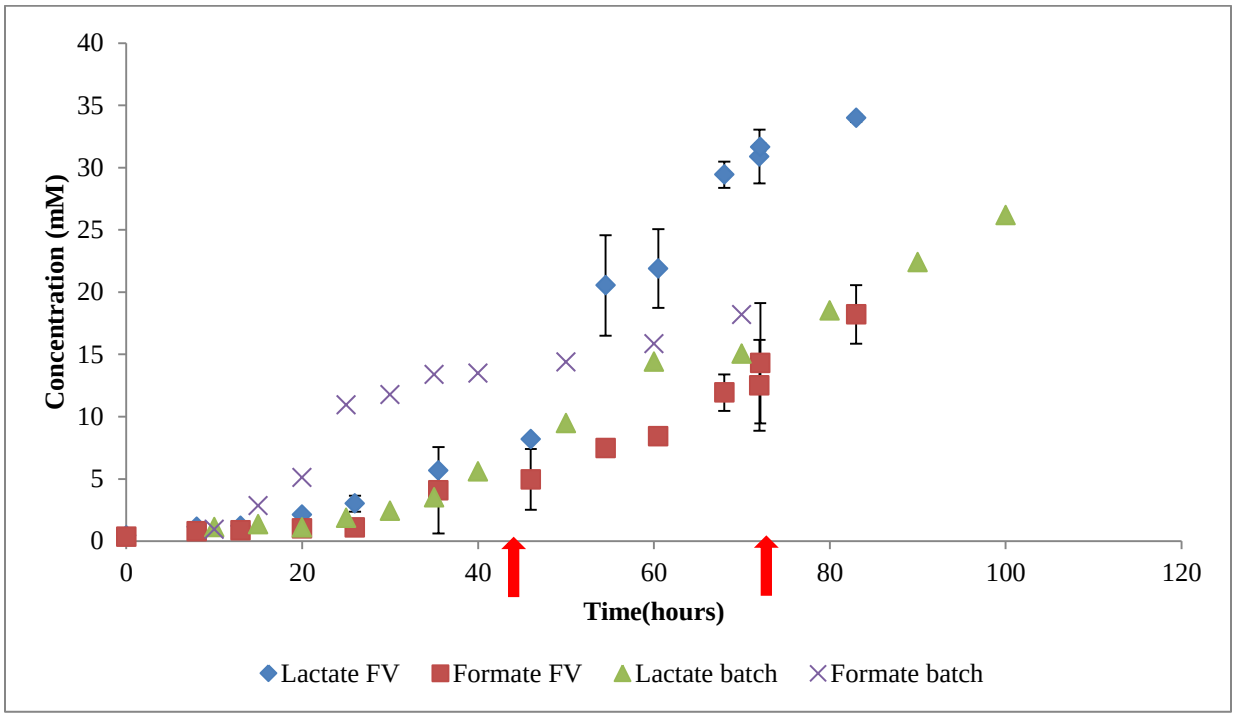


Figure 3.12 Lactate and formate concentrations in the fixed volume fed-batch and batch fermentation cultures. The arrows indicate the time points where cellulose was added to the reactor (43 hours and 72 hours).

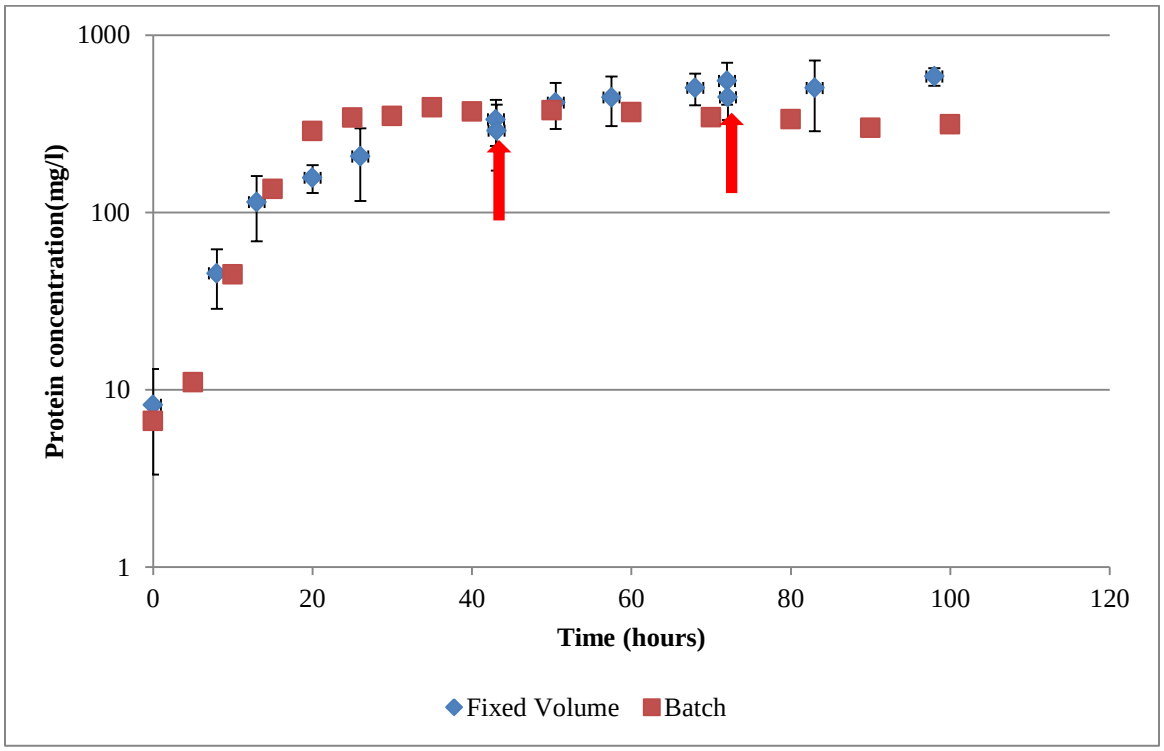


The cell growth before the first addition of cellulose to the fixed volume fed-batch fermentation culture was similar to cell growth observed in the batch fermentation culture (Figure 3.13). This is expected as the growth conditions were identical prior to the first cellulose addition. After the first cellulose addition at 44 h pi, the cell concentration in fixed volume fed-batch fermentation increased slightly. It is clear that with higher cellulose concentrations, the cells continued to grow. Even though the growth rate was slower after 44 h pi, it did not plateau as observed in the batch fermentation culture.

3.3.3.1 Nutrient Concentrations

Fresh supplies of nutrients were not added to the fixed volume fed-batch fermentation throughout the run. Consequently, there was a possibility that certain nutrients could become limiting. To verify this, the metal ion concentrations were measured at the final time point (Table 3.2). This analysis revealed which nutrients were present in excess and which ones had a lower uptake. Magnesium and iron had the highest uptake when compared to other ions. A number of studies have shown the pivotal role of magnesium on various microorganisms (Dombek et al. 1986; Jasper et al. 1977). Magnesium ions act as a cofactor for a number of glycolytic enzymes such as hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, and enolase. In addition, certain components of the cell wall and cell membrane contain magnesium. Therefore, low concentrations of magnesium ions could affect glycolysis, assimilation and H_2 synthesis capacity (Wang et al. 2007).

Figure 3.13 Total protein concentrations extracted from *C. thermocellum* cell pellets in the fixed volume fed-batch fermentation and batch fermentation cultures. The arrows indicate the time points where cellulose was added to the reactor (43 hours and 72 hours).



Iron concentrations also play an important role in the metabolic pathways of *Clostridium* species. In the metabolic pathway used by *C. thermocellum*, H_2 is produced through the reaction $2H^+ + 2e^- \leftrightarrow H_2$. This reaction is catalyzed through NAD(P)H-dependent or ferredoxin-dependent hydrogenase enzymes that consist of iron-based metal clusters (Adams et al. 1989). A study done on *Clostridium acetobutylicum* showed that hydrogenase specific activity was decreased by 40% under iron limitation (Junelles et al. 1988). Therefore, limitation of iron can have a severe impact on H_2 synthesis.

The analysis of the metal ion concentrations suggests that limitations in the magnesium and iron concentrations could have an impact on H_2 and ethanol concentrations after 96 h pi. Nevertheless, none of the nutrients were completely depleted, and therefore nutrient depletion cannot be considered as the sole reason for the drop in the production rate of the desired end-products.

3.3.4 Yields and Productivities

Variation in the carbon balances from the value of 1 could be due to the evaporation of ethanol in the reactor or during the analysis or errors during the measurement of residual cellulose (Table 3.3). Another reason for the carbon balances to be lower than 1 is the accumulation of amino acids as reported by Holwerda et al. (2014) which was not accounted for in analysis of end-products in the current study. The O/R ratios close to 1 confirm the accuracy of the end-product measurements. All three parameters used to compare the fermentations [fermentation end-product yields (mmol/mmol hexose equivalent), the productivity (mmol/grams dry cell. hour), and the production rate (mmol/L/hour)] indicate that batch fermentation performed far better than the other two feeding strategies. Table 3.3 also shows that the main

reason for the poor performance of fed-batch fermentations when compared to the batch fermentation is the increase in lactate production observed in the fed-batch fermentations.

Another indication of the low efficiency of the fed-batch fermentation was the high concentration of soluble sugars produced compared with the batch fermentation culture, where only trace amounts were detected. The soluble sugars consisted of glucose, xylose, or cellobiose and it was difficult to distinguish between them due to their identical retention time in the HPLC analysis. The percentage of soluble sugars from the total amount of cellulose consumed was 12.7 ± 1.1% (mM of soluble sugar / mM hexose equivalent of total cellulose consumed) for variable volume fed-batch fermentation and 18.1 ± 0.53% for the fixed volume fed-batch fermentation culture. This corresponds to 3.5 ± 0.3 g/L and 5.16 ± 0.5 g/L of soluble sugars in variable volume fed-batch fermentation and fixed volume fed-batch fermentation, respectively.

The xylose concentrations were mainly due to the impurities in the form of hemicellulose present in the cellulose substrate. Although *C. thermocellum* can break down hemicellulose (xylan), it cannot consume the resulting xylose or other xylose oligomers (Demain et al. 2005). Therefore, the xylose gets accumulated in the fermentation culture media.

Studies of *C. thermocellum* cultured with high cellulose concentrations have also reported high quantities of soluble sugars (Jiang et al. 2012; Wang et al. 1983). Wang et al. (1983) cultured C. *thermocellum* in cellulose concentrations between 15 g/L and 30 g/L, and reported total residual sugar concentrations as high as 8 g/L, consisting of glucose, cellobiose, xylobiose and xylose. Jiang et al. (2012) also reported soluble sugars, consisting of 4.3 mM glucose, 11.5 mM cellobiose, and 2.2 mM xylose, detected in cyclic fed-batch cultures of *C. thermocellum* LQR1.

Table 3.2 Initial and final metal ion concentrations of in the fixed volume fed-batch fermentation culture.

Time (h pi)	Metal concentration (mg/l)				
	Ca	**Mg**	**Na**	**P**	**Fe**
0	46.51	206.90	801.70	858.58	0.26
94	44.70	133.20	739.10	775.40	0.15

Table 3.3 Summary of batch fermentation, variable volume fed-batch, and fixed volume fed-batch fermentation performance.

Fermentation Feeding Strategy	**Carbon balance**	**O/R**	**Yields (mmol/mmol hexose consumed)** [a]			**Productivity (mmol/g dry cell.h)** [b]			**Production rate (mmol/L/hour)** [b]		
			Ethanol	**H_2**	**Lactate**	**Ethanol**	**H_2**	**Lactate**	**Ethanol**	**H_2**	**Lactate**
Batch	0.83	1.00	1.016	0.529	0.19	4.413	2.298	0.83	5.544	2.887	0.23
Variable Volume	0.93	1.02	0.442	0.420	0.58	0.956	0.908	1.26	1.790	1.700	0.41
Fixed Volume	0.86	1.03	0.833	0.401	0.30	2.425	1.242	0.86	4.610	2.361	0.35

[a] When calculating the yields, the total cellulose consumed was considered and not the total amount of cellulose added.

[b] These values were calculated based on the total time of operation. This is the time between the time of inoculation to the time taken before the increase in the ethanol concentration between consecutive sampling points become statistically insignificant.

3.4 Conclusions

The objective of these experiments was to study the performance of *C. thermocellum* under high cellulose concentrations under different feeding strategies. The results show that the batch fermentation culture yielded far superior results. However, data from the variable volume fed-batch and fixed volume fed-batch fermentations provided insight to the causes of the poor performance of *C. thermocellum* in these cultures. The variable volume fed-batch revealed that cellulose concentrations above 25 g/L had an adverse effect on the culture due to multiple reasons. First, the deviation of the carbon flow away from the ethanol synthesis to the lactate synthesis resulted in causing sharp increases in lactate concentration. Second, high cellulose concentrations caused physical hindrances in the bioreactor, such as the settling of the cellulose at the bottom in the form of a bed, making the cellulose inaccessible for the microorganisms and creating end-product saturation within the cellulose bed. Finally, the results of the variable volume fed-batch fermentation showed that the step-wise increase in the volume did not have a positive impact on the culture, as the smaller working volume had a negative impact on the cell growth rate. Also, it is possible that the step-wise addition of fresh medium that varied from ideal composition identified for the maximum cell productivity and end-product yields, and which also resulted in significant increases in culture volume, resulted in less than ideal growth conditions.

The fixed volume fed-batch fermentation and the batch fermentation cultures had almost identical end-product synthesis profiles, apart from the higher lactate concentration produced by higher cellulose loads in the fixed volume fed-batch fermentation. Due to this reason, the yields and productivity of the fixed volume fed-batch fermentation were lower than the batch fermentation culture. Other reasons for the plateauing of ethanol and H_2 concentrations at 100 h

pi was an effect of high concentrations of ethanol and low concentrations of essential nutrients, such as magnesium and iron.

Other studies of various *C. thermocellum* strains cultured with high cellulose concentrations have also reported the plateauing of end-products at ethanol concentrations of 4 g/L or lower, even when excess concentrations of cellulose were present (Argyros et al. 2001; Jiang et al. 2012; Saddler et al. 1982; Tailliez et al. 1989; Wang et al. 1983). In order to increase the cellulose consumption and the ethanol concentration, many of these studies used mutant strains of *C. thermocellum* (Tailliez et al. 1989; Wang et al. 1983), metabolic engineering (Argyros et al. 2011), or different feeding strategies (Jiang et al. 2012; South et al. 1993). In contrast, Holwerdra et al. (2014), found that *C. thermocellum* DSMZ 1313 consumed up to 93 g/L of cellulose (from an initial concentration of 100 g/L cellulose) and produced up to 14 g/L of ethanol, with only 5 g/L of lactate.

H.E Spinnler (1986) performed a fermentation similar to a fixed volume fermentation where he maintained the cellulose concentrations at approximately 30g/L by adding cellulose to the culture every 20 hours. In this work, the highest ethanol concentration observed was 8.5 g/L which was obtained at 90 hours and a cellulose hydrolysis rate of approximately 0.55 g/l.h was observed. In our fixed volume fed-batch fermentation, within 90 hours 5.48g/l of ethanol was produced and the cellulose hydrolysis rate was 0.31 g/l/h. The main difference between the current work and Spinnler's work is that the reactor in Spinnler's work was sealed hermitically. Therefore, the dissolved hydrogen and carbon dioxide would have reached super-saturation levels which lead the electron flow away from the hydrogen producing pathways and more towards ethanol producing pathways.

In conclusion, the results from the two fed-batch fermentation strategies show the following improvements for the development of the next feeding strategy:

1) Cellulose concentration must be maintained lower than 25 g/L (Based on the results obtained in variable volume fed-batch fermentation).

2) Inhibitory end-products must be removed from the system before the culture reaches stationary phase.

3) Nutrients must be replenished periodically to maintain nutrient at the optimum concentrations (Table 3.2).

In order to satisfy all these requirements, a semi-continuous system could be designed where the benefits of fed-batch fermentation and continuous fermentation are combined. In a semi-continuous system, the reactor is fed intermittently like in the fixed volume fed-batch fermentation culture, and once the end-products reach inhibitory levels, the exhausted media is removed and replaced by fresh media. Chapter 4 will discuss the details of the semi-continuous system.

Chapter 4

Comparison of semi-continuous fermentation with fed-batch and batch fermentation

4.1 Introduction

A semi-continuous fermentation is defined as a system where a portion of the fermented media is withdrawn intermittently and fresh media is added to the system. The portion of the media left in the reactor becomes the inoculum for the second cycle (Seishu, 2013). With this technique, an ideal semi-continuous culture can be maintained indefinitely (Roehr, 2001). Semi-continuous cultures are commonly used in waste water digesters where the floating and settling characteristics of the waste make it difficult to continuously withdraw from the digester (Nijaguna, 2002). This is a common problem also faced in continuous fermentation of high concentrations of cellulose. The settling characteristics of the cellulose particles cause operational difficulties in pumping and mixing, making the overall process inefficient. A number of studies conducted with continuous fermentation of cellulosic biomass have reported operational difficulties at concentrations as low was 8 g/L (Lynd et al. 1989; Magnusson et al. 2009). Therefore, a semi-continuous culture is an ideal system that gives the benefits of longer operation times of a continuous fermentation with the compatibility of handling higher cellulose concentrations. The longer operational time eliminates non-productive idle time for cleaning and re-sterilization, which usually makes batch operations economically less desirable.

4.2 Materials and methods

In this work, two types of semi-continuous fermentations were carried out. In the first type, the cellulose concentration was maintained between 10 g/L – 15 g/L while in the second

the cellulose concentration was maintained between 20 g/L – 25 g/L. Throughout this chapter, the first semi-continuous fermentation will be referred to as the "semi-continuous fermentation with low cellulose concentrations", while the latter will be referred to as the "semi-continuous fermentation with high cellulose concentrations". The medium composition, reactor set-up and inoculation, and end-product analysis in both fermentations are as described in Chapter 2, Sections 2.1, 2.2, and 2.3 respectively.

The feeding strategies for both runs were developed based on the gas end- product (H_2 and CO_2) profiles. The gas end-products were selected for this because of the ease of measuring them in real time. Also, previous studies conducted with *C. thermocellum* have shown that gas end-product profiles closely correlate with the cell growth profiles (Islam et al. 2006). The reactor was started with a 4 L working volume and a cellulose concentration of 15 g/L in both low and high concentration semi-continuous runs. In order to keep the cellulose concentration at the desired level, cellulose was fed intermittently, similar to the technique used in fixed volume fed-batch fermentation described in Chapter 3, Section 3.2.2. For the purpose of preventing the nutrient depletion observed in fixed volume fermentations, 250 mL of concentrated media was added periodically to the system, along with the cellulose. The degree of concentration was in correlation to the amount of cellulose added to the system. For example, with 30 g of cellulose, 8 times concentrated media was added, while for 60 g of cellulose 16 times concentrated media was added. The rationale for this was to ensure that the nutrient to cellulose ratio of the feed streams was similar to the nutrient to cellulose ratio of the initial media composition.

Once the cumulative concentration of gaseous end-products no longer increased (i.e. plateaued), the mixing of the medium in the reactor was stopped for one hour to allow the medium to settle. After 1 hour, the clear liquid above the settled cellulose was removed through

the sampling port using a peristaltic pump (Masterflex, L/S Series peristaltic pumps). The tubing used was a Materflex L/S 25 silicon tube. Once 2 L of the fermented broth was removed, the peristaltic pump was stopped and 2 L of fresh media was added. The fresh media was previously prepared in 1 L bottles (Belco Co.), and gassed and degassed prior to autoclaving. Filter sterilized vitamin and urea solutions were added to the reactor immediately after media addition. After the addition of fresh media, the flow rate of nitrogen gas (N_2) was increased to 40 mL/min for 30 minutes to remove any oxygen that may have entered the system during the feeding. Table 4.1 and Table 4.2 summarise the feeding strategy employed in the semi-continuous fermentations with low and high cellulose concentrations, respectively.

Table 4.1 Feeding strategy employed in semi-continuous fermentations with low cellulose concentrations.

Time (h pi)*	Feeding
0	Reactor initiated with 4L working volume and cellulose concentration of 15 g/L
38	30 g of cellulose in total and 250 ml of 8 times concentrated media was added
72	2 L of the clear medium post settling was removed and replenished with 2L of fresh medium
86	30 g of cellulose was fed
140	30 g of cellulose and 250 ml of 8 times concentrated medium was added

*h pi, hours post-infection

Table 4.2 Feeding strategy employed in semi-continuous fermentation with high cellulose concentrations.

Time (h pi)*	Feeding
0	Reactor initiated with 4L working volume and cellulose concentration of 15g/L
35	60 g of cellulose in total and 250 ml of 16 times concentrated media was added
114	60 grams of cellulose was added
136	2L of clear medium post settling was removed and replenished with fresh media
204	60 g of cellulose and 250 ml of 16 times concentrated media was added

*h pi, hours post-infection

4.3 Results and Discussion

4.3.1 Development of a semi-continuous feeding strategy

The semi-continuous feeding strategy was an extension of fixed volume fed-batch fermentation developed with the following objectives: 1) Avoid high cellulose concentrations, which have been shown to have a detrimental effect on the ethanol and H_2 production in fixed-volume and variable-volume fed-batch fermentations due to the carbon flow directed towards lactate production; 2) Avoid end-product inhibition from the accumulated ethanol. In the batch fermentation and fed-batch fermentation, the inhibition initiated when the ethanol concentration reached approximately 100 mM; 3) Avoid the reduction of essential nutrients such as magnesium and iron; and 4) Achieve longer operation times to avoid down-time observed in batch fermentations (Roehr, 2001).

The problem of end-product inhibition was diminished through the removal of 50% of the spent media and its replacement with fresh media. Also, additional fresh nutrients were added periodically to avoid any potential nutrient depletion. In order to avoid high lactate production induced by high cellulose concentrations, the reactor was initially started with only 15 g/L.

In both liquid and gas product comparison graphs, the total molar amounts (mmol) of end-products produced were taken into consideration instead of the volumetric concentrations of each end-product (mmol/L = mM). The dilution of the culture at decanting caused the concentration curves to dip, and comparisons of the fermentation end-products produced by batch reactions versus semi-continuous reactions could not be easily visualized through curves showing the volumetric concentrations.

4.3.2 Semi-continuous fermentations with low cellulose concentrations (10 g/L - 15 g/L).

The semi-continuous fermentation reactions showed promising results, with a steady increase in H_2 production throughout the 200 hour operation time (Figure 4.1). It is important to note that during the first 100 hours post inoculation (h pi), the rate of production was slightly higher than that of batch fermentation, despite the lower cellulose concentration used in the media.

At 70 h pi, both H_2 and CO_2 production started to plateau, owing to the build-up of ethanol in the media. After decanting and replenishing the media at 70 h pi the H_2 production increased. Nevertheless, the rate of production did not significantly increase until about 20 hours after the media addition. This lag-time in the production rate may be due to cell lysis caused by the high ethanol concentrations before decanting. Ethanol concentration at the time of decanting was 4.6g/L which is sufficient for almost 15% of the cells to lyse (Kundu et al. 1983; Wang et al. 1983). A similar trend was observed in batch fermentation where the pellet protein concentration started to decrease after 60 hours when the ethanol concentration reached 4.7g/L (Figure 4.6). The protein data of the semi-continuous fermentation confirms that cell lysis had taken place in the culture after 56 h pi. The percentage of the pellet protein concentration to supernatant protein concentration increased from 11.8% to 32.1% in the time between 56 h pi to 70 h pi, indicating a significant amount of cell lysis. In the calculation of supernatant protein, the initial protein concentration from the added yeast extract was subtracted to ensure that only *C. thermocellum* protein was calculated. This cell lysis could be avoided by decanting the media at an earlier time before the product rates begin to decrease.

The CO_2 production rate did not continue to increase after the decanting. Nevertheless, the production of CO_2 continued until the end of the reactor run (Figure 4.2). The rate of

production of ethanol was slightly lower in semi-continuous fermentation when compared to batch fermentation in the first 100 h pi (Figure 4.3). Also, unlike the H_2 production profile, the ethanol production rate decreased significantly after the decanting and replenishing the culture media. Therefore, it can be concluded that the semi-continuous fermentation system favoured H_2 production. Previous studies conducted with different concentrations of cellulose also showed a shift to higher H_2 and lower ethanol production in fermentation reactions with higher cellulose concentrations (Islam et al 2009). The reason behind this is that in high concentrations there is poorer mass transfer within the solid bed of settled cellulose causing localized super-saturation of dissolved hydrogen (Blunt et al. 2014). This in turn will hinder the reoxidation of NADH through the reaction, $NADH + H^+ \rightarrow NAD^+ + H_2$. As a result, a metabolic shift occurs from the acetate synthesis pathway to reoxidation of NADH through acetyl CoA reduction to ethanol (Lamed et al. 1980). Therefore, the lower concentrations of cellulose in the media in semi-continuous fermentation, when compared to batch fermentation, could be the reason behind the higher rates of H_2 and lower rates of ethanol production.

Acetate production rate was also slightly lower in the semi-continuous fermentation reactions compared to batch fermentations. Similar to other liquid end-products, acetate production rate did not increase after the media was decanted and replaced (Figure 4.3). Lactate production, however, was much lower in the semi-continuous fermentations compared with both the fixed-volume and the variable-volume batch fermentations (Figure 4.4). In contrast, high formate concentrations were observed in the semi-continuous fermentations after lactate production plateaued at 95 h pi. Other studies of *C.thermocellum* also reported the synthesis of high concentrations of formate during stationary phase. For example, Sparling et al. (2006) reported that of the 123.4 mmoles of end-products produced, formate accounted for 22.9%.

Figure 4.1 Total hydrogen (H_2) produced by *C. thermocellum* in semi-continuous fermentations with low cellulose concentrations versus batch fermentation reactions. The time point of decanting is indicated by the arrow (70 h).

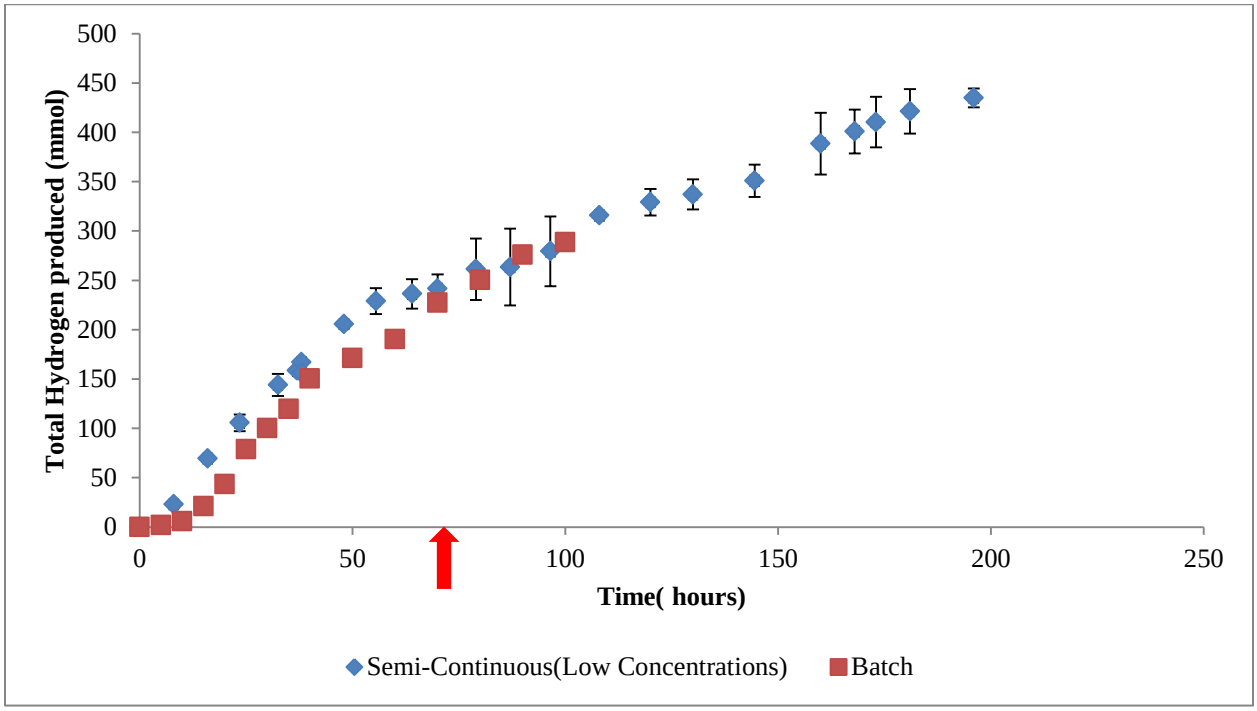


Figure 4.2 Total Carbon dioxide (CO_2) produced by *C. thermocellum* in semi-continuous fermentations with low cellulose concentrations versus batch fermentation reactions. The time point of decanting is indicated by the arrow (70 h).

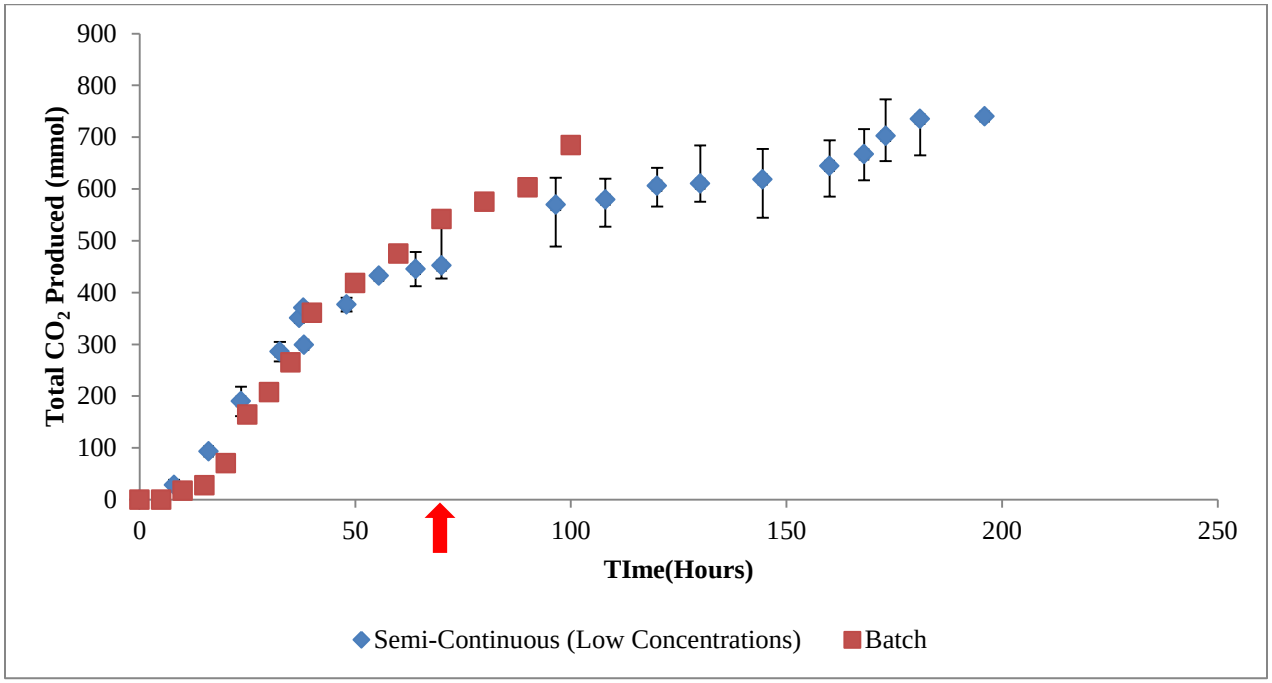


Figure 4.3 Total ethanol and acetate produced by *C. thermocellum* in the semi-continuous fermentations with low cellulose concentrations versus batch fermentation reactions. The time point of decanting is indicated by the arrow (70 h).

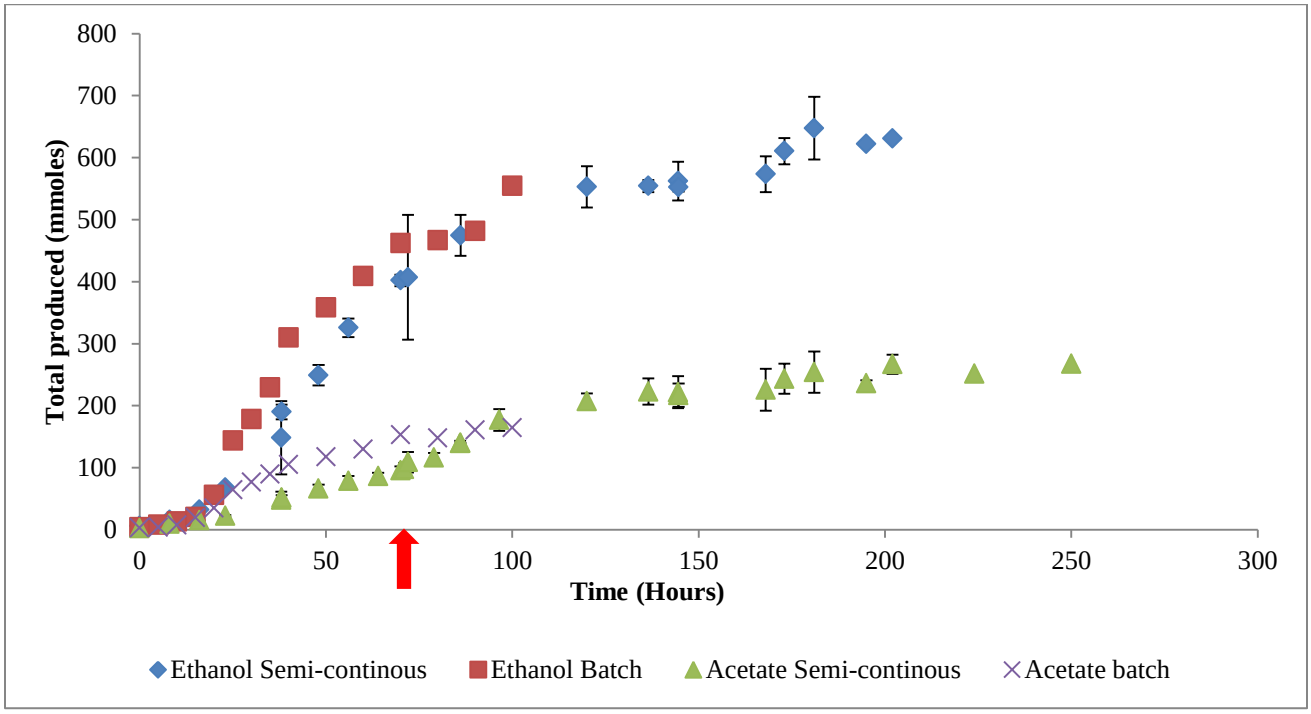
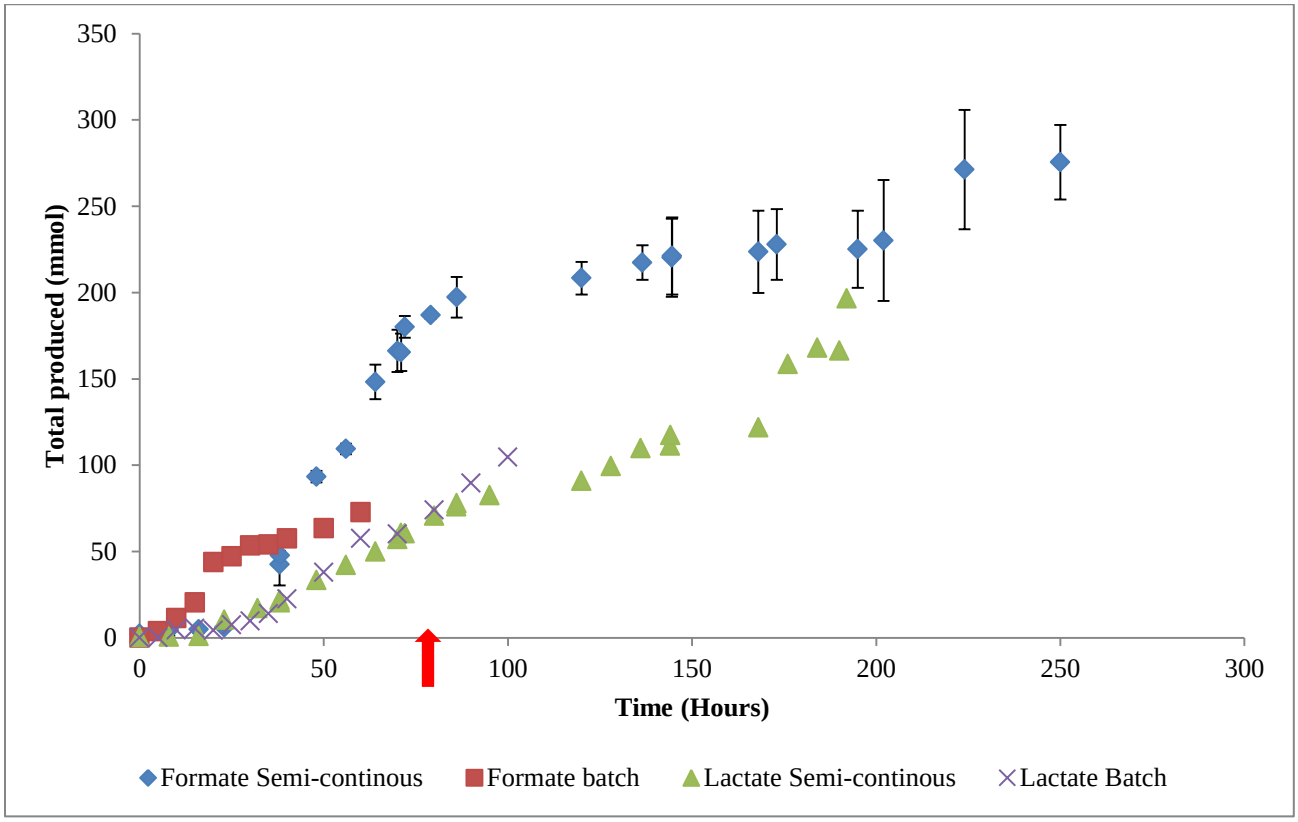


Figure 4.4 Total lactate and formate produced by *C. thermocellum* in the semi-continuous fermentations with low cellulose concentrations versus batch fermentation reactions. The time point of decanting is indicated by the arrow (70 h).



In comparison, formate accounted for only 6.9 ± 1.2% of the total end-products synthesized by *C. thermocellum* in the semi-continuous fermentation. Lower formate production is a desirable outcome, as formate production competes with the H_2 synthesis pathways.

The protein data showed that *C. thermocellum* was in exponential phase until 24 h pi in the semi-continuous fermentation reactions with low cellulose concentrations, as opposed to the batch fermentation reactions where the exponential phase was observed for only 20 h (Figure 4.5). Higher protein concentrations (700 mg/L) were observed at the end of the stationary phase in the semi-continuous fermentation with low cellulose concentrations when compared to the stationary phase protein concentration in batch fermentation. The reason for this could be the addition of extra nutrients in the semi-continuous fermentation after the initial exponential phase that allowed *C. thermocellum* to achieve higher cell densities, and thus a higher protein concentration.

The efficiency of any semi-continuous fermentation will be highly compromised if viable cells are removed along with the removal of the spent media. During the sampling, once the media removed was spun down, the resulting pellet contains cells that were attached to the small amount of cellulose that was not settled during the settling period, as well as unattached cells in the media. The protein in the supernatant represents lysed cell protein and removal of this protein will not have negative effects on the culture. Therefore, high pellet protein concentrations from the removed media indicate that decanting the media removed viable cells, making the process less efficient. In the semi-continuous fermentation, 10.2% of total protein in the culture prior to settling was recovered in the pellet derived from centrifugation of the decanted supernatant. This is not an extremely high amount, but nevertheless may be considered significant.

The residual cellulose concentration showed that the concentration of cellulose did not exceed 15 g/L at any time. Also, consistent with the end-product profiles, the cellulose hydrolysis rates decreased over the fermentation reaction time (Figure 4.6 and Figure 4.7). The overall volumetric rate of consumption of cellulose between the three cellulose additions are, 0.21g/L.h (0 -38 h), 0.21 g/L.h (38h-86h), 0.14 g/L.h (86h-142h) and 0.14 g/L.h (142h-188h).

Figure 4.5 Protein concentrations extracted from *C. thermocellum* in the semi-continuous fermentations with low cellulose concentrations versus the batch fermentation reactions. The time point of decanting is indicated by the arrow (70 h).

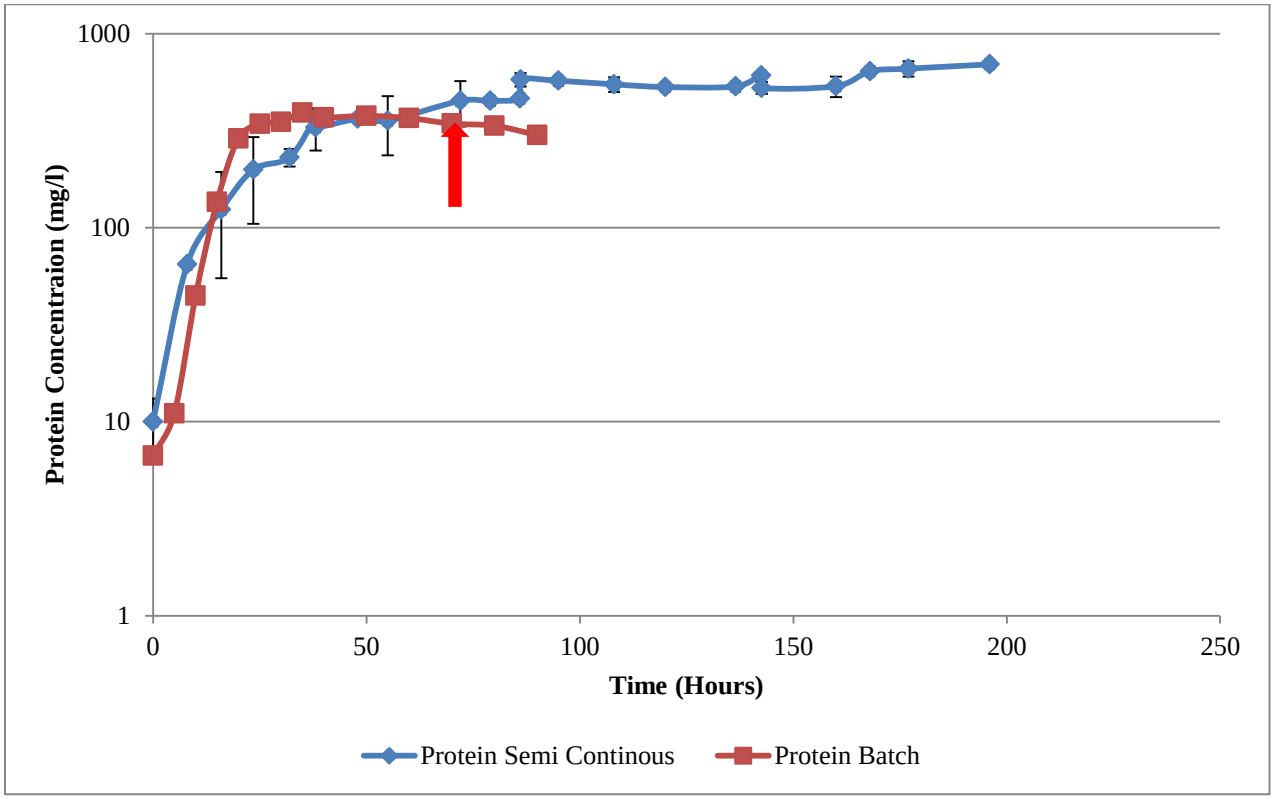


Figure 4.6 Residual cellulose concentrations in the *C. thermocellum* semi-continuous fermentations with low cellulose concentrations.

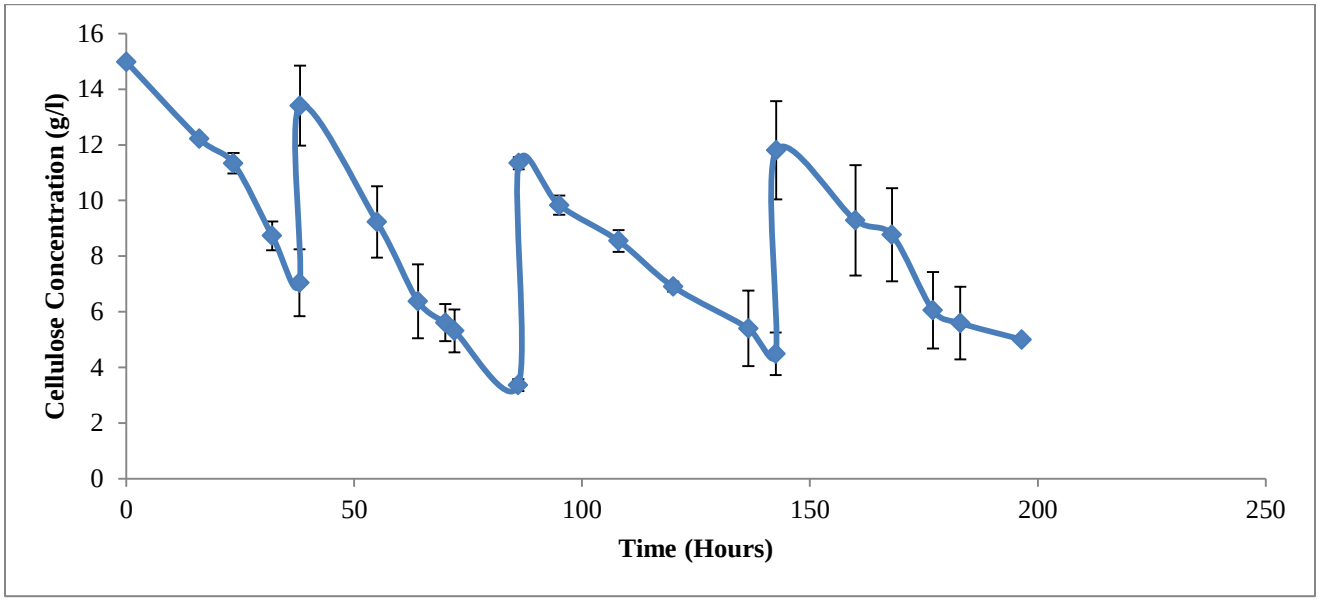
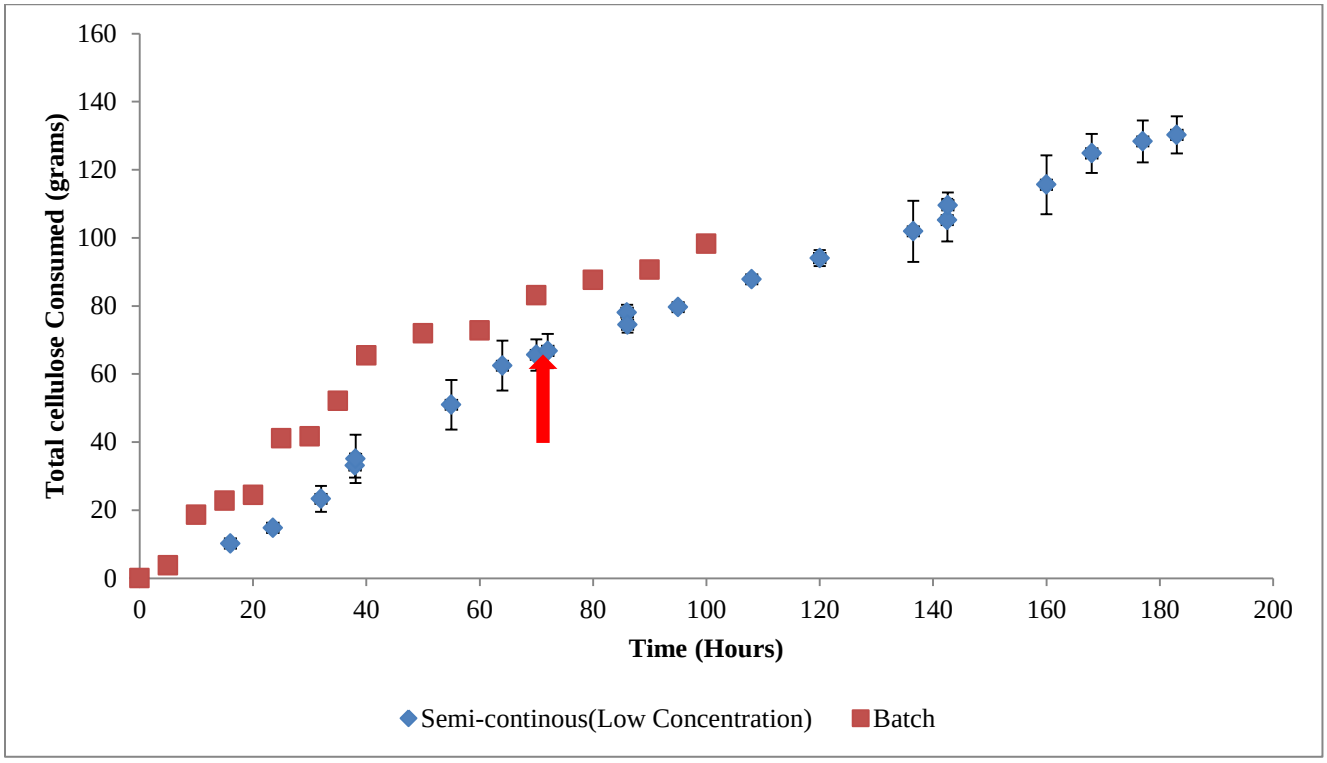


Figure 4.7 Cellulose consumption by *C. thermocellum* in the semi-continuous fermentations with low cellulose concentrations versus the batch fermentation reactions. The time point of decanting is indicated by the arrow (70 h).



4.3.3 Semi-Continuous fermentation with high cellulose concentrations (20 g/L - 25g/L)

The steadily increasing end-product production profiles in the semi-continuous fermentations with cellulose concentrations between 10 g/L and 15 g/L led to the next set of experiments using higher cellulose loadings. In order to keep the batch portion of the fermentation reactions similar in both trials, the high cellulose semi-continuous reactions were started with 15 g/L. In each addition after that, 60 g of cellulose was added as opposed to the 30 g added in the low concentration fermentation reactions. The gaseous end-products were not observed to plateau until 135 h pi, which is almost twice as long as previously observed for the semi-continuous fermentations with low cellulose concentrations.

The H_2 production rate in the semi-continuous fermentations with high cellulose concentrations were slightly lower than that of the batch fermentation in the first 100 h pi, while the CO_2 production showed a similar profile (Figures 4.8 and 4.9). Nevertheless, after the decanting, the H_2 production rate did not increase to its previous rate until approximately 200 h pi. The reason for this is not clear, as other end-products did not show a similar trend. The standard deviations for the gaseous end-products are not available after 60 h pi, due to a failure in gas chromatography during the second trial.

Due to the lower production rate of ethanol, the culture did not reach inhibitory levels of ethanol until 136 hours at which the ethanol concentration was 120 mmol/L. The ethanol production rate increased soon after decanting of the media and stabilized again at approximately 200 hours, when the culture once again reached inhibitory levels of ethanol (Figure 4.10).

When compared to the semi-continuous fermentations with low cellulose concentrations, the high cellulose concentration reactions appear to have undergone a shift in production of lactate and formate (Figure 4.11). Higher formate (275 mmol) and lower lactate (196 mmol)

production were observed in the low cellulose concentrations fermentations. In contrast, higher lactate (547 mmol) and a lower formate (270 mmol) production were observed in the high cellulose concentrations fermentation reactions. High lactate production was a common feature observed in all fermentation reactions carried-out with a cellulose concentration above 20 g/L.

The duration of the *C. thermocellum* exponential growth phase in the semi-continuous fermentation with high cellulose concentrations was approximately 20 h, which was similar to the duration of the exponential phase observed in the low concentration reactions. However, *C. thermocellum* achieved a higher cell density in the high cellulose concentration fermentation reactions, as the protein concentration was approximately 1000 mg/L, compared with protein concentrations achieved from the batch and the low concentration reactions, which achieved only 400 mg/L and 700 mg/L respectively (Figure 4.12). Again this can be attributed to the high concentrations of nutrients that were supplied to the reactor after the exponential phase.

The amount of protein lost during the decanting was 7.8 ± 1.4%, which was lower than the amount of protein lost during decanting of the media from the low cellulose concentration reactions. This could be due to the high cellulose concentration in the media that facilitated better settling during the one hour settling time.

One of the interesting observations in both the low and high cellulose concentration semi-continuous fermentations was that despite the higher cell concentrations compared to the batch fermentations, the cellulose consumption rates were lower in the first 100 h pi, and this rate dropped further towards the end of the fermentation reactions. In fact, the mean cellulose consumption rates for batch, low cellulose concentration semi-continuous and high cellulose concentration semi-continuous were 1.31 g/hours, 0.794 g/hours, and 0.80 g/hours, respectively. Therefore, the rate limiting step that caused lower end-product formation for the semi-continuous

fermentation reactions was at the hydrolysis of cellulose to soluble sugars. A possible reason for this may be that the *C. thermocellum* cells showed less inclination to attach to newly added cellulose due to the presence of other end-products in the media.

Xu et al. (2010) demonstrated the influence of organic acids on the activity of the cellulosome. They purified unbound cellulosomes from the supernatants of *C. thermocellum* JYT01 cultures. *C. thermocellum* JYT01 is a derivative of *C. thermocellum* LQRI, which was evolved to tolerate high ethanol concentrations (up to 30 g/L). Xu et al (2010) showed that small concentrations of acetate, formate, and lactate in the media promoted the activity of *C. thermocellum* JYT01 cellulosomes up to a critical concentration, beyond which the organic acids had a negative impact on the cellulosome activity. The minimum inhibitory levels of formate, acetate, and lactate for this modified strain were found to be 1000 mM, concentrations that were much higher than the observed concentrations in our work. However, *C. thermocellum* JYT01 was evolved to tolerate high concentrations of ethanol, and since ethanol and the organic acids are synthesized concomitantly, the *C. thermocellum* JYT01 cellulosomes were putatively also more resistant to organic acids. The inhibitory concentrations of organic acids on the celluloses hydrolysis of the wild type *C. thermocellum* 24750 cellulosome is not available in literature. Assuming that the inhibitory concentrations of organic acids are lower for *C. thermocellum* 24750, this could explain the lower cellulose hydrolysis rate found in all fed-batch fermentation reactions.

Figure 4.8 Total hydrogen (H_2) produced by *C. thermocellum* in the semi-continuous fermentations with high cellulose concentrations versus the batch fermentation reactions. The time point of decanting is indicated by the arrow (136h).

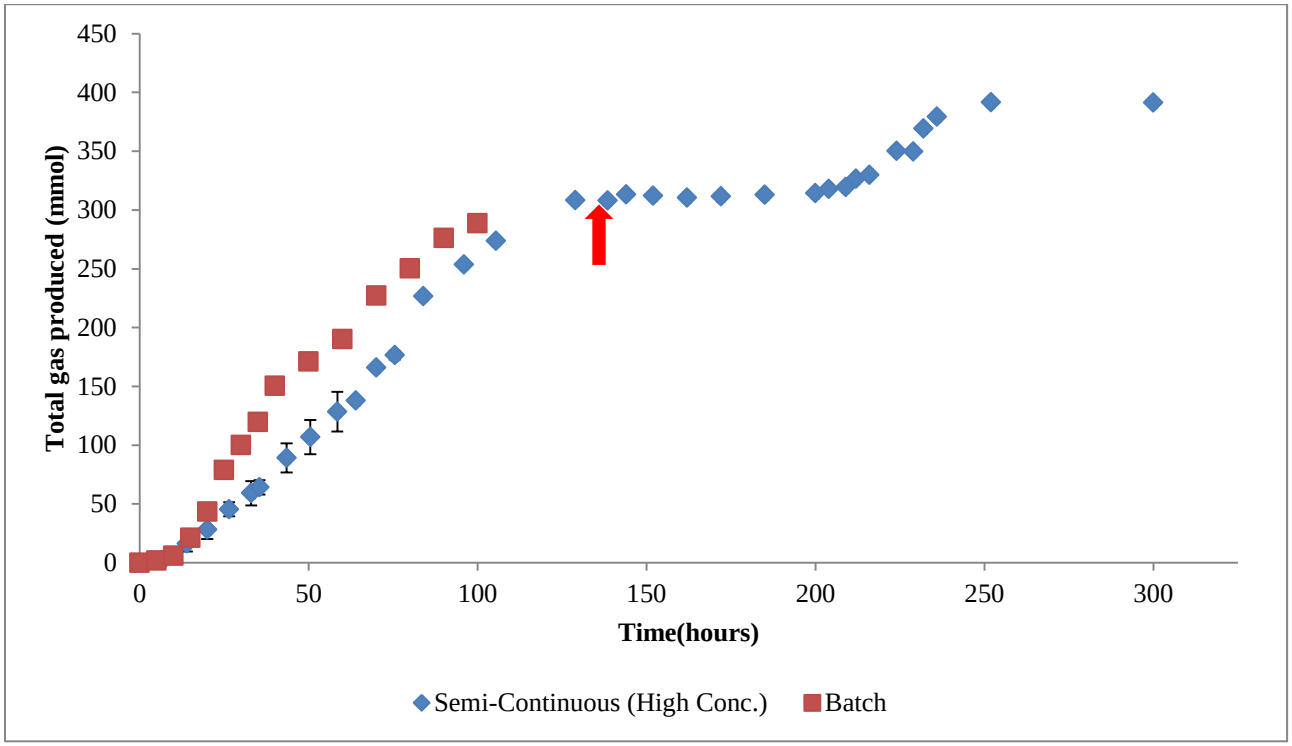


Figure 4.9 Total Carbon dioxide (CO_2) produced by *C. thermocellum* in the semi-continuous fermentations with high cellulose concentrations versus the batch fermentation reactions**.** The time point of decanting is indicated by the arrow (136h).

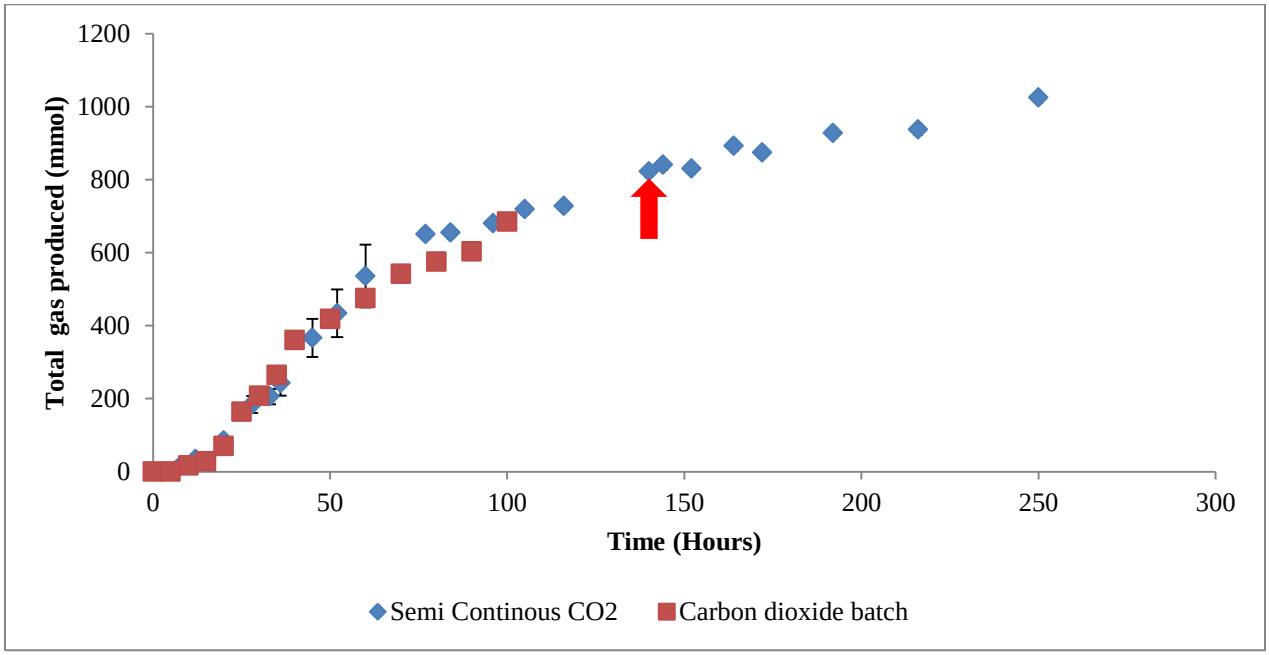


Figure 4.10 Total ethanol and acetate produced by *C. thermocellum* in the semi-continuous fermentations with high cellulose concentrations versus the batch fermentation reactions. The time point of decanting is indicated by the arrow (136h).

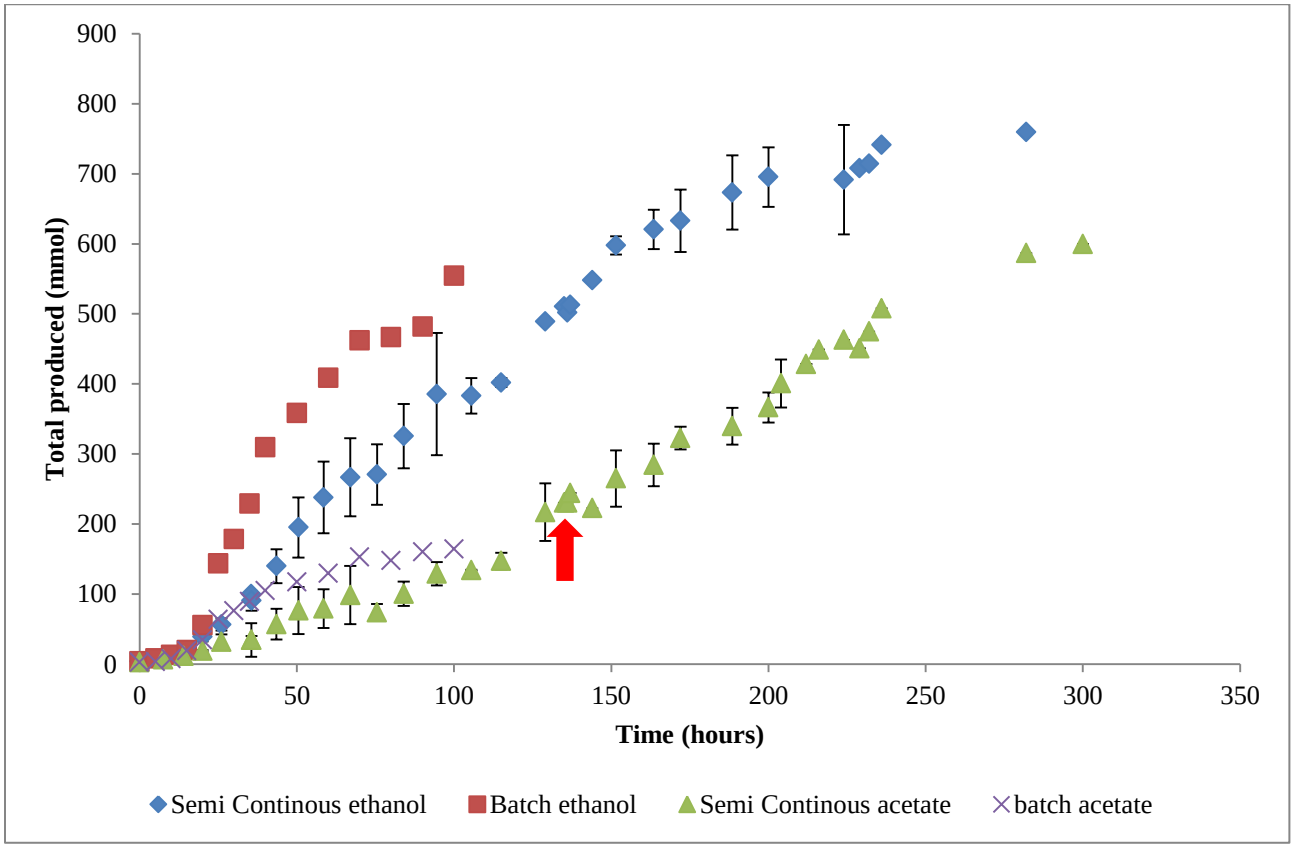
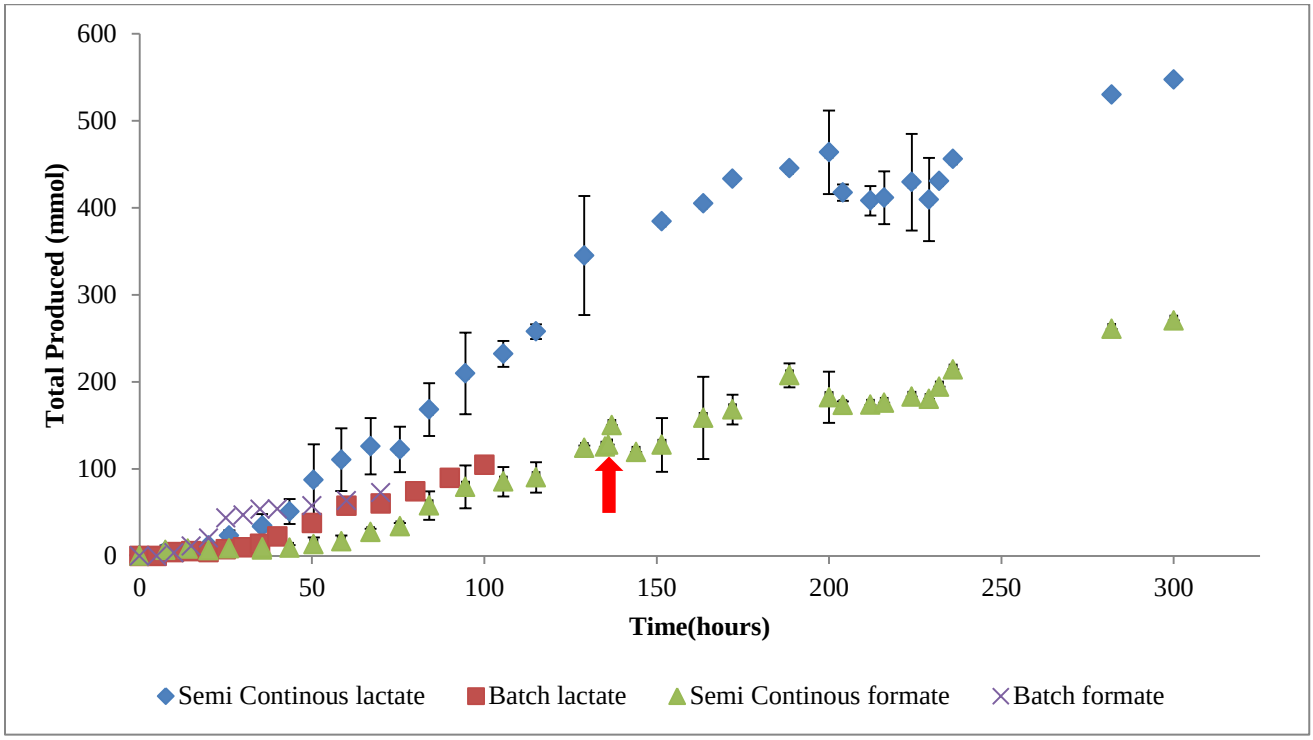


Figure 4.11 Total lactate and formate produced by *C. thermocellum* in the semi-continuous fermentations with high cellulose concentrations versus the batch fermentation reactions. The time point of decanting is indicated by the arrow (136h).



4.3.4 Nutrient uptake

The fixed-volume fed-batch fermentation reaction showed a reduction depletion of essential nutrients such as Mg^{++} and Fe^{++} towards to end of the fermentations. In order to avoid this, fresh nutrients were provided periodically in the semi-continuous fermentation reactions. As observed in the fixed-volume fermentation reactions, Mg^{++} and Fe^{++} were the nutrients with the highest up-take rates in both semi-continuous fermentation reactions (Table 4.3). Prior to the decanting, both semi-continuous reactions consumed almost 50% of the Mg^{++} added. Nevertheless, all nutrients were present in excess at the end of both semi-continuous fermentation reactions, indicating that the slow growth rates associated with the second cycle were not due the depletion of any essential nutrients.

Figure 4.12 Protein concentrations in the *C. thermocellum* semi-continuous fermentation with high cellulose concentrations versus batch fermentation reactions. The time point of decanting is indicated by the arrow (136h).

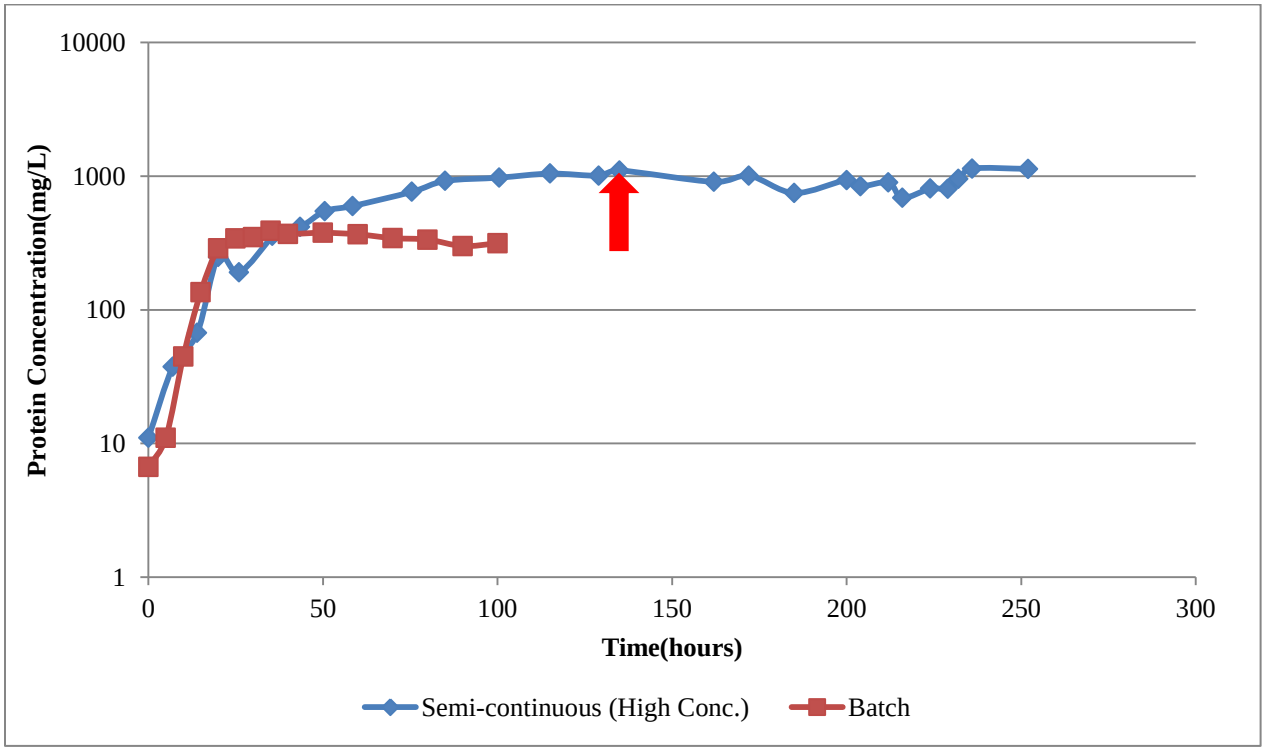


Figure 4.13 Cellulose consumption by *C. thermocellum* in the semi-continuous fermentations with high cellulose concentrations versus batch fermentation reactions.

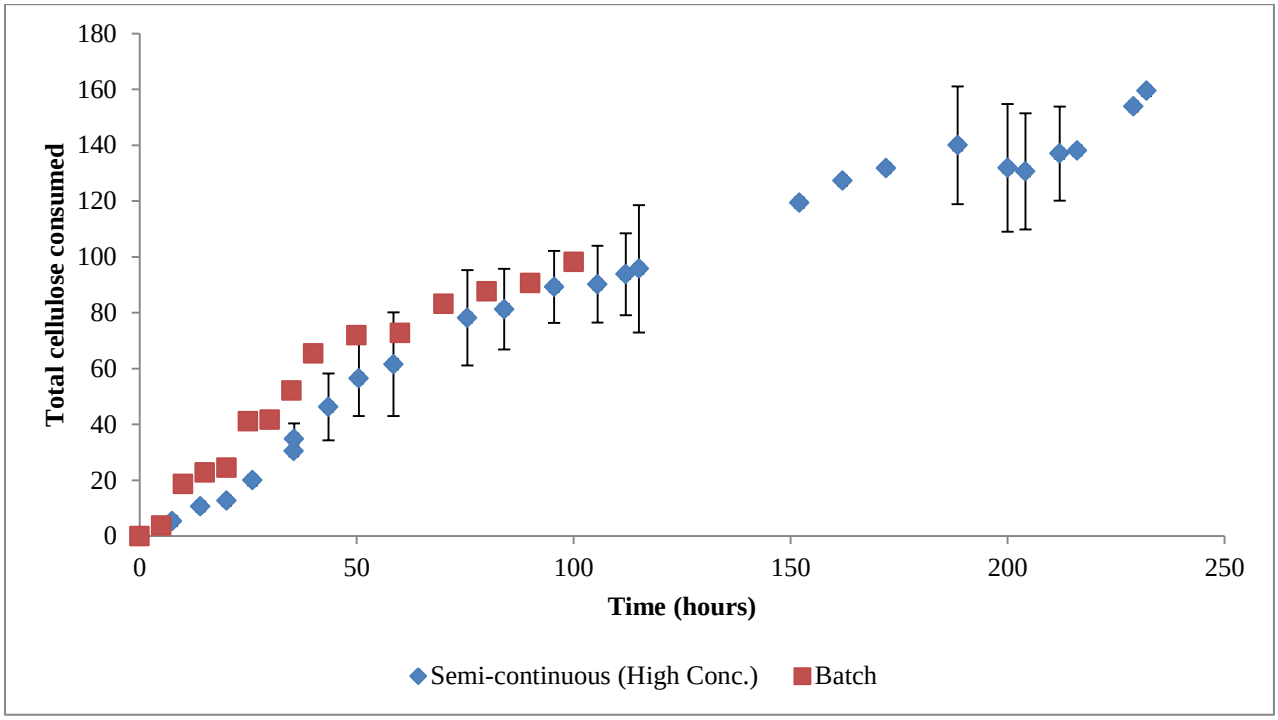


Table 4.3 The nutrient concentrations at the time of inoculation, time of decanting, and final sampling point in semi-continuous fermentation reactions.

		Semi-Continuous with low concentrations (mg/L)		**Semi-Continuous with high concentrations (mg/L)**	
Metal ion	**Initial Concentration (mg/L)**	**Prior to decanting (70 h pi)**	**Final concentration (250 h pi)**	**Prior to decanting (135 h pi)**	**Final concentration (250 h pi)**
Ca^{++}	46.5	45.3	83.1	59.6	56.1
Mg^{++}	206.9	100.5	255.8	101.2	263.0
Na^{+}	801.7	535.3	1104.0	722.5	1068.3
PO_4^{-}	858.6	636.1	1118.1	704.1	944.2
Fe^{++}	0.26	0.19	0.50	0.53	0.22

4.4 Conclusions

Carbon balance analyses of the semi-continuous fermentation reactions indicated an approximately 10-20% variation from the ideal values. This is consistent with other studies of *C. thermocellum* cultured with high cellulose concentrations (Holwerda et al. 2014; Jiang et al. 2013). Yet, the calculated O/R ratios from the semi-continuous the high cellulose concentration fermentations were near one, which confirms the accuracy with which the fermentation end-products were, measured (Table 4.4).

For a fermentation process to be economically feasible, it is vital to have a high production rate and a high substrate specific yield of the desired end-product. Figure 4.14 demonstrates the change of production rate and the change of cellulose specific yields with time for the production of ethanol in the two semi-continuous fermentations. Both fermentations show a similar trend where there was a steady growth in the production rate and substrate specific yield prior to decanting followed by a steep decrease after decanting (The decanting occurred at 72 h pi for semi-continuous fermentation with low cellulose concentrations and 136 h pi for semi-continuous fermentation with high cellulose concentrations) . Due to its bi-phasic production profiles, for all performance comparison purposes, the semi-continuous fermentations were divided as cycle 1, corresponding to the fermentation before the decanting and cycle 2 corresponding to the fermentation after the decanting (Table 4.4).

The cycle 1 of semi-continuous fermentation with low cellulose concentrations has demonstrated the most superior performance in terms of production rates and cellulose specific yields when it comes to both ethanol and hydrogen production. Nevertheless, the decrease in performance in the cycle 2 brings the overall performance quite similar to batch fermentation but still superior to the performance of fixed volume fermentation and variable volume fermentation.

The semi-continuous fermentation with high cellulose concentrations has shown a similar trend between cycle 1 and 2. Although the production rates are lower in the high cellulose run compared to batch and low concentration fermentation, the yields were quite similar in cycle 1.The reason for the drop in production rates and yields of ethanol and hydrogen in the second cycle could be attributed to the diversion of carbon and electrons towards lactate and formate synthesis, and away from the ethanol and H_2 synthesis pathways.

4.4.1 Metabolic shift towards lactate and formate production

A common feature seen in all feeding strategies is that once the culture reaches stationary phase, there is a shift towards lactate and formate production. While the fermentation continues at a significant rate, this shift results in a lower production of our desired end-products of ethanol and hydrogen. This is consistent with other studies done with *C.thermocellum* under excess carbon loading where the carbon and electron flow directs towards lactate and formate production pathways during the stationary phase (Islam et al. 2006, 2009; Levin et al. 2006). Most of these previous studies were carried out in batch cultures without pH control and the synthesis of lactate initiated when the media pH dropped below the optimum level of 7. Despite the pH being maintained at the optimum level throughout the reactor runs in this study, a significant amount of carbon diverted towards lactate production resulting lower productivity and yield of the desired end-products. The rate of lactate production differs among the feeding strategies with the highest rate of production in the variable volume fed-batch and the semi-continuous fermentation with high cellulose concentrations (Table 4.5). One of the common features of both these feeding strategies is having cellulose concentrations between 25g/L-30g/L during stationary phase. Therefore, it can be concluded that with higher cellulose loadings, the

carbon flux will shift towards lactate production regardless of maintaining the pH at the optimum value.

4.4.2 Process economics

Due to the shift towards lactate and formate production observed over time in both semi-continuous fermentations, it will not be economically feasible to operate the fermentation for a longer time or a higher number of cycles (Figure 4.14). This poses the question at which time the fermentation should be stopped and a new fermentation should begin to gain the highest economic benefit. The economic feasibility would depend on many factors but mainly on the hours of operation and the requirement substrate and nutrients (Wingren et al. 2003). Therefore, in order to get a simpler comparison between the fermentations, a calculation was performed to find the required number of days and required amount of cellulose to achieve a given quantity of ethanol and hydrogen. For this purpose, the arbitrary value of 5000 mmol of ethanol and 5000 mmol of hydrogen was selected. Throughout this calculation it was assumed that the down time between 2 batches is 24 hours (Table 4.6).

Table 4.6 shows that considering the required operation time and cellulose requirement , the cycle 1 in the semi-continuous fermentation with low cellulose concentration has shown a much favourable outcome for both hydrogen and ethanol production. Overall, the semi-continuous with low cellulose concentration run has shown the most promising results as a fed-batch fermentation technique. As seen in Table 4.5 these two feeding strategies led to the lowest production of lactate and hence most of the carbon and electron flow directed towards the production of the desired end-products. For a more informed comparison between the fermentation techniques, case-specific data are required.

Table 4.4 Summary of the performance of batch, fed-batch, and semi-continuous fermentation reactions.

	Total time (h)[a]	**Carbon balance**	**O/R**	**Total Produced (mmol)**		**Yields (mmol/mmol hexose)**		**Productivity (mmol/g dry cell.h)**		**Production rate (mmol/hour)[b]**	
				Ethanol	**H_2**	**Ethanol**	**H_2**	**Ethanol**	**H_2**	**Ethanol**	**H_2**
Batch	96	0.83	1.00	554.43	288.71	1.02	0.53	4.41	2.30	5.54	2.89
Variable volume	171	0.93	1.03	336.43	364.1	0.44	0.42	0.96	0.91	1.79	1.70
Fixed volume	96	0.86	1.38	477.16	231.59	0.83	0.40	2.42	1.24	4.61	2.36
Semi-Continuous Low concentrations											
Cycle 1	70	0.95	0.96	411.45	227.01	1.13	0.62	2.04	1.13	5.88	3.24
Cycle 1 & 2	181	0.80	0.95	698.03	443.94	0.94	0.60	1.67	1.06	3.86	2.45
Semi-Continuous high concentrations											
Cycle 1	136	0.94	1.22	406.98	311.98	0.93	0.57	1.00	0.61	3.75	2.29
Cycle 1 & 2	236	0.96	0.96	741.19	387.12	0.84	0.44	1.27	0.66	3.14	1.64

[a] The total time of operation. This is the time between the time of inoculation to the time taken before the increase in the ethanol concentration between consecutive sampling points become statistically insignificant. This is valid for all end points shown on Table 4.4 except for the cycle 1 end point of the semi-continuous fermentations where the time of decanting was considered;

[b] The overall production rate within the time of operation.

Table 4.5 Lactate production in different feeding strategies during exponential phase and stationary phase

Fermentation strategy	Growth phase durations (hours)		Total Lactate Produced(mmol)		Lactate Production rate(mmol/hours)		Lactate yield (mmol/mmol hexose consumed)	
	Exponential phase	Stationary phase	Exponential phase	Stationary phase	Exponential phase	Stationary phase	Exponential phase	Stationary phase
Batch	0-25	25-96	7.28	82.31	0.29	1.16	0.03	0.19
Variable Volume	0-15	15-171	7.07	343.83	0.47	2.20	0.04	0.58
Fixed Volume	0-15	15-96	8.49	127.42	0.57	1.57	0.07	0.30
Semi-Continuous (low cellulose)	0-24	24-186	8.56	86.19	0.36	0.53	0.02	0.22
Semi-Continuous (high cellulose)	0-20	20-231	12.20	418.51	0.61	1.98	0.15	0.55

Figure 4.14 The Ethanol production rate (mmol/hours) and ethanol yield (mmol/mmol hexose) fluctuation with time in semi-continuous fermentation with low cellulose concentrations (SC-low) and semi-continuous fermentation with high cellulose concentrations (SC-high).

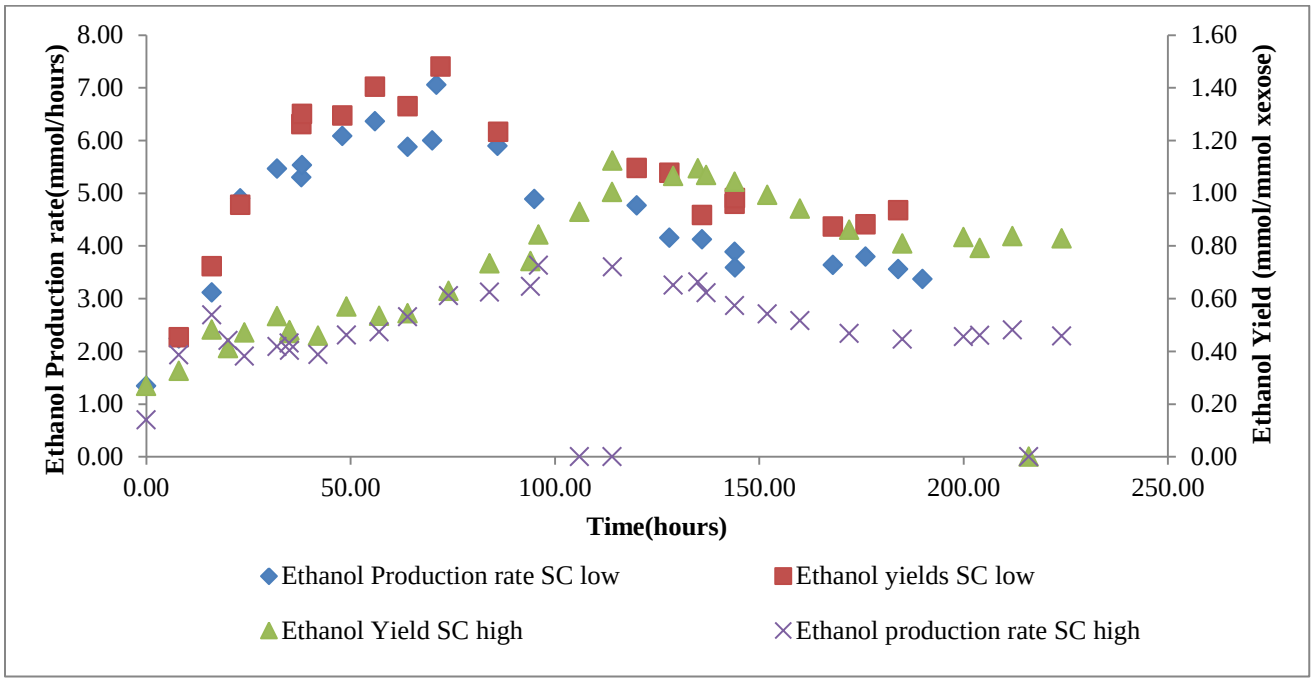


Table 4.6 Time and substrate requirements for the production of 5000 mmol of ethanol and 5000 mmol of hydrogen using different fermentation techniques.

	Batches required to produce 5000 mmol		**Total time required (Without down time)-(days)**		**Total time required (With down time)-(days)**		**Cellulose required to produce 5000 mmol (g)**	
	Ethanol	**H_2**	**Ethanol**	**H_2**	**Ethanol**	**H_2**	**Ethanol**	**H_2**
Batch	9	17	36	69	44	86	886	1701
Variable volume	15	14	106	98	120	111	2037	2145
Fixed volume	10	22	42	86	51	107	1081	2242
Semi-Continuous Low concentrations								
Cycle 1	12	22	35	64	47	85	797	1445
Cycle 1 & 2	7	11	54	85	60	95	957	1504
Semi-Continuous high concentrations								
Cycle 1	12	16	70	91	81	106	963	1576
Cycle 1 & 2	7	13	66	127	72	139	1076	2060

Chapter 5

Conclusions

5.1 Take home messages

This research was carried-out to study the potential of improving the direct conversion of cellulose to ethanol and hydrogen through fed-batch fermentation techniques. By using different feeding strategies, we were able to identify the fermentation techniques that offer greater potential for larger scale deployment. This study also gave us an insight in to the behaviour of *C. thermocellum* under high cellulose loadings. Following are some of the key learning outcomes from the current study:

- Batch fermentation and fixed-volume fed-batch fermentation processes generated similar fermentation end-product profiles, suggesting that the growth arrest observed at end of the batch fermentation was not solely attributed to the depletion of cellulose. Instead, a combination of nutrient depletion, cell death due to ethanol inhibition, and depletion of cellulose may all contribute to the cell growth arrest observed;
- The Variable-volume fed-batch fermentation process demonstrated that cellulose concentrations above 25 g/L had a severe negative impact on the production rates and substrate specific yields of ethanol and hydrogen. This may be due to the physical hindrance caused by improper mixing and the diversion of the carbon and election flow towards lactate and formate synthesis.
- The semi-continuous fermentation process demonstrated that better ethanol and hydrogen production rates were achieved by maintaining cellulose at relatively lower concentrations of 10-15 g/L, as opposed to 20-25g/L. Nevertheless, the semi-continuous fermentation reactions did not show potential for long-term operation over multiple

cycles, as the production rates and yields severely dropped with each cycle due to excessive lactate and formate production. From an economic point of view, it was observed that shorter operation times with intermittent cellulose and nutrient feeding, as demonstrated in the first cycle of the semi-continuous reactions could result in the highest economic gains. With shorter operation times, we could minimize the time the culture stays in the stationary phase where high amounts of lactate and formate are produced.

5.2 Future Directions

This study revealed that ethanol inhibition and high lactate production were key contributors to reduced hydrogen and ethanol production rates in the fed-batch fermentation processes. In order to circumvent this, fed-batch fermentations could be carried-out with modified strains that have higher ethanol tolerance and lower tendency for lactate production. The strains used in batch studies done in Herrero et al. (1980), Biswas et al. (2014), Tailliez et al. (1988), and Xu et al. (2010) are some examples of modified strains.

Future studies could also concentrate on one of the desired end-products, ethanol or hydrogen, in order to achieve higher cellulose consumption while avoiding lactate production. This is especially important as the electron and carbon flow dictates that higher production of ethanol will result in lower production of hydrogen and vice versa (Islam et al. 2006, Rydzak et al. 2011). Previous studies carried-out with the sole objective of increasing higher ethanol yields in sealed reactors, with no continuous gas sparging, have shown to have significantly higher ethanol production rates and higher cellulose hydrolysis rates (Wang et al. 1983).

Chapter 6

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Appendix –I

Calculations of gas end-products for the reactor

Table: Symbols used for the calculation of gas end-products produced in the reactor

	Symbol	**Unit**
Molar percentage of CO_2 in head space at time point 't'	M_{CO_2}(t)	%
Molar percentage of H_2 in head space at time point 't'	M_{H_2}(t)	%
Molar percentage of N_2 in head space at time point 't'	M_{N_2}(t)	%
Total output flow rate from the reactor at time 't'	F_{Total}(t)	mL/min
Output flow rate of H_2 at time 't'	$F_{H_2}(t)$	mL/min
Cumulative of total H_2 purged out at time 't	$C_{H_2}(t)$	mL
Head space volume	(V_H)	L
Total H_2 in the head space	$H_{H_2}(t)$	mL

Molar percentage of CO_2, H_2 and N_2 in the head space at time point 't' is M_{CO_2}(t), M_{H_2}(t) and M_{N_2}(t) respectively. These percentages are obtained from the gas chromatography.

Total output flow rate from the reactor at time 't' [F_{Total}(t) mL/minute] when the N_2 sparging rate is 10 ml/min :

$$F_{total}(t) = \frac{10\ ml/\ \text{min} \times 100}{M_{N2}(t)}$$

Output flow rate of H_2 at time 't' from the reactor [$F_{H_2}(t)$ mL/min]:

$$F_{H_2}(t) = F_{Total}(\text{t}) \times M_{H_2}(\text{t})$$

Cumulative of total H_2 purged out at time 't'[$C_{H_2}(t)$mL]:

$$C_{H_2}(t) = \left\{\frac{F_{H_2}(t) + F_{H_2}(t-1)}{2} \times [(t) - (t-1)] \times 60\right\} + C_{H_2}(t-1)$$

't' and '(t-1)' correspond to two consecutive time points measured in hours post inoculation.

Head space volume (V_H)

Total H_2 in the head space [$H_{H_2}(t)\ ml$]: = $V_H \times 1000 \times M_{H_2}$

Total volumetric H_2 produced at time 't' = $H_{H_2}(t) + C_{H_2}(t)$ mL

Using ideal gas equation laws, the mols of 1 mol of gas at room temperature at atmospheric pressure ($V_{ideal}\ ml/mmol$)

Total production of H_2 in at time 't' in mmoles = $\frac{H_{H_2}(t) + C_{H_2}(t)}{V_{ideal}}$

Similarly, the total production of CO_2 can be calculated.