Effect of Substrate Loading on Metabolic Flux of *Clostridium thermocellum* During Fermentative Hydrogen Production using Cellulosic Substrates

Submitted by

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A thesis submitted to
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of

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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"Effect of Substrate Loading on Metabolic Flux of *Clostridium thermocellum* During Fermentative Hydrogen Production using Cellulosic Substrates"

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ABSTRACT

Significant amounts of hydrogen (H_2) may be produced from cellulosic feedstock such as straw, wood chips, grass residue, paper waste, saw dust, etc. using anaerobic thermophilic fermentation process and can contribute to reducing greenhouse gas emissions alleviating global warming. This study investigated the effects of substrate loading on cell-growth, metabolic flux distribution among various end products, and hydrogen production potential from cellulosic substrates (such as cellobiose, pure cellulose, paper, delignified wood) relative to a soluble substrate, cellobiose. Rates of cell growth were unaffected by different substrate concentrations. H_2, carbon dioxide (CO_2), acetic acid, formic acid, lactic acid, pyruvic acid and ethanol were the main products of fermentation. Formic acid detection as fermentation end product was a surprising observation but not unexpected as *C. thermocellum* has the gene pyruvate-formate-lyase present in its sequence. It was concluded that *C. thermocellum* is capable of i) maintaining carbon flow to acetic acid and H_2-production under limited, sufficient and excess-substrate conditions, ii) balancing oxidation/reduction (O/R) ratios over the entire period of fermentation being independent of substrate concentrations. Under excess-substrate conditions Carbon flow shifted away from acetate pathway as H_2 partial pressures exceeded 50 kPa and pH dropped below 7.0. *Clostridium thermocellum* possess a very active cellulase system to hydrolize insoluble cellulose and is capable of obtaining a competitive yield of 1.6 mol H2 /mol glucose equivalent producing H_2 at a high specific rate of 14.6 mmol /g dry cell/h. Sustainable H_2 production from cellulosic biomass by *C. thermocellum* may be possible if bioreactor conditions were able to maintain a near neutral pH (approx. 7.0) and gas products (H2 and CO2) are removed rapidly to maintain a low enough H_2 partial pressure.

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Chapter 1: Literature Review

1.1 Introduction

Environmental concerns about reducing greenhouse gases (GHGs) have stimulated the search for clean energy sources. Biologically derived organic materials and residues currently constitute a large source of waste biomass (Giallo *et al.* 1985). These include solid and liquid municipal wastes, manure, lumber and pulp mill wastes, and forest and agricultural residues. Waste biomass from agricultural and forestry sectors, as well as from municipal sewage and municipal mixed solid waste, also contribute a major part to GHG emissions by natural decomposition (AAFC 2000; Environment Canada 2000). Use of these biomass-rich resources for bioenergy and related bioproducts could contribute to the displacement of fossil fuels as our primary energy source and could reduce GHG emissions.

Hydrogen is an environment friendly energy substitute for fossil fuels, producing water as its only by-product when it burns. The higher heating value of hydrogen is 3042 cals/m^3, the highest of all known fuels (Das and Veziroğlu 2001; Levin *et al.* 2004). Currently 90% of H_2 is produced from methane reformation or electrolysis of water and contribute to only 3% of energy total consumption. These processes are both energy intensive and not environment friendly (Das and Veziroğlu 2001).

Research on biological hydrogen production for practical application has being carried out for over a quarter century (Hallenbeck and Benemann 2002). A wide variety of biological hydrogen production processes have been investigated and include direct biophotolysis, indirect biophotolysis, photo-fermentations and dark-fermentation. Comparison of process economy and rates of hydrogen production by various bio-hydrogen systems suggests that dark-fermentation systems offer the best potential for

practical application and integration with emerging hydrogen and fuel cell technologies (Levin *et al.* 2004).

Most of the previous studies conducted on anaerobic hydrogen fermentation have involved mesophiles (Lay 2001, Fang *et al.* 2002) or mixed microflora (Lay 2000, Lee *et al.* 2002) using sugars as substrates and no commercial high-rate biohydrogen generation processes are yet established for practical application. Anaerobic thermophilic fermentation of cellulosic biomass to hydrogen has a great potential to reduce waste accumulation, to displace fossil fuels and to reduce the release of greenhouse gases (CO_2 and CH_4). Anaerobic thermophile *Clostridium thermocellum* possesses the most powerful cellulose degrading extracellular multi-enzyme complex, called cellulosome (Schwarz 2001). While this organism has been mainly studied for its ethanol production capability (Balusu *et al.* 2004, Wood and Ingram 1992), its hydrogen synthesis potential has received little attention. This underlines the requirement to concentrate more research on fermentative hydrogen production processes in this organism.

1.2 Dark-Fermentation Process

Hydrogen can be produced by anaerobic bacteria, grown in the dark on carbohydrate-rich substrates. Fermentation reactions can be operated at mesophilic (25-40°C), thermophilic (40-65°C), extreme thermophilic (65-80°C), or hyperthermophilic (> 80°C) temperatures. Bacteria known to produce hydrogen include members of the following genera, *Bacillus, Escherichia, Enterobacter, Ruminococcoi* and *Clostridium* (Nandi and Sengupta 1998; Claassen *et al.* 1999). Some anaerobic bacteria ferment cellulosic substrates via the glycolytic (Embeden-Meyerhof-Parnas) pathway and produce a variety of solvents and shorter chain fatty acids such as ethanol, acetic acid, formic acid, butyric acid, lactic acid as well as hydrogen and CO_2. A schematic of dark-fermentation for hydrogen production is shown in Figure. 1.1.

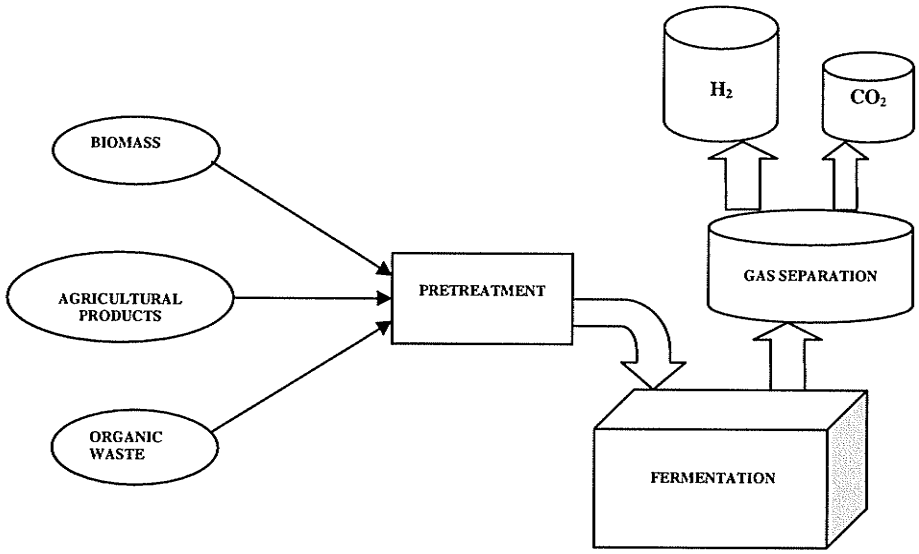


Figure 1.1 Production of hydrogen utilizing cellulosic wastes in dark-fermentation process and separation of the product gases (Hallenbeck and Benemann 2002).

1.3. Microbiology of Fermentation

1.3.1 Microbial Metabolism

During metabolism, microbial cells take in nutrients, convert them into cell components, obtain energy from them, and excrete waste products (products of fermentation: acids, ethanol, CO_2, H_2) into the environment. Metabolism involves two classes of chemical transformations (Madigan *et al.* 2000),

i. Anabolism: building up processes

ii. Catabolism: breaking down processes which involve production of

- energy for biosynthesis
- energy for mobility, transport of nutrition
- fermentation products: acids, alcohols, CO_2, H_2

1.3.1.1 Assimilative and Dissimilative metabolism: All organisms need sources of N, S and C for growth. When inorganic compounds such as NO_3^-, SO_4^{2-}, or CO_2 are reduced only enough to satisfy the need of nutrient for growth, the process is called assimilative metabolism. In dissimilative metabolism, a comparatively large amount of the electron acceptor is reduced for energy metabolism and much of the reduced product is excreted into the environment.

1.3.2 Energy Conservation

In living organisms, energy is conserved in the form of high energy phosphate bonds and transmembrane ion motive force. The most important high energy phosphate compound, which serves as the prime energy carrier in living organisms, is adenosine triphosphate (ATP). Derivatives of coenzyme A (e.g., acetyl-CoA) are the other high energy compounds, which yield sufficient free energy upon hydrolysis. The oxidation of organic compounds in a fermentation process is coupled to the subsequent reduction of an organic compound generated from the initial substrate. So, no external electron acceptor is required. ATP is formed during fermentation by a process called substrate-

level-phosphorylation. In substrate-level-phosphorylation, ATP if formed during specific enzymatic steps in the catabolism of the organic compound, as shown in Figure. 1.2.

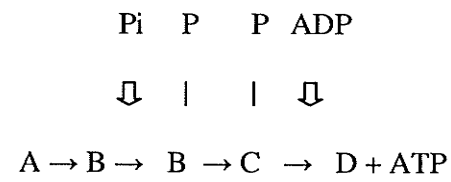


Figure 1.2: In fermentation, a phosphate group gets added to some intermediate in the biochemical pathway and eventually gets transferred to ADP to form ATP (Madigan *et al.* 2000).

1.3.3 Electron Balance by Hydrogen Production

In heterotrophic organisms, the anaerobic mode of growth poses special problems for the cell with respect to the disposition of electrons from energy-yielding oxidation reactions. This is particularly so when the overall reducing power required for biosynthetic activity activity is much lower than the amount of oxidation of organic matter needed for ATP synthesis, required as energy source for cell maintenance and biosynthesis. Such organisms dispose of excess electrons (e-) in the form of reduced organic end products, and molecular hydrogen (H_2) through the activity of various hydrogenases. An iron-sulfur protein in the organism called ferredoxin is a very electronegative electron career. Various hydrogenase enzymes catalyze the transfer of electrons from different electron carrying cofactors such as NADH, NADPH, $FADH_2$, or ferredoxin to H^+. Thus electron balance is maintained in part by the production of molecular hydrogen (Madigan *et al.* 2000).

1.4 Fermentation Pathways and Thermodynamics

The majority of microbial hydrogen production is driven by the anaerobic metabolism of pyruvate, formed during the catabolism of various substrates by glycolysis as shown below:

Embden-Meyerhof pathway

$$C_6H_{12}O_6 + 2\,NAD^+ \longrightarrow 2CH3COCOOH + 2NADH + 2H^+ \quad (1\text{-}1)$$

In glycolysis, NADH is created during the biochemical conversion of glucose to pyruvate. Re-oxidation of this NADH is another route of hydrogen evolution according to the following reaction (Das and Veziroğlu 2001):

ferredoxin oxidoreductase

$$\text{i.} \quad NADH + H^+ \longrightarrow H_2 + NAD^+ \quad (1\text{-}2)$$

Decomposition of pyruvate is catalyzed by one of two enzyme systems (Hallenbeck and Benemann 2002):

ii. Pyruvate : formate lyase (PFL)

PFL Formate hydrogenlyase

$$Pyruvate + CoA \longrightarrow acetyl\text{-}CoA + formate \longrightarrow CO_2 + H_2 \quad (1\text{-}3)$$

iii. Pyruvate : ferredoxin (flavodoxin) oxidoreductase (PFOR)

PFOR Hydrogenase

$$Pyruvate + CoA + 2Fd\,(ox) \longrightarrow acetyl\text{-}CoA + CO_2 + 2Fd(red) \longrightarrow H_2 \quad (1\text{-}4)$$

1.4.1 Hydrogen Yield and Thermodynamics of Fermentation

The yield per mole of glucose has been found to be maximally 4 mol hydrogen when acetic acid is the only fermentation end-product (Solomon *et al.* 1995; Kengen *et al.* 1996):

$$C_6H_{12}O_6 + 4H_2O \longrightarrow 2CH_3COO^- + 4H_2 + 2HCO_3^- + 4H^+ \quad (1\text{-}5)$$

The $\Delta G'_o$ of this reaction is -206 kJ/mole, which is sufficient to allow microbial growth. This value of $\Delta G'_o$ changes, however, with rise in temperature and becomes more and more favorable and more product is formed to reach the equilibrium state. The remainder of the potential hydrogen is contained in by-product, acetic acid. When butyrate is the end product, a theoretical maximum of only 2 moles H_2/mole glucose are obtained:

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CH_2\,CH_2\,CH_2COOH + 2H_2 + 2CO_2 \qquad (1\text{-}6)$$

The major issue is the feasibility of a dark fermentation reaction yielding close to the stoichiometric 12 mol of H_2 stored in each molecule of glucose metabolized (Hallenbeck and Benemann 2002). A near theoretical maximum yield of 11.6 mol H/mol glucose was achieved when the enzymes of the oxidative pentose phosphate cycle were coupled to hydrogenase purified from the bacterium, *Pyrococcus furiosus* (Woodward *et al.* 2000). Essentially no energy is obtained from the complete conversion of glucose to hydrogen and CO_2 because the $\Delta G'_o$ = +3.2 kJ/mole (Thauer 1976).

$$C_6H_{12}O_6 + 12H_2O \longrightarrow 12H_2 + 6HCO_3^- + 6H^+ \qquad (1\text{-}7)$$

At moderate temperatures, the conversion of acetate to hydrogen according to:

$$CH_3COOH + 2\,H_2O \longrightarrow 4H_2 + 2CO_2 \qquad (1\text{-}8)$$

is thermodynamically unfavorable ($\Delta G`_o$ = +104.6 kJ/mole) and is strongly determined by the hydrogen partial pressure. Acetic acid oxidation to hydrogen is thermodynamically feasible at hydrogen partial pressure of 10-100 Pa and at temperatures of 40°C and higher (Classen *et al.* 1999). The problem is that no cellulolytic syntroph has ever been isolated, and there are no feasible engineered means

of maintaining such low partial pressures except via other hydrogen consuming microorganisms.

1.5 Advantages Over Other Bio-hydrogen Processes

The evolution of hydrogen by dark-fermentation has several advantages for industrial production (Das and Veziroğlu 2001; Levin *et al.* 2004),

i. Reduce green-house gas emission by utilizing cellulosic biomass waste;

ii. Produce hydrogen constantly through day and night from organic substrates;

iii. Reduce the size of the bioreactor, as no aeration is required;

iv. Growth of microorganisms is suitable for supply to the production system;

v. Fermentative bacteria have very high evolution rate of hydrogen;

vi. Stable processes are capable of using non-sterile feedstock.

1.6 Major Challenges

There are some technical limitations to overcome to make the dark-fermentation process more promising. Major technical challenges are,

i. H_2 acts to repress H_2 synthesis enzymes;

ii. H_2 concentration in gas mixture is low (25%-50%);

iii. H_2 purification and storage;

iv. Integration with proton exchange membrane fuel cell (PEMFC).

1.7 Fermentative Hydrogen Production

1.7.1 Selection of Feedstock

Thermodynamically, carbohydrates are the preferred organic carbon source for hydrogen fermentation under pure culture condition. The majority of research on fermentative hydrogen production has been conducted with pure and expensive sources of substrates such as glucose and sucrose (Kumar *et al.* 2001; Oh *et al.* 2002; Kataoka *et*

al. 1997). For sustainable hydrogen production, the feedstock must (Hawkes *et al.* 2002),

i. be produced from sustainable sources (cellulose or starch-based organics);
ii. be sufficiently concentrated so that fermentative conversion and energy recovery is energetically favorable (with highest possible % of total solids);
iii. require minimum pre-treatment (e. g. delignification, size reduction); and
iv. be available at low or no cost.

Cellulosic biomass is in compliance with all the above mentioned criteria.

1.7.2 Choice of Microorganisms

A microorganism, suitable for practical applications in bio-hydrogen production process, must have the capability to,

i. produce hydrogen at high rate and yield;
ii. grow well on inexpensive renewable sources of feedstock (such as cellulosic biomass);
iii. metabolize at high temperature which allows,
    - utilization of non-sterile substrates;
    - prevention of mesophilic, hydrogen consuming methanogens;
    - inhibition of mesophilic fermentative organisms;
    - lower hydrogen partial pressure in fermentation broth;
iv. be amenable to genetic engineering; and
v. be nonpathogenic

C. thermocellum meets all the above mentioned criteria.

1.7.3 Hydrogen Producing Microorganisms

Most studies on hydrogen production by anaerobic fermentation have involved mixed micro flora. Ueno *et al.* (1995, 1996) obtained maximum production yields of

2.59 mol H_2 /mol-hexose from sugary wastewater and 2.4 mol H_2/mol-hexose from cellulose containing wastewater using anaerobic microflora. From acclimated anaerobic sewage sludge, the specific hydrogen production rate was 19 mmol H_2/g of cell/h, with a maximum yield of 1.7 mol H_2/mol glucose (Lin and chang 1999). Lay (2001) reported a maximum hydrogen yield of 0.58 mmol H_2/mmole of hexose. The highest hydrogen yields were found for a *Clostridium* sp. as 2.36 mole H_2/ mole of hexose (Taguchi et al. 1995).

Mixed cultures present a number of problems, such that,

i. it is difficult to optimize due to the variation of hydrogen producing organisms have different pHs and temperature ranges for growth;
ii. they require pretreatment to exclude hydrogen consuming methanogens;
iii. their metabolic pathways are difficult to control and follow; and
iv. they cannot be manipulated by metabolic engineering to improve rates or yields of H_2 production.

Fermentation with pure cultures has been studied for a number of different hydrogen producing organisms. Many investigations involving pure cultures for fermentative hydrogen production used principally glucose as feed stock. Comparison of rates and yields of hydrogen production obtained from various studies is not straight forward as they used different operating conditions. Some of the yields and rates of H_2 production by different hydrogen producing organisms are summarized in Table 1.1.

Table 1.1: Hydrogen yields and rates from different fermentative organisms

Organisms	Substrates	Maximum Rate (mmol H_2/g cell h)	Maximum yield (mol H_2/mol substrate)	References
Enterobacter cloacae DM11	Glucose	-	3.8	Kumar *et al.* 2001
Rhodopseudomonas palustris P4	Glucose	29.9	2.76	Oh *et al.* 2002
Citrobacter sp. Y19	Glucose	32.3	2.49	Oh *et al.* 2003
Citrobacter intermedius	Cellulose, starch, glucose	9.5	1.14	Brosseau and Zajic 1982
Enterobacter cloacae IIT-BT 08	Glucose	29.6	2.2	Kumar and Das 2000
Clostridium butyricum st. SC-E1	Sucrose	Nd	1.4 - 2.3	Kataoka *et al.* 1997
Clostridium thermocellum LQ8	Cellulose and cellobiose	Nd	0.85 2.25[a]	Weimer and Zeikus 1977

Notes:
[a]Obtained from co-culture of *C. thermocellum* with *M. thermoautotrophicum*

1.8 Microbial Cellulose Utilization

1.8.1 Cellulose and Microorganisms

Cellulose is the single most abundant biopolymer on the earth. Cellulose is a chemically homogeneous linear polymer of up to 10,000 D-glucose molecules. Microbial cellulose utilization is responsible for the huge flow of carbon in the biosphere (Lynd *et al.* 2002). Although a large number of microorganisms are capable of degrading cellulose through the production of cellulase enzymes, only a few of these microorganisms produce it in significant amount. Cellulose degradation occurs through the synergistic action of three types of enzymatic activities: (1) endoglucanases or 1,4-β-D-glucan 4-glucanohydrolases, which act at random in the polymeric chain and produce new ends; (2) exoglucanases, which include both 1,4-β-D-glucan glucanohydrolases, liberate D-glucose from β-glucan and cellodextrins, and 1,4-β-D-glucan cellobiohydrolases, which liberate D-cellobiose from β-glucan in a processive manner; and (3) β-glucosidases or β-D-glucoside glucohydrolases, which release D-glucose units from soluble cellodextrins and a variety of glycosides (Schwarz, 2001).

1.8.2 Cellulosome

An extracellular multi-enzyme complex, called cellulosome, was found in the cellulolytic bacterium *C. thermocellum* as a cellulose-binding and degradating factor (Bayer *et al.* 1983). Cellulosomes are cell protuberances which tightly bind to crystalline cellulose during hydrolysis (Lamed *et al.* 1987; Mayer 1987). Cellulosomes can easily be isolated from cultures, fully grown on microcrystalline cellulose or cellobiose, by affinity chromatography to cellulose and gel filtration. They are macromolecular machines specially designed for the hydrolysis of insoluble polysaccharides, and mediate a close association between cell and substrate, and thus minimize diffusion losses of hydrolytic products, which is a major advantage for

attached cells. Specific activity of cellulosome or rates of enzymatic hydrolysis of cellulose depend on substrate concentration, enzymes-binding affinity and capacity and concentration of binding sites on cellulose. The binding affinity of *C. thermocellum* was over 100-fold higher than *T. ressei* (Table 1.2). A segment of the cellulosome, called the dockerin domain, is responsible for the correct physical alignment of the scaffold bind to crystalline cellulose (Lamed *et al.* 1987; Mayer *et al.* 1987).

Table 1.2: Binding affinity and specific activity for multi-component cellulose complexes (from Lynd *et al.* 2002).

Organism (Growth temperature)	**Substrate**	**Binding affinity (Liters/g)**	**Specific activity (mol/min/mg)**
Trichoderma reesei			
(50°C)	Avicel	1.04	0.83
(40°C)	Cellulose in pretreated wood	1.82	1.16
C. thermocellum			
(60°C)*d*	Avicel	246	13.2
(60°C) *c,d*	Pretreated wood	344	

*c*Dilute-acid-pretreated wood prepared at 220°C;
*d*Calculated quantities based on a specific activity of 2.4 mol/mg/min and a molecular mass of 2.1 $\times 10^6$ Da.

1.8.3 *Clostridium thermocellum*

Around 5 to 10% of cellulosic materials are degraded anaerobically and among cellylolytic bacteria, clostridia play an important role in such processes (Laschine 1995). The most complex and the best investigated cellulosome is that of *Clostridium thermocellum* (Bergquist *et al*, 1999; Felix *et al*, 1993), a moderately thermophilic bacterium (55–65 °C), repeatedly isolated from hot springs and wet, rotting biomass. *C. thermocellum* is highly specialized for growth on cellulose and cellodextrins as carbon and energy source and, surprisingly, it is hardly (and only after a long lag phase) able to grow on glucose or fructose as carbon source (Nochur *et al.* 1992). Minimum doubling time of *Clostridium thermocellum* was reported (Weimer and Zeikus, 1976) as 11 h on cellulose and 2.1 h on cellobiose. Cellobiose and soluble cellodextrins are taken up by a common transport system and hydrolyzed intracellularily by cellobiose and cellodextrin phosphorylase (Arai *et al.* 1994; Strobel *et al.* 1995; Tanaka *et al.* 1995). This hydrolysis is an energy-efficient way to yield glucose-1-phosphate and glucose. The formation of cellulosomes is induced by growth on cellulose and also on cellobiose (a major product of cellulose hydrolysis).

1.8.4 Fermentation of Cellulose by *C. thermocellum*

Bacteria often preferentially metabolize one substrate over another, and preferred substrates many times support faster growth rates and higher growth yields. Some investigations showed that *C. thermocellum* grown in batch culture preferred cellobiose over glucose (Ng and Zeikus 1982). Intracellular phosphorylases of cellobiose and cellodextrin suggest that oligomers (a polymer that consists of two, three, or four monomers) are metabolized within the cell (Alexander 1972; Zhang and Lynd 2005). Zhang and Lynd (2005) concluded that *C. thermocellum* assimilates primarily cellotetraose during growth on cellulose. One ATP is required per cellobiose to form

two activated glucose molecules by phosphorylysis. Thus, 0.5 ATP per activated hexose is theoretically conserved through phosphorylytic cleavage. This comparison predicts that bacterial yield would be greater on cellobiose than on glucose. *C. cellulolyticum*, a mesophilic and cellulolytic bacterium, (Desvaux *et al.* 2000) was detected to be well adapted and even restricted to a low carbon flow characterizing growth on its to its natural substrate, cellulose.

Kinetic studies on *C. thermocellum* indicated that cellobiose and larger cellodextrins were taken up by a common mechanism, while glucose entered via a separate mechanism (Strobel *et al.*, 1995). Fermentation parameters were essentially the same for dilute-acid-pretreated mixed hardwood and Avicel, and was higher in continuous culture compared to batch culture (Lynd *et al.* 1989). Co-culture of *C. thermocellum* with *Methanobacterium thermoautotrophicum*, produced more H_2 (2.25 mole/mole glucose equivalent) than cultures of *C. thermocellum* only (0.85 mole/mole glucose equivalent) co-cultures also produced more acetic acid and less ethanol than monocultures (Weimer and Zeikus 1977). Growth yields were greater on cellobiose were found higher than on glucose, and an even greater yields were obtained on cellotetraose. Glucose grown cells had a four-fold higher maintenance energy requirement than cellobiose-grown cultures. Also, the carbohydrate utilization patterns in continuous culture are different from those in batch culture (Strobel 1995). Pathways of different metabolites formed during cellulose fermentation (as understood prior to 2004) by *C. thermocellum* are shown in Figure 1.3.

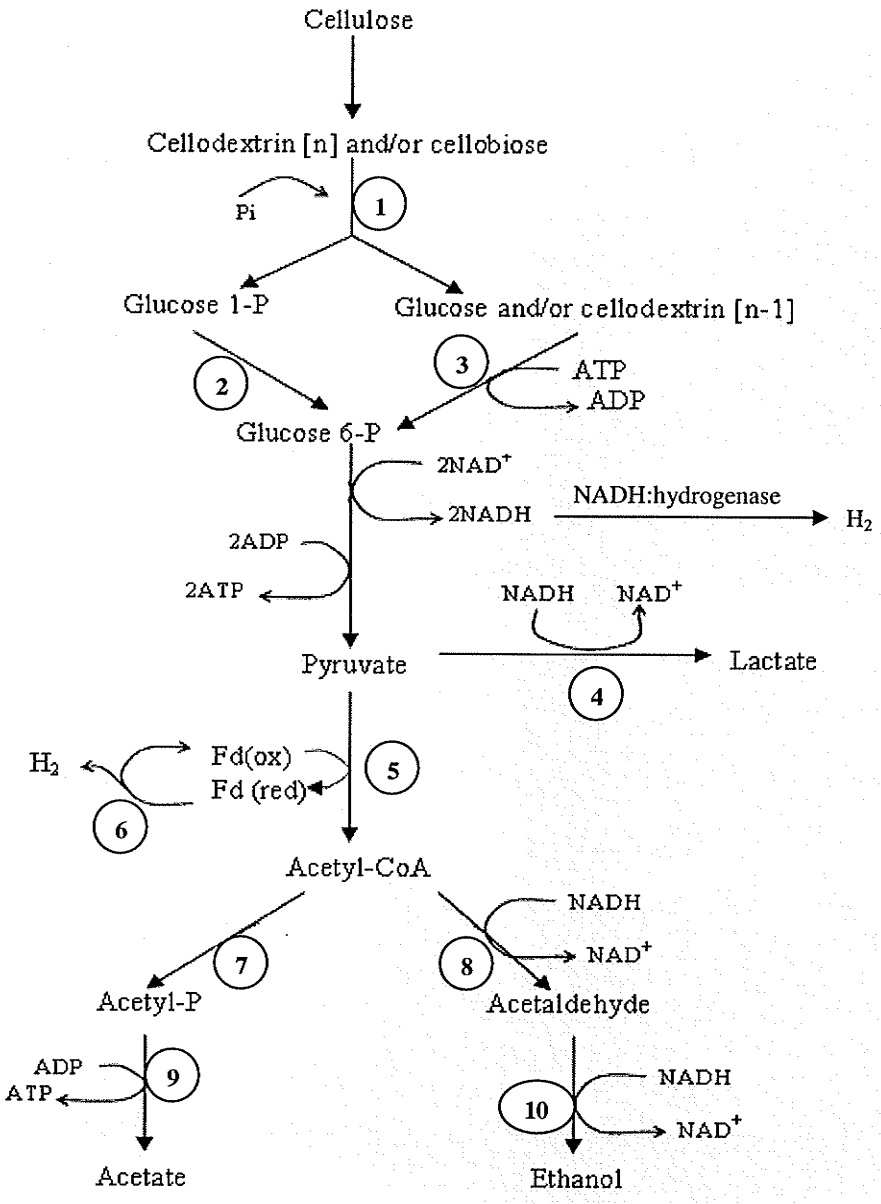


Figure 1.3 Pathway-model of different metabolites based on literature review of cellulose fermentation by *Clostridium thermocellum* (Lynd *et al.* 1989; Weimer and Zeikus 1977). 1, cellodextrin phosphorylase and cellobiose phosphorylase; 2, glucokinase; 3, phosphoglucomutase; 4, Lactate dehydrogenase (LDH); 5, pyruvate ferredoxin oxidoreductase (PFO); 6, Fd:hydrogenase; 7, phosphotransacetylase; 8, acetaldehyde dehydrogenase; 9, acetate kinase; 10, alcohol dehydrogenase; p, phosphate; Fd, ferredoxin; ox, oxidatized; red, reduced; (Partially adapted from Guedon *et al.* 2002).

1.9 Factors Effecting Fermentative Hydrogen Production

Rates and yields of hydrogen production by fermentative anaerobic processes are influenced by a large number of environmental and physico-chemical factors. These are culture medium, pH, partial pressure of hydrogen, hydraulic retention time (HRT), agitation rate, concentration of substrates and end-products.

1.9.1 Culture Medium

Fermentation-medium needs to be chosen carefully for carbohydrate feedstocks for optimum performance. Ethanol was found to be a major product under phosphate limited conditions, and insufficient iron (less than 0.56 mg/l) limited the growth of *C. pasteurianum* with significant lactic acid production (Dabrok *et al.* 1992). Iron limitation also results in lowered hydrogenase activity, as it is a component of hydrogenase enzymes (Hawkes *et al.* 2002). Growth of *C. thermocellum* ATCC 27405 in medium MJ containing urea and MOPS [3-(n-morpholino) propanesulphonic acid] buffer provided the most active and stable cellulosome (Halliwell *et al* 1995). *C. cellulolyticum* consumed threefold higher cellobiose when cultured in a synthetic medium compared with a complex medium, and NADH/NAD^+ ratios dropped from 57 to 2.08. Carbon flow was well regulated on synthetic medium compared to complex medium where intracellular metabolites (NADH) accumulated (Guedon *et al.* 1999).

1.9.2 pH

Fermentative hydrogen production is accompanied by synthesis of organic acids, such as acetic acid, lactic acid, butyric acid, etc. Anaerobic cellulolytic bacteria, like most fermentative microbes, grow within a fairly narrow pH range. Cellulolytic anaerobes do not grow at pH below 6.0. For thermophilic species, the optimal pH was identified as 6.5 for the maximum yield of 102 mL H_2/g cellulose (Hong *et al.* 2004). Hydrogen production by *Clostridium* sp. proceeds at neutral pH, but is inhibited in the

pH range 4-5 (Bahl *et al.* 1986). This observation, however, is at odds with the observation that cellulose removal in some anaerobic mixed cultures occurs at pH as low as 4.5 with an optimum near pH 5 (Lee *et al.* 2002). The activity of iron-containing enzyme hydrogenases, is affected by low pH which was reported as one of the most influential factors in hydrogen fermentation (Khanal *et al.* 2003). An increase in the intracellular acid concentration shifted a continuous culture of *Clostridium acetobutylicum* from acetate and butyrate to acetone and butanol production (Grupe and Gottschalk, 1992).

1.9.3 Partial Pressure of Hydrogen

In some cases, growth can be inhibited by the accumulation of intracellular, reduced nucleotides. This may occur at high concentrations of dissolved hydrogen in the fermentation broth, which inhibit the reoxidation of NADH produced during glycolysis. As H_2 concentrations increase, rate of H_2 synthesis decreases and metabolic pathways shift to production of more reduced substrates such as lactate, ethanol, acetone, butanol, or alanine decreasing the yield of H_2 per mole of glucose equivalent utilized (Kengen and Stams 1994). Continuous hydrogen synthesis requires the partial pressure of H_2 to be < 50 kPa at 60 °C (Lee and Zinder 1988), and < 20 kPa at 70°C (Van *et al.* 2002).

As the concentration of dissolved hydrogen increases with the headspace gas pressure, continuous removal of headspace gas is also required. An intermittent pressure release method (Owen method) and a continuous venting method (respirometric method) were investigated to improve the hydrogen production. Under otherwise identical conditions, the respirometric method produced 43% more hydrogen than the Owen method (Logan *et al.* 2002). If the partial pressure of hydrogen can be lowered enough (below 50 Pa), the acetate pathway will be more thermodynamically

favorable. Table 1.3 presents the improvement in H_2 yield, approaching the maximum (2 mol H_2 / mol glucose) when butyric acid was a major fermentation end-product.

Table 1.3: Effect of lowering dissolved H_2 concentration (Mizuno *et al.* 2000)

Products	Non-sparging	Sparging
H_2 yield (mol/mol glucose)	0.85	1.43
Acetic acid (mg/L)	773	785
n-butyric acid(mg/L)	1742	1929

1.9.4 Hydraulic Retention Time (HRT)

When organisms are grown in a continuous culture, hydraulic retention time can affect the rate of H_2 production to a great extent. If the dilution rate is too high, microbes will be washed out of culture and the consumption rate of substrate will decrease quickly. One study (Strobel 1995) with *C. thermocellum* observed that at dilution rates ranging from 0.04 to 0.25/h, more than 95% of the carbohydrate was consumed, but as the dilution went higher, substrates began to accumulate in reactor.

1.9.5 Substrate Conditions

Substrate concentration, solubility and accessibility, have significant influence on fermentation product. Initial substrate concentrations appear to have a profound influence on the H_2 yield. In continuous fermentation, maximum substrate conversion (80-90%) was obtained at feed substrate concentrations ranging from 4 to < 7.5 g/L (Lynd *et al.* 1989). Batch testes performed by a later study achieved the maximum production rate (4.9 ml H_2 /g COD) at a substrate concentration of 40 g/L and a sustained hydrogen production rate of 3.5-4.0 ml H_2 /g COD at F/M ratio in the range of 2 to 4 (Fan *et al.*2004). Under continuous fermentation conditions, cell density leveled

off at approximately 7.6 g of cellulose liter^{-1} at steady state condition (Desvaux *et al.* 2001).

Carbon flow towards cell-mass was approximately two-fold higher while *C. cellulolyticum* was growing on soluble substrate (cellobiose) compared to growth on highly insoluble cellulose (Desvaux *et al.* 2001). Maximum growth rates on soluble sugars were usually several-fold higher than on crystalline cellulose (Lynd *et al.* 2002).

1.9.6 Stirring Effect

Lay (2000) found that the agitation speed of a continuous chemostat-fermentor increasing from 100-700 rpm promoted a three-fold increase in H_2-production. A previous study on fermentation of cellulose by *C. thermocellum* showed a stirring rate of more than 150 rpm is needed for H_2 transfer to the gas phase (Lamed *et al.* 1988). Hydrogen production increased in the stirred culture from 215 μmol to 600 μmol. This suggests that stirring facilitated H_2 transfer to the gas phase, which relieved the inhibition of acetate formation caused by the high H_2 concentration in the medium. For the same reason care should be taken to avoid developing dead zones in the fermentor. Very rigorous mixing may create excess foaming which makes it difficult to keep the microbes suspended in the culture.

1.9.7 Liquid to Gas Mass Transfer

Anaerobic processes can suffer from severe liquid-to-gas mass transfer limitations. Mass transfer of highly soluble gases is not limited in usual conditions that occur in anaerobic processes. In contrast, the interphase mass transfer for poorly soluble gases, such as hydrogen, can be severely limited because of low mass transfer efficiency. Supersaturation of hydrogen, to as much as 80 times the thermodynamic equilibrium concentration, has been observed (Pauss *et al.* 1990). This will greatly

decrease the rates and yields of hydrogen production. Mass transfer of hydrogen from culture to the headspace may be improved by installing an efficient gas-liquid contactor.

1.9.8 Concentration of Ethanol

The ethanol tolerance of *C. thermocellum* has been investigated extensively among. *Clostridium thermocellum* has a low alcohol tolerance for growth (<2% [vol/vol]) (Burdette *et al.* 2002). Ethanol exerted an inhibitory effect on fermentation of cellulose by *C. thermocellum* and caused a cessation of gas production (Weimer and Zeikus, 1977). Inhibition of *C. thermocellum* by ethanol has been attributed to a blockage in glycolysis associated with ethanol induced changes in the cell membrane (Herrero *et al.* 1985). These results indicate that removal of ethanol produced in the fermentation process could accelerate the growth rate and cellulose degradation.

1.9.9 Effect of Carbon-dioxide

Depending on the availability of CO_2, *Enterobacter aerogenes* produces succinate and formate using NADH, which decreases the yield of hydrogen (Tanisho *et al.* 1998). By removing CO_2 from the culture liquid, it is possible to reduce the production of formate and succinate. This will undoubtedly increase the yield of hydrogen due to greater availability of NADH. The estimated yield increased from 0.5 to 1.6 mol hydrogen/mol glucose by *Enterobacter aerogenes* when CO_2 was removed by argon bubbling.

Above mentioned factors affecting the fermentation pathways for hydrogen production are basically correlated to one another and manipulation of one factor may influence others to a great extent. A thorough understanding of the microorganism and extensive analyses are required to optimize fermentative hydrogen process.

1.10 Metabolic Engineering

Achieving high product yields is a primary focus of organism development for biotechnological applications. Although substantial amounts of several organic end products are produced under most conditions by cellulolytic anaerobes, several lines of evidence suggest that organisms in this category are capable of metabolizing cellulose with near-exclusive production of a single end product (Lynd *et al.* 2002). In glycolysis, the acetate pathway is competing with the ethanol pathway. Therefore, acetaldehyde dehydrogenase and alcohol dehydrogenase could be two potential genes as knockout targets to redirect metabolic flux toward hydrogen production. Metabolic engineering of *C. cellulolyticum,* by expressing pyruvate decarboxylase and alcohol dehydrogenase from *Zymomonas mobilis,* improved cellulose consumption by 150% and acetate production by 93% (Guedon *et al.* 2002). The use a type of strain whose a known genome sequence can permit a re-evaluation of the pathway branches present in the organism of interest which would allow for a more thorough and thoughtful approach to genetic engineering.

1.11 Objectives of This Thesis

C. thermocellum has enormous potential for hydrogen synthesis from cellulosic substrates and is very suitable for practical applications. Development of a sustainable hydrogen production process requires a thorough understading of metabolic flux responses of *C. thermocellum* under various culture conditions. This is essential for the development of a system that can control the fermentation dynamics and direct it toward high sustainable hydrogen yields that are sufficient for practical applications. The primary objective of this study is to investigate the hydrogen production potential by *C. thermocellum* strain *27405* from cellulosic substrates. More specifically, the objectives of this study were to determine:

i. The effects of substrate loading on
   - Cell growth rate,
   - Carbon flow distribution among various end products, and
   - Hydrogen production potential;

ii. Hydrogen production from insoluble cellulosic substrates (such as pure cellulose, paper, delignified wood) relative to from soluble substrate (such as cellobiose); and

iii. The relative performance of various cellulosic materials as substrate for hydrogen production.

1.12 Thesis Organization

The rest of the thesis is arranged according to the following sequential order:

- Chapter 2 contains the description of materials and analytical methods used for all the experiments performed.
- Chapter 3 includes comparison of different cellulosic substrates (cellobiose, α-cellulose, DLC and paper) for hydrogen production potential.
- Chapter 4 describes the results obtained from batch experiments of *C. thermocellum* on cellobiose.
- Chapter 5 describes the results obtained from batch fermentation of α-cellulose, by *C. thermocellum.*
- Chapter 6 presents a general discussion based on overall results, conclusions, engineering significance of this study, ongoing investigations and directions for future research.

Chapter 2: Materials and Methods

2.1 Sources of Chemicals and Substrates

All chemicals and reagents for media and substrates were obtained from Sigma Chemical Co. The delignified cellulose or wood fibers (DLC) used in these experiments was provided by Lignol Innovations Inc., Vancouver, BC.

2.2 Microorganism and Media

Clostridium thermocellum 27405 was obtained from the American Type Culture Collection (ATCC) and was employed for all growth experiments. Fresh cultures were maintained by routinely transferring 10% (v/v) inocula into fresh 1191 media (obtained from ATCC) containing 5 g/L cellulose or cellobiose. This complex medium contained (per liter of distilled deionized water): KH_2PO_4, 1.5 g ; $Na_2HPO_4.12H_2O$, 4.2 g ; NH_4Cl, 0.5 g ; $MgCl_2 . 6H_2O$, 0.18 g ; Yeast Extract (BD 212750), 2.0 g ; Resazurin (0.1%), 1.0 ml ; 0.25 mg/ml Resazurin , 4.00 ml; vitamin solution (10X), 0.50 ml; mineral solution (10X); 1.00 ml.

Vitamin Solution contained the following (per 1000 ml): Biotin, 5.0 mg ; P-Aminobenzoic acid, 5.0 mg ; Folic acid, 5.0 mg ; Nicotinic acid, 5.0 mg ; Thiamine, 5.0 mg; Riboflavin, 5.0 mg ; Lipoic acid (thioctic acid), 5.0 mg; Cyanocobalamin, 1 mg. Mineral solution contained (grams per litre): Trisodium nitrilacetate 2.02; $FeCl_3.6H_2O$, 0.21; $CoCl_2.6H_2O$, 0.20; $MnCl_2.4H_2O$, 0.10; $ZnCl_2$, 0.10; $NiCl_2.6H_2O$, 0.1; $CaCl_2.2H_2O$, 0.05; $CuSO_4.2H_2O$, 0.05; $Na_2MoO_4.2H_2O$, 0.05. Reducing Solution was prepared under nitrogen using sodium sulphide crystals in distilled water to a final concentration of 200 mM.

2.3 Experimental Design

Experimental design and scheduling are described in Appendix-B. Section B1 describes the experimental setup for Chapter-3, section-B2 includes experimental approach for Chapter-4 and section-B3 contains design and setup for Chapter-5.

2.4 Analytical Procedures

2.4.1 Growth Measurement

Cell-growth on cellobiose was measured as a function of optical density by spectrophotometry (Biochrom, Novaspec II) at 600 nm (OD_{600}) immediately after briefly vortexing the tube. A dry weight measurement of approximately 0.5 g L^{-1} was found to be correlated with an OD_{600} of 1, in agreement with previous observations (Payot *et al.*, 1998).

Growth on insoluble cellulosic substrates such as α-cellulose and wood fiber was determined by a modification of the Bradford dye method (Bradford 1976) as follows. A sample (10 ml) was centrifuged (8000 × g for 15 min) and supernatant was removed from the top. The pellet was washed with 0.9% (wt/vol) NaCl and was resuspended in 2 ml of 0.2N NaOH. This suspension was boiled for 10 min in a water bath. After cooling the sample was centrifuged as above and the supernatant was collected for protein assay (Desvaux *et al.* 2000).

2.4.2 Conversion of Cell Biomass

The elemental biomass composition, denoted by $C_4H_7O_2N$, based on a stoichiometric conversion of cellobiose in cell material is:

$$C_{12}H_{22}O_{11} + 3NH_3 + 23.8\ ATP \Longrightarrow 3C_4H_7O_2N + 5H_2O$$ (Guedon *et al.* 1999)

A molecular weight of 101 g, corresponding to the composition of cellobiose, was used to calculate cell biomass in moles.

2.4.3 Gas Measurement

Product gas composition (H_2 and CO_2) was measured using a gas chromatograph (Gow Mac model-580) with a thermal conductivity detector. Hydrogen measurements were conducted with a stainless steel (1/8 inch x 8 ft.) column packed with molecular sieve 5A (60/80 mesh), and nitrogen as the carrier gas. For CO_2 analysis, a stainless steel (1/4 inch x 8 ft.), Porapak Q column was used, with helium as the carrier gas. Gas concentrations were quantified by comparing peak height values to those of a standard curve prepared on the same day from known concentrations of H_2 and CO_2. The average amount of gas produced was then calculated taking temperature and atmospheric pressure into account (Appendix D). All gas measurements were corrected by calculating their solubilities in water (Sander, 1999), and for CO_2, the bicarbonate equilibrium was taken into account.

2.4.4 Sugar and End-Product Analyses

Cellobiose and glucose were measured by a high-pressure liquid chromatography (HPLC) with an anion-exchange CarboPac-PA1 analytical column (4 X 250 mm) installed. An IonPac AS11-HC anion-exchange column (Dionex Corporation, Sunnyvale, CA, USA) was used to measure acetate, lactate, formate and pyruvate. Ethanol was measured by a GC packed AT-1000 column (6ft x 1/8" outer diameter) with 15% H_3PO_4 on 100/120 Chromosorb W-AW (Alltech Associates). Standards for acids, sugars and ethanol were prepared on 1191 medium to correct the background.

2.4.5 Measurement of Residual Cellulose

Residual cellulose was measured by taking the dry weight of remaining cellulose. 1 ml of culture was dried in a micro-centrifuge tube (pre-weighted) in 70°C oven for one day and weighted after cooling down to the room temperature. This

process was repeated until a constant weight was obtained. 1 ml of 1191 medium was used as the control. Weight of this 1 ml medium (dried) was deducted from each sample to achieve the dry weigh of cellulose.

2.5 Calculation of Hydrogen Yield

Yield of hydrogen ($Y_{hydrogen}$; moles of hydrogen produced per moles of glucose equivalent utilized per tube) for cellulosic substrates were calculated based on total amount of substrates converted to products. Total amounts of cellulosic material per culture-tube were first converted into moles of glucose equivalents.

2.6 Calculation of Specific Rates of Hydrogen Production

Specific rates of hydrogen synthesis were calculated by dividing the differential amounts of hydrogen produced (mmoles) between two consecutive time points with the average of cell-mass present (gram of dry cells) at that time in the culture.

Chapter 3: Hydrogen Production from Various Cellulosic Substrates Using *Clostridium thermocellum*

3.1 Introduction

Significant amounts of hydrogen (H_2) may be produced from cellulosic feedstocks (straw, wood chips, grass residue, paper waste, saw dust, etc.) using conventional anaerobic digester technology under conditions that favor H_2-producing acetogenic bacteria and inhibit methane-producing bacteria (Sparling *et al.* 1997; Valdez-Vazquez *et al.* 2005). *C. thermocellum* is a thermophilic (optimum growth temperature: 60°C), anaerobic bacterium, which possesses the highest rate of cellulose degradation of all known cellulose degrading microorganisms (Lynd *et al.* 2002). Because of its high rate of cellulose degradation and propensity to synthesize H_2, carbon dioxide (CO_2) and acetate, *C. thermocellum* offers the potential for producing H_2 from cellulosic waste biomass. In Addition, the optimal operating temperature 60°C for *C. thermocellum* precludes the growth of many other microorganisms, and allows for *C. thermocellum* enriched cultures to be maintained even when non-sterile substrates are used.

Batch experiments were conducted with various cellulosic substrates and cellobiose with different initial substrate-concentrations using *Clostridium thermocellum* strain 27405. The goal of this study was i) to develop a basis for metabolic flux analysis during fermentative hydrogen production, ii) to observe the impact of substrate-loading on hydrogen production potential and iii) to compare a soluble substrate, cellobiose, with insoluble cellulosic materials.

Some hypotheses, based on literature review, are as follows:

i. More hydrogen will be produced from cellulosic substrates than from cellobiose

ii. All cellulosic materials will display similar performances as substrate for hydrogen production

iii. Major fermentation products will be acetate and ethanol

3.2 Materials and Methods

Material and methods for this experiment are described in sections 2.1 to 2.5 of chapter 2 of this thesis. Initial and terminal samples were collected in triplicate for analyzing end-point products. Cultures containing 0.1, 1.1 and 4.5 g l^{-1} batches were incubated at 60°C for 3 days, 8 days and 11 days, respectively.

3.3 Results

3.3.1 Gas Production

Figure 3.1 displays the total production of H_2 and CO_2 from cellobiose and three cellulosic substrates. Cellulosic substrates produced comparatively higher amounts of H_2 and CO_2 than cellobiose in cultures with 0.1 and 1.1 gl^{-1} substrate. At substrate concentrations of 4.5 gl^{-1}, DLC produced the maximum amount of H_2, and gas production from α-cellulose and filter-paper was less than that from cellobiose. Estimated amounts of gases were 10 to 45% higher than their measured values. For example, 43 micromoles of H_2 were estimated from DLC (0.1 g/l batch) compared with its measured value of 25 micromoles.

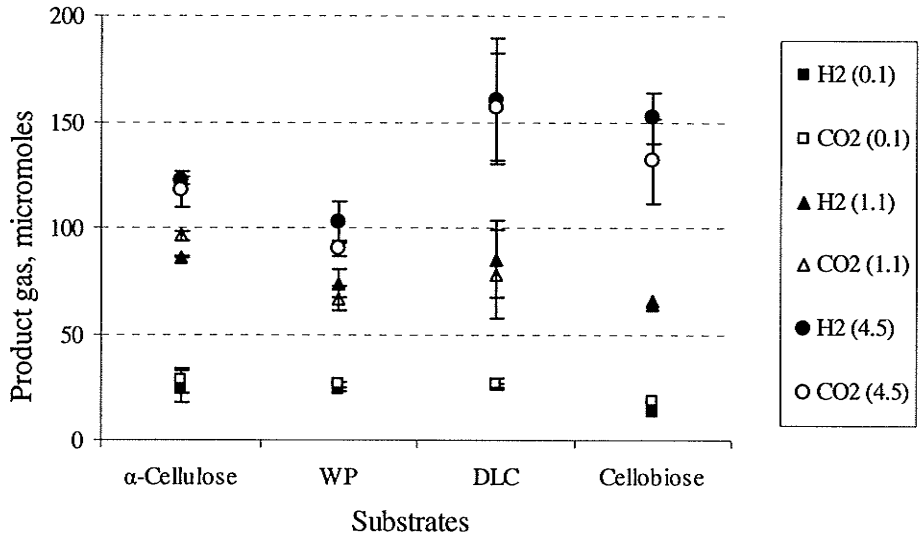


Figure 3.1: H_2 and CO_2 produced from α-cellulose, whatman paper (WP), delignified cellulose (DLC) and cellobiose at .1, 1.1 and 4.5 g l^{-1} concentrations. Gas amounts are expressed as total micromoles per 10 ml of culture.

3.3.2 Gas Ratios

Ratios of H_2/CO_2, calculated using the measured gas quantities for different substrates, are presented in Table 3.1. H_2/CO_2 ratios varied from 0.9 to 1.15 for all substrate concentrations except the lower value for 0.1 g/l cellobiose containing cultures (0.76). Estimated values based on fermentation end-products for all four substrates were very similar (≈ 1.0) and almost no variation in H_2/CO_2 ratios was observed in culture with different substrate concentrations.

Table 3.1 The H_2 /CO_2 ratios for four different substrates under different initial substrate conditions.

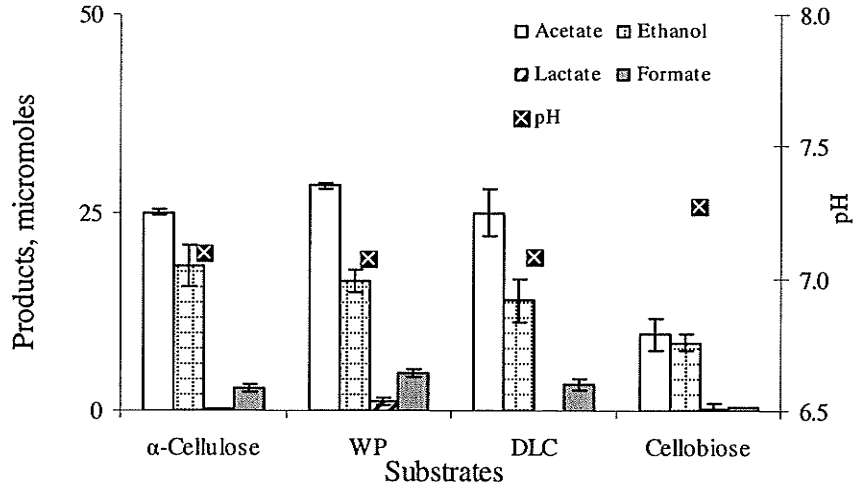
Substrates	0.1 g/L	1.1 g/l	4.5 g/l
		H_2 /CO_2	
Alpha-cellulose	0.90 (± 0.13)	0.90 (± 0.01)	1.04 (± 0.06)
Whatman filter-paper	0.91 (± 0.07)	1.10 (± 0.02)	1.13 (± 0.06)
De-lignified wood fibres	0.95 (± 0.05)	1.09 (± 0.06)	1.02 (± 0.08)
Cellobiose	0.76 (± 0.04)	0.97 (± 0.07)	1.15 (± 0.08)

3.3.3 Acids and Alcohol Production

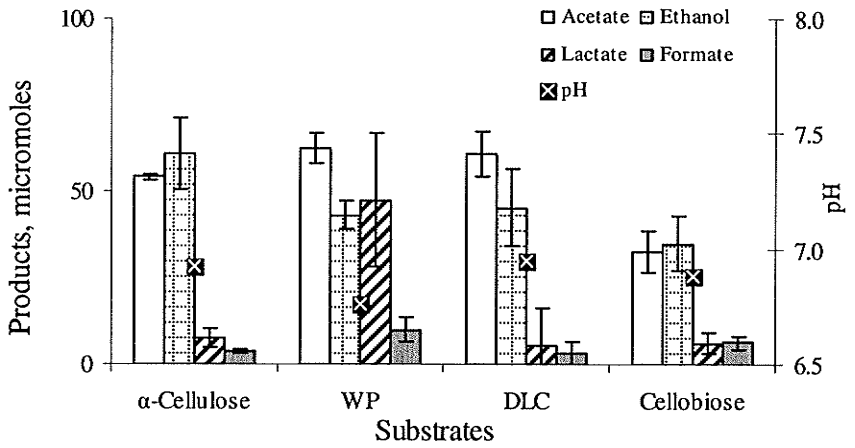
Acetate, ethanol and lactate were major end products of fermentation by *C. thermocellum* for all substrates (Figure 3.2). Formate production was observed and constituted from 4 to 8% of fermentation products for all four kinds of substrates utilized and was not influenced by various substrate-conditions. Experimental errors were within acceptable range.

Proportion of acetate production decreased by 25 to 30% as substrate condition changed from 0.1 to 4.5 g l^{-1}. Lactate production which was undetectable under 0.1 g l^{-1} substrate-concentration, increased linearly with the increase in substrate flow. At 4.5 g l^{-1} concentrations, lactate constituted almost 37% of total products with a 2 to 3% contribution from extracellular pyruvate (data not shown).

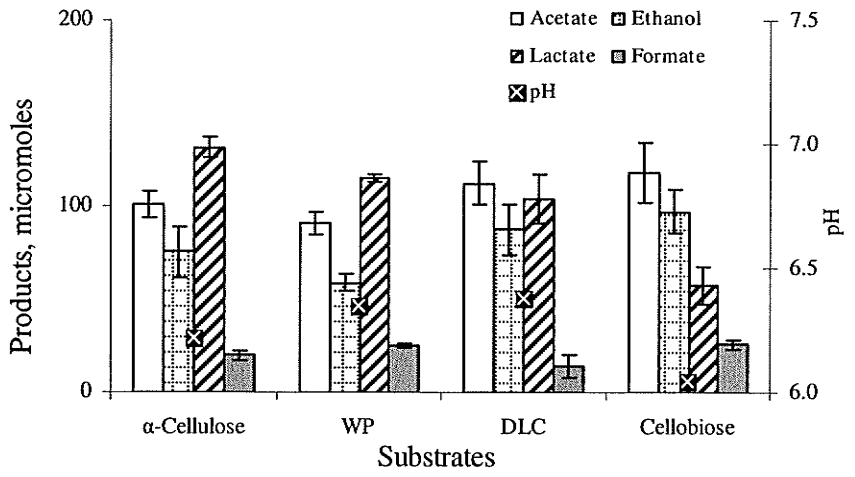
In cultures with 4.5 g l^{-1} substrate, unusually high concentrations of lactate were observed for filter-paper substrate. This result was consistent with the lower amounts of gas measured for the same set of samples conditions (Figure 3.1).


50
25
0
Products, micromoles
Acetate
Ethanol
Lactate
Formate
pH
8.0
7.5
7.0
6.5
pH
α-Cellulose
WP
DLC
Cellobiose
Substrates

(a)


100
50
0
Products, micromoles
Acetate
Ethanol
Lactate
Formate
pH
8.0
7.5
7.0
6.5
pH
α-Cellulose
WP
DLC
Cellobiose
Substrates

(b)



(c)

Figure 3.2 Fermentation end products of *C. thermocellum* and corresponding pH values for cellobiose, α-cellulose, whatman paper (WP) and delignified cellulose (DLC), at 0.1 g l^{-1}, (a); 1.1 g l^{-1}, (b); and 4.5 g l^{-1}, (c) substrate concentrations. Amounts of products are expressed in total micromoles produced per 10 ml of culture.

3.3.4 Effect of Substrate Concentrations on Yields

Figure 3.3 illustrates the differences in yields of H_2 under different substrate concentrations for the various cellulosic substrates. In general, yields of H_2 on cellulosic substrates were higher than on cellobiose in 0.1 and 1.1 g l^{-1} batches. The only exception was cultures containing 1.1 g l^{-1} of filter-paper and this low yields may be correlated with high lactic acid productions in those cultures. In carbon-excess batch, yields on cellobiose reached the highest value among all carbon sources. A maximum of yield 1.55 mol H_2/mol glucose-equivalent was obtained from DLC at 1.1 g l^{-1} concentrations.

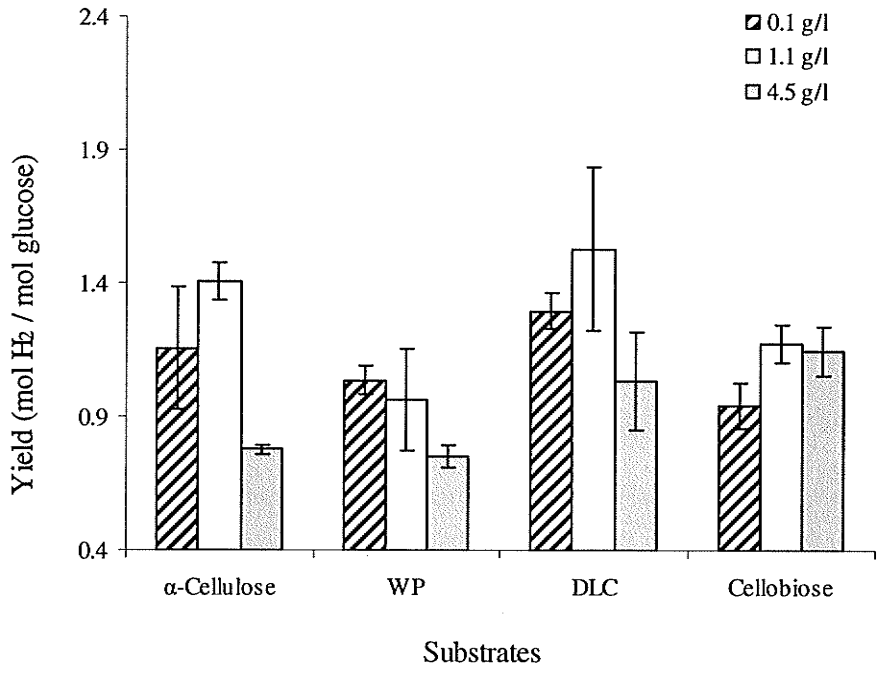


Figure 3.3: Effect of different substrate concentrations on yields of H_2-production from cellobiose, α-cellulose, whatman filter-paper (WP) and delignified cellulose (DLC).

Table 3.2 summarizes a comparison between fermentation products from cellobiose and delignified cellulose (DLC) for the three different substrate-conditions. Lower ethanol/acetate ratios were obtained from DLC under all conditions, which corresponded to higher amounts of hydrogen. Formate levels were similar in 0.1 g/l and 1.1 g/l concentrations. Lactate levels, however, were almost two-fold higher for DLC in cultures containing 4.5 g l^{-1} indicating alterations in the regulation of flow of substrate to the various branches of the fermentation pathway. Extracellular pyruate production, detected at 4.5 g l^{-1} substrate concentrations, is consistent with the use of pyruvate as a carbon overflow product.

Table 3.2 Fermentation products of cellobiose and delignified cellulose or wood-fibers

Conditions	**0.1 g l^{-1}**		**1.1 g l^{-1}**		**4.5 g l^{-1}**	
Substrate →	**DLC**	**CB**	**DLC**	**CB**	**DLC**	**CB**
Products ↓	**µmoles per 10 ml of culture**					
Acetate	24.98	10.78	55.78	36.77	112.52	117.84
Ethanol	13.94	9.65	49.61	39.2	87.77	97.36
Formate	3.36	0.60	3.14	7.01	14.14	28.99
Lactate	0.07	0.39	5.58	6.73	104.26	64.20
Pyruvate	ND	ND	ND	ND	4.52	3.77
Ethanol/Acetate	0.6	0.9	0.75	1.07	0.78	0.83
Hydrogen	25.28	13.56	85.32	63.93	159.80	151.83
Carbon dioxide	26.63	18.43	78.11	65.63	156.83	131.51
H_2/CO_2	0.95	0.76	1.09	0.97	1.02	1.15
pH	7.08	7.28	6.95	6.88	6.38	6.04
O/R	1.07	1.14	0.91	1.09	0.99	0.90

*ND indicates amounts of product not detectable.
Oxidation reduction (O/R) indices were between acceptable ranges of 0.81 to 1.09 indicating that no other major products were overlooked during the analyses of culture liquid and headspace gas.

3.4 Discussions

H_2-production from all cellulosic substrates confirmed that *C. thermocellum* 27405 has a very active cellulase system capable of hydrolyzing various type of cellulosic biomass. Among cellulosic substrates, the highest H_2-production was obtained from DLC. As lingo-cellulosic biomass comprises a huge portion of our organic waste, fermentation by *C. thermocellum* offers potentials for converting cellulosic waste materials to hydrogen gas.

Results from the 0.1 g l^{-1} and 1.1 g l^{-1} batches showed that *C. thermocellum* produced more gas and end-products from long chain cellulosic materials compared to cellobiose as substrate. This was observed in previous investigations (Strobe, 1995; Ng and Zeikus 1982), where *C. thermocellum* grown on cellotetraose had a higher biomass yields than when grown on cellobiose. Schafer and King (1965) found increased cell yields of *Cellvibrio gilbus* as dextrin length increased.

Changing from low to high initial substrate conditions showed that the proportion of acetate formation decreased with increasing initial substrate concentrations. Around 50% of carbon flowing toward acetate shifted to lactate production when conditions changed from low to high substrate concentration. Extracellular pyruvate production was an evidence of overflow of incoming nutrient caused by inhibited carbon-flow down toward acetate production and induction of the alternative pathway to lactic acid production. High concentrations of lactate were generally observed in cultures where the pH dropped below 6.8.

A dramatic rise in lactate level in high substrate conditions indicate the production of H_2 (through the reaction $NADH + H^+ \rightarrow NAD^+ + H_2$) was extremely low or completely stopped in the stationary phase. Trapping of hydrogen under

insoluble substrates may have occurred as the cultures were not stirred and such highly localized hydrogen concentrations in these cultures may have provided unfavorable thermodynamic conditions shifting the flow of carbon away from acetate or ethanol production to lactate production. Under high-substrate conditions, the H_2 partial pressures ranged between 20 to 24 kPa (measured at 19°C) and at 60°C, were over 60 kPa. But continuous H_2-synthesis requires the partial pressure of H_2 to be < 50 kPa at 60 °C (Lee & Zinder, 1988). Therefore, high H_2 concentrations could be the factor responsible for inhibiting H_2-synthesis in high-carbon conditions.

In past research, super-saturation of culture with trapped H_2 between cells and settled cellulose has been observed in unstirred cultures and resulted higher ethanol/acetate ratios (Lamed et al. 1988). This may explain the higher H_2/CO_2 ratios obtained for soluble cellobiose (1.15) compared with DLC (1.02) in high-substrate conditions.

Hydrogen yields for cellulosic substrates obtained from this study reached a maximum value of 1.55 ± 0.31 mol H_2/mol glucose. This value is comparable with many previous studies such as, yields of 1.7–1.9 mol H_2/mol hexose were achieved from pure sucrose was reported by Hussy *et al.* (2005) through nitrogen sparging. Batch fermentation of starch by *Clostridium beijerinckii* AM21B resulted in H_2 yields of 1.3–2.0 mol H_2/mol hexose (Taguchi *et al.* 1992) while a mixed microflora in sludge compost produced yields as high as 2.4 mol H_2/mol-hexose (Ueno *et al.* 1995).

Overall, the performance of insoluble cellulosic substrates was better than soluble cellobiose at 0.1 and 1.1 g/l concentrations. Under all conditions, delignified wood was found to be the best substrate for H_2 production.

Chapter 4: Metabolic Flux Analysis During Fermentation of Cellobiose by *C. thermocellum*

4.1 Introduction

C. thermocellum has been studied extensively for its potential to produce ethanol from cellulosic biomass (Weimer & Zeikus, 1977; Lamed & Zeikus, 1980; Lynd *et al.*, 1987, 1989). Information on carbon flow distribution among by-products (acetate, ethanol, lactate etc.) under various growth conditions (carbon-limited and carbon-excess) is necessary to control metabolic pathways and have not been investigated thoroughly. Moreover, no studies focusing on H_2-production by *C. thermocellum* have been conducted to date.

The impact of substrate type and initial-loading on fermentation end-products and hydrogen yields, described in previous section (Chapter 3), confirmed the necessity for a detailed investigation of the kinetics of cellulose fermentation. Because it is difficult to follow cell-growth and carbon flow regulation in cultures using pure cellulose or cellulosic materials as substrate, these initial experiments have used cellobiose, a soluble cellodextrin released during cellulose hydrolysis.

This part of the study was mainly aimed to investigate the effect of initial substrate (soluble) loading on cell-growth and carbon flow directed to various end-products and the effect on H_2-production by *C. thermocellum* strain 27405. This chapter includes results and analyses on production of H_2, CO_2, organic acids, and ethanol by *C. thermocellum* cultured on cellobiose at low-carbon (0.1 g l^{-1}), medium-carbon (1.1 g l^{-1}), and high-carbon (4.5 g l^{-1}) conditions.

Several hypotheses, based on earlier investigations and the results from Chapter 3, are as follows:

i. Generation time of cells on cellobiose will be approximately two hours

ii. Acetate will be the main product in low and medium-substrate cultures

iii. Pyruvate-overflow may occur in high-substrate cultures

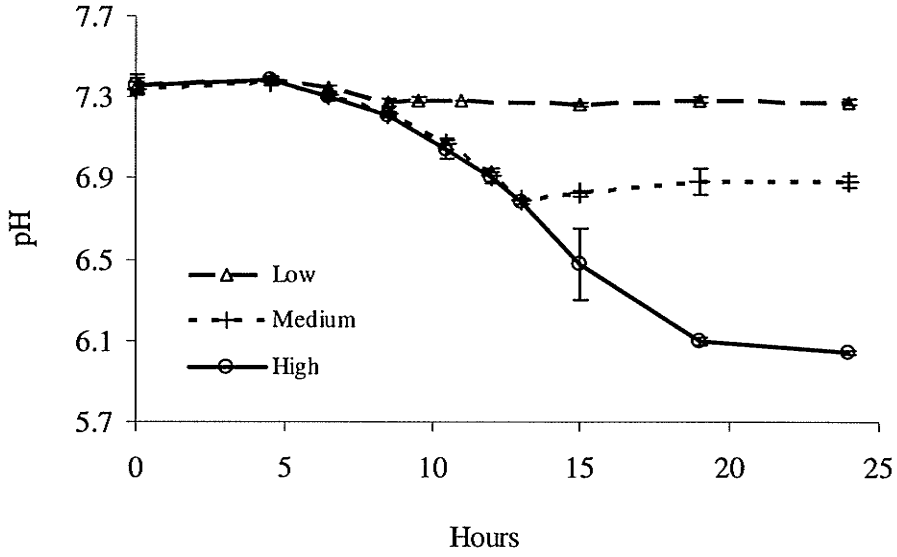
4.2 Materials and Methods

Material and methods for this experiment are described in sections 2.1-2.3 and 2.5- 2.7 of chapter 2 of this thesis.

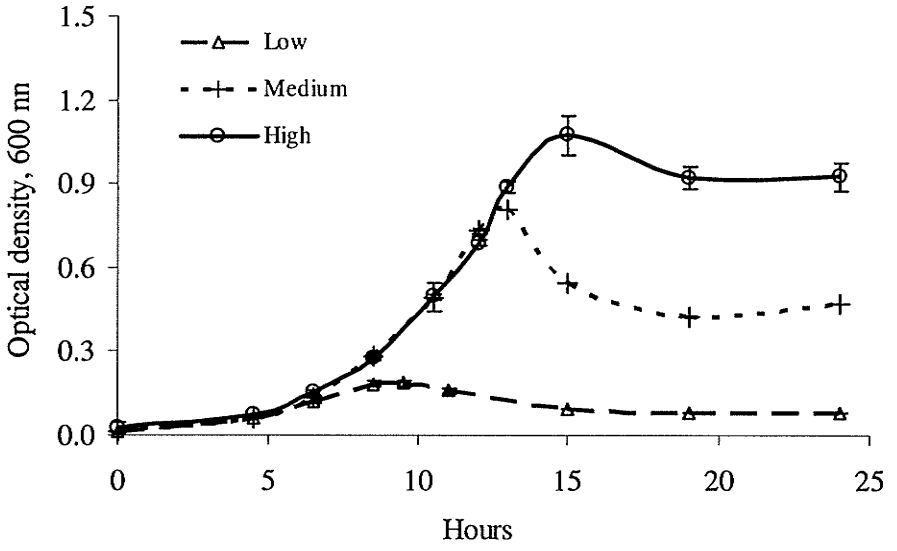
4.3 Results

4.3.1 Batch Fermentation of Cellobiose

Figure 4.1 (a and b) illustrates the growth of *C. thermocellum* under low (0.1g L^{-1}), medium (1.1g L^{-1}), and high-carbon (4.5g L^{-1}) conditions and the corresponding changes in pH. The growth rate of *C. thermocellum* was the same at all substrate concentrations (low, medium and high). The final population densities, however, were significantly affected by substrate availability. *C. thermocellum* grew to a very low density under low-substrate conditions and very little change in pH was observed. Medium-substrate conditions supported cell-growth to an OD_{600} of 0.8, accompanied by a rapid decline in pH to pH 6.7 at the end of growth. At high-substrate concentrations, cell-growth continued to a maximum OD_{600} of approximately 1.1, with a decline of pH to 6.48 as the culture reached stationary phase. An OD_{600} of 1.1 corresponds to a cell dry weight of 0.55 g L^{-1}. Fermentation, however, continued while the cells were in stationary phase, reducing the pH down even further (pH 6.04).



(a)

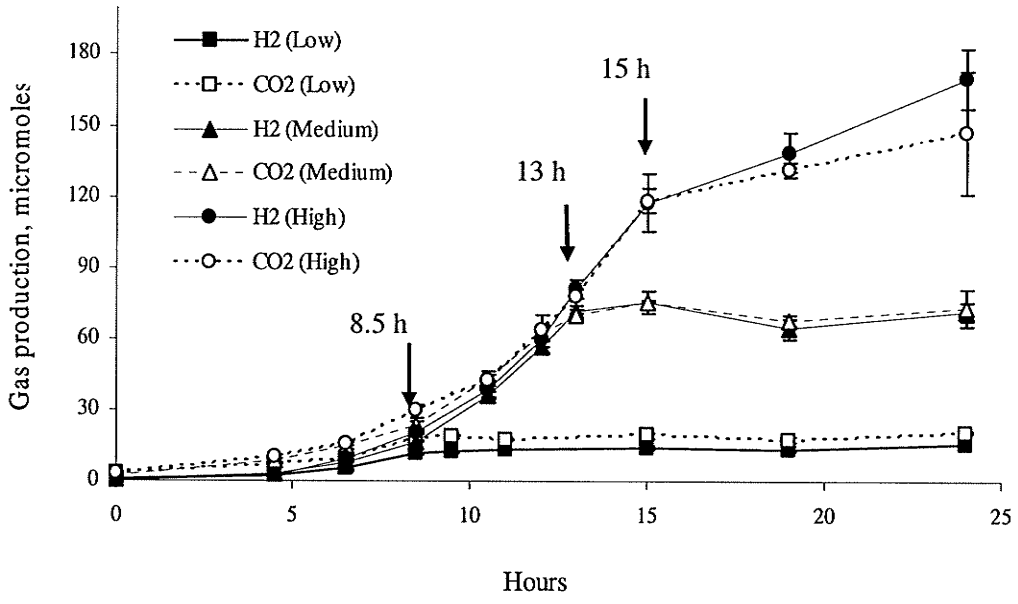


(b)

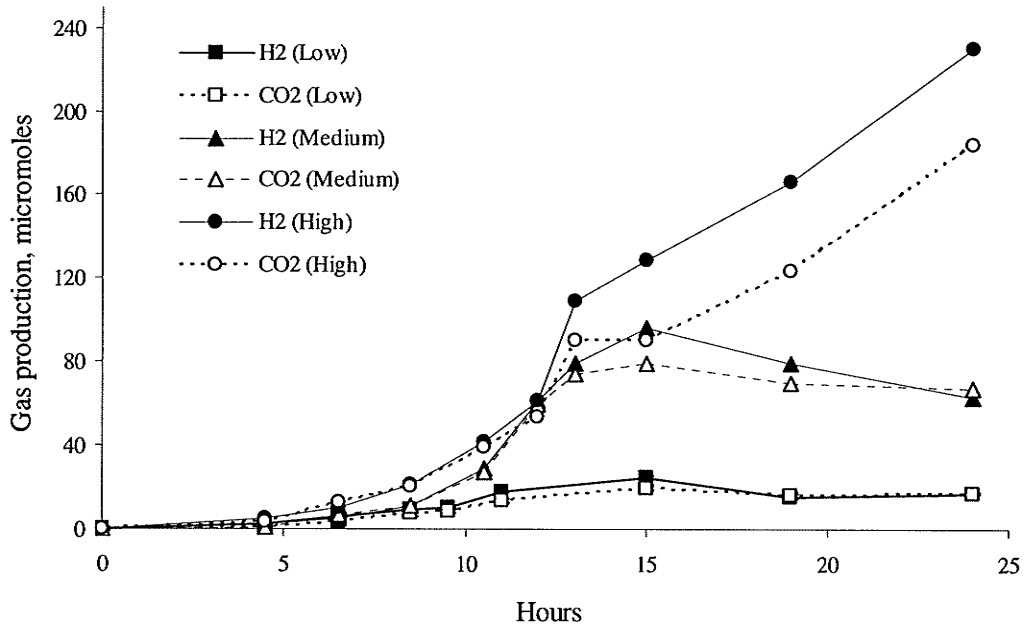
Figure 4.1 Growth, (a); and corresponding changes in pH, (b); when of *C. thermocellum* was cultured on cellobiose at low, medium and high substrate concentrations.

4.3.2 Gas Production

Figure 4.2 illustrates the total gas production (μmoles) measured (a) and estimated (b) per culture tube at different phases of growth. Production rate of both H_2 and CO_2 were unaffected at low, medium and high-substrate concentrations. Cessation of gas production correlated with termination of cell growth for both low and medium-carbon conditions. Under high-carbon conditions, H_2 and CO_2 production continued, even after the cells entered stationary phase. The rate of gas production during the stationary phase, however, was lower than during the exponential growth phase.



(a)



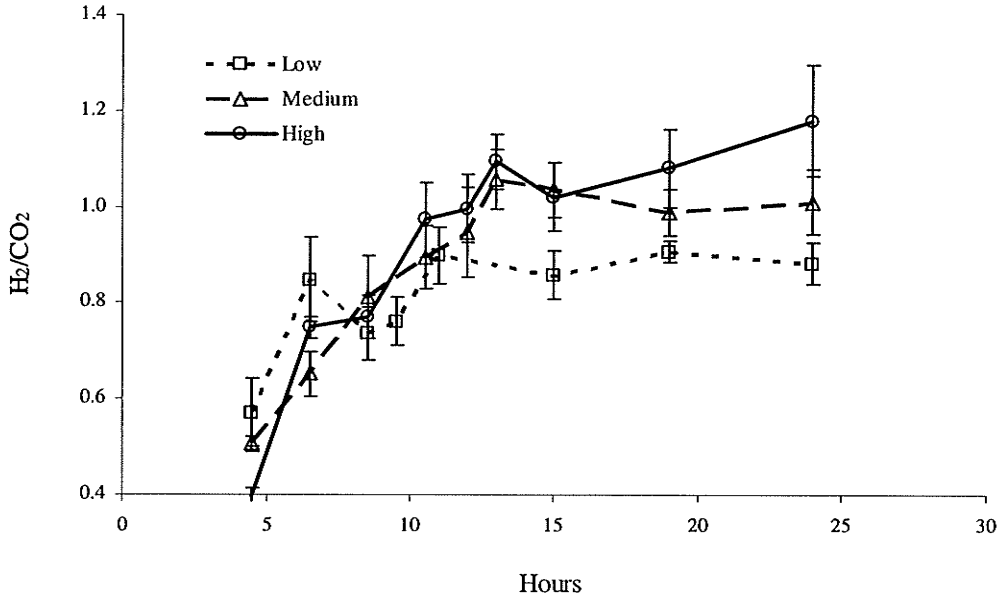
(b)

Figure 4.2 Cumulative H_2 and CO_2 production from batch cultures at low, medium and high-substrate concentrations. Data-points were obtained from (a) measurement of cumulative gas production and (b) estimated amounts based on fermentation end-products. Each time-point represents the total gas produced up to that point. Arrows of 8.5h, 13h and 15h are pointing to time-points when growth reached their stationary phases in low, medium and high-carbon cultures respectively.

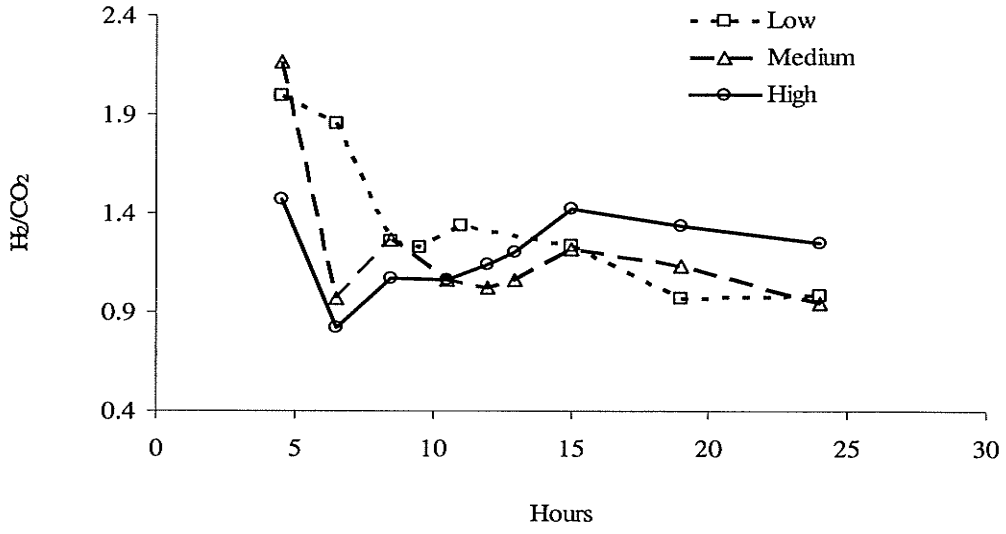
Calculated values of hydrogen were up to 46% higher than the measured values.

Figure 4.3(a) presents the ratios of H_2/CO_2 calculated from the total gas accumulation measured at each time-point for each substrate concentration. Apparently, the H_2/CO_2 ratios displayed an increasing trend during the exponential growth phase. Figure 4.3 (b), ratios based on estimated amounts of gases considering fermentation end products, presents high H_2/CO_2 values at the beginning of

exponential phase for all substrate concentrations. These ratios varied between 1 and 1.4 for the rest of fermentation period.



(a)



(b)

Figure 4.3 Effect of substrate concentration on H_2/CO_2 ratios. Ratios were obtained from cumulative gas production (a) measured and (b) estimated (based on fermentation end-products).

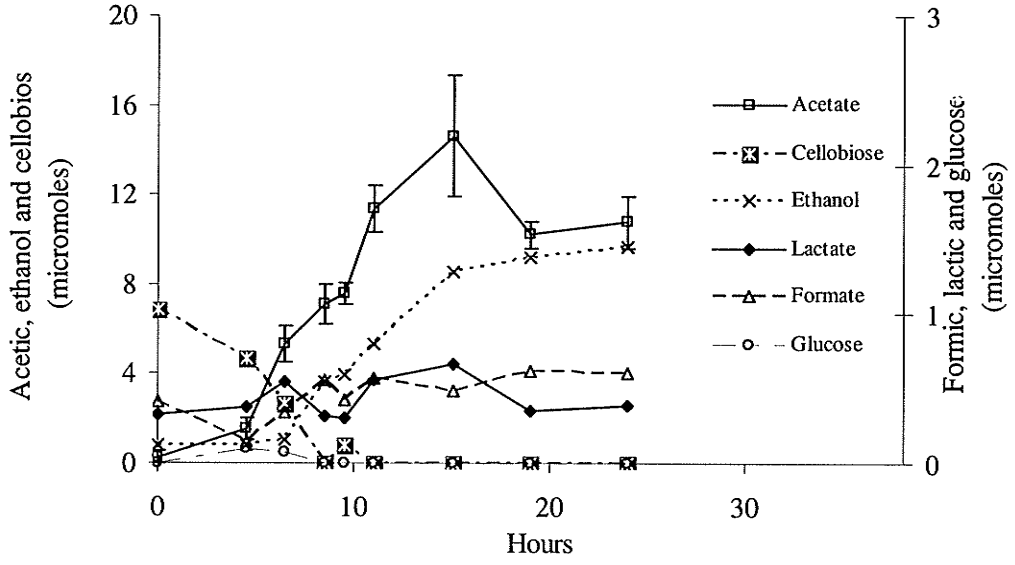
4.3.3 Production of Organic Acids and Ethanol

Acetate, formate, lactate and ethanol were major fermentation metabolites (Figure 4.3-a, 4.3-b, and 4.3-c). Under high-carbon conditions, pyruvic acid was also produced, starting when cells approached stationary phase. Under low-carbon conditions (Fig. 4.3-a), around 90% of the total carbon flow was directed toward production of acetic acid during early-exponential phase of growth which was reflected accordingly by H_2/CO_2 ratios estimated (Fig. 4.3-a) for that period. As cellobiose concentrations approached zero, 75% of carbon flow shifted from acetic acid to ethanol production. No significant increase in lactate or formate level was found above the amount transported with inocula.

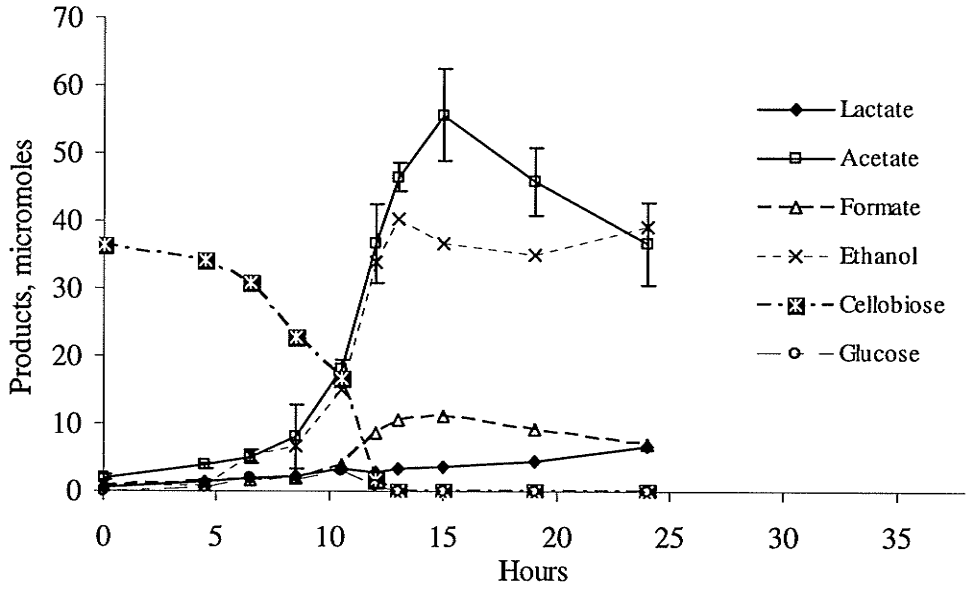
Under medium-carbon conditions (Fig. 4.3-b), measurable concentrations of glucose appeared during exponential phase. As the cells neared stationary phase, cellobiose was completely utilized, followed by a total consumption of the extra-cellular glucose. Acetate and ethanol production increased proportionally with the utilization of cellobiose and continued until cellobiose levels reached zero. High-substrate supported greater cell growth and carbon flow was directed to two primary end-products, acetate (47-58%) and ethanol (30-44%). The rest was distributed among formate (5 to 10%) and lactate (3-5%).

At high-substrate cultures (Fig. 4.3-c), carbon flow was converted to acetate (55% to 70%) and ethanol (30% to 55%) until the pH dropped below 7.0. Overall production of lactic acid and formic acid were very low during exponential phase, but increased dramatically and captured over 36% and 18% of carbon respectively as growth reached the stationary phase and the pH dropped below 6.7. Extracellular pyruvate production was observed at low concentrations but slowly increased with time of fermentation. A transient increase in glucose levels was also detected during

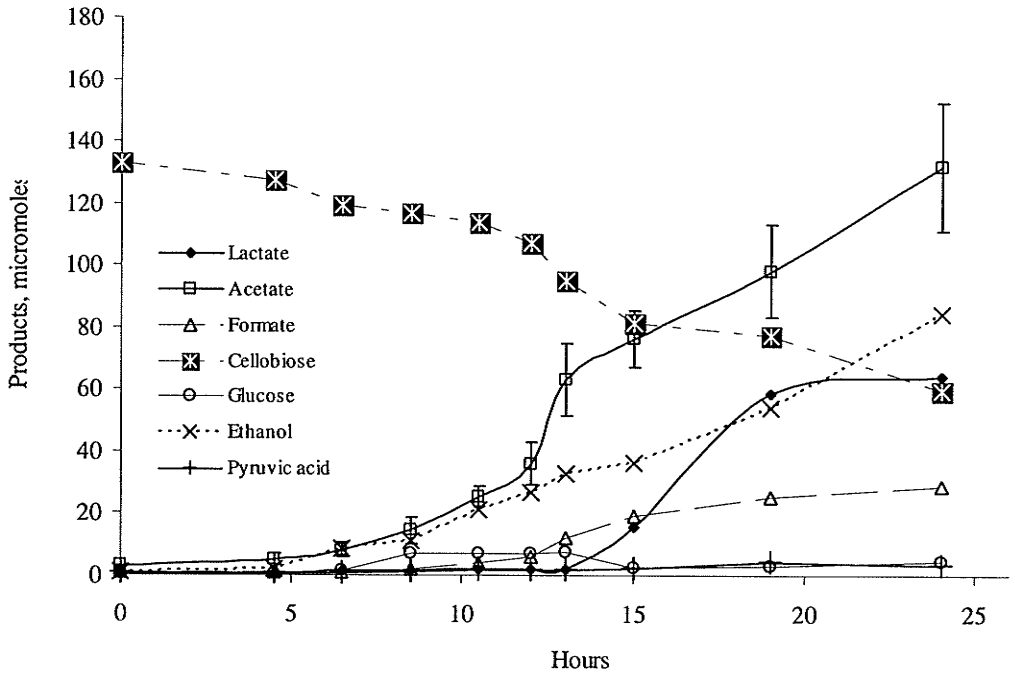
exponential-growth, decreasing, but not completely disappearing, as fermentation continued during stationary phase.


Acetic, ethanol and cellobios (micromoles)
Formic, lactic and glucose (micromoles)
Hours
Acetate
Cellobiose
Ethanol
Lactate
Formate
Glucose
0
4
8
12
16
20
0
1
2
3
0
10
20
30

(a)


Products, micromoles
Hours
Lactate
Acetate
Formate
Ethanol
Cellobiose
Glucose
0
10
20
30
40
50
60
70
0
5
10
15
20
25
30
35

(b)



(c)

Figure 4.4 Utilization of substrates and production of organic acids and ethanol by *C. thermocellum* under low (a), medium (b) and high (c) substrate concentrations.

4.3.4 Yields of Hydrogen Production

The effects of substrate concentration on cumulative H_2-yield by *C. thermocellum* culture are illustrated in Figure 4.5. The yield of H_2 was calculated on the basis of the total amount of H_2 (moles/mole of glucose) produced at a time point and the corresponding conversion of substrates up to that timepoint. Increase in H_2 yields during exponential growth showed a correlation with H_2/CO_2 ratios and varied around 1.0 mole H_2/mole of glucose from the mid-exponential growth phase. At high-substrate concentrations, however, H_2-production reached a bit higher in late exponential phase and a maximum of 1.4 mole H_2 /mole of glucose was obtained.

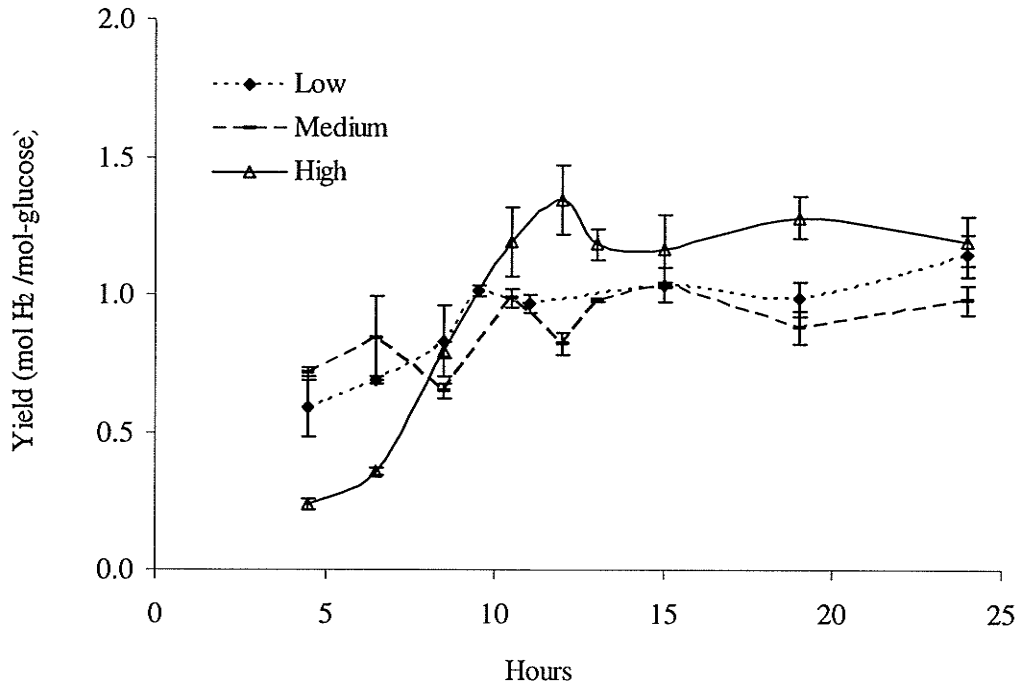


Figure 4.5 Yield of hydrogen obtained from *C. thermocellum* cultures at low (0.1 $g.l^{-1}$), medium (1.1 $g.l^{-1}$) and high (4.5 $g.l^{-1}$) cellobiose concentrations.

4.3.5 Specific H_2-Production Rates

Specific rates of H_2-production during fermentation of cellobiose by *C. thermocellum* at the three different initial substrate concentrations are illustrated in Figure 4.6. The specific H_2-production rates were calculated on the basis of the amounts of H_2-produced per hour between two consecutive time points and the corresponding cell dry weight at those time points. Yields were very similar for all conditions at the beginning of growth and were more or less constant during the exponential phase. However, the maximum specific H_2-production rates were observed as 8.4, 12.9 and 14.6 mmoles.g dry $weight^{-1}.h^{-1}$ for low, medium, and high substrate concentrations, respectively during late-log phase. These data are not contradictory to H_2/CO_2 ratios and yields from the same phase of growth.

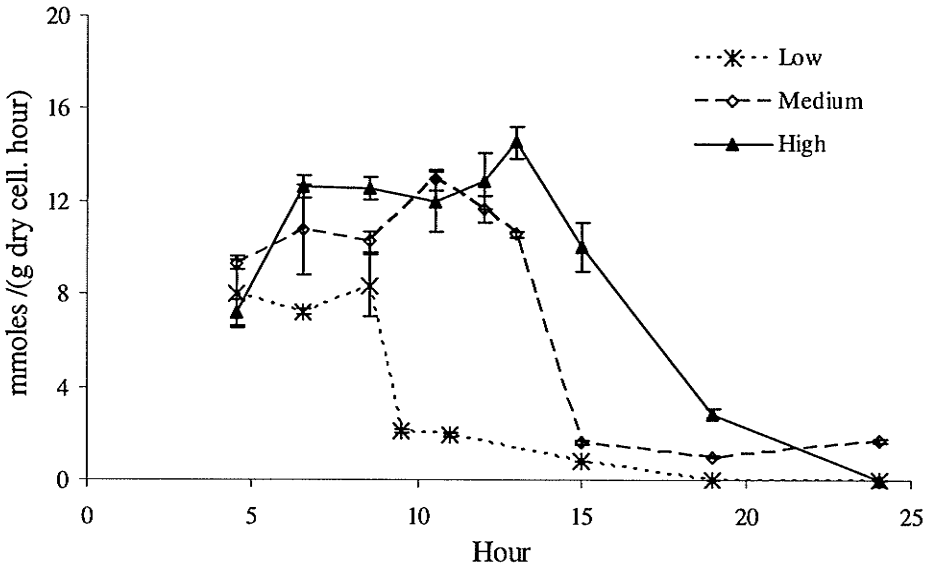


Figure 4.6 Specific rates of hydrogen production during fermentation of cellobiose by *C. thermocellum* at different initial substrate concentration.

4.3.6 Carbon Recovery and Redox Balance

For carbon balance during fermentation, all measurable end-products, substrates, and cell dry weight were considered (Table 4.1). Percentages of carbon recovery obtained varied between 80 to 112%. The oxidation/reduction (O/R) ratios were very close to 1 for the entire period of fermentation for high-carbon conditions, indicating no significant under-estimation of products. For low-carbon and medium-carbon conditions, higher O/R ratios (2 to 4) were obtained for the first two time-points (during early exponential phase, data not shown). Such results are most likely caused by the fact that the amounts of H_2 produced in the early stages of fermentation (in case of low and medium substrate concentrations, in Fig. 4.2) were two- to three-fold lower than the values predicted on the basis of theoretical amounts calculated (data not shown) and the O/R balance of organic product measured. The under-

estimation of H_2 produced during the early exponential phase could partly be attributed to the reduced sensitivity of the gas analysis tools for low concentration gas mixtures.

Table 4.1 Calculated percentages of carbon recovery and oxidation/reduction (O/R) index based on fermentation end-products, residual sugars and cell biomass

Conditions	Acetate	Formate	Lactate	Pyruvate	H_2	CO_2	Ethanol	Glucose equivalent Utilized	Cell biomass	%C recovery	O/R index
	µmoles produced per 11 ml of culture							µmoles	µmoles[a]		
Low	7.07	0.55	0.31	0	11.43	18.30	5.64	13.88	10.22	92.65	1.37
Medium	36.75	8.67	2.67	0	56.61	62.47	33.83	36.52	40.60	85.59	1.04
High	63.15	12.20	1.92	2.14	81.36	77.56	32.98	68.69	49.35	93.20	1.09

Notes:

Presented data are from late exponential phases of all conditions (8.5h, 12h and 13h time-points of low, medium and high substrate concentrations, respectively).

[a]Cell-biomass was calculated based on the elemental composition of cells ($C_4H_7O_2N$) which corresponds to a molecular weight of 101 g mol^{-1}.

4.4 Discussions

This chapter presents data on gas production (H_2 and CO_2) and end-product synthesis by *C. thermocellum* from cultures containing low (0.1 g/l), medium (1.1g/l) and high (4.5 g/l) concentrations of cellobiose. It was noticed that cell growth and product synthesis stopped as soon as substrate was consumed completely in 0.1 g/l cellobiose. At 1.1 g/l cellobiose, substrate was exhausted as cell growth reached stationary phase. At 4.5 g/l batch, cell growth reached stationary phase before the substrate was exhausted. Based on these observations, cultures of 0.1 g/l concentrations can be defined as substrate-limited, those with 1.1 g/l as substrate-sufficient and those with 4.5 g/l as substrate-excess conditions.

There is evidence suggesting that metabolic flux responses are influenced by the rate of carbon assimilation, changes in pH and redox potential (Sridhar & Eitman, 2001), and accumulation of metabolic end-products (Desai *et al.*, 1999). In *Clostridium* species cultured under carbon-limiting conditions, catabolism is tightly coupled to anabolism, resulting in high biomass yields (Russel & Cook, 1995; Dauner *et al.*, 2001a,b). Carbon-excess conditions, on the other hand, can result in high rates of carbon flux through catabolic pathways, low biomass yields with low energetic growth efficiencies (Neijssel *et al.*, 1996; Teixeira de Mattos & Neijssel, 1997), and "overflow metabolism", where carbon or electron flow are shifted to less efficient pathways and/or "energy-spilling" reactions (Russel & Cook, 1995; Neijssel *et al.*, 1996; Teixeira de Mattos & Neijssel, 1997).

Metabolic flux has been studied in *C. cellulolyticum*, a mesophilic, cellulose-degrading anaerobe. In continuous cultures with cellobiose, excess carbon flowed to

glycogen (overflow storage compounds) through Gluc-1-P, while excess carbon from Gluc-6-P stimulated the production of a variety of alternative end-products including pyruvate, lactate, and ethanol (Guedon *et al.*, 1999a, 1999b, 2000). High carbon flow into cells resulted in high NADH/NAD+ ratios (>1.5), which were correlated with inhibition of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and low growth rates (Payot *et al.*, 1998). When *C. cellulolyticum* was cultured with cellulose (carbon-limiting conditions), on the other hand, the NADH/NAD+ ratio was always < 1.0, the major fermentation product was acetate (with H_2 evolution), and ATP synthesis was maximized (Desvaux *et al.*, 2001a,b). Expression of exogenous pyruvate decarboxylase and alcohol dehydrogenase in recombinant *C. cellulolyticum* shifted metabolic flux toward acetate and resulted in both increased acetate synthesis and increased cellulose consumption (Guedon *et al.*, 2002).

We see a different pattern of metabolic flux in response to carbon availability in *C. thermocellum*. Unlike *C. cellulolyticum*, cell densities and growth rates of *C. thermocellum* were unimpaired in the presence of carbon-excess, and carbon flow (>60%) was directed to acetic acid synthesis with concomitant release of H_2, and very little carbon was directed to over-flow molecules during exponential phase. Formic and lactic acid were produced at low levels, which increased slowly with the time of fermentation, until the cellobiose was completely used or as cells approached stationary phase. Also glucose was observed transiently during exponential phase, which decreased again as cells approach stationary phase.

H_2/CO_2 ratios showed an increasing trend over the exponential growth period which was contradictory to calculated H_2/CO_2 values based on amounts of end-products

(acids and ethanol) formed. Poor mass transfer rates of H_2 from liquid to gas phase (Pauss *et al.* 1990) could be responsible for this which is also correlated to the rate of gas production in solution.

Detection of formic acid was an unexpected result. The presence of formate as a fermentation product of *C. thermocellum* has not been reported previously (Patni & Alexander, 1971a,b; Ng *et al.*, 1977; Weimer & Zeikus, 1977; Lamed & Zeikus, 1980; Lynd *et al.*, 1987, 1989). Nevertheless, DNA sequences consistent with the presence of a formate-producing pathway (using pyruvate-formate-lyase) are present in the genome of *C. thermocellum* (DOE, 2003). Since formate-synthesis competes stoichiometrically with H_2-synthesis, its presence in significant amounts has an impact on total H_2-production.

The specific H_2-synthesis rates were found directly related to the availability of substrate as they reached their highest maximum (14.6 mmoles.g dry weight^{-1}.h^{-1}) under substrate-excess culture compared to substrate-sufficient limited (12.94 mmoles.g dry cell^{-1}.h^{-1}) and substrate-limited (8.38 mmoles.g dry cell^{-1}.h^{-1}) cultures. This high rate dropped sharply under substrate-excess conditions as the H_2 partial pressures (pH_2) increased to 17.8 kPa (measured at 19°C) and at 60°C, these pH_2 were over 57 kPa. Continuous H_2-synthesis requires the partial pressure of H_2 to be < 50 kPa at 60 °C (Lee & Zinder, 1988). At H_2 partial pressures > 50kPa, H_2 inhibits NADH reoxidation, when electron flow via ferredoxin becomes thermodynamically unfavorable. Thus, high H_2 partial pressures may be a principle reason for decrease in H_2 synthesis rates under substrate-excess conditions, where no carbon availability was not limited.

CHAPTER 5: Metabolic Flux Analysis During Fermentation of α-Cellulose by *C. thermocellum*

5.1 Introduction

Chapter 4 provided basic information on metabolic flux of *Clostridium thermocellum* 27405 during the growth on soluble substrate (cellobiose). It also provided an insight to what to expect from cellulose utilization by this organism as cellobiose is one of the major soluble products from cellulose hydrolysis. Therefore, experiments were conducted with commercially available crystalline cellulose (α-cellulose) which is closely related to substrates found in the natural ecosystem of *C. thermocellum.*

The goal of this study were to observe the effect of α-cellulose loading on, i) growth rates, ii) H_2 production potential, and iii) carbon flow distribution among various end products produced by *C. thermocellum.*

Based on previous results several hypotheses can be derived for this experiment. Compared with parameters of cellobiose fermentation by *C. thermocellum,*

i. fermentation end products on α-cellulose will be similar,

ii. growth rates and gas-production rates will be slower on α-cellulose, and

iii. amounts of gases and end-products will be higher

5.2 Materials and Methods

Description of materials and methods used for this set of experiments are included in the method sections (sections 2.1-2.3, 2.5 and 2.6 in Chap-2).

5.3 Results

5.3.1 Cell Growth

Batch fermentation experiments of α-cellulose were carried out under limited (0.1 g/L), sufficient (1.1 g/L) and excess (4.5 g/L) initial concentrations. Cell growth rates (Figure 5.1) were found independent of initial substrate concentrations and only final total protein amounts differed significantly depending on the substrate availability. Each time point was collected from an average of triplicates which were within acceptable range of error.

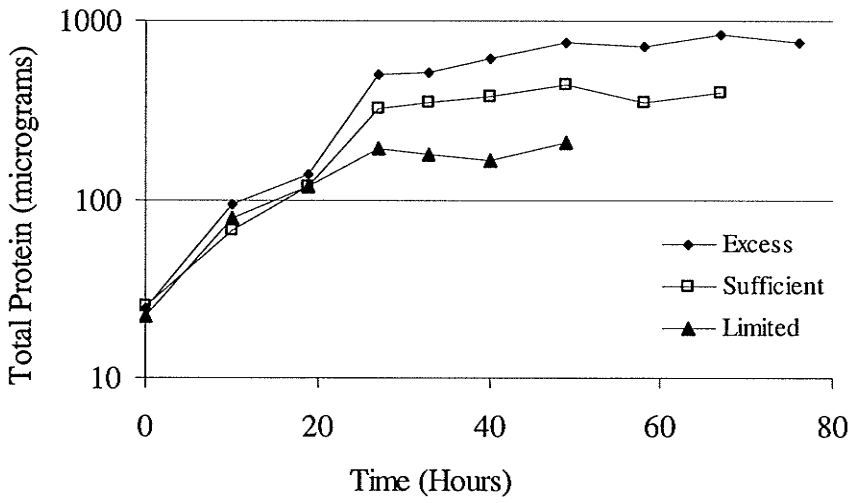


Figure 5.1 Growth of *C. thermocellum* under different initial concentrations of α-cellulose. Total protein in 10 ml of culture was quantified in micrograms (μg) at each time point of the growth curve.

Doubling time during exponential growth of cells varied between 3.5 (early-exponential phase) and 6 hours (late-exponential phase). Substrate-limited culture had a very low growth (maximum = 200 μg protein/culture) compared to cultures under substrate-excess (maximum = 835 μg protein/culture) where growth was terminated by other factors. Declining pH (Fig. 5.2) was also observed as an indicator of growth.

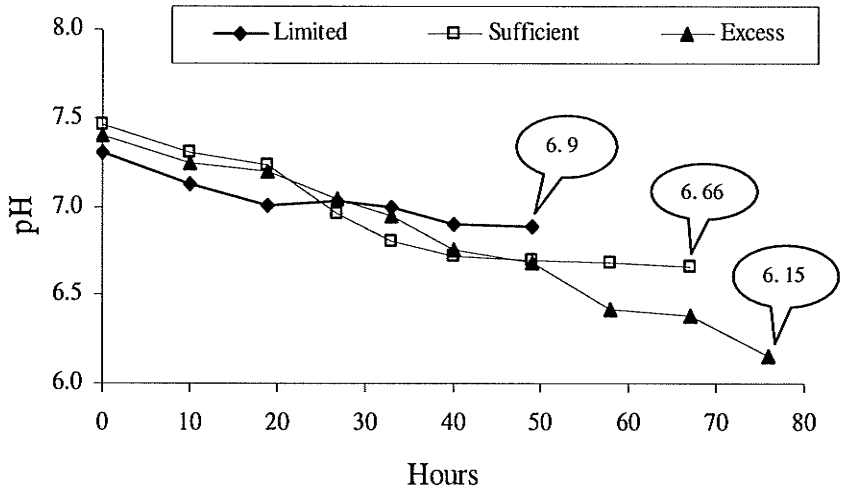


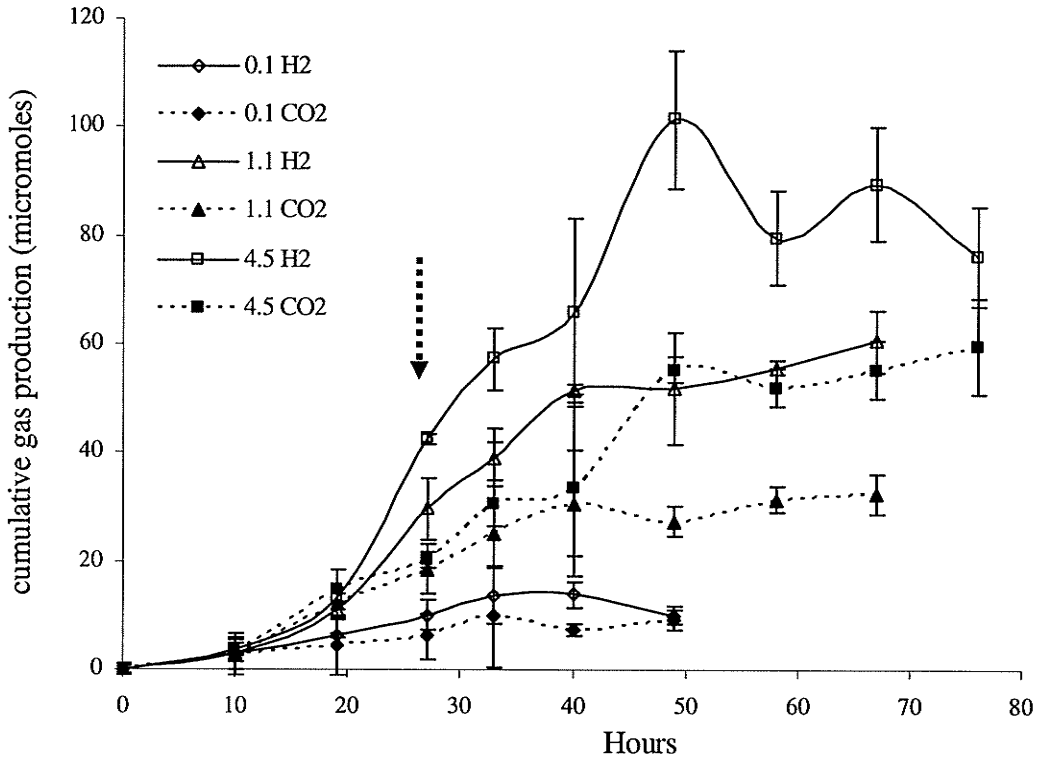
Figure 5.2 Change in pH values with the growth of *C. thermocellum* under different initial cellulose concentrations.

Only a small change in pH occurred in substrate-limited cultures compared to substrate-excess cultures where pH values dropped to near 6. The onset of stationary phase correlated with a pH dropped below 7 in all cultures.

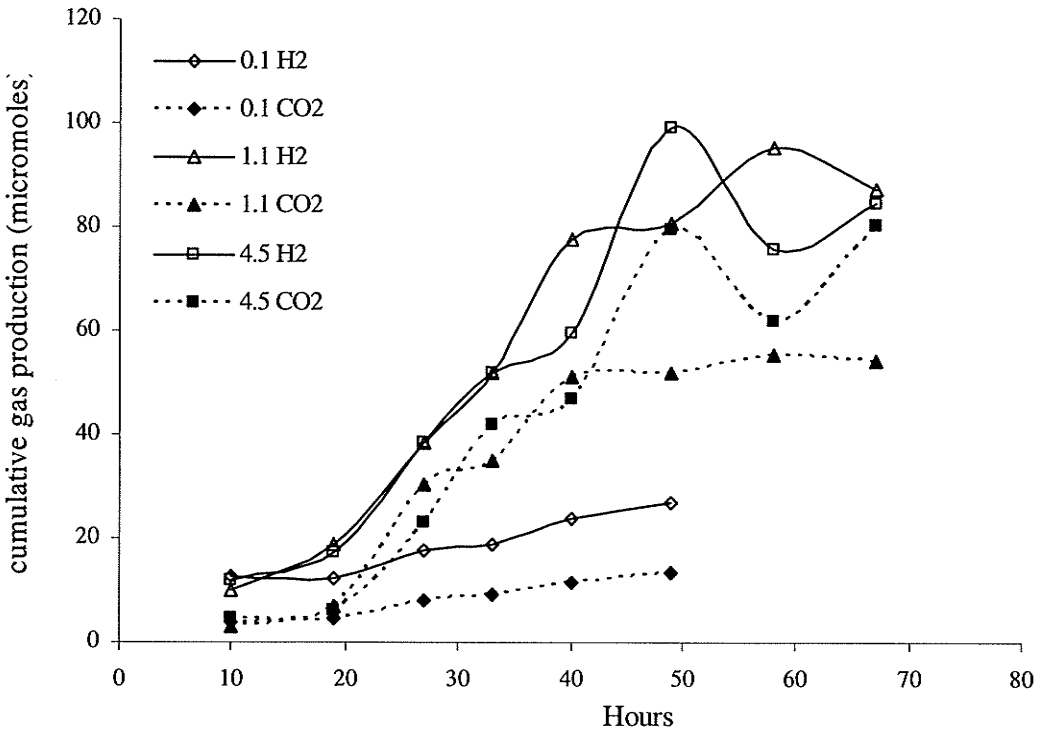
5.3.2 Gas Production

Figure 5.3 (a and b) presents the cumulative production of H_2 and CO_2 during α-cellulose fermentation until each time-point samples were collected. During the early-log growth, rates of gas production were not influenced by substrate concentrations. In substrate-limited conditions, gas production stopped as soon as the growth ceased. In substrate-sufficient cultures, gas production decreased with the cessation of growth but continued until the substrate was completely depleted. In substrate-excess cultures, cumulative H_2 production continued even after growth reached stationary phase, and

leveled off as pH dropped below 6.7. Estimated quantities of H_2 were 10 to 55% higher than measured quantities. For CO_2 this underestimation ranged from10 to 25%.



(a)

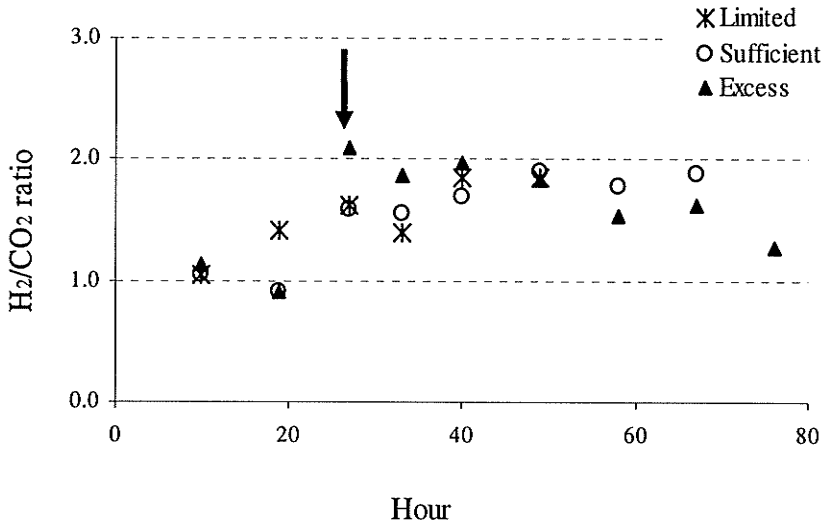


(b)

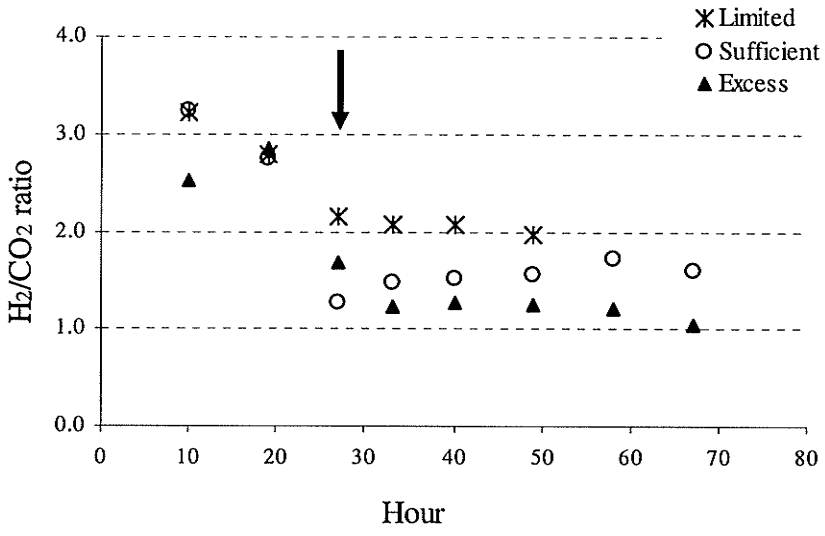
Figure 5.3 (a), measured and (b), estimated H_2 and CO_2 produced from fermentation of α-cellulose under substrate-limited, substrate-sufficient and substrate-excess conditions. Cell-growth entered stationary phase 27 hour after fermentation started (marked with the broken arrow). Gas amounts are expressed in total accumulation up to each time-point from 10 ml of *C. thermocellum* culture.

Ratios of H_2/CO_2 (Figure 5.4-a, b) were calculated from gas measured (a) from the headspace of each culture, considering dissolution of H_2 and CO_2 in water and estimated (b) based on end-products of fermentation. Ratios (Figure 5.4-a) varied from 0.9 to 2.1 depending on the end-products of fermentation. Increasing trends were observed for substrate-limited and substrate-sufficient conditions. Ratios of estimated gases (Figure 5.4-b) were well above 2 at the early exponential phase of growth which

could be an effect of formate production. In stationary phase they decreased to ≈ 2 for substrate-limited, ≈ 1.5 for substrate-sufficient and ≈ 1.2 for substrate-excess batches.



(a)



(b)

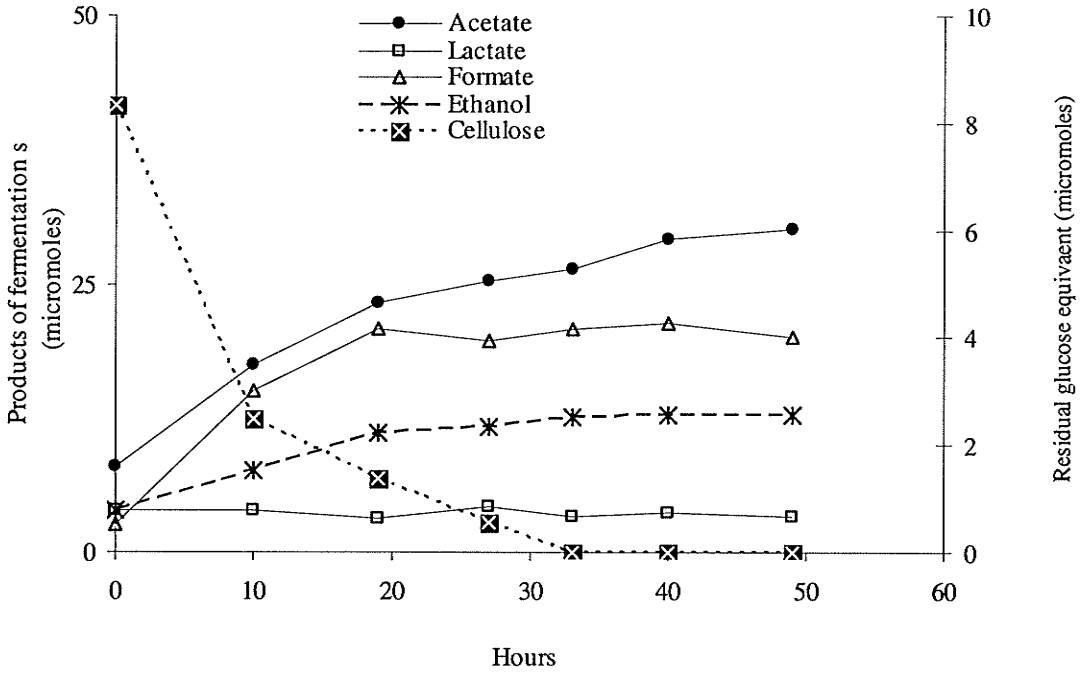
Figure 5.4 H_2/CO_2 ratios observed in *C. thermocellum* cultures under different substrate conditions. Ratios were calculated based on cumulative amounts of product-gas (a)

measured and (b) estimated. The arrow is pointing to the time-point when cell-growth entered stationary phase.

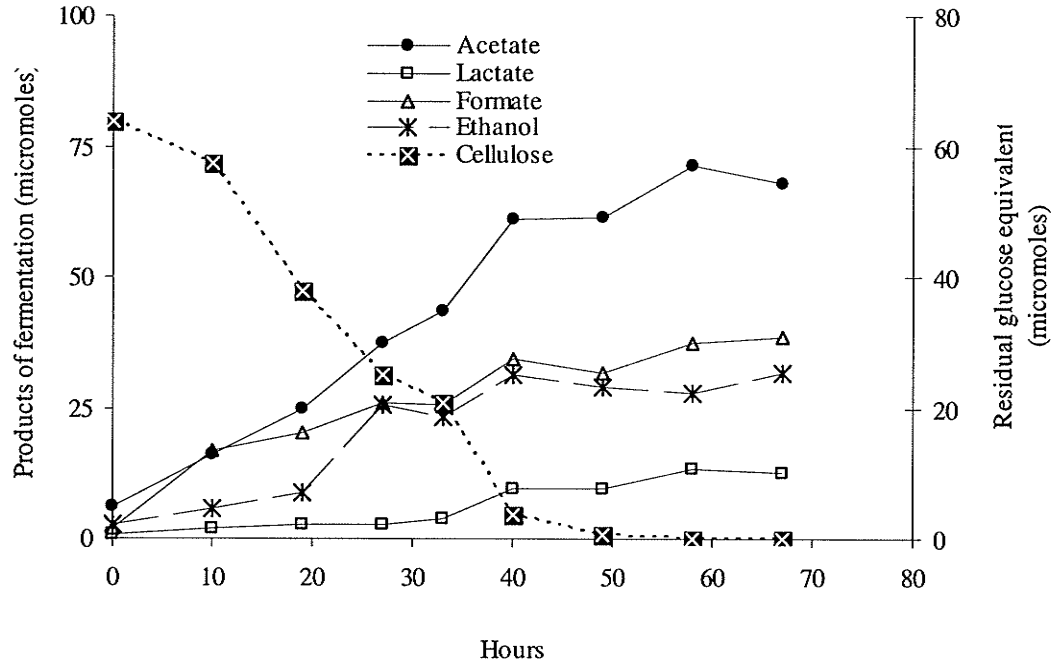
Observed amounts of product gases (H_2 and CO_2) quantified were found lower than amounts calculated based on fermentation end product measured and were very significant for hydrogen. In limited cultures, measured H_2 was found to correspond only up to 40% of total H_2 produced (calculated based on end-products of fermentation).

5.3.3 Acids and Alcohol Production

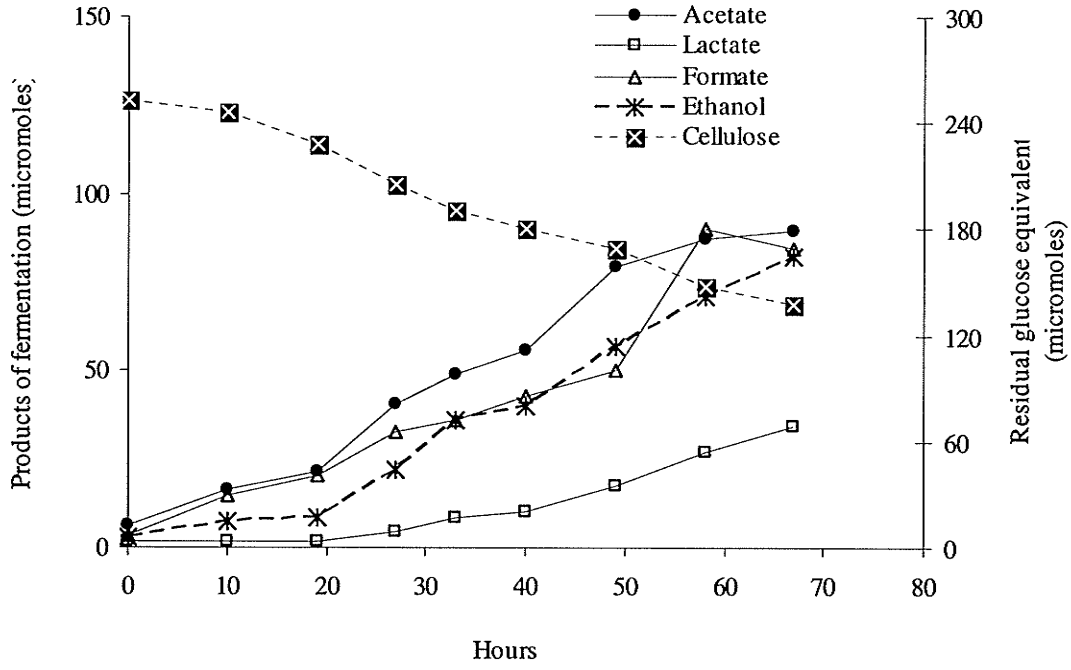
Acetate, formate, ethanol and lactate were major fermentation end products of *C. thermocellum* for various concentrations of α-cellulose (Figure 5.5 a, b and c).



(a)


Acetate
Lactate
Formate
Ethanol
Cellulose
Products of fermentation (micromoles)
Residual glucose equivalent (micromoles)
Hours
0
25
50
75
100
0
20
40
60
80
0
10
20
30
40
50
60
70
80

(b)


Acetate
Lactate
Formate
Ethanol
Cellulose
Products of fermentation (micromoles)
Residual glucose equivalent (micromoles)
Hours
0
50
100
150
0
60
120
180
240
300
0
10
20
30
40
50
60
70
80

(c)

Figure 5.5 End-products of batch fermentation of α-cellulose by *C. thermocellum* under limited (a), sufficient (b) and excess (c) substrate conditions. Amounts of products are expressed in total micromoles produced per 10 ml of culture.

Under substrate-limited conditions, acetate and formate were major soluble end-product comprising more than 80% of the product stream. A small increase in ethanol level was detected after growth stopped but no lactate production was detected. The amount of lactate detected was most likely transferred with inoculums. Over 90% of added cellulose was consumed during exponential-growth of cells.

Carbon flow in substrate-sufficient cultures was mainly directed to acetate and formate (almost 70%) production during log-growth phase while ethanol captured the rest. During the late log period, formic acid production leveled off and lactate appeared as a fermentation end-product. Carbon flow diverted away from acetate in stationary period and ethanol contributed up to 57% to total products and substrate approached zero.

In cellulose-excess cultures acetate and formate were the major products (70% to 80%) during exponential-growth phase. During late log phase ethanol production increased sharply and diverted the product stream away from acetic acid by 40% to 50%. Lactate formation was not detected during log-growth but initiated at the onset of late log phase and increased until the end of fermentation process. Overall consumption of initially added α-cellulose was close to 50% by the end of fermentation period. Rates of cellulose consumption with time were unaffected by different initial concentrations.

As no significant amount of glucose or cellobiose were detected in later time-point samples (49 and 58 hours). It was assumed that soluble sugar was consumed by cells as soon as they were produced from α-cellulose hydrolysis during fermentation.

5.3.4 Specific Hydrogen Production Rates

Figure 5.6 illustrates the specific rates of H_2 production under different initial substrate concentrations. Specific rates were calculated based on differential production of hydrogen between two time points and the average amount of cell dry weight at those time points. For all conditions, the maximum specific rates of hydrogen production occurred during early-exponential phase of growth.

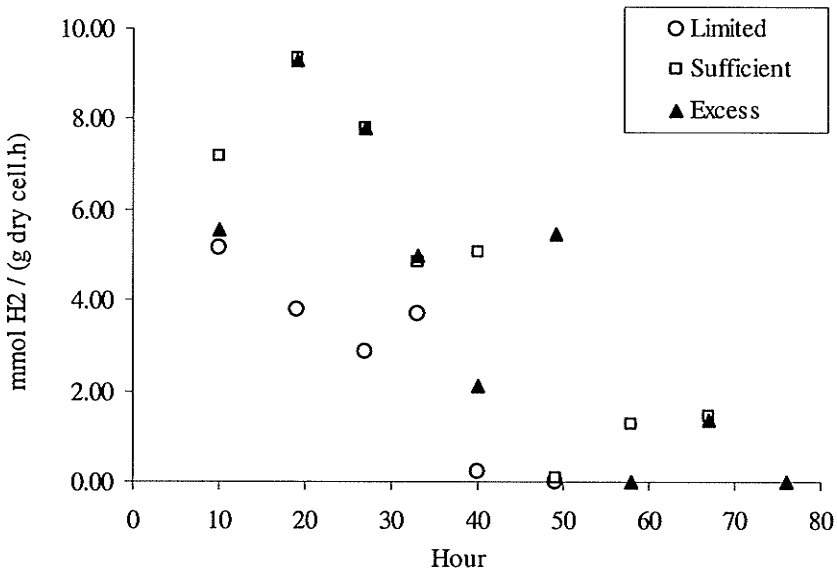


Figure 5.6 Effect of different substrate concentrations on specific rates of H_2-production from α-cellulose fermentation by *C. thermocellum.* Experimental errors were within acceptable statistical ranges.

A lower maximum specific hydrogen production rate of 5.17 mmol H_2 / (g dry cell .h) was obtained under substrate-limited conditions. The highest maximum rate, 9.35 mmol H_2 / (g dry cell .h), was observed in substrate-sufficient cultures which was similar to the maximum rate (9.31 mmol H_2 / (g dry cell .h)) obtained in substrate-excess conditions. These rates dropped sharply as the cell-growth reached stationary phase.

5.3.4 Oxidation-Reduction (O/R) Values

Oxidation-reduction potentials were calculated based on fermentation end-products (Table 5.1). O/R values ranged between 1.32- 2.22, during mid-exponential growth phase for all substrate-concentrations. This may have resulted from the underestimation of total hydrogen production which affects all results, most significantly at the beginning of growth.

Table 5.1 Oxidation-reduction ratios obtained under various substrate conditions

Substrate conditions	Lactate	Acetate	Formate	Ethanol	H_2	CO_2	Glucose equivale-nt utilized	O/R index
	Total µmoles per culture							
Limited	0	15.33	18.30	7.4	6.15	4.33	8.31	1.12
Sufficient	0.01	37.64	23.35	20.81	39.13	25.10	43.15	0.88
Excess	4.82	42.56	33.19	33.31	57.24	30.60	61.96	0.8

Notes:

Presented data represent late exponential phases of all conditions (19h, 27h and 33h time-points of limited, sufficient and excess conditions, respectively).

5.4 Discussions

Presented data indicate that *C. thermocellum* strain 27405 are able to efficiently maintain carbon flow without significant overflow to extracellular pyruvate under even substrate-excess conditions. Carbon flow distribution into various end products at different stages of growth was influenced by initially added substrate concentrations. Acetate and formate were major fermentation end-products and their synthesis was correlated with hydrogen production under different initial substrate conditions. Formic acid was found to be the second major product during cellulose fermentation. This is a noel discovery as formate production has not been reported in earlier studies performed with *C. thermocellum* (Ng *et al.*, 1977; Weimer & Zeikus, 1977; Lamed & Zeikus, 1980; Lynd *et al.*, 1987, 1989, Zhang and Lynd 2005a).

Cell-growth rates were not affected by various initial cellulose concentrations and only final cell densities were linked to the availability of substrate. This is not surprising because conversion of insoluble cellulose has been determined to be the rate–limiting factor (Pavlostathis *et al.* 1988). Substrate-excess cultures supported a much higher final density of cells compared to substrate-limited and substrate-sufficient conditions. For initial cellulose concentrations below 6.7 g liter^{-1}, accessible sites for cellulases decreased as the cellulose-substrate is hydrolyzed (Desvaux *et al.* 2000). This may be a reason for identical cellulose degradation rates observed under limited (0.1g l^{-1}), sufficient (1.1g l^{-1}) and excess (4.5 g l^{-1}) conditions.

Acetate and formate were mainly produced in cellulose-limited cultures. Half of the maximum theoretical amount of hydrogen was lost due to formic acid production.

Carbon flow shifted from acetate to ethanol production significantly in substrate-sufficient and substrate-excess cultures during late exponential phase.

Significant differences between measured and calculated amounts of H_2 were detected which suggest the culture medium was supersaturated with H_2. Supersaturaion of H_2 was observed in earlier studies of *C. thermocellum*. H_2 trapped between cells and settled cellulose was assumed to be responsible for super-saturation of culture with H_2 in unstirred conditions and resulted higher ethanol/acetate ratios (Lamed et al. 1988). The underestimated amounts of H_2 found in these studies were very similar to the present study. Low mass transfer rates of poorly soluble gas like hydrogen can also contribute to supersaturation (Pauss *et al.* 1990). Underestimation of product gases caused by supersaturation and trapping could have resulted O/R ratios significantly higher than 1.0 obtained during exponential cell-growth even after considering high formic acid production.

Specific rates of hydrogen synthesis reached their maximum values during exponential growth for all initial cellulose concentrations. This was quite expected as the fermentation parameters such as pH, H_2 partial pressure, and concentration of products were favorable for cell-growth and H_2-production thermodynamics. As fermentation progressed, high H_2 partial pressures and hydrogen super-saturation increased, resulting in decreased and eventually cessation of H_2 synthesis. Substantial improvements in the rates and yields of H_2 production from cellulose fermentation may be possible by rapid removal of product gases from the bioreactor.

CHAPTER 6

6.1 General Discussions

Substantial amounts of H_2-production from cellobiose (Chap. 4) and α-cellulose (Chap. 5) confirmed that the strain of *Clostrudium thermocellum* 27405 used has the ability to synthesize hydrogen from cellulose-substrates representing cellulosic biomass. As expected from published results, with a very active cellulase system, *C. thermocellum* was able to hydrolyze and metabolize insoluble crystalline cellulose. High H_2-production was observed from both cellobiose, a major product of cellulose hydrolysis, and from insoluble α-cellulose under various initial substrate conditions. This suggests potential future success for *C. thermocellum* fermentation processes to convert cellulosic waste materials to hydrogen gas.

Considerable differences in fermentation parameters during batch cultivation of *C. thermocellum* with cellobiose and α-cellulose were observed; including such as cell-growth rates, pH, amounts of product formed and carbon flow distribution. This is consistent with the assumption that cellobiose is not the only and major soluble hydrolysis product (Zhang and Lynd 2005a) during growth on cellulose cellulose as considered by other studies (Ng and Zeikus 1982; Strobel 1995; Strobel *et al.* 1995). A more recent study concluded that *C. thermocellum* assimilates cellodextrines with $n \approx 4$ when grows on cellulose (Zhang and Lynd 2005b). Thus, these variations may be attributed to the direct uptake of longer chain polysaccharides. Also the effective concentration of soluble substrate is much lower when cells are growing on cellulose as noted in Chapter 5.

Detection of formic acid as a major fermentation end-product was a novel discovery. Presented data indicate that carbohydrate utilization patterns and metabolite synthesis by *C. thermocellum* using cellobiose and cellulose are different. The presence of a gene encoding formate pyruvate lyase in the *C. thermocellum* genome database is in compliance with formate production (Figure 6.1). this is surprising since formate generation would have been predicted on the basis of the genome sequence from this strain.

Minimum generation time of cells was much faster on cellobiose (1.5 hours) than that on cellulose (6 hours), which are shorter than the minimum doubling times observed by Weimer and Zeikus (1977). Shorter doubling times (3.5 hours), at the beginning of log-growth on cellulose, could be an effect of soluble sugars transported to cellulose-cultures with inoculums. The change in pH was negligible (from 7.35 to 7.2) in cellobiose-limited cultures where as in cellulose-limited cultures displayed greater changes in pH (from 7.4 to 6.9) as more acidic-end-products were produced.

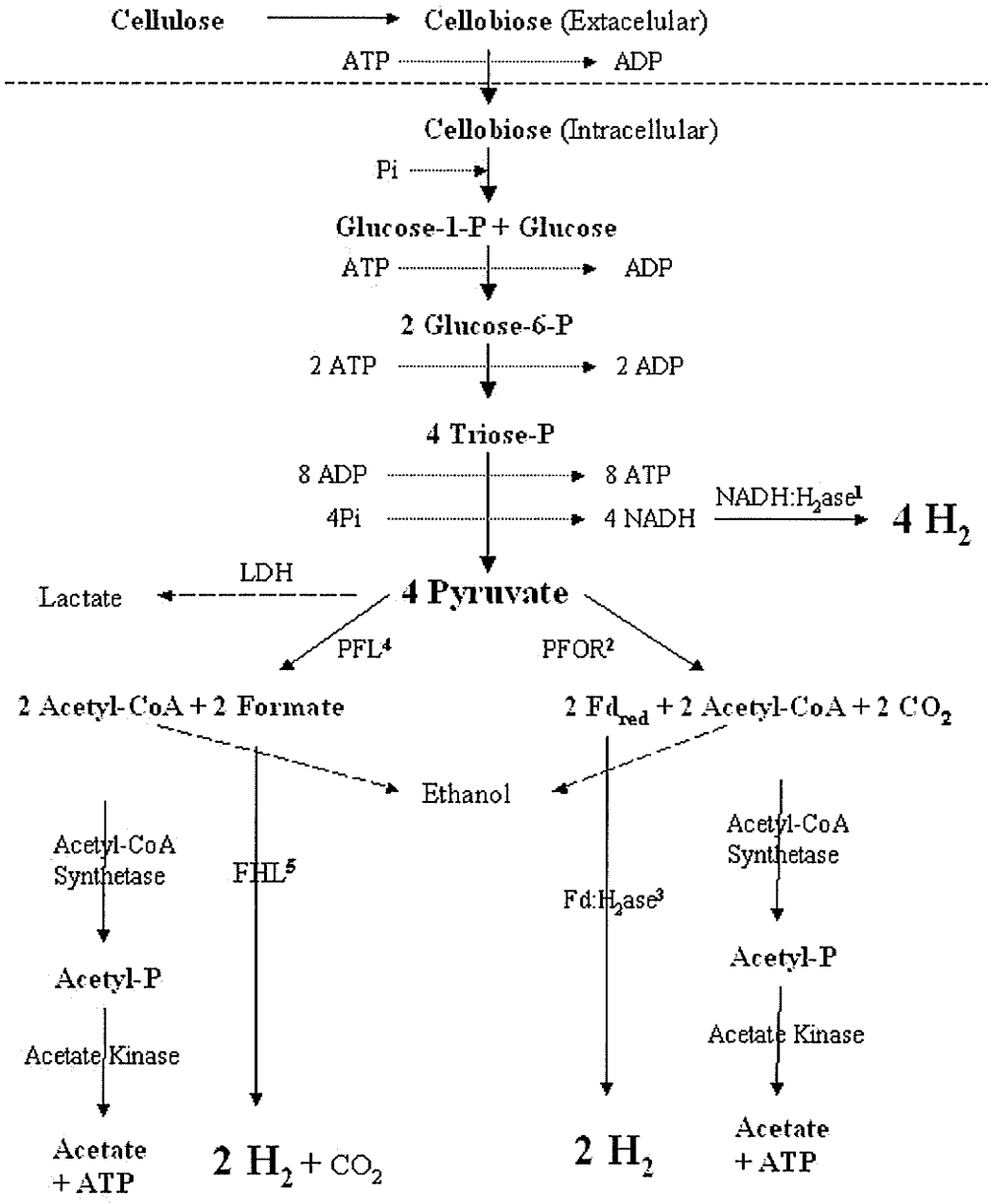


Figure 6.1 Pathway-model of different metabolites based on findings from this study on cellobiose and cellulose fermentation by *Clostridium thermocellum.* 1, Hydrogenase; 2, Pyruvate-formate-oxidoreductase (PFOR); 3, Ferredoxin-hydrogenase; 4, Pyruvate-formate-lyase; 5, Formate-hydrogen-lyase; LDH, Lactate dehydrogenase.

Cumulative gas production (H_2 and CO_2) calculated based on end products were also much higher on cellulose cultures (40 micromoles) than those from cellobiose cultures (26 micromoles) under substrate-limited conditions (Chap. 3). Previous observations of greater growth yields on higher oligomers and greatest variation in yield at low dilution rate (Strobel, 1995) are in agreement with these findings. Greater amounts of H_2 were produced from 1.1 g/l cellulose cultures than 1.1 g/l cellobiose cultures but this phenomenon was opposite in 4.5 g/l cultures. This may be due to H_2–supersaturation which was more severe in 4.5 g/l cellulose cultures than that in 4.5 g/l cellobiose cultures because of gas trapping (Lamed *et al.* 1988) and poorer mass transfer rate (Pauss *et al.* 1990). These factors most likely induced shift of metabolic pathways from hydrogen production to solvent production. Considerable amounts of ethanol were produced during fermentation of both cellobiose and α-cellulose and around 50% of substrate flowing toward acetate shifted mainly to ethanol production during late log phase. Removal of H_2 could be a possible solution to this problem as suggested by Wolin (1974). An opposing argument was presented by Weimer and Zeikus (1977), who stated that the production of ethanol by NADH oxidation can also make H_2-syntesis thermodynamically unfavorable. Inhibition of *C. thermocellum* 27405 growth by ethanol (Kundu *et al.* 1983) suggested that concentrations of ethanol above 10 g L^{-1} suppressed cell growth because of toxicity. The maximum concentration we observed was 1.5 g L^{-1}. thus, ethanol inhibition is unlikely to be a factor in the present study.

Production of formate was low in cultures containing cellobiose until late exponential growth phase. In α-cellulose cultures, however, formate was the second major product during the exponential growth phase. A low concentration of extracellular

pyruvate was observed during cellobiose fermentation and was not detected in α-cellulose fermentation. Pyruvate is an evidence of carbon-overflow which caused by inhibited carbon-flow toward acetic acid production and induction of carbon-flow to lactate and ethanol production. Absence of pyruvate in α-cellulose culture indicates that overflow of carbon was regulated more efficiently on cellulosic substrates.

High concentrations of lactate were generally observed in cultures where the pH dropped below 6.8. Batch experiments with *C. pasteurianum* resulted high level of lactate production caused by iron limitation (Dabrock *et al.* 1992). Continuous increase in lactate level in 4.5 g/l cultures indicates the production of H_2 through the reaction $NADH + H^+ \rightarrow NAD^+ + H_2$, decreased as fermentation progressed. Once again, removal of H_2 from culture could be a possible solution to this problem as suggested by Wolin (1974).

Maximum specific rate of H_2 synthesis obtained 4.5 g/l cellobiose cultures (14.6 mmoles.g dry weight^{-1}.h^{-1}) was much higher than that obtained from 4.5 g/l α-cellulose cultures (9.35 mmol H_2.g dry weight^{-1}.h^{-1}). Maximum H_2-yield from cellobiose (1.4 mole/mole of glucose) was above the maximum yield observed on α-cellulose. This value is comparable with many previous studies such as, 1.7–1.9 mol H_2/mol hexose from pure sucrose (Hussy *et al.* 2005) through nitrogen sparging. Batch fermentation of starch by *Clostridium beijerinckii* AM21B resulted in a yield range of 1.3–2.0 mol H_2/mol hexose (Taguchi *et al.* 1992) and 2.4 mol H_2/mol-hexose (Ueno *et al.* 1995) was obtained using mixed microflora in sludge compost.

In the first set of experiments (Chap 3) yields of hydrogen (mol H_2 /mol glucose) were higher in cultures containing insoluble cellulosic substrates compared with those containing cellobiose under carbon-limited and carbon-sufficient conditions. Subsequent

experiments with α- cellulose (chapter 5) did not obtain the same results. Possible reasons for this could be the differences in experimental design. Culture tubes in experiments discussed in Chapter-3 were subjected to regular shaking and longer incubation periods. These parameters may have facilitated releasing trapped gas from cultures.

6.2 Concluding Remarks

Batch fermentations established that this strain of *C. thermocellum* 27405 can be considered for efficient conversion of cellulosic biomass to hydrogen even under non-optimized culture conditions. Metabolic flux response of *C. thermocellum* changed depending on different substrate-loading conditions which noticeably influenced rates and yields of H_2-production. Specific conclusions based on this study are,

i. *C. thermocellum* is capable of maintaining carbon flow to acetic acid and H_2-production under limited, sufficient and excess-substrate conditions,

ii. Formic acid was a fermentation end-product. This was a surprising observation but not unexpected as *C. thermocellum* contains the gene encoding pyruvate-formate-lyase present in its genome.

iii. Under substrate-excess conditions carbon flow shifted away from acetate pathway for

   a. increased H_2 partial pressures

   b. drop of pH below 7.0

iv. *Clostridium thermocellum* possess a very active cellulase system to hydrolize insoluble cellulose and is capable of

   a. obtaining a competitive yields of 1.6 mol H_2 /mol glucose,

   b. producing H_2 at a high specific rate (14.6 mmol /g dry cell/h)

v. Sustainable H_2 production from cellulosic biomass by *C. thermocellum* may be possible if

   c. bioreactor conditions were able to maintain a near neutral pH (approx. 7.0)

d. gas products (H_2 and CO_2) are removed rapidly to maintain a low enough H_2 partial pressure.

6.3 Ongoing Investigations

A baseline methodology has been developed throughout batch experiments performed in this study culturing *C. thermocellum* on cellulosic substrates for a long term investigation on bio-hydrogen production. Continued research will be directed toward the following issues:

i. Evaluating effects of fermentation products on rate of hydrogen synthesis

ii. Designing a continues-feed, pilot-scale, bio-fermenter with

   a) gas removal system to keep the pH_2 low enough

   b) optimized pH control and agitation rate

ii. Genetic engineering for avoiding diversion of metabolic flux from hydrogen producing pathways by

   a) improving the carbon flow toward acetate + H_2,

   b) shifting carbon flow from reduced end products (e.g. ethanol and lactate), and

   c) enhancing the pathway (used by formate-hydrogen-lyase) of formic acids conversion into hydrogen.

6.4 Potential Areas of Future Research

In spite of a great potential to generate high quality fuel from little or negative value cellulosic waste biomass, fermentative H_2 -production processes have received very little attention. In order to establish a continuous and high-rate process, extensive and continued investigation is required. For further improvement in design and operation of a bio-fermentor following recommendations can be suggested,

(i) Optimizing and controlling the operating conditions (such as pH, stirring) in hydrogen fermenter for a sufficient high production rate.

(ii) Developing a continuous fermentation process and scale-up of the process from the laboratory level to pilot plant level.

(iii) Developing an effective membrane system for continuous and efficient (>90%) removal of dissolved hydrogen from the culture.

(iv) Developing a gas separation system to achieve highly pure (99.99%) hydrogen.

Also from microbiological point of view, areas for further investigation and improvements include,

(i) Effect of culture medium (such as, complex and defined mineral medium) on metabolic flux response of *C. thermocellum* under various culture conditions during cellulosic digestion.

(ii) Influence of fermentation products on the response of hydrogen synthesizing enzymes.

   a. improving the carbon flow toward acetate $+H_2$

b. shift carbon flow from reduced end products (e.g. ethanol and lactate)

c. Introduce/improve a pathway for conversion of formate into hydrogen

6.5 Engineering Significance

To develop a continuous and scaled-up bio-fermenter, a large number of environmental and operational parameters which are interrelated need to be understood. It is very difficult and sometime unrealistic to control and optimize all those factors simultaneously without having a detailed knowledge about their effect. Lab-scale batch-fermentation studies allow for the evaluation of key design and operational parameters and the extent of their influence on the process. Batch fermentations of cellobiose and cellulosic substrates performed in this study provides a strong background for fermentative thermophilic H_2-production processes using pure cultures.

Most important observation from this work was the influence of substrate loading on fermentation metabolites which allows selecting an optimum start-up concentration of substrate in a scaled-up process. A better understanding of dilution rates and corresponding reactor volume can be obtained from the present data for design optimization and process economy, can also be obtained from the presented data.

An efficient gas-removal system was determined to be a necessary design requirement. To overcome major obstacles of hydrogen synthesis, involving high H_2-partial pressure and H_2-supersaturation, a proper gas-liquid contactor or gas extraction devices need to be incorporated. Other operational parameters such as pH control and mixing were also determined to be crucial factors effecting H_2-production yields and rates. This research establishes a foundation for further engineering optimization of bio-hydrogen production through fermentation of cellulosic waste biomass.

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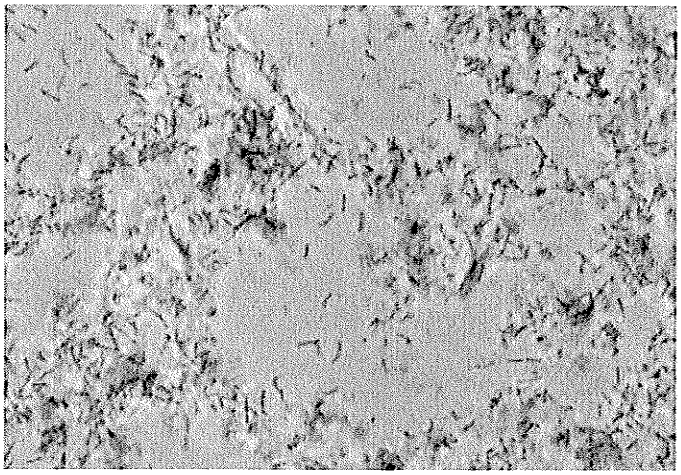
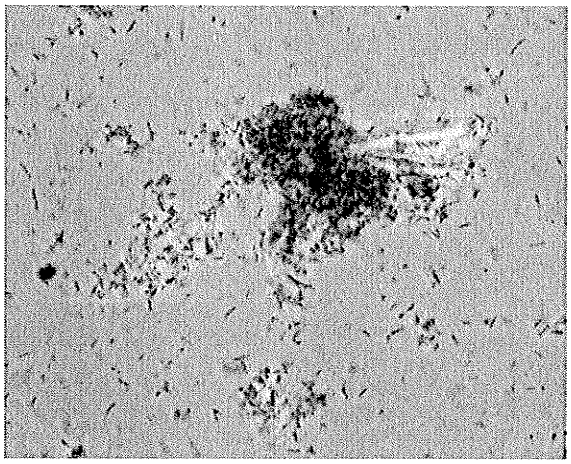
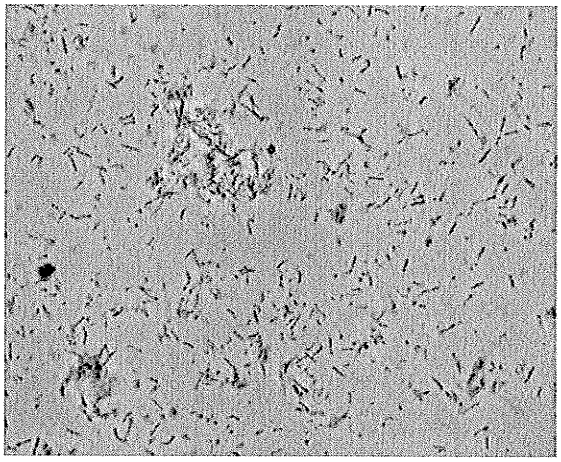
Appendix A



(a)





(b) **(c)**

Figure A.1 Live culture of *Clostridium thermocellum* on shredded filter-paper substrate after 4, 5 and 8 days of incubation presented in (a), (b), and (c), respectively.

Appendix B

Experimental Design

B.1 Hydrogen Production from Cellobiose and Cellulosic Materials Using Three Different Concentrations 4.5 g/l, 1.1 g/l and 0.1 g/l by *Clostridium thermocellum*

B.1.1 Preparation of Cellobiose Solution

B.1.1.1) Preparation of Sterile 125 ml Serum Bottles

- Insert butyl-rubber stopper into four (4) clean 125 ml serum bottles
- Add metal seals and crimp
- Autoclave

B.1.1.2) Preparation of Cellobiose Solution (120 g/L,)

- 6 g Cellobiose was added to a 100 ml beaker containing 30 ml distilled, deionized, water (ddH_2O);
- Stirred until dissolved;
- Transferred to graduated cylinder and brought the final volume to 50 ml;
- Flushed with N_2 (100%) for 20 minutes;
- Filter sterilized into sterile 125 ml serum bottles;
- Degassed : gassed (5': 1'), N_2 (100%) (5':1'), 4 times with sterile hoses
- Added 0.25 ml sterile 200 mM Na_2S to each bottle

B.1.2 Preparation of 1191 Medium

➢ Following chemicals were added to a 1000 ml beaker containing 700 ml ddH_2O,

Component	Amount required (mg/L)
KH_2PO_4	1.50 g
$Na_2HPO4:2H_20$	4.20 g

NH_4Cl	0.50 g
$MgCl_2$: $6H_20$	0.18 g
Yeast Extract	2.00 g
Resazurin	8.00 ml(0.25 mg/ml)
10X Vitamin Mix	0.50 ml
10X Mineral Mix	1.00 ml

- Stirred until dissolved
- Adjusted pH → 7.2 with NaOH pellets (weigh the pellets before adding)
- Flushed solution with N_2 (100%) 30 minutes
- Transferred to graduated cylinder and bring volume to 1000 ml
- Returned solution to beaker and check pH; restore pH → 7.2 with NaOH pellets, if required
- Dispenseed 10 ml of 1191 medium into each Balch tubes (27 ml)
- Inserted butyl-rubber stoppers;
- Sealed with metal crimp
- Degassed/gassed (5':1'), 4 times using N_2 (100%)
- Added 0.1 ml of 200 mM Na_2S solution to Balch tubes
- Autoclaved

B.1.3 Experimental Approach (Chap-3)

B.1.3.1) Preparation of inoculum and sampling schedule

- Inoculated one-liter source-bottles of cellobiose and α-cellulose with actively growing cells.

- Incubated until source bottles with cellobiose reached an OD600≈ 0.5 and bottles with α-cellulose growed for 24 hours.
- Added cellobiose solution to nine tubes at concentrations of 0.1,1.1 and 4.5 g/l
- Triplicates of each concentration for each substrate (cellobiose, paper, DLC and α-cellulose) were inoculated.
- Cellobiose tubes were inoculated for 24 hours
- All 0.1 g/l cellulosic-substrate containing tubes were incubated for 3 days
- All 1.1 g/l cellulosic-substrate containing tubes were incubated for 8 days
- All 4.5 g/l cellulosic-substrate containing tubes were incubated for 11 days

B.2 Hydrogen Production from Cellobiose Using Three Different Concentrations 4.5 g/l, 1.1 g/l and 0.1 g/l by *Clostridium Thermocellum*

B.2.1 Preparation of Cellobiose Solution

Same as section B.1.1 and B.1.2.

B.2.4 Experimental Approach (Chap-4)

B.2.4.1) Preparation of inoculum and sampling schedule

- Inoculated three one-liter bottles containing 1191 with cellobiose (5 g/L) from an actively growing source
- Only used the bottles of which the OD_{600} has reached at about 0.5 (within first 5-8 hours) for the inoculation to be sure that we are adding exponentially growing cells.

  - calculated the volume of cells required to give the same OD_{600} (around 0.05) in each of the experimental tubes

- Inoculated three batches of tubes (containing 0.1, 1.1 and 4.5 g/l cellobiose)with *C. thermocellum* from the freshly growing culture in 1191-cellobiose medium

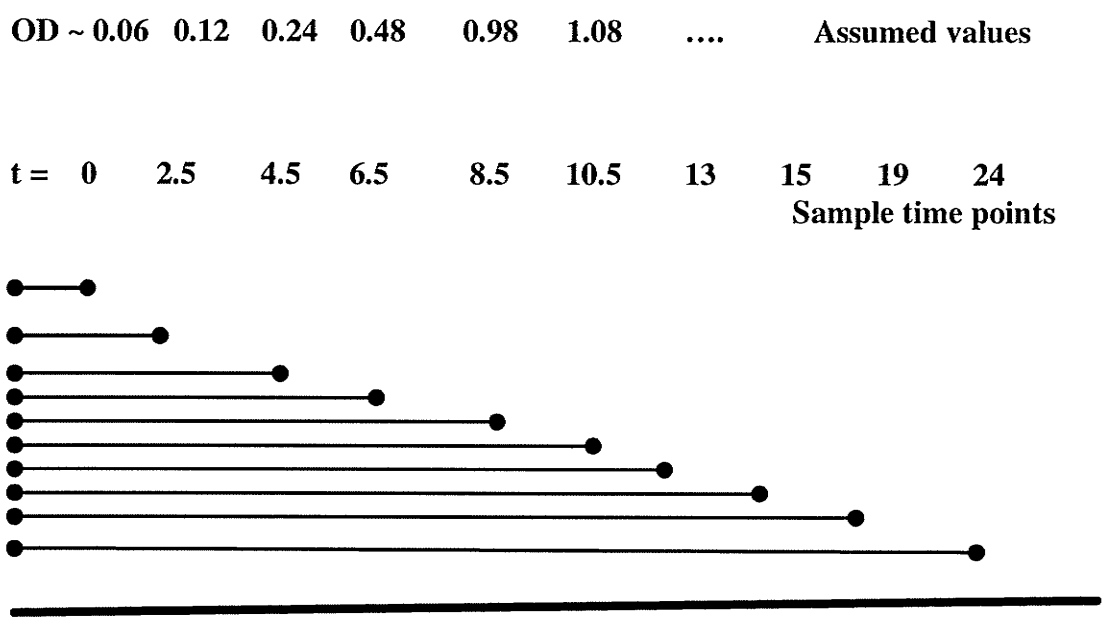


Figure B.1: Sampling schedule for batch experiments described in chapter-4.

B.2.5 Processing Tubes at Each Time Point

- Measured at each time point,:
  - OD_{600} of each tube (for cellobiose)
  - Take 3 tubes for each time point and each concentration out (9 in total)
  - Then place the tubes at 4 °C if the gas composition is not done right way

- After all time points are collected:
  - Gas content (CO_2 and H_2)

- Protein quantification
- Dry mass (will be measured using 50 ml of cultures grown in 125 ml serum bottles)

B.2.4.3) Processing the supernatants

- determined the content of the supernatants once all the time point samples were taken,
- Measured:
  - pH
  - organic acids (lactate, acetate, formate)
  - Ethanol
  - sugars (glucose, cellobiose)

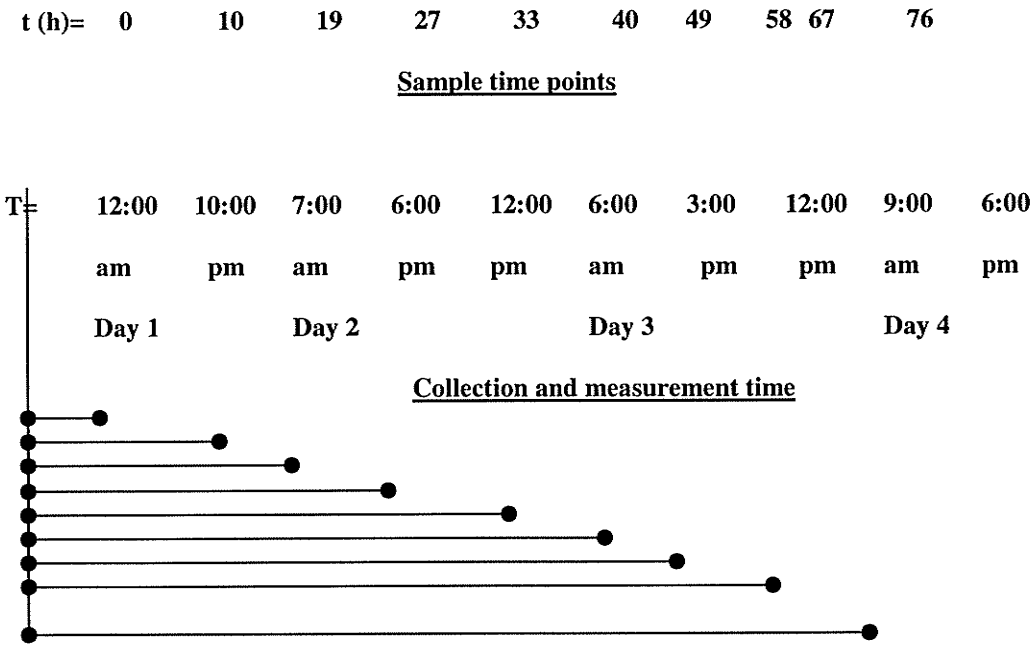
B.3 Hydrogen Production from Alpha-Cellulose Using Three Different Initial Concentrations (0.1 g/l, 1.1 g/l and 4.5 g/l) by *Clostridium Thermocellum*

B.3.1 Preparation of Cellobiose Solution and 1191 Medium

Same as described in sections B.2.1 and B.2.2 . Only exceptions were, α-cellulose was introduced as substrate instead of cellobiose before sealing and autoclaving tubes.

B.3.2 Preparing Inoculums

1) Inoculated 2 serum bottles containing 1 g/l α-cellulose with 10% (v/v) log-phase inoculum.
2) Watched growth visually
3) Transferred 40 ml of log phase culture into a 1 litre bottle
4) Watched growth by protein assay
5) Inoculated three batches of tubes when growth reached the mid log phase.



Notes:

1) In each time point 3 tubes for products analysis and 2 tubes for protein assay was collected
2) 0.1 g/L batch continued upto 49h timepoint
3) 1.1 g/L batch continued upto 67h timepoint
4) 4.5 g/L batch continued upto 76h timepoint

B.3.3 Processing the Tubes at Each Time Point

- At each time point:
    - Protein assay on 2 tubes (6 total)
    - Take 3 tubes for each time point and each concentration out (9 in total)
    - Place at 4 °C if the gas composition is not done right way
- After all time points are collected:
    - Complete Gas analysis (CO_2 and H_2)
    - Open tubes and collect supernatant
    - Dry mass of pellets will be measured for residual cellulose
        - Add formic acid for cell lysing
        - Place pellet in pre-weighted microcentrifuge tube
        - Dry at 60°C until constant weight is achieved
        - Take the difference as the residual cellulose weight

<u>Processing the supernatants</u>

- Once all the time point samples are taken, determine the following contents of the supernatants

- Measure:

    - pH
    - organic acids (lactate, acetate, formate)
    - alcohols (ethanol)
    - sugars (glucose, cellobiose)
    - reducing sugars (cellotriose, cellotetraose etc.)

B.3.4 Protein assay (Modified Bradford Method)

- centrifuge 2 ml culture at 14000 rpm for 10 min
- separate supernatant from the pellet
- wash the pellet with 0.9% NaCl solution once
- resuspend the pellet in 2 ml of 0.2 N NaOH solution
- centrifuge for 5 min
- use the supernatant for protein assay
    - Prepare BSA protein standards (10 ug/ml to 100 ug/ml) on 0.2N NaOH
    - Filter the Bradford reagent (stored at 4°C)
    - Add 1.8 ml of Bradford reagent to 0.2 ml of sample (supernatant)
    - Wait 5 min for color development
    - Measure the absorbance at 595 nm

Appendix C

Measurement of Gas Production

C.1 Measurement of Headspace Gas

C.1.1 Creation of Standard Curve for Analysis of H_2 peak areas (GC) -May 19, 2005

Example: 10 mL H_2 in 1 L of gas is 1% standard and this volume is never constant. It will depend upon changing environmental conditions.

Standards and Conversions
standard temp (0*C)=273 K
101.325 kPa=760 mm Hg
1 kPa=7.50 mm Hg
102.5 kPa*(7.50 mm Hg/kPa)=768.
standard pressure=760 mm Hg
Convert to STP

= 10 mL*(273K/273+temp)*(pres/760
=H2 (uL/mL)***(mL injected)**/22.4 uL/umol=0.4096
= umol H2 injected from the 1% standard

Convert to *u*mol
gas constant=22.4 L/mol --> 22.4 uL/umol
volume injected into GC
H2 standards (In 162 ml bottles):
When making these, be sure to first remove the volume of air in the bottle that corresponds to the volume of hydrogen you wish to add. A new standard curve must be created each day that samples are taken from the given cultures
Attenuation: 8 used in GC

Table D.1 Standard Curve Data - May 19, 05

Umol H2	Peak Height
0	0
0.2048	0.65
0.4096	1.28
0.8192	2.5
2.0480	7.2

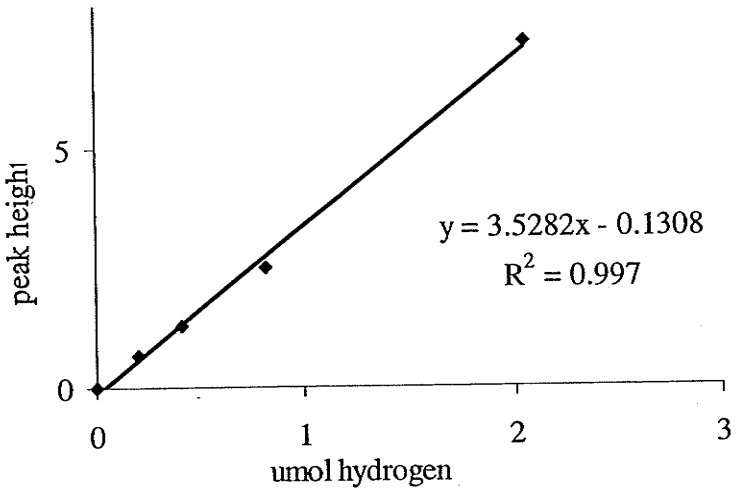


Figure C.1 Standard curve of hydrogen correlating micromoles of hydrogen to peak height.

C.2 Measurement of Soluble Gas

C.2.1 Equilibrium Gas Concentrations

Henry's law as a function of temp, $k_H = k^{\circ}_H \times \exp((-\Delta_{soln} H)/R)(1/T - 1/T^{\circ}))$

Dimensionless ratio between aqueous-phase (Ca) and gas phase (C_g) concentrations, $k^{cc}_H = C_a/C_g$ and $T \times k_H = 12.2 \times k_H^{cc}$

Here, $(-\Delta soln\ H)/R = -\ d\ lnk_H\ /d(1/T)$

C.2.2 Calculation of equilibrated bicarbonate in culture

Handerson-Hasselbach equation: $pH = pKoverall + \log_{10} \frac{[HCO^-_3]}{[CO_2(d)]}$

$Koverall = K_a \times K_h$
$pKoverall = -\log (Koverall)$

Appendix D

Protein Quantification by Bradford Method

D.1 Preparation of standards using 1 mg/ml BSA solution

A dilute BSA solution was prepared (1000 µl BSA+ 9000 µl dd water). From this dilute stock solution, 5 standards were prepared as follows:

i. 10 µg/ml → 100 µl of BSA + 900 µl dd water
ii. 20 „ → 200 µl of BSA + 800 µl dd water
iii. 50 „ → 500 µl of BSA + 500 µl dd water
iv. 80 „ → 800 µl of BSA + 200 µl dd water
v. 100 „ → 1000 µl of BSA

D.2 Bradford Assay

A. The Bradford solution was always filtered before using. 200 µl of each standard was mixed with 1800 µl of Bradford solution;
B. Three replicates were prepared for each standard;
C. Centrifuged 1 ml of culture and drained the supernatant;
D. 1ml of Bradford solution was added to extract the protein from cells;
E. Prepared three replicates of a sample (200 µl of sample + 1800 µl of Bradford solution);
F. Spectroscopic readings were taken at 595 setting taking distilled deionized water as blank