Characterization of cell growth, substrate utilization, end-product synthesis and gene expression patterns in cellulose degrading co-cultures of *Clostridium termitidis* CT1112 and *Clostridium intestinale* URNW

by

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A thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfilment of the requirements of the degree of

MASTER OF SCIENCE

Department of Biosystems Engineering

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Winnipeg

Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

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Abstract

Co-cultures of selected fermentative bacteria have been shown to improve rates of substrate conversion and yields of some fermentation end-products. We have tested this hypothesis using a co-culture of the mesophilic, cellulolytic bacterium *Clostridium termitidis* CT1112 and the mesophilic, saccharophylic bacterium, *Clostridium intestinale* URNW. *C. termitidis* can utilize cellulose as a sole carbon source. It releases glycosyl hydrolases that hydrolyze cellulose, cellobiose and glucose. *C. intestinale* URNW, which was isolated as a contaminant from a cellobiose stock bottle of *C. termitidis* (Ramachandran et al., 2011), is not capable of hydrolyzing cellulose, but can utilize the cellobiose and glucose released by cellulose hydrolysis to grow in co-culture with *C. termitidis*. Based on the faster doubling-time of *C. intestinale* URNW on cellobiose, it was expected that the "soluble sugar free" environment will stimulate *C. termitidis* to hydrolyse cellulose at faster rate and which in-turn will result in increased substrate utilization and end-product synthesis compared to the monoculture of *C. termitidis*. The designed co-culture was characterized in depth with the use of microbial quantification studies (multiplex quantitative Real Time Polymerase Chain Reaction – qPCR) and 'Omics techniques to understand the population dynamics and gene product expression in each species in the co-culture versus their respective monocultures at the molecular level. Inoculation of co-culture with diffferent initial ratios of the *C. termitidis* and *C. intestinale* resulted a fixed ratio of approximately 13:1 (*C. termitidis* : *C. intestinale*) at 168 hour post-inoculation (h pi). A statistical difference in substrate utilization and total cell mass production, but not end-product cocentrations, was observed at 168 h pi in cultures with an initial *C. termitidis* : *C. intestinale* ratio of 1:1 and 1:0.2). No statisical differences in substrate utilization, biomass accumulation, or end-product synthesis concentrations were observed for all other initial *C. termitidis* : *C. intestinale* ratios, or for co-cultures in where the *C. termitidis* : *C. intestinale* ratio was 1:25. Thus, the hypothesis that synergistic

interactions between species in co-cultures can stimulate substrate consumption and end-product synthesis was supported under very limited conditions in this co-culutre system. Unlike other co-cultures reported in literature, the co-culture of *C. termitidis* and *C. intestinale* did not show large increases in substrate utilization or end-product concentrations synthesized when cultured on 2 g/L α-cellulose. This may be due to the slower cellulose degradation ability of *C. termitidis* in the co-culture, (compared to the cellulose degraders in other co-cultures such as *C. thermocellum*), which is the main contributor to the growth of *C. intestinale* in the co-culture and the competition for the same substrate (cellobiose) by both the species in the co-culture.

Acknowledgments

First and foremost I thank my supervisor Dr. David Levin for accepting me into his research group, supporting and motivating me throughout my tenure. Additionally I would like to thank my committee members Dr. Richard Sparling and Dr. Nazim Cicek for their expert advice, insight and positive encouragement. The continuous support from my committee members helped me to improve my knowledge in various areas of my research and kindled my passion towards further research.

At this time, I would like to extend my gratitude to the Minister of Advanced Education and Literacy, Manitoba for selecting me as a recipient of Manitoba Graduate Scholarship which helped me to ease my immigration process and continue my further education in Manitoba.

I would also like to thank John Schellenberg and Alan Froese for their help and assistance in developing and optimizing molecular tools which helped me to answer most of my research questions. I also like to thank other team members especially, Warren Blunt, Scott Wushke, Riffat Munir, Eftekhar Hossain, Valery Agbor, Preethi Kumaravelayutham, Charushi Panditharatne, Jilagamazhi Fu and Marcel Taillefer for guiding me whenever I requested.

Finally I like to thank the people in my personal life for their constant support and love. Specifically this goes out to my mom, dad, Niresh Peranantham, and Saranya Rajendran.

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Chapter 1

Introduction to Cellulosic Biofuels and Co-cultures for Cellulosic Biofuel Production

1.1 The need for alternative energy

The increasing human population demands an increased use of fossil fuels, with unprecedented increases in the quality of life, and equally unprecedented levels of global pollution. The energy requirements in the present scenario are met through the exploitation of readily available, inexpensive, non-renewable resources such as: oil, coal and natural gas. Oil can be considered as the black blood running through the modern energy system (Hook, 2009). Fossil fuels are non-renewable resources, and recent studies show that fossil fuel consumption has reached a state of "peak oil" in which reserves of low cost "sweet crude-the crude oil which has less sulphur content" are becoming scarce. Renewable resources such as food or water will last indefinitely as long as the amount we use does not exceed the amount regenerated by the nature. However in the case of non-renewable resources like fossil fuels or minerals, the availability of the resources completely depends on the rate of their discovery and depletion. As these resources become limited, the ways of obtaining them becomes highly expensive and less safe (Bardi, 2008).

1.1.1 Hubbert Curve – Peak Oil Concept

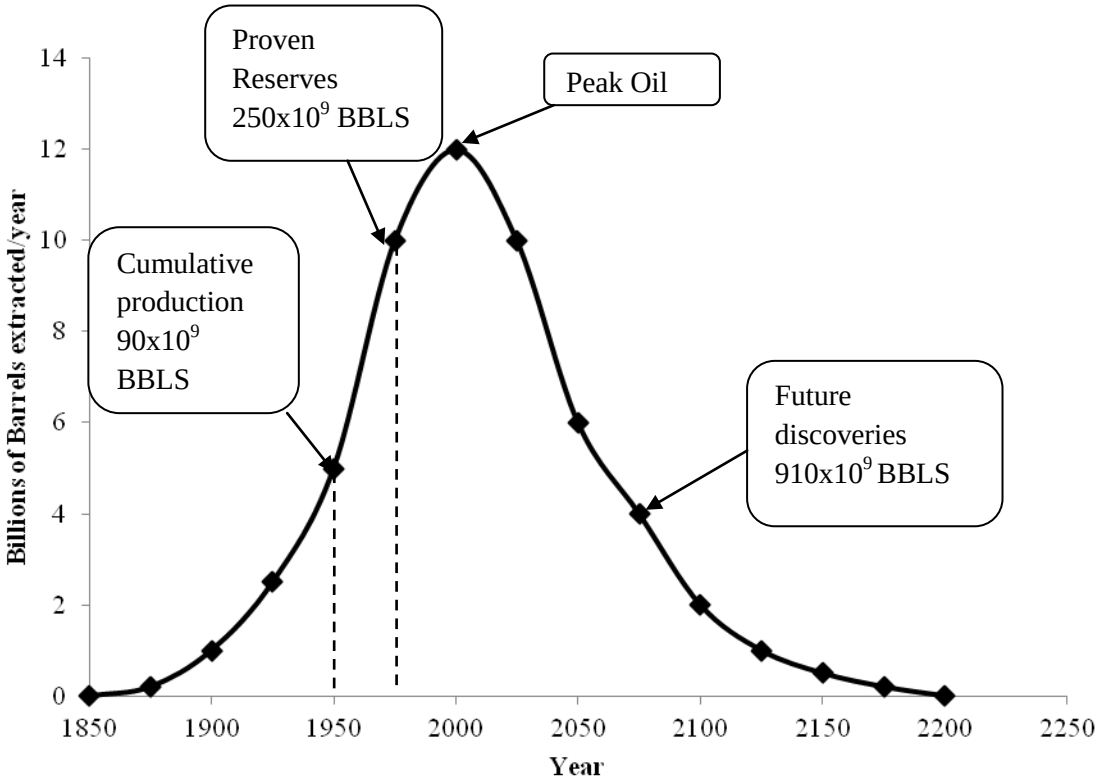
Marion King Hubbert in 1956 explained the concept of peak oil, which implies that the production of crude oil in the market economy will reach a maximum and then declines to zero. Hubbert had proposed that the production curve is bell shaped and this peak occurs when half of the non renewable oil is extracted (Bardi, 2008). The Hubbert curve is a basic mathematical function, which is a bell shaped curve that starts at zero, rises to a peak and then drops off to zero. The rising part of the curve is of "S" shaped and has three parts: (1) an upwardly bending "lag phase", (2) a rapidly raising "log phase" and (3) a plateau that levels

off. When Hubbert originally presented his hypothesis in 1956, he predicted that the production of oil in the United States will reach a peak in late 1960s and worldwide about 2005. These predictions are popularly known as the "Peak Oil" concept (Figure 1.1). However several recent predictions and models show that the production of oil in the US will reach a peak only by 2020 (Maggio and Cacciola, 2009).

1.1.2 British Petroleum Deep Water Horizon Explosion

Although there are still large reserves of petroleum in the world, they are of much lower quality and in locations that are very difficult and dangerous to access. On April 20, 2010, an explosion occurred on a British Petroleum Deep Water Horizon platform at the Gulf of Mexico. After two days, the platform completely collapsed, releasing the oil into the Gulf. This tragedy claimed the lives of eleven crew members and affected many individuals in the surrounding area as well as the fragile ecosystem. The cause for this disaster can be directly attributed to the increased oil demand in America, which forced the company to raise the oil production rate from 50,000 gallons per day to 1 million gallons per day without developing a functional safety culture (Deepwater Horizon Study Group, 2011). This incident clearly explains the risk involved in extracting oil from deep wells, which are not easily accessible. In addition, the extraction processing and combustion of fossil fuels contribute to greater levels of environmental pollution by the emission of toxic green house gases. Green house gases include carbon dioxide (CO_2), methane (CH_4), nitrous oxides and chlorofluorocarbons (CFCs) which causes severe environmental degradation. These factors have created an urgent need for the development of alternative energy sources, which are renewable and environmentally friendly.

Figure 1.1 Concept of Hubbert's curve. Ultimate world crude oil production based upon initial reserves of 1250 billion barrels (Hubbert, 1956).



1.2 Biofuels as alternative energy

Biofuels are looked as a promising alternative to reduce the fossil fuel dependency and to bring down environmental degradation levels. Biofuels can be defined as a form of energy derived from organic matter obtained directly from plants or indirectly from agricultural, commercial and domestic waste materials (Demirbas, 2011). Biofuels may be liquids (ethanol, biodiesel) or gaseous (hydrogen) which causes less damage to our environment compared to fossil fuels (Carere et al., 2008). Among them, hydrogen (H_2) can be called a "green fuel" because it gives only water as the combustion product, which does not harm the environment. Also, H_2 possesses the highest gravimetric energy density, 122 KJ g^{-1}. Hydrogen can be produced by electrolysis of water, steam reformation of methane, or through biological fermentation process (Levin and Chahine, 2010). Among them, H_2 production through fermentation of waste materials is gaining more popularity and interest, as it is not energy intensive as the other process (Levin et al., 2006) and it also gives value to the waste materials. Bioethanol is the most widely used liquid biofuel in the world. The largest producers of bioethanol in the world are Brazil (37%), the United States (33%) and Asia (14%). In Brazil, bioethanol is mainly produced from sugarcane and it accounts for nearly 18% of the country's automotive fuel needs (Carere et al., 2008). Ethanol is commonly used as an additive to the fossil fuels such as gasoline to reduce emissions of carbon monoxide, nitrous oxides and hydrocarbons which are harmful to the environment.

1.2.1 First-Generation Biofuels

First generation biofuels refer to the fuels which are derived from sources like starch, sugar, animal fat and vegetable oil (Singh et al., 2011). The most popular types of first generation biofuels are: 1) Biodiesel – the most common type of biofuel in Europe produced by transesterfication; 2) Vegetable oil from soybean or canola; 3) Biogas – produced by the anaerobic digestion of organic materials; 4) Bioalcohols – produced by the fermentation of

sugars or starch; and 5) Syngas – produced by pyrolysis. The major statement against first generation biofuels is that the raw materials used for producing these fuels are also food commodities. Hence, commercializing this type of fuel is always looked and blamed to be the main reason for shortage of food and increase in food prices (Naik et al., 2010) and it also raises the food versus fuel debate.

1.2.2 Second Generation Biofuels

Plants through the process of photosynthesis capture the energy from sun and store it as carbohydrates. There is a great potential to derive this energy from plants and use it as an alternative energy source for the future. Second generation biofuels are also called as advanced biofuels that can be derived from various types of non-edible plant biomass. In addition to plant materials, forestry residues, municipal waste materials and dedicated biomass crops have also been investigated as raw materials for second generation biofuels (Lynd et al., 2002 and Sivakumar et al., 2010).

1.3 Cellulosic Substrates for Biofuel Production

Cellulose is the most abundant polymer on earth and forms a large fraction of the degradable waste materials discarded. It is estimated that 7.5×10^{10} tons of cellulose is synthesized annually through photosynthetic process of plants. Besides being stored in plants, cellulose is also used as a raw material in paper and textile industries. Organisms that are capable of degrading cellulose and utilizing it as the carbon source are of great importance as they can be used for the dual purpose of energy production and waste management. Lignocellulosic biomass directly obtained from plants has a complex structure consisting of three polymers: 1) cellulose – the primary constituent of the plant cell wall; 2) hemicellulose – which is meshed into cellulose fibres; and 3) lignin – which fills the pores in the mesh of cellulose and hemicellulose and provides the plant its mechanical strength (Agbor et al., 2011b; Levin et al., 2009). The complex structure of the cellulosic materials is a great

challenge for developing cellulosic biofuels as most celluloytic microbes cannot metabolise lignin. Therefore, pretreatment of lignocellulosic biomass is considered as an essential step to achieve high yields of cellulosic biofuels. A number of different pretreatment techniques (physical, chemical and mechanical approaches) have been investigated to achieve this goal. Various cellulolytic bacteria can degrade cellulose into H_2O and CO_2 under aerobic conditions, whereas under anaerobic conditions, methane and hydrogen are produced (Carere, 2008). Hence, anaerobic *Clostridia* have been given more importance for H_2 production from cellulose rich waste materials. These bacteria are ubiquitous in anaerobic soil environments, and have the capability to form endospores. Several species digest cellulose through an enzyme complex called "cellulosome", which appear as polycellulosomal aggregates on the cell surface and help the bacteria to get attached to the cellulose and break down into simpler sugars. Once the cells get attached to the cellulose fibre, the degradation is accomplished through the action of enzyme complex, which includes endo-1,4-β-glucanases and exo-1,4-β-glucanases (Carere et al., 2008). Micro-organisms such as *Clostridum thermocellum* and *Clostridium termitidis* are selected and extensively studied in our research group for cellulosic biofuel production because of their ability to hydrolyse cellulose to produce hydrogen and ethanol.

1.4 Consolidated Bioprocessing System (CBP)

Consolidated bioprocessing system (CBP), using *Clostridia* is gaining more importance for biohydrogen production. Unlike normal fermentation process, in CBP cellulose hydrolysis and glucose fermentation are achieved in a single-step by in situ produced enzymes which simplifies the process design and reduces the cost. Ethanol and H_2 production via direct cellulosic fermentation, using a CBP approach has been investigated and proved to be effective in substrate utilization and end-product synthesis (Islam et al., 2006, 2009; Levin et al., 2006; Ramachandran et al., 2008). CBP significantly brings down

the cost of feed stock processing for biofuel production, lower energy inputs and increased substrate conversion efficiencies. CBP is an attractive short-term goal for developing third generation biofuels from lignocellulosic materials (Carere et al., 2008). Many species of *Clostridium* such as *C. cellulolyticum, C. phytofermentans,* and *C. saccharolyticus* have also been investigated as alternate candidates for CBP for biofuel production.

1.5 Co-cultures and CBP

One of the main challenges associated with lignocellulosic biofuel production is making the lignin covered cellulose and hemicellulose accessible to the pure cultures of cellulolytic bacteria and their enzymes. In their natural habitats, bacterial species are engaged in numerous interactions with other species through various mechanisms, such as commensalism, mutualism, competition or parasitism to carry on their metabolic functions and to degrade the substrates (Jagman et al., 2012). Consortia in the gut of various termite species are capable of degrading lignocellulose effectively (Brune, 2006). The first step in biohydorgen production from complex polymers (cellulose or starch) is their hydrolysis to release a concentrated sugar solution (Ozmihci and Kargi, 2011). Acetogenic anaerobic bacteria, such as *Clostridia sp* or *Enterobactor sp* can ferment these free sugars anaerobically to release hydrogen and acetate. In industries, the initial hydrolysis of sugars is achieved by the use of enzymes or acids, this considerably increase the pre- treatment time/ cost. This problem can be simplified by the use of co-cultures, in which one pure culture hydrolyses the polymer and the other utilises the free sugars to produce hydrogen and ethanol. Besides, the microbes in the co-culture can interact with each other through quorum sensing circuits and control their ratios and biofilm formation (Lee et al., 2007). This may result in several advantages to biofuel production by providing an additional level of physiological response in the form of self-modulating activators and inhibitors (Zuroff et al., 2012). It is also expected that one of the partners in the co-culture can influence the metabolism and the

growth of the other to contribute towards increased end-product synthesis and substrate utilization by utilising the residual substrate or regulating the electron flow in a particular direction (genetic regulation) or by synthesizing end-products which may be utilised by the other species. This has accelerated the research towards developing designer co-cultures, which can bring about effective degradation of substrates and end-product synthesis in CBP for biofuel production.

1.5.1 Examples of Co-cultures used for Biofuel Production

Chen et al. (2008) developed a co-culture system for bio-hydrogen production, in which the initial starch hydrolysis was carried out by *Caldimonas taiwanensis* which can hydrolyse starch under the optimal condition of 55 ^{o}C and pH 7.5 giving a yield of 0.46 - 0.53 g reducing sugar /g starch. The free sugar is utilised by *Clostridium pasteurianum* to produce hydrogen at a rate of up to 118 ml/g VSS /h. It must be noted that *Clostridium pasteurianum* cannot assimilate starch but can only utilise the free sugar released by *Caldimonas taiwanensis* for its growth and efficient hydrogen production.

Liu et al. (2008) conducted a study to examine the interactive co-operation between thermophilic *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17 isolated from rotten wheat straw. Although the pure culture of *C. thermocellum* JN4 could effectively degrade microcrystalline cellulose to produce hydrogen, ethanol, acetic acid and lactate, it could not completely utilise the by-products of cellulose degradation (cellobiose and glucose). On co-culturing *C. thermocellum* JN4 with *T. thermosaccharolyticum* GD17 significant increase in substrate utilization and end-products synthesis were observed. At the end of fermentation, the co-culture showed nearly two-fold increased hydrogen production compared to the monoculture of *C. thermocellum* JN4.The demand for soluble sugars in the co-culture might have stimulated *C. thermocellum* JN4 to hydrolyse cellulose at faster rate. When this co-culture was grown on raw substrates, the

hemicellulose, which was not metabolized effectively by the monoculture of *C. thermocellum* JN4 was completely utilised by *T. thermosaccharolyticum* GD17 presented in the co-culture. This is achieved due to the combined metabolic activity of both the species in the co-culture. Though the cellulosome complex of *C. thermocellum* JN4 could hydrolyse the xylan to xylobiose and xylose, it cannot utilise them for growth. However these free sugars were completely utilised when *T. thermosaccharolyticum* GD17 was co-cultured with *C. thermocellum* JN4. This thermophilic co-culture was also cultured on complex natural substrates such as corn cob powder and proven to show an increase in substrate utilization and end-product synthesis when compared to the monocultures.

Lu et al. (2013) studied the effect of the addition of a non-celluloytic bacterium W2-10 (*Geobacillus sp*) on the cellulolytic activity of a cellulolytic bacterium CTL-6 (*C. thermocellum*). The study was conducted in peptone cellulose solution containing cellulose materials such as paper and straw under aerobic conditions. In the co-culture, it was observed that the W2-10 helped to the growth of CTL-6 by maintaining a stable pH and eliminating the oxygen to create an anaerobic environment for its growth. The co-culture showed a 72% and 38% increase in the utilization of filter paper and wheat straw when compared to the mono-culture of CTL-6. The acetate and the propionate production of the co-culture were 2.58 and 4.45 times higher than the monoculture of CTL-6. This co-culture can be considered as an example, where the presence of one of the organisms favour symbiotic growth of the other to contribute towards increased substrate utilization and end-product synthesis. This change in metabolism of the individual species in the co-culture can be further analysed by the use of advanced molecular sequencing techniques such as transcriptomics and proteomics.

Li and Zhu (2011) developed a co-culture of *C. thermocellum* and *Thermoanaerobacterium aotearoense* in equal proportions (1:1) to hydrolyse cassava pulp and to generate glucose for ethanol production by the subsequently inoculated

Saccharomyces cerevisiae. The glucose generated by the co-culture was 1.75 and 1.17 times greater than that generated by the monocultures of *C. thermocellum* and *T. aotearoense* respectively. Subsequently the hydrogen produced by the co-culture was also 1.54 and 2.09 times greater than that generated by the monocultures of *C. thermocellum* and *T. aotearoense* respectively. The main by-products of the monocultures of *C. thermocellum* and *T. aotearoense* were mainly acetic acid and lactate which significantly reduced the pH of the medium. The pH of the co-culture significantly dropped to 4.90 and accumulation of lactate and acetate was also increased. In this study, the growth of several initial inoculum ratios of *C. thermocellum* and *T. aotearoense* (2:1, 1:1, 1:2, 1:4 and 1:9) were tested by measuring the pH and the residual sugars at the end of fermentation at 96 hours. The glucose accumulated was 13.85 g/L, which was significantly higher when compared to that generated by the initial inoculum ratios 1:9 (8.02 g/L glucose) and 2:1 (8.90 g/L glucose). This experiment was conducted as a preliminary study and based on this, the initial inoculum ratio which provided the maximum glucose yield (1:1) was analysed further. The reason for the increase in substrate utilization and end-product synthesis by the 1:1 co-culture was unclear as the study did not involve the population dynamics and ‘omics studies. The increment may be also due to the increased biomass concentration in the co-culture when compared to the monoculture.

A co-culture of ethanologenic cellulolytic *C. thermocellum* strain YM4 and non-celluloytic bacterium *Thermoanaerobacter thermohydrosulfuricus* YM3 was obtained from volcanic solid, showed a stable population of both the species and produced ethanol in higher amounts from cellulose (Mori 1990). Both these bacterial species as monocultures required yeast extract (up to 2%) for their growth and cellulose degradation to produce ethanol. But it was observed that the co-culture could decompose cellulose even in the absence of yeast extract which indicates a type of mutualism established among these species in the co-culture. This mutualism was analysed by a study in which the cell free supernatants of

Thermoanaerobacter thermohydrosulfuricus YM3 and *C. thermocellum* strain YM4 were used to substitute the yeast extract to basal medium. The individual cultures showed significant growth even in the absence of yeast extract which indicates that both the species synthesize by-products which favour the growth of the other co-culture partner. It was observed that the cell free supernatant of *Thermoanaerobacter thermohydrosulfuricus* YM3 had significant amounts of vitamin B_6, vitamin B_{12} analogs, *p*-aminobenzoic acid and folic acid which were necessary for the growth of *C. thermocellum* YM4. Similarly, the strain YM4 produced niacin-active compounds, thiamine and methionine which were mandatory for the growth of *Thermoanaerobacter thermohydrosulfuricus YM3*. This co-culture can be considered as a good example for mutual-supporting mechanism developed among these species under nutritionally deficient conditions, which brings in the increase of end-products and substrate utilisation.

Co-cultures of *C. thermocellum* strain JW20 and *Thermoanaerobactor pseudoethanolicus* showed efficient utilization of xylose and higher ethanol production than their monocultures (Liu et al., 2008). These studies indicate that defined co-cultures can synergistically increase substrate utilization and end-products synthesis. Co-culture system is a promising approach to design a consolidated bioprocessing system with organisms which have the ability to produce hydrogen from sugars but do not have the ability to hydrolyse complex polymers.

1.6 Co-culture of *Clostridium termitidis* and *Clostridium intestinale*

From the above examples it can be concluded that fermentative biofuel production can be improved by using co-cultures by taking advantage of the interactive co-operation between the co-culture partners. Co-cultures of bacteria can result in synergistic effects on substrate consumption and end-product synthesis patterns that differ from those of the individual mono-cultures. Hydrogen producing bacteria which lack the ability to utilise

complex substrates can be co-cultured with bacteria which can hydrolyse the complex material and aid in its growth and enhanced hydrogen production. This research work is mainly focused on developing a mesophillic co-culture of *Clostridium termitidis* and *Clostridium intestinale* URNW for biofuel (Hydrogen and Ethanol) production from cellulosic materials. Experiments were designed to check the hypothesis that the co-culture will yield an increased substrate consumption and end-product synthesis compared to the monocultures. Techniques such as quantitative PCR, Transcriptomics, and Proteomics were used to quantify the individual bacteria in the co-culture and to understand the changes in the metabolism of the individual species.

1.6.1 *Clostridium termitidis*

Clostridium termitidis isolated from the gut of termite *Nasutitermes lujae* is an anaerobic, mesophilic, spore-forming, cellulolytic bacterium (Hethener et al., 1992; Ramachandran et al., 2008). It displayed a doubling time of 6.5 h and 18.9 h when cultured on 2 g/L cellobiose and α-cellulose respectively. The major end-products synthesized on cellobiose were H_2, CO_2, acetate, lactate, formate and ethanol, whereas on α-cellulose the major end-products consisted of only H_2, CO_2, acetate and ethanol (Figure 1.2) (Hethener et al., 1992; Ramachandran et al., 2008). Ethanol produced was slightly higher on cellobiose when compared to α-cellulose grown cultures. When cultured on cellulose, acetate and H_2 synthesis were favoured over ethanol synthesis indicating that the carbon flow towards ethanol and formate was blocked. *Clostridium termitidis* can be considered as an excellent candidate for CBP for biofuel production from cellulosic waste materials. However, the growth rate of *C. termitidis* on cellulose is very slow (doubling time 18 hours on 2 g/L α-cellulose) when compared to other thermophilic celluloytic bacteria such as *C. thermocellum* (Lynd et al., 2002) which has a doubling time of 4-6 hours on 2 g/L α-cellulose. This problem maybe overcome by co-culturing *C. termitidis* with a partner, which can effectively utilise the

soluble sugars or the end-products released by *C. termitidis* during its growth on α-cellulose and stimulate for faster cellulose degradation.

1.6.2 *Clostridium intestinale* URNW

Clostridium intestinale URNW is a gram positive, anaerobic, mesophillic, non-celluloytic bacterium. *Clostridium intestinale* URNW was isolated from a contaminated *Clostridium termitidis* stock bottle and further characterized on cellobiose as a hydrogen and butyrate producing bacterium. This non-celluloytic bacterium displayed a doubling time of 1.5 hours on cellobiose and has the ability to synthesize H_2 at a faster rate compared to other mesophillic celluloytic species (Ramachandran et al., 2011). *C. intestinale* showed a yield of 1.3 mol H_2 per mole of glucose equivalent and a specific H_2 production rate of 0.45 mol h^{-1} g^{-1} dry cell mass with 2 g/L cellobiose as substrate. *C. intestinale* strain URNW was capable of utilizing cellobiose, hexose sugars and sugar alcohols with a cell generation time of 1.5 h and produced H_2, CO_2, acetate, butyrate, formate, lactate, pyruvate, and ethanol as fermentation end-products (Figure 1.2). The faster doubling time of *C.intestinale* on soluble sugars can be taken as an advantage for CBP.

Co-culturing the celluloytic *C. termitidis* and non-celluloytic *C. intestinale* URNW may be a novel approach to improve biohydrogen production from cellulosic materials. *C. intestinale* is not capable of hydrolysing the complex cellulose, but can grow faster on the free sugars released by *C. termitidis* in the co-culture. Therefore, the co-culture of *Clostridium termitidis* and *Clostridium intestinale* URNW can be considered as an example for a mesophillic co-culture system that uses cellulose to produce hydrogen. *Clostridium termitidis* can hydrolyze cellulose to release cellobiose, glucose and other free sugars. In this research, the above mentioned co-culture is analysed in detail to observe any changes in the end-product synthesis pattern or substrate consumption, when compared with that of their monocultures. The key points which make these two species excellent co-culture partners are:

1) both the organisms are hydrogen producers; 2) they grow actively at mesophillic temperatures; and 3) though *Clostridium intestinale* URNW cannot grow on cellulose, *Clostridium termitidis* can degrade cellulose and release soluble sugars such as cellobiose and glucose to support its growth (Figure 1.3). It is also necessary to develop the co-cultures in a ratio at which they grow synergistically to contribute towards increased substrate conversion and end-product synthesis compared to the monocultures.

1.7 Co-culture metabolism

In *Clostridia* and other hydrogen producing organisms, the yield of hydrogen per mole of glucose will vary based on the pathway and the end products synthesized. When acetate is the end product, a theoretical maximum yield of 4 moles of H_2 per mole of glucose is obtained. When butyrate is the end product, a theoretical maximum yield of 2 moles of H_2 per mole of glucose is obtained. It should be noted that when the electron flow is completely directed towards the ethanol pathway, the reducing equivalents generated are consumed and no H_2 is generated (Levin et al., 2004). It can be clearly stated that we are facing a trade off between hydrogen and ethanol production. On comparing the delta G values for the three pathways, it can be concluded that glucose to butyrate production pathway is the most favoured one when compared to the ethanol production pathway (Fig 1.4). The previous growth studies of *C. termitidis* suggested that there is an accumulation of excess cellobiose in the culture medium. In the co-culture of *C. termitidis* and *C. intestinale*, it was expected that the left over cellobiose would be consumed by *C. intestinale* which can pull the electrons towards the butyrate pathway and generate H_2 as it produces less ethanol compared to *C. termitidis*.

Figure 1.2 Maximum concentrations of end-products synthesized by monocultures of *C. intestinale* and *C. termitidis* on 2 g/L cellobiose and 2 g/L α-cellulose, respectively (Ramachandran et al., 2008; 2011).

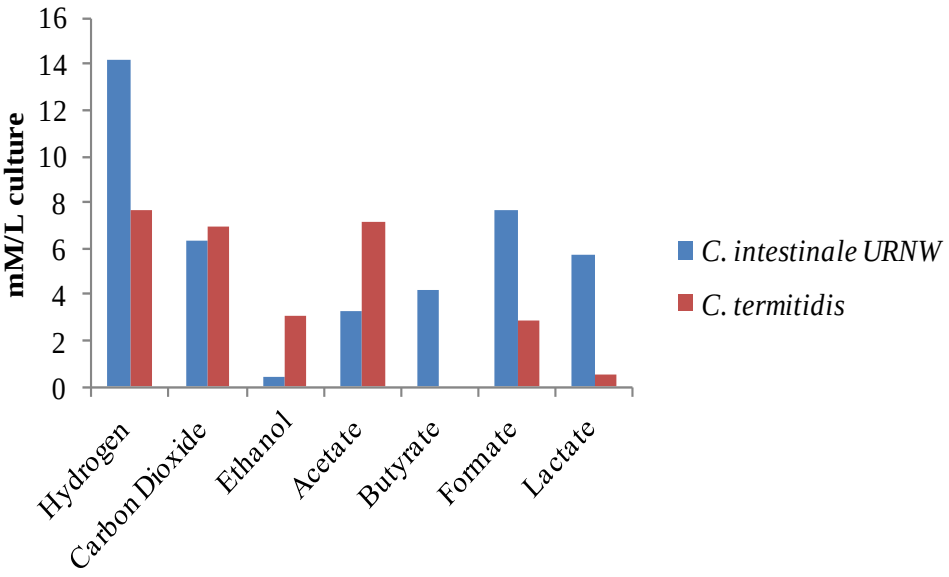


Figure 1.3 Diagrammatic representation of the co-culture of *C. termitidis* and *C. intestinale.*

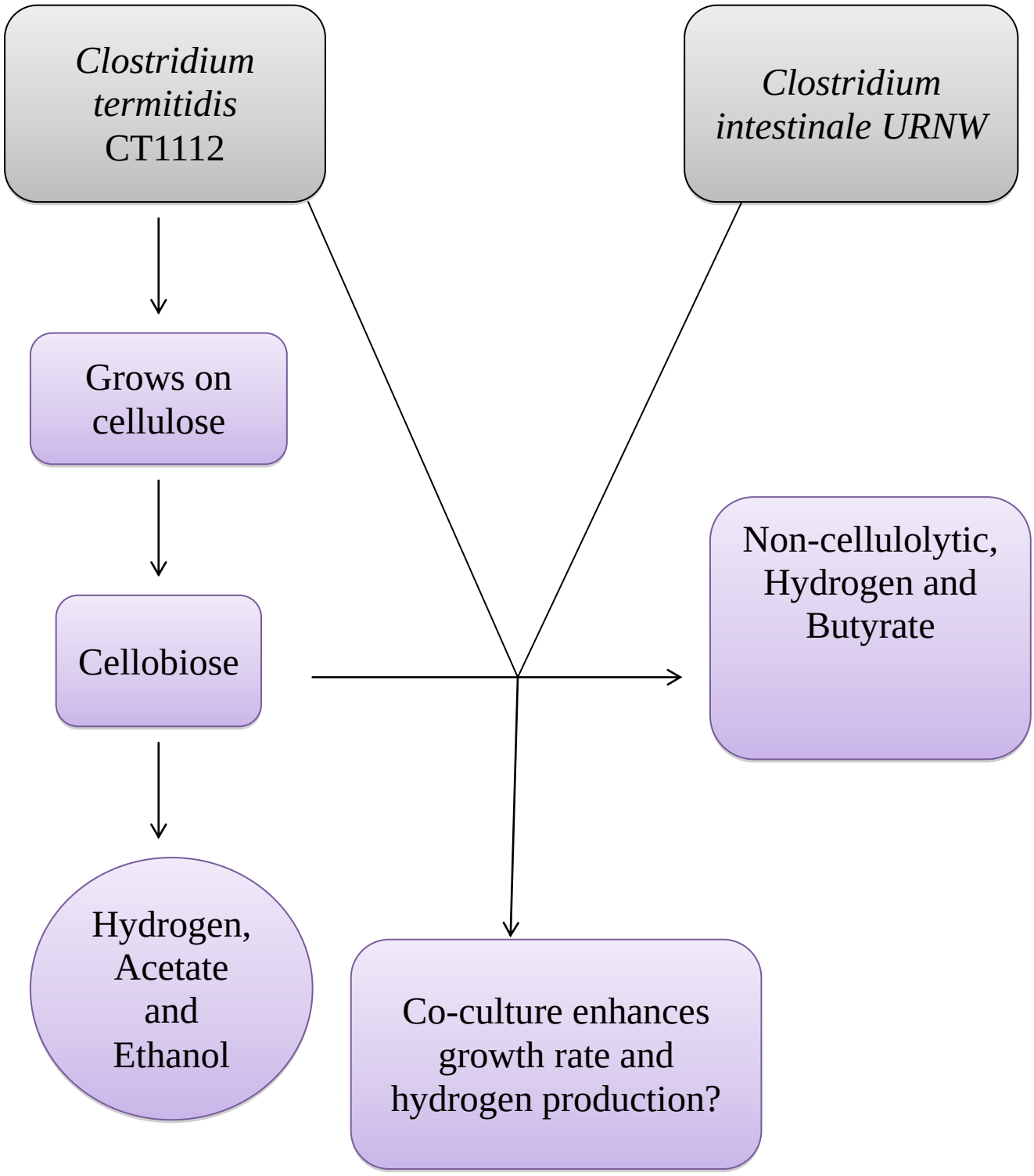
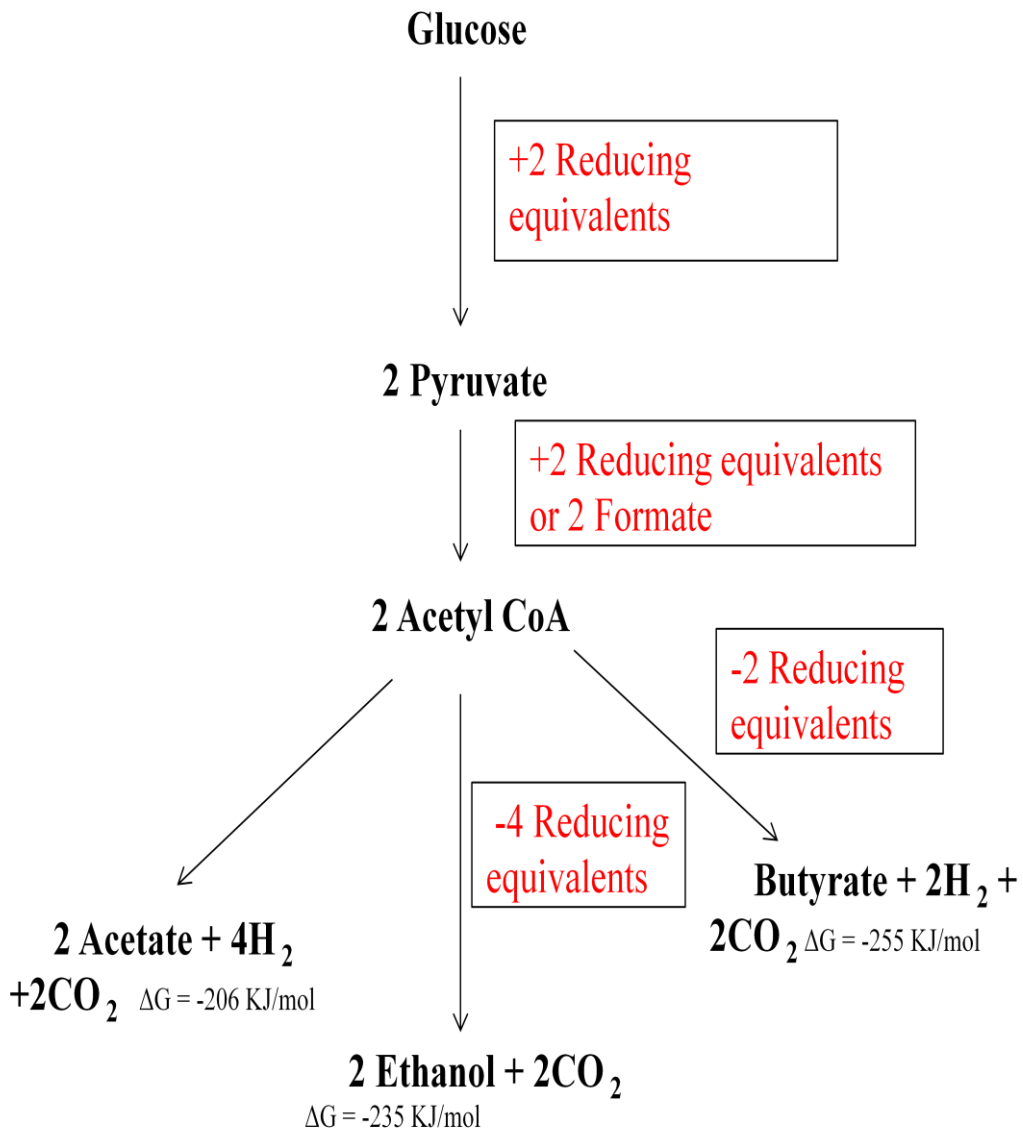


Figure 1.4 Co-culture metabolism (Thauer et al., 1977)



1.7 Quantification of Individual species in the co-culture

From the above mentioned examples of the co-culture systems it is evident that quantification of the individual species during the growth and at the end of growth is essential to understand the mutualism or symbiotic relationship established among the species. Quantitative molecular methods are more accurate and selective than traditional methods as they do not depend on the ability of the bacteria to grow in a selective medium. In addition, DNA based methods of quantification provide us the advantage of storing the samples until they are ready for analysis (Castillo et al., 2006). Real time or Quantitative Polymerase Chain Reaction (qPCR) has been effectively used in the fields of viral quantification, gene expression quantification, DNA damage measurement, pathogen detection and genotyping (Arya et al., 2005, Castillo et al., 2006). Real time PCR offers the reliable detection and quantification of the PCR products developed at the end of each cycle which is directly proportional to the amount of template present at the beginning of the reaction. In the case of the co-culture of *C. termitidis* and *C. intestinale* it is mandatory to develop a method which provides a way of quantifying both the species present in the co-culture. The term multiplex real time PCR describes the use of two or more fluorogenic probes specific for the individual amplicons in the sample. The main advantage of this multiplexing is the reduced reagent costs and preservation of the samples (Arya et al., 2005).

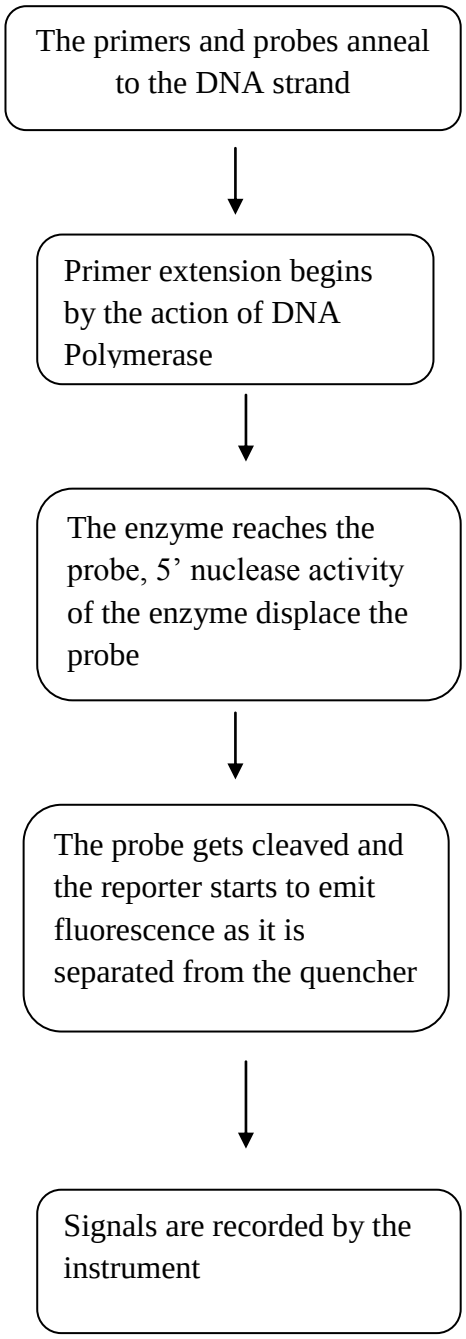
1.7.1 Steps involved in developing a Multiplex qPCR for the quantification of individual species in the co-culture of *C. termitidis* and *C. intestinale*

The major challenge involved in developing a Multiplex qPCR method to quantify the individual species in a sample is the selection of primer/probe target regions which are specific to each species. It is mandatory that the primers and the probes selected for one of the species in the co-culture is not amplifying the other species during multiplexing to avoid any inaccurate or biased results. Though the use of 16S rDNA has been reported to provide

useful information on the identification and discrimination of closely related species, it did not provide unambiguous identification as several closely related species had identical 16S rDNA sequences. Recently it has been reported that the *cpn60* (also known as GroEL or HSP60) sequences are variable even among the closely related bacterial species and can be used to discriminate them compared to 16S rDNA (Brousseau et al., 2001; Goh et al., 1997, 2000). It has also been reported that the *cpn60* gene can be used as a "universal target (UT)" for molecular phylogenetics for the characterization of microbial communities (Jian et al., 2001; Hill et al., 2002, 2005). The *cpn60* gene encodes the 60 kDa chaperonin protein that is found in all eubacteria, and in the plastids and mitochondria of all eukaroyotes. Hill et al. (2004) introduced a cpnDB, which is a curated collection of *cpn60* sequence data obtained from public databases and/or generated by researchers using *cpn60* sequences as target regions in their microbial discrimination studies. *cpnDB* contains approximately 2000 records from eukaroyotes, bacteria and archea and the major taxonomic groups defined by the 16S rRNA "backbone tree". Based on the above literature search and the uniqueness of the *cpn60* gene among the closely related species, primers and probes for the quantification of *C. termitidis* and *C. intestinale* in the co-culture were designed with the data from cpnDB and Joint Genome Institute's Integrated Microbial Genomes (JGI-IMG) Database. Once the primers were designed, the specificity of the primers was checked by the use of iQ SYBR Green Super Mix. The primers for *C. termitidis* were added to the genomic DNA from *C. intestinale* and vice versa to check the primer specificity and to confirm that there was no cross amplification. This step is very crucial as the cross amplification between the species will result in inaccurate or biased quantification. The DNA probes were labelled with different fluorophores at their 5' end (FAM for *C. termitidis* probe and HEX for *C. intestinale* probe) and black hole quencher at the 3' end for the simultaneous quantification of both the

species in the sample at the same time. When the probe is intact it does not emit fluorescence as the signals are absorbed by the quencher (Figure 1.5).

Figure 1.5 Major steps involved in the qPCR



1.8 Characterization of the co-culture with Transcriptomics

Based on the examples mentioned above in section 1.5.1, it is evident that the individual species in a co-culture may involve in number of interactions and have regulatory mechanisms which may influence the growth pattern and end-product synthesis of one another. Though the quantification of the individual species in the co-culture is made possible by the use of qPCR, in order to analyse the co-culture further some advanced techniques such as Transcriptomics and Proteomics are essential. Characterizing and comparing the transcriptome and the proteome of the individual bacteria in the co-culture and as a monoculture will provide a clear picture of the regulations that take place at the molecular level of the individual bacteria the way each bacteria sense the other's presence in the co-culture. The transcriptome is the complete set of transcripts in a cell, and their quantity at a specific growth phase or physiological condition (Wang et al., 2009). Understanding the transcriptome is mandatory for interpreting the functional elements of the genome of each species in the co-culture and compare them to the monocultures. RNA-Seq is an advanced technique developed to revolutionize the transcriptome profiling method and have altered our view of the extent and the complexity of transcriptomes. The key aims of transcriptomics involve: 1) cataloguing all species of transcripts (mRNAs, non-coding RNAs and small RNAs); 2) determining the structure of genes, splicing patterns and other post-transcriptional modifications; and 3) quantifying the change in expression levels of each transcript at different conditions (Snyder et al., 2009). RNA-Seq has a number of advantages over the hybridization-based techniques (Table 1.1), such as: annotation independent detection of transcription, enhanced sensitivity and dynamic range (Thomson et al., 2010). RNA-Seq uses the advanced deep second generation sequencing technologies to characterize the transcriptome in an unbiased manner without altering the experimental design in accordance to the differences in genome sequence. There are several advantages which make RNA-Seq

the ideal technique for studying the complex transcriptomes, which includes: 1) the ability to study the non-model organisms whose genome sequences are yet to be determined; 2) the ability to locate the precise location of the transcription boundaries; and 3) the ability to reveal the connectivity between two or multiple exons. In addition to RNA-Seq, the proteome from the co-culture was characterized for further analysis.

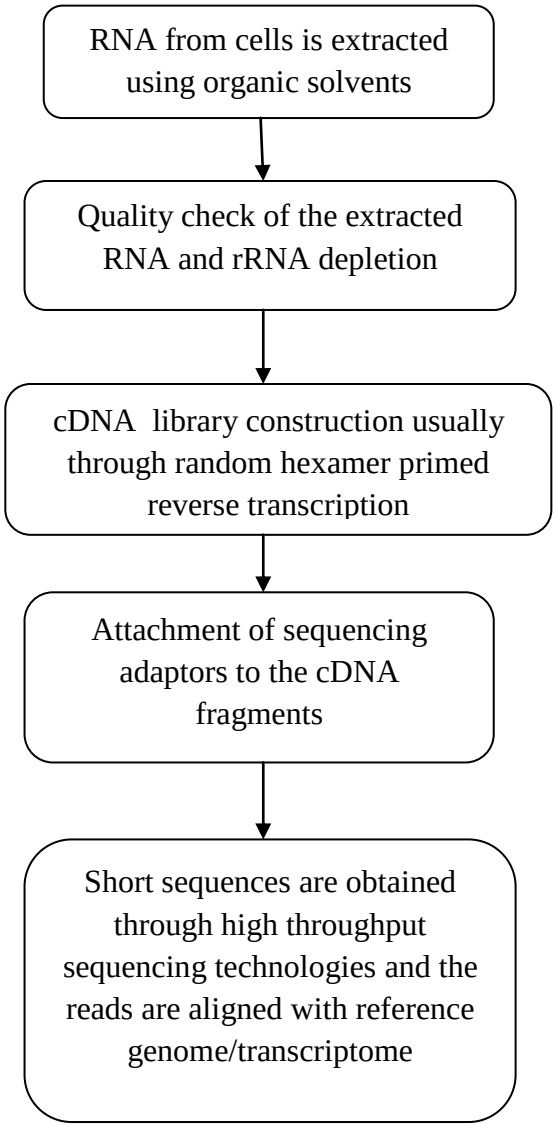
1.8.1 Steps involved in a general RNA-seq experiments

RNA is extracted and purified (to avoid proteins and DNA) from the cells using the commercially available kits or organic solvents. However care should be taken to ensure that the method does not affect the quality/integrity of the RNA (Thomson et al., 2010). The method should also yield high quantity of the starting material, as more RNA is needed for constructing a cDNA library compared to the microarray experiments (Boor et al., 2009). The extracted RNA is then converted to a library of cDNA with adaptors attached to one or both ends. Each molecule is then sequenced from one end or both ends and read (typically 30-400 base pairs) depending on the sequencing technology used. Once sequenced, the reads are then aligned to a reference genome or reference transcripts or assembled *de novo* without the genomic sequence to generate a genome scale transcription map (Figure 1.5) which represents the transcriptional structure and sometimes the expression levels for each gene in the transcriptome (Snyder et al., 2009).

Table 1.1 Advantages of RNA-Seq compared with other transcriptomics methods (Snyder et al., 2009).

Technology	**Tiling Microarray**	**cDNA or EST sequencing**	**RNA-Seq**
Principle	Hybridization	Sanger sequencing	High-throughput sequencing
Resolution	From several to 100 bp	Single base	Single base
Throughput	High	Low	High
Reliance on genomic sequence	Yes	No	In some cases
Background noise	High	Low	Low
Application			
Simultaneously map transcribed regions and gene expression	Yes	Limited for gene expression	Yes
Dynamic range to quantify gene expression level	Up to a few-hundredfold	Not practical	>8,000-fold
Ability to distinguish different isoforms	Limited	Yes	Yes
Ability to distinguish allelic expression	Limited	Yes	Yes
Practical issues			
Required amount of RNA	High	High	Low
Cost for mapping transcriptomes of large genomes	High	High	Relatively low

Figure 1.6 A typical RNA-seq Experiment. RNA extracted is first converted into a library of cDNA fragments through RNA fragmentation or DNA fragmentation. Sequencing adaptors are attached to the cDNA fragments and short sequencing is obtained through the high-throughput sequencing technology. The resulting reads are aligned with the reference genome or transcriptome to generate a genome scale transcription map (Snyder et al., 2009).



1.9 Summary and Research Objectives

The long term objective of this research is to develop a stable co-culture of *C. termitidis* and *C. intestinale* which can hydrolyse cellulose at a faster rate and provide an increase in the substrate utilization and the end-product synthesis (hydrogen and ethanol). The literature search on co-cultures for biofuel production yielded several interesting examples and in most reports, co-cultures displayed increased substrate utilization and end-product synthesis when compared to the monocultures. The research reported in this thesis is based on the same hypothesis and has the following short term objectives:

1) To find the best ratio of the co-culture partners which yields an increased substrate utilization and end-product synthesis when compared to the monoculture of *C. termitidis*;

2) To develop and optimize a method to quantify the individual species in the co-culture by the multiplex qPCR;

3) Analyse and compare the transcriptome and the proteome of the co-culture with that of the monocultures; and

4) Investigate the natural ratio developed between the species on different substrates

Chapter 2

Materials and Methods

This chapter contains a description of the general materials and methods that were common to all experiments described in subsequent chapters.

2.1 Micro-organisms, Media and Substrates

Clostridium termitidis CT1112 was obtained from the American Type Culture Collection (ATCC 51846). *Clostridium intestinale* URNW was isolated as a contaminant within a *C. termitidis* culture with cellobiose as the carbon source. The contaminant was subsequently characterized by Ramachandran et al. (2011). *C. termitidis* and *C. intestinale* cultures were cultured in 1191 medium (Ramachandran et al. 2008; Ramachandran et al. 2011), which was prepared as follows (per liter of milliQ water): KH_2PO_4, 1.5 g; Na_2HPO_4, 3.35 g; NH_4Cl, 0.5 g; $MgCl_2.6H_2O$, 0.18 g; yeast extract, 2.0 g; resazurin (0.25 mg/mL), 2.0 mL; 10X Vitamin Solution, 0.50 mL; 10X Mineral Solution, 1.0 mL. The vitamin solution consisted of the following ingredients (per liter of deionized water): 20 mg of each biotin and folic acid; and 50 mg of each p-aminobenzoic acid, nicotinic acid, thiamine, riboflavin, lipoic acid; and 10 mg of cyanocobalamin. The mineral solution was prepared from (per liter of deionized water): 20.2 g nitrilotriacetate; 2.1 g $FeCl_3.6H_2O$; 2.0 g $CoCl_2.6H_2O$; 1.0 g of each $MnCl_2.4H_2O$, $ZnCl_2$ and $NiCl_2.6H_2O$; 0.5 g of each $CaCl_2.2H_2O$, $CuSO_4.2H_2O$ and $Na_2MoO_4.2H_2O$. L-Cysteine Hydrochloride monohydrate at a concentration of 1 g/L was used as a reducing agent. All required chemicals were purchased from Sigma Aldrich. The final pH of the medium was adjusted to 7.2 for *C. termitidis* and 7.4 for *C. intestinale*.

Pure cultures of *C. termitidis* CT1112 and *C. intestinale* were maintained in 50 mL serum bottles, by transferring a 10% (v/v) aliquot to fresh medium 1191 containing 2 g/L α-cellulose and 2 g/L cellobiose, respectively. Serum bottles with a working volume of 125 mL were used for maintaining the cultures. To maintain anaerobic and sterile environment, the

bottles containing 50 mL medium were air-sealed with butyl-rubber stoppers, crimped with aluminium seals (Bellco Glass, Vineland, NJ), gassed and degassed (1:4 min) four times with 100% nitrogen (N_2) (Ramachandran et al., 2008).

For *C. intestinale* URNW cultures, sterile, anaerobic cellobiose was added after gassing-degassing and sterilization to a final concentration of 2.0 g/L. 50 g/L cellobiose stock was prepared and filter sterilized into an anaerobic, sterile serum bottle sealed with butyl rubber stopper and aluminium crimp top. Culture purity was checked before each experiment by Ribosomal Intergenic Spacer Analysis (RISA), which involves PCR amplification of a region of the rRNA gene operon between the small 16S and large 23S subunits called the intergenic spacer region (ISR). The forward and the reverse primer sequences used are 5'-GT CGTAACAAGGTAGCCGTA-3' and 5'-GTCGTAACAAGGTAGCCGTA-3', respectively. DNA for this analysis was extracted from the fully grown cultures using InstaGene^{TM} Matrix (BioRad).

2.2 Experimental Setup

2.2.1 Batch Experiments

The pre-experimental cultures were first grown in 125 mL serum bottles. *C. termitidis* was grown on 2 g/L α-cellulose, whereas *C. intestinale* was grown on 2 g/L cellobiose. Mid-late exponential phase cultures (65-72 hours for *C. termitidis* and 5-6 hours for *C. intestinale*) were selected and the protein content per mL of the culture (Biomass) was quantified using the Bradford Assay. The ratio of *C. termitidis*: *C. intestinale* in the co-culture at inoculation was calculated based on the protein content measured by the above method. Balch tubes (Bellco Glass Co.) with a working volume of 27 mL were used for all co-culture tests. To maintain anaerobic and sterile conditions, tubes containing 9 mL medium 1191 with 2 g/L α-cellulose or milled wheat straw were air-sealed with butyl-rubber stoppers, crimped with aluminium seals, gassed and degassed (1:4 min) with 100% nitrogen (N_2) (Ramachnadran et

al., 2008). The control tubes were inoculated with 10% v/v fresh, mid-late exponential *C. termitidis* cultured on 2 g/L α-cellulose. The co-culture tubes were inoculated with *C. termitidis* and *C. intestinale.* The volume of the individual cultures required to make different ratios (*C. termitidis*: *C. intestinale*) in the culture were calculated based on the protein concentration of the pre-cultures. The biomass concentrations in the co-culture tubes (two cultures in experimental ratios) were equivalent to the biomass concentrations in the control tubes, which were inoculated with only *C. termitidis*. Balch tubes inoculated with the monoculture and the co-culture were incubated at 37 ^{o}C throughout the experiment. Three independent replicate tubes from the monoculture and the co-culture were sacrificed at time points (based on the experiment).

2.2.2 Analytical Methods

Gas samples (1 mL) were collected from the headspace of each tubes and the gas composition was analysed with the Varian 490 Micro-GC gas chromatograph. Liquid samples (1 mL) were collected from each tube and centrifuged at 4000 g to separate the supernatants and the pellets. The supernatants and the pellets were stored at -20 ^{o}C until analysis. Supernatants were used to quantify the soluble fermentation end-products such as cellobiose, glucose, xylose, lactate, formate, acetate, ethanol and butyrate by a HPLC with a Model 1515 pump, Model 2707 autosampler, and a Model 2414 refractive index detector (Waters, Milford, MA). A 300 mm x 7.8 mm resin-based column was used for separation (Aminex HPX-87H, Bio-Rad Laboratories, Mississauga, ON). Standards of the soluble sugars, ethanol and butyrate were prepared in deionized water and stored in screw cap vials at -20 ^{o}C before being diluted to make standard solutions ranging from 0.1 mM to 50 mM prior to HPLC analysis.

2.3 Quantitative Real Time PCR (qPCR) to quantify individual bacterial species

DNA was extracted from the cell pellets using Instagene™ Matrix to quantify the individual cell population by multiplex qPCR. Primers and DNA probes were designed for both species targeting the *cpn60* region. The forward and the reverse primer sequences used to target the *cpn60* region of *C. termitidis* are, 5-GAAGGGTTTACAGAAAGC-3' and 5'-CTACCACATCATCATTAGC-3'. The forward and the reverse primer sequences used to target the *cpn60* region of *C. intestinale* are 5'-CCTGTTAATGGTAGAGAAG-3' and 5'-CATGGATTTAGATTCTTCAAC-3'. DNA probes for the individual species were designed to be complementary to a sequence of the *cpn60* gene located between the two primers. The 5'-end of the probe specific to the *C. termitidis cpn60* gene was labelled with FAM and the 5'- end of probe for *C. intestinale cpn60* gene was labelled with HEX, with a black hole quencher (BHQ) at the 3'-end. The BHQ was used to facilitate quantification of the individual bacterial genome populations from the same co-culture samples at the same time. The primers were synthesized at Alpha DNA, whereas the probes were ordered from Biosearch Technologies (Petaluma, CA). Specificity of the primers was assayed using the SYBR green fluorescent dye (Bio-Rad Cat # 172-5996) in the qPCR mixture. To make the standards, DNA was extracted from 1 mL of fully grown cellobiose cultures and the DNA concentration was quantified using NanoDrop 1000 spectrophotometer (Thermo Scientific). The genome copy number in the stocks was calculated using the following relation:

$$\text{Copy \#} = \frac{6.023E23 * [DNA]\ g/\mu L * X\ \mu L}{(\text{size of genome in bp})\ (660\ g/mol)\ (\#\text{of copies of the gene in the genome})}$$

where: 6.023E23 is the Avagadro Number; [DNA] is the concentration of DNA measured by NanoDrop 1000; X uL is the amount of template to be added into the reaction mixture; and 660 g/mol is the average molecular weight of a base pair of DNA.

The genome sizes of the individual organisms were found from the JGI-IMG website

From the stock, dilutions were prepared to have known copy number and are used as standards for the qPCR. The reaction mixture contained 10 µL iQ™ Multiplex Powermix (BioRad), 1.5 uL of each forward and reverse primers (10 µM), 0.8 µL of each DNA probes (10 µM), and 2µL of the DNA template. The final volume was brought to 20 µL by adding ddH_2O. Master mix of the PCR mix was prepared based on the number of samples to be analysed. Two (2) µL of the template was first loaded to the qPCR reaction wells and then the master mix was dispensed into the wells. The CFX Connect™ Real-Time PCR Detection System was used for the qPCR runs. The qPCR reaction involved the following steps. Reactions were subjected to an initial denaturation at 95 °C for 3 minutes (min); followed by 39 cycles each of denaturation at 95 °C for 10 seconds (s); annealing at 62 °C for 10 s; and extension at 72 °C for 30 s. At the end of each extension step, the signals from the wells were read by the instrument.

2.4 Sample Preparation for Transcriptomics and Proteomics

Omics study involves growing the microbial cells in 1000 mL serum bottles with a working volume of 500 mL. Fifty (50) mL of the mid-log and stationary phase cells were collected in RNase free falcon tubes. The sample was centrifuged at maximum speed for 20 min and the supernatant was discarded. The cell pellets were re-suspended in 1 mL of 1X PBS buffer and washed to remove any residual medium present with the cells. The cell pellets for RNA extraction were then re-suspended in 1 mL of RNAlater (Life Technologies) – RNA stabilization solution, and stored at -80 ^oC until they are ready for protein and RNA extraction. The cell pellets for protein extraction were stored at -80 ^oC without the addition of any stabilization agents.

2.4.1 RNA Extraction and Quality Analysis

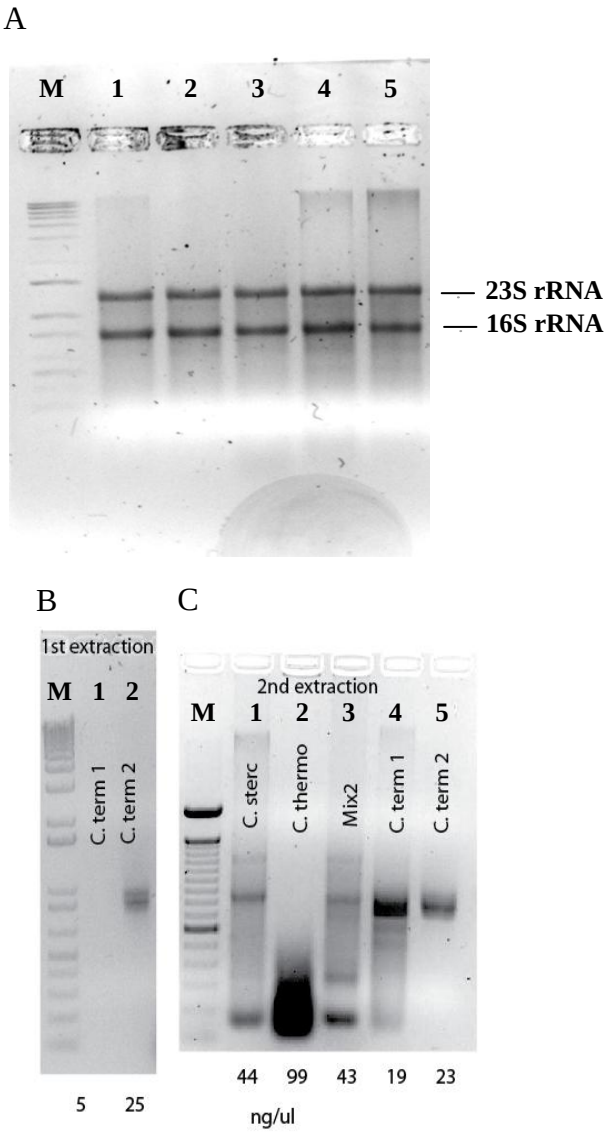
Prior to extraction, the frozen cells were centrifuged to remove the RNA stabilization solution. The extraction procedure was carried on with ChargeSwitch Nucleic Acid

Purification kit (Life Technologies), with a magnetic rack following the kit guide lines. After the addition of the lysis buffer, the sample was divided into three portions (1 mL each) to ease sample handling and to have technical replicates. At all stages of the sampling and the extraction process, RNase free tubes, tips and solutions were used to prevent the denaturation of the RNA. The quality and the quantity of the extracted RNA were checked with NanoDrop 1000, by measuring the absorption at 230 nm, 260 nm, and 280 nm. In addition the extracted samples were run on a 1% agarose gel and Experion^{TM} Automated Electrophoresis System (BioRad) to check the integrity of the extracted RNA (Figure 2.1). The samples were shipped frozen to Genome Quebec for sequencing.

2.4.2 Protein Extraction

The samples for protein extraction were thawed and re-suspended in 2 mL of SDS lysis buffer (8% of SDS in 200 mL Tris-HCl, 200 mM DTT). The samples were divided into small volumes (0.5 mL each) in microcentrifuge tubes and sonicated for 30 s at the power level 35%. The supernatant was collected and the quantity and quality of the extracted proteins were checked with SDS PAGE (NuPAGE Bis-Tris precast gels 4-12%, 1.0 mm, 10 well (Invitrogen, cat. NP0321 Box). Based on the gel results 500 µL of the samples and 12 mL of Urea Tris buffer (8M Urea in 100 mM Tris, pH 8.5) was added to the filter of the Ultrafiltration centrifugal units: Amicon Ultra-15 with Ultracel-10 Membrane NMMW 10000 (Millipore, cat. UFC901024). The tubes were centrifuged at 4000 g for 20 minutes at 25 ^{o}C and 12 mL of Urea Tris buffer was added to the filter. This process was repeated three times and each time after the centrifugation, the volume in the filter was made sure to be below the 1000 µL mark on the filter. The volume in the filter was adjusted to 1 mL with UA and then 2X IAA solution (0.05 M Iodoacetamide prepared in Urea-Tris Solution) was added and mixed with the pipette tip. Care should be taken not to touch the filter membrane. The tubes were stored in dark for 10 minutes and centrifuged at 4000 g for 5 minutes and the flow

Figure 2.1 Quality control of RNA extracted for RNA Seq analysis. A) High quality RNA extracted from *C. termitidis* cells in mid-log phase showing intact 23S and 16S rRNA bands; B) Degraded RNA extracted from two different cultures of *C. termitidis*. 1) First extraction of RNA from *C. termitidis* culture C.term 1; 2) First extraction of RNA extracted from *C. termitidis* culture C.term 2 in stationary phase; C) RNA extracted from cultures of different species. 1) C.sterc, *Clostridium stercorarium*; 2) C.thermo, *Clostridium thermocellum*; 3) Mix 2, RNA extracted from a mixture of *C. stercorarium* and *C. thermocellum*; 4) Second extraction of RNA from *C. termitidis* culture C.term 1; 5) Second extraction of RNA from *C. termitidis* culture C.term 2. molecular size marker (1 Kb ladder, Life Technologies). Numbers under panels B and C indicate the concentrations of RNA in each extract measured by NanoDrop 1000.



through was discarded. Twelve (12) mL of Urea-Tris buffer was added and centrifuged for 4000 g for 40 minutes at 25 ^{o}C and the final volume in the filter was adjusted to be 1 mL. 100 µL sample was taken from the filter to estimate the protein concentration by Bicinchoninic acid assay. After taking the sample for protein estimation, 10 mL of ABC buffer (0.05 M Ammonium Carbonate) was added to the filter and centrifuged for 30 min at 4000 g at 25 ^{o}C. Based on the protein assay, sufficient amount of Trypsin is added to the filter (Protein: Trypsin = 50:1) and the tubes were incubated overnight in dark.

All the washing steps were performed to remove the SDS, as it can interfere with the sequencing. Next day, the filter units were transferred to a new falcon tube and centrifuged for 15 minutes at 4000 g, 25 ^{o}C. One (1) mL of 0.5 M NaCl was added to the filter and the flow through was also collected. The protein concentration in the total flow through was estimated with NanoDrop 1000 and the samples were desalted with the HPLC. The purified samples were sequenced with 1D MS.

2.4.3 RNA/Protein Identification and Statistical Analysis

Reads from RNAseq and peptide MS/MS spectra from proteomic analysis were identified by mapping them to the IMG/ER version of the *C. termitidis* and *C. intestinale* genomes. For peptides, raw WIFF files were converted into Mascot Generic Format (MGF) using the Mascot.dll script bundled with Analyst QS2.0 (Perkins et al., 1999). The resulting CID spectra were analyzed using our in-house graphic processor unit (GPU) based peptide identification engine (McQueen et al., 2012) with standard QTOF settings. Candidate peptide masses and CID parent masses must fall within a fixed tolerance, typically 20 PPM for the TripleTOF 5600 mass spectrometer. The raw data from the RNAseq builds a non-redundant alignment database with a maximum of 3000 100-mer alignment elements per gene (and 3000 of their reverse-complement counterparts) using a Monte Carlo approach of random numbers. Raw Illumina R2 paired-end read sets are exactly aligned against this database via

Perl associative arrays, and the alignments counter for the *C. termitidis* and *C. intestinale* member genes are incremented. The in-house developed simple platform UNITY which is used to map the multi-omic expression values into a common analysis matrix. Columns contain log2 expression values for a given experiment, while rows encode the source proteins/genes, their annotation descriptions. The resulting data is subjected to a simple filtering: RNAseq values must have at least two alignment counts, and proteins must have at least two peptides with expectation values of log(e)< -1.5 each. The data are then analysed/compared by constructing simple scatter plots, counting, calculating the differences and filtering functions. The protein total ion counts (TIC) were used as a relative measure of the protein expression in an experimental run and expressed in log2 scale. For transcriptomics, log2 of the sum of 100-mer gene alignment counts were used for the analysis. The difference in expression values of genes of each species in the co-culture was compared to its monoculture data to identify any significant up/down regulation.

Chapter 3

Testing Different Ratios of the Co-culture of *C. termitidis* and *C. intestinale* to Find the Optimum Ratio for Biofuel Production

3.1 Introduction

Fermentative biofuel production can be improved by using co-cultures of bacteria. Co-cultures of selected anaerobic bacteria have been shown to display greater rates of substrate conversion and end-product synthesis than monocultures of the same species (He et al., 2011; Kriedelbaugh et al., 2013). The co-culture of *Clostridium termitidis* and *Clostridium intestinale* URNW can be considered as an example for a mesophillic system that uses cellulose to produce H_2. *C. termitidis* can hydrolyze cellulose to release cellobiose, glucose, and other free sugars. *C. intestinale,* which is not capable of hydrolyzing cellulose, can utilize the cellobiose and glucose released by cellulose hydrolysis for growth and synthesizes H_2 and other end products (Ramachandran et al., 2008 and 2011).

A study was conducted to analyse the effect of different inoculation ratios of *C. termitidis* and *C. intestinale* on the growth of these two bacteria in a co-culture, and to determine if a particular ratio results in increased end-product synthesis and/or substrate conversion, relative to a *C. termitidis* monoculture. Samples were collected at two points (100 and 168 hours post-inoculation, which corresponds to the mid-log and stationary phase of the monoculture of *C. termitidis* on 2 g/L α-cellulose) for analyses of end-product concentrations, substrate consumption and quantification of each species by real-time quantitative polymerase chain reactions (qPCR). The inoculation ratio of the two species which generated a co-culture with end-product synthesis and substrate consumption patterns similar to or better than, the monoculture of *C. termitidis* would be considered for the next experiments.

3.2 Materials and Methods

The microorganisms and medium preparation used for this study were described in Chapter 2, Section 2.1.The experiment was performed in Balch tubes containing medium 1191 plus 2 g/L α-cellulose. The initial inoculation ratios of *C. termitidis* and *C. intestinale* used (1:1, 1:0.2, 1:0.1, 1:0.05, 1:0.02) were prepared according to the biomass/protein estimation results. To ease the handling of small volumes of individual inocula and to prevent contamination, a "master mix" of 20 mL was prepared for each co-culture ratio and inoculated into the tubes.

The tubes with different co-culture inoculation ratios and the monoculture of *C. termitidis* were incubated at 37 ^{o}C. At 100 hours post-inoculation (h pi) and 168 h pi, three of the Balch tubes from each initial inoculation ratio and 3 Balch tubes of the *C. termitidis* monoculture were taken for analysis. Gas samples and liquid samples were analysed as described in Chapter 2, Section 2.2.2. The residual cellulose was estimated by dry cell mass, as described in Chapter 2. Ratios of *C. ternitidis* and *C. intestinale* genome copy numbers (as an indicator of cell numbers) in the co-culture were quantified at each time point by the method described in Chapter 2, Section 2.3. The variances in end-product synthesis, substrate utilization, and gene copy numbers among the different co-culture inoculum ratios and the monoculture were statistically analysed by one way ANOVA (p value = 0.05) using Microsoft Excel 2007 and SAS 9.3.

3.3 Results

3.3.1 Growth Characteristics

The increase in total (*C. termitidis* and *C. intestinale*) biomass concentration for each initial inoculation ratio and the *C. termitidis* monoculture was estimated by measuring the protein content. Statistical analysis by one way ANOVA indicated that two of the cultures

(1:1 and 1:0.2) among all the tested cultures (including the monoculture) showed significantly different biomass content (Figure 3.1).

3.3.2 Gas Production

Hydrogen and CO_2 concentrations generated by co-cultures resulting from the different initial inoculation ratios of *C. termitidis* and *C. intestinale* were compared with gas concentrations resulting from the *C. termitidis* monoculture. The H_2 and CO_2 produced by the co-cultures and the monocultures showed no significant differences as indicated by the ANOVA (Table 3.2 and Figure 3.2).

3.3.3 Liquid End-Product Analyses

Concentrations of liquid phase, soluble end-products generated by co-cultures resulting from the different initial inoculation ratios of *C. termitidis* and *C. intestinale* were compared with those detected in the liquid phase of the *C. termitidis* monoculture are shown in Figure 3.3. The major soluble end-products included: 1) acetate, which was produced at significantly higher concentrations compared to other liquid end-products; 2) ethanol; and 3) butyrate. *C. termitidis* synthesizes acetate and ethanol, but not butyrate, while *C. intestinale* synthesizes buytrate, acetate and trace amounts of ethanol. The differences in liquid end-product concentrations were not statistically different among the co-culture ratios and the monoculture (Table 3.1). As butyrate is produced only by *C. intestinale*, it is essential to perform ANOVA to understand the difference in butyrate synthesis among the five initial inoculation ratios of the co-culture. The results of ANOVA ($F_{4,10;0.05} > F_{critic}$) clearly showed that there was no statistical difference in butyrate production among the co-cultures, which shows that the growth of *C. intestinale* is restricted to a fixed number regardless of the starting ratio.

3.3.4 Substrate Utilization

Substrate (α-cellulose) consumption in each sample set was calculated by drying and weighing the cellulose left at 100 and 168 h pi. These values were normalized with the estimated protein to calculate the cellulose consumed (Table 3.1). The data showed that there was a significant difference in substrate consumption by the co-cultures, compared with the *C. termitidis* monoculture, when the initial inoculation ratio of the co-culture was 1:1 or 1:0.2. This may be due to the higher cell number of *C. intestinale* in these ratios when compared with that of the other ratios investigated. The higher *C. intestinale* cell numbers might have created a higher competition for the residual cellobiose and which might have significantly affected the cellulose degradation by *C. termitidis* in these ratios.

Table 3.1 Cellulose consumption by the *C. termitidis* (*Cter*) and *C. intestinale* (*Cint*) co-cultures and the *C. termitidis* monoculture at 168 hours post-inoculation (h pi). * Statistcally significant differences.

Culture	Cellulose consumption at the end of 168 h pi (µg/mL)	Standard Deviation
Control (*Cter*)	1477.00	6.32
1:1 (*Cter:Cint*)	1261.00	60.02*
1:0.2 (*Cter:Cint*)	1249.76	51.33*
1:0.1 (*Cter:Cint*)	1560.35	43.60
1:0.05 (*Cter:Cint*)	1436.77	13.50
1:0.02 (*Cter:Cint*)	1516.03	16.00

Table 3.2 Analysis of differences (ANOVA) in biomass production, end-product concentrations, substrate utilization, and genome copy numbers in co-cultures generated from all different initial inoculation ratios compared to the *C. termitidis* mono-culture.

Source	F-Critic	F-Test
Biomass	3.11	4.06*
End-products		
H_2	3.11	0.05
CO_2	3.11	1.09
Ethanol	3.11	1.76
Acetate	3.11	0.95
Substrate utilization	3.11	36.2*
Genome copy number		
C. termitidis	3.11	1.18
C. intestinale	3.48	3.44

H_0 = the Null Hypothesis, in which the means are all equal; H_A = Hypothesis, in which at least one of the means is different. If the F-test value > the F-Critic value, then the Null Hypothesis is rejected and we can conclude that one (1:1) of the means is significantly different, with 95% confidence. * - statistically significant differences

Figure 3.1 Protein concentrations extracted from co-cultures resulting from different initial inoculation ratios and the *C. termitidis* monoculture cultured on 2 g/L α-cellulose, as determined by the Bradford Assay. This figure shows one representative replicate of the three biological replicate experiments performed in this study. Error bars represent standard deviation about the means of three biological replicates. ➡ – Statistically significant difference.

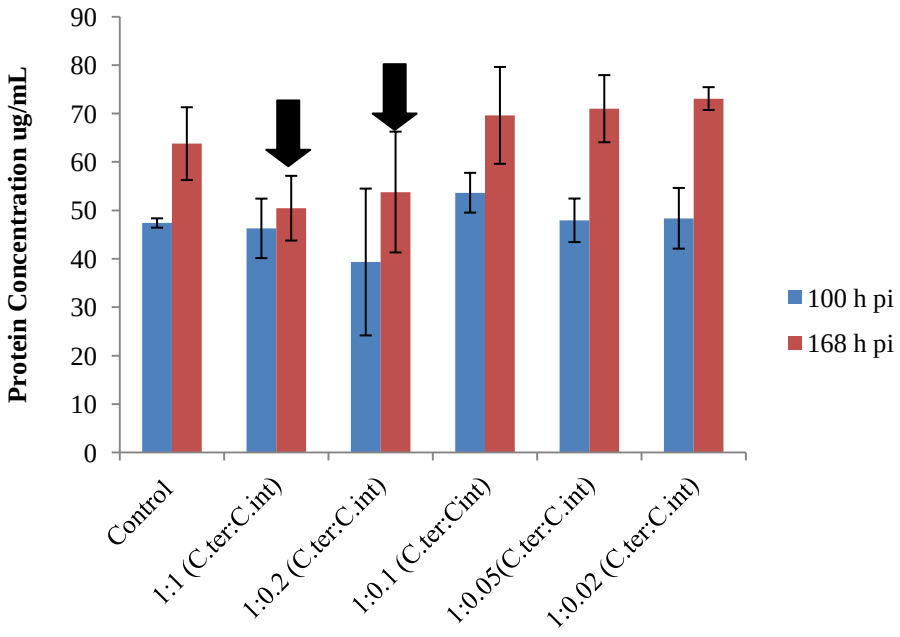
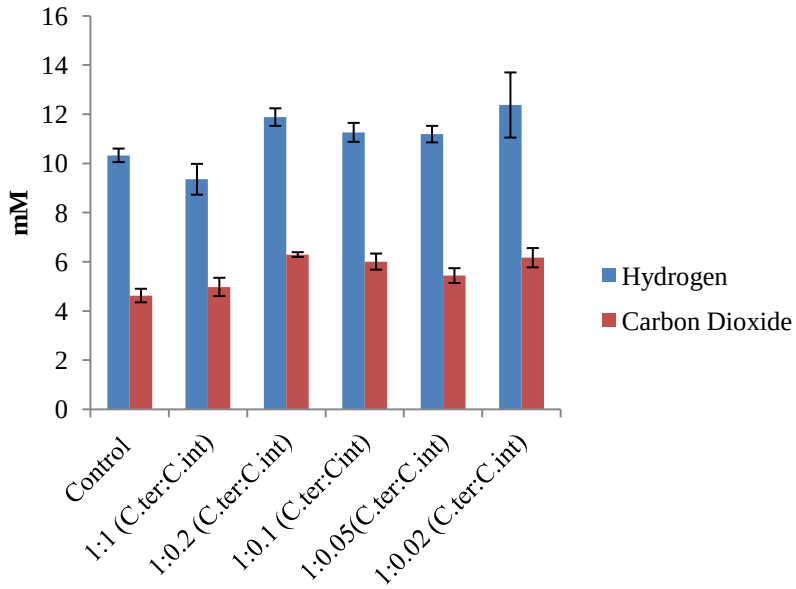


Figure 3.2 Gas composition analysis for the *C. termitidis* - *C. intestinale* co-cultures and the *C. termitidis* mono-culture at the end of A) 100 h pi and B) 168 h pi. bars represent standard deviation about the means of three biological replicates.

A)



B)

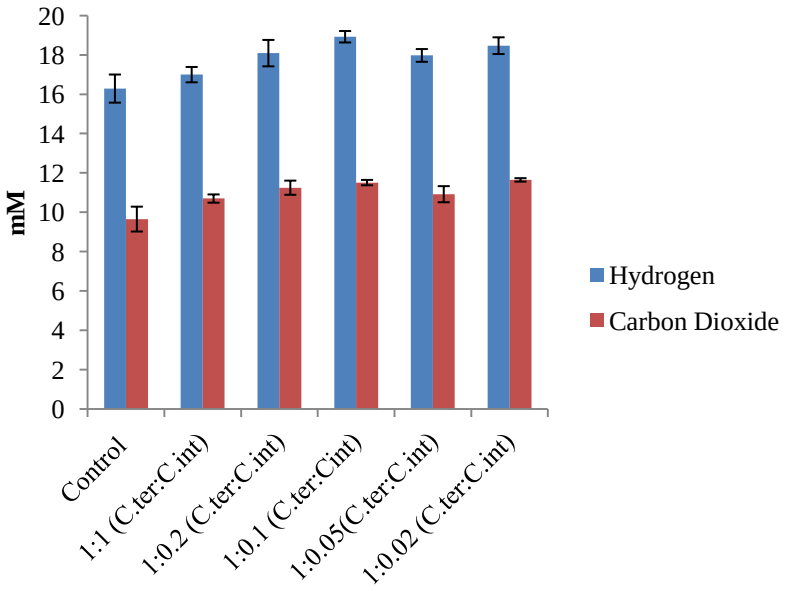
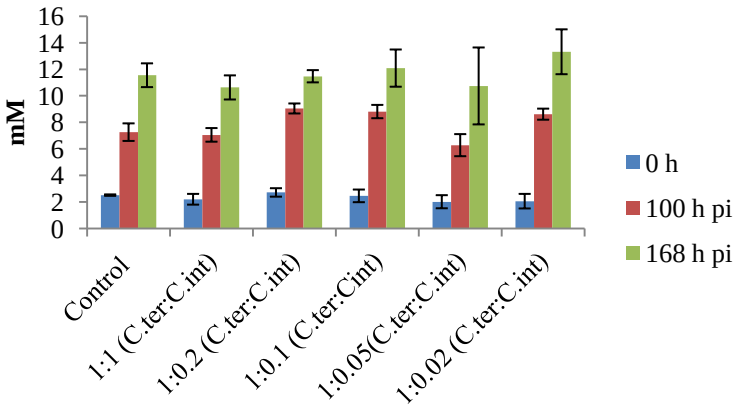
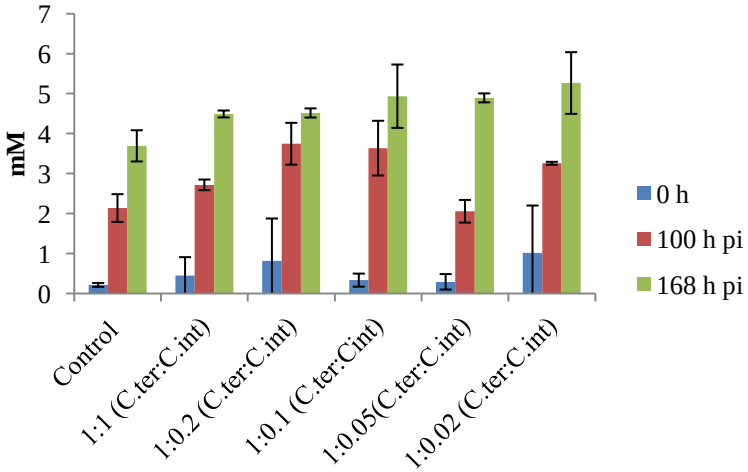


Figure 3.3 Concentrations of liquid end-products. A) Acetate; B) Ethanol, and C) Butyrate at 0, 100, and 168 h pi. Error bars represent standard deviation about the means of three biological replicates**.**

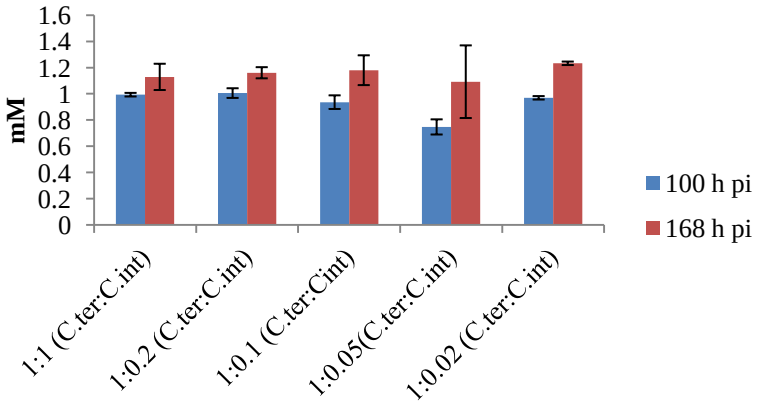
A)



B)



C)



3.3.5 Quantification of Individual Microbial Cells by qPCR

The ratios of *C. termitidis* and *C. intestinale* genome copy numbers (as an indicator of cell number) resulting in the co-cultures generated from different initial inoculation ratios and in the *C. termitidis* monoculture were quantified by qPCR at 100 and 168 h pi (Table 3.3 and Figure 3.4). The results of the statistical analysis (ANOVA) showed that the copy numbers were not significantly different among the co-cultures and the monoculture tested. In addition, it was observed that the copy number of *C. intestinale* was limited to a specific level (approximately log 6) in all the co-culture ratios, regardless of the initial inoculum ratio. This shows that there may be a hindrance for the growth of *C. intestinale* in the co-culture beyond a level of growth.

3.4 Discussion and Conclusions

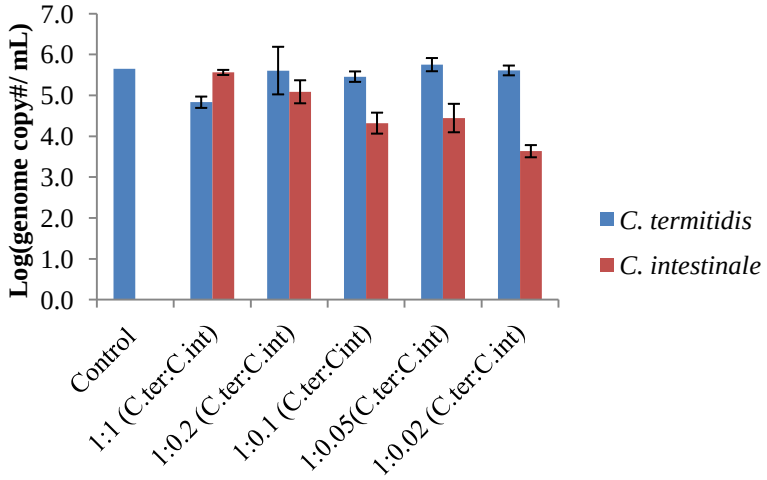
Co-cultures have been reported to enhance both substrate conversion and end-product synthesis by synergistically stimulating metabolic pathways (He et al., 2011; Kriedelbaugh et al., 2013). It was hypothesized that the cellulolytic *C. termitidis* would hydrolyse cellulose and release free soluble sugars for *C. intestinale* consumption and that the co-cultures would display increased end-product synthesis and substrate utilization relative to the *C. termitidis* monoculture. The data from this study does not support this hypothesis.

The results of one way ANOVA suggests that the co-cultures generated from initial innoculation ratios of 1:1 and 1:0.2 showed a significant difference (lesser than the control) in substrate consumption and total biomass produced compared to all other cultures: i.e. co-cultures generated with other initial inoculation ratios, as well as the *C. termitidis* monoculture. This may be due to the increased copy numbers of *C. intestinale* in this inoculum compared to other ratios, which hindered the cellulose degrading ability of *C. termitidis.* Though statistically significant difference in substrate utilization and biomass production were observed for the co-cultures with initial inoculation ratios of 1:1 and 1:0.2,

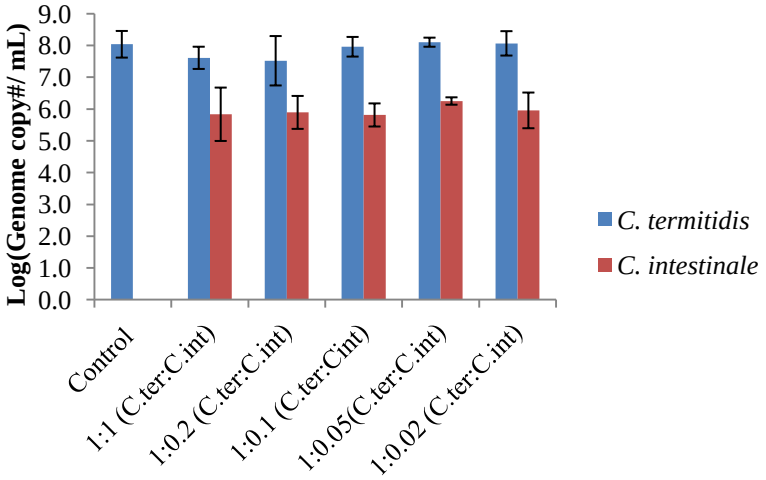
no significant differences were observed in end-product concentrations or substrate utilization in these cultures, relative to the monoculture, as reported in previous literature where a two-fold increase in end-product synthesis was observed in the co-culture compared to the monoculture (Liu et al., 2008). The growth of *C. intestinale* is limited to a certain value (log 6 genome copy#/mL) regardless of the starting inoculation ratio and it may be due to the effect of *C. termitidis* growing in the co-culture which might have a hindrance towards the growth of *C. intestinale*. *C. intestinale* was isolated and characterized as a contaminant from a *C. termitidis* stock bottle and it is expected that these species will develop a natural ratio as other mixed cultures in the natural environment such as wheat straw. So the following chapters will focus on identifying the natural ratio established between these bacteria over repeated passaging and analysing the transcriptome and the proteome of these individual species in the co-culture and as a monoculture to characterize the metabolic changes.

Figure 3.4 Genome copy numbers of *C. termitidis* and *C. intestinale* in A) each initial inoculum ratio mix; B) at 100 h pi; and C) 168 h pi. Error bars represent standard deviation about the means of three biological replicate.

A)



B)



C)

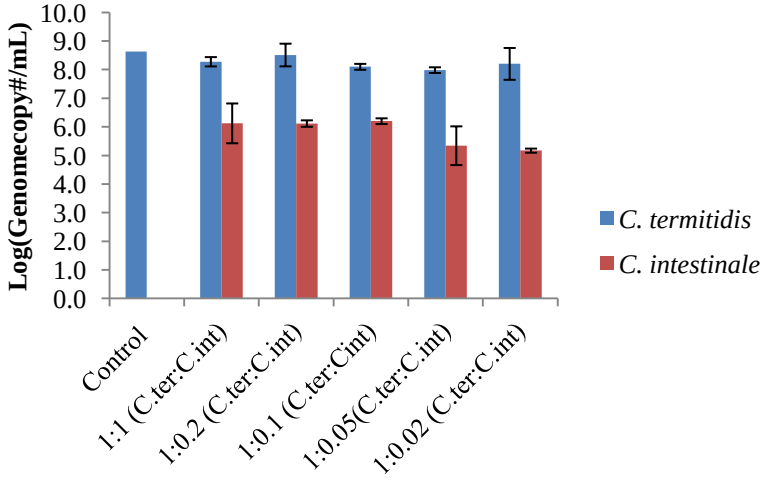


Table 3.3 Quantification of individual bacterial species in the different inoculum ratios, and the ratio established at 0 hour, 100 h pi and 168 h pi.

Ratio of inoculum by Protein Assay	**Log (genome copy#/mL *C. termitidis*)**			**Log (genome copy#/mL *C. intestinale*)**			**Ratio =Genome copy #/mL *C. termitidis*/Genome copy #/mL *C. intestinale***		
	0 Hour	**100 h pi**	**168 h pi**	**0 Hour**	**100 h pi**	**168 h pi**	**0 Hour**	**100 h pi**	**168 h pi**
Control	5.6	8.0	8.6	-	-	-	-	-	-
1:1	4.8	7.6	8.3	5.6	5.8	6.1	0.2	63.1	158.5
1:0.2	5.6	7.5	8.5	5.1	5.9	6.1	3.2	39.9	251.2
1:0.1	5.5	8.0	6.9	4.3	5.8	6.2	15.9	158.5	5.1
1:0.05	5.8	8.1	8.0	4.4	6.3	5.3	25.1	63.1	501.2
1:0.02	5.6	8.1	8.2	3.6	6.0	5.2	100	125.9	1000

Chapter 4

Comparison of the Growth and End-product synthesis of *Clostridium termitidis* on α-cellulose and Glenn cultivar Wheat Straw

4.1 Introduction

In the previous chapters, growth pattern and end-product synthesis of *Clostridium termitidis* and the different inoculum ratios of the co-culture were analysed on α-cellulose. Lignocellulosic agricultural waste materials are considered as suitable and inexpensive sources of cellulose for cellulosic biofuel production. However the complex structure of these raw materials is a serious hurdle for the microorganisms to utilise cellulose effectively in their metabolic pathways. In this study, the wheat straw from Glen cultivar was pre-treated with superheated steam and used as the carbon source for the growth of *C. termitidis*. The compositional analysis of the Glen cultivar wheat straw showed that the raw material has 9.56% of lignin, 42% of cellulose and 25.39% of hemicellulose. *C. termitidis* may be very attractive for raw material degradation because it is capable of both cellulose and hemi-cellulose degradation (Hethener et al., 1992). The raw material was initially grounded and treated with superheated steam which is described in section 4.2.1 in detail. The growth pattern and the end-product synthesis of *C. termitidis* on the pre-treated wheat straw were compared with that of α-cellulose and ground wheat straw to analyse the effect of pre-treatment. The initial study was conducted as an end-point study in 150 mL serum bottles where the cultures were allowed to grow for up to 240 hours. The growth of *C. termitidis* on these substrates were analysed by measuring the final pH, and the end products concentrations. Based on the results from this study a time point experiment was designed to conduct a detailed study.

4.2 Materials and Methods

4.2.1 Collection and pre-treatment of wheat straw

The wheat straw obtained was initially grounded using a coffee grinder and sieved using a 0.355 mm sieve. Then a few grams of the sample was placed in an iron container covered with mesh cage and boiled in a pressure cooker for 30 minutes. The sample was retrieved and placed in a mesh cage and exposed to 200 ^{o}C superheated steam, flowing over it at a constant flow rate for 15 minutes as per the protocol developed and optimized by Dr. Cenkowski's group at the University of Manitoba.

4.2.2 Micro-organisms and Medium

The micro-organisms and the medium preparation used for this study were described in Chapter 2, Section 2.1. The preliminary end point study was conducted in 150 mL serum bottles and the time point study was performed in 27 mL Balch tubes. The study was conducted on 2 g/L of the substrate (α-cellulose or ground wheat straw or pre-treated wheat straw). *C. termitidis* was also cultured on 4.8 g/L of wheat straw to account for 2 g/L of cellulose based on the compositional analysis of the wheat straw. Mid-log phase culture of *C. termitidis*, cultured on 2 g/L α-cellulose was used as the source of inoculum for this study. The experimental tubes and bottles were inoculated with 10 % (v/v) of the inoculum and incubated at 37 ^{0}C. Samples were collected at the end of growth (240 hour post inoculation) for the preliminary end-point study and every 24 hours for the time point study.

4.2.3 Analysis of the Gaseous end-products

The composition of the gas samples collected was analysed by the method described in Chapter 2, Section 2.2.2.

4.2.4 Analysis of the Liquid End products

The composition of the liquid samples collected was analysed by the method described in Chapter 2, Section 2.2.2.

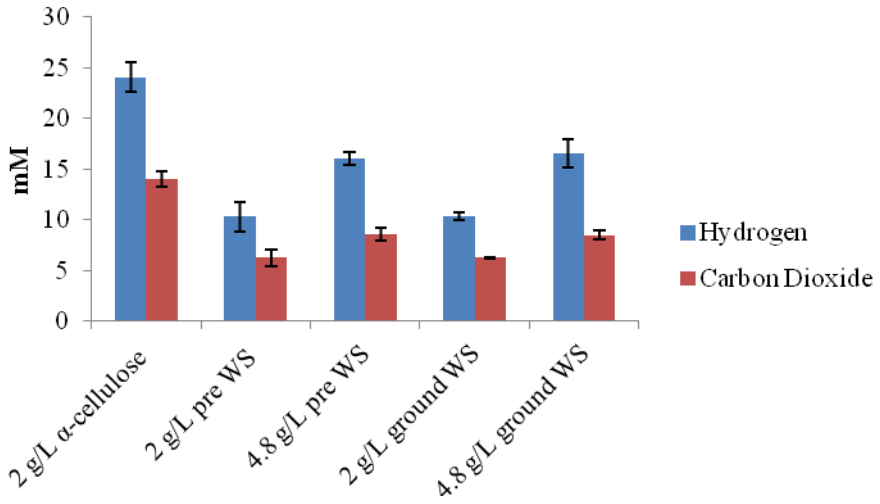
4.3 Results

4.3.1 Growth Characteristics

The results of the gas measurement, liquid end-product analysis and pH at 240 h pi showed that the culture of *C. termitidis* on α-cellulose showed the highest growth. The hydrogen production by *C. termitidis*, which is an indicator of its growth on ground wheat straw and steam pre-treated wheat straw, showed no significant differences indicating that the steam pre-treatment had no effect on increasing the cellulose availability to *C. termitidis* (Figure 4.1). Compositional analysis of the liquid products yielded the same results (Figure 4.2). Since the growth of *C. termitidis* was similar on both types of wheat straw, ground wheat straw was taken for the time point growth study. The growth pattern of *C. termitidis* on 2 g/L α-cellulose was compared to the growth on ground wheat straw. Samples were collected at every 24 hours for growth measurements. On the ground wheat straw, the cells were in the lag phase for 48 hours and the growth entered into the stationary phase at 120 h pi (Figures 4.3 and 4.4).

Figure 4.1 A) Hydrogen (H_2) and carbon dioxide (CO_2) produced by *Clostridium termitidis* on α-cellulose and different concentrations of super heated steam treated wheat straw (pre WS) and ground WS. B) pH drop measured on each substrates at 240 h pi. Error bars represent the standard deviation.

A)



B)

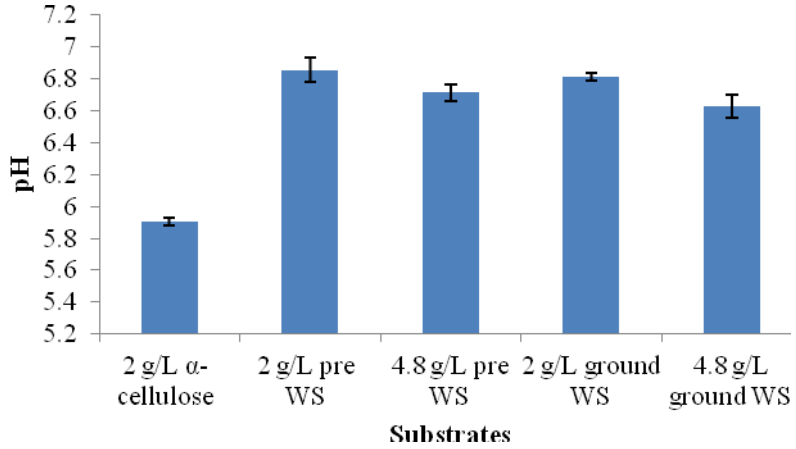
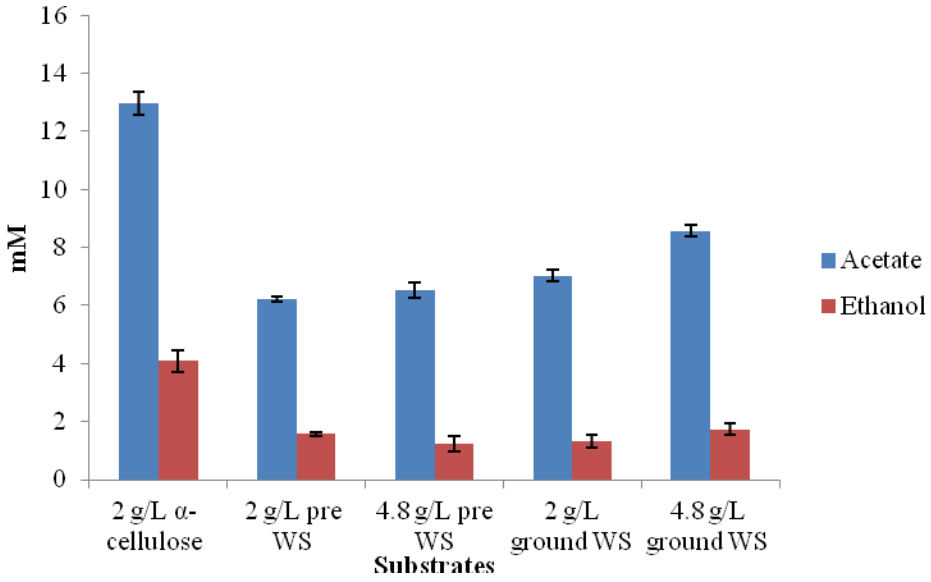


Figure 4.2 A) & B) Liquid end-products produced by *Clostridium termitidis* on α-cellulose and different concentrations of super heated steam treated wheat straw (pre WS) and ground WS. Error bars represent the standard deviation of the biological replicates.

A)



B)

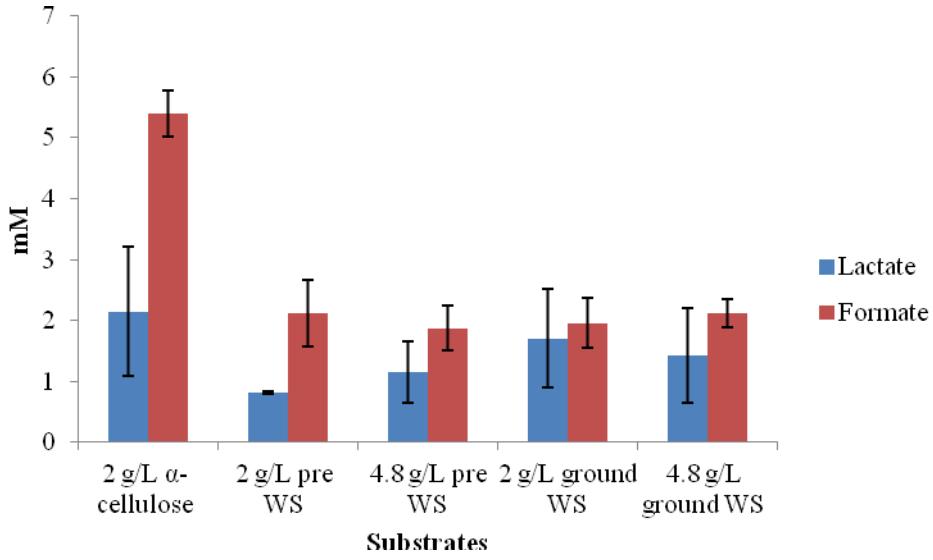
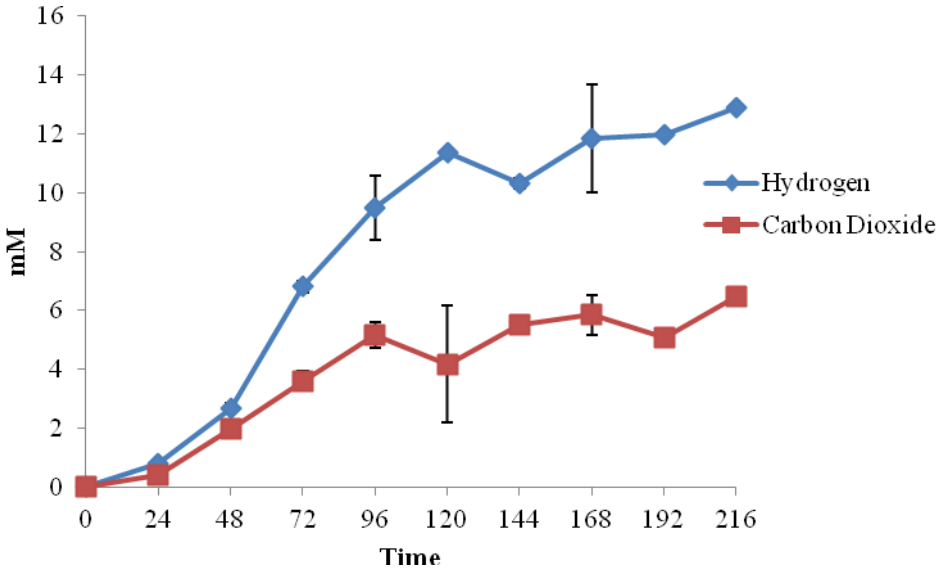


Figure 4.3 A) Gas production by *C. termitidis* on 2 g/L α-cellulose. B) Change in pH during the growth of *C. termitidis* on 2 g/L α-cellulose. Error bars represent the standard deviation of the biological replicates.

A)



B)

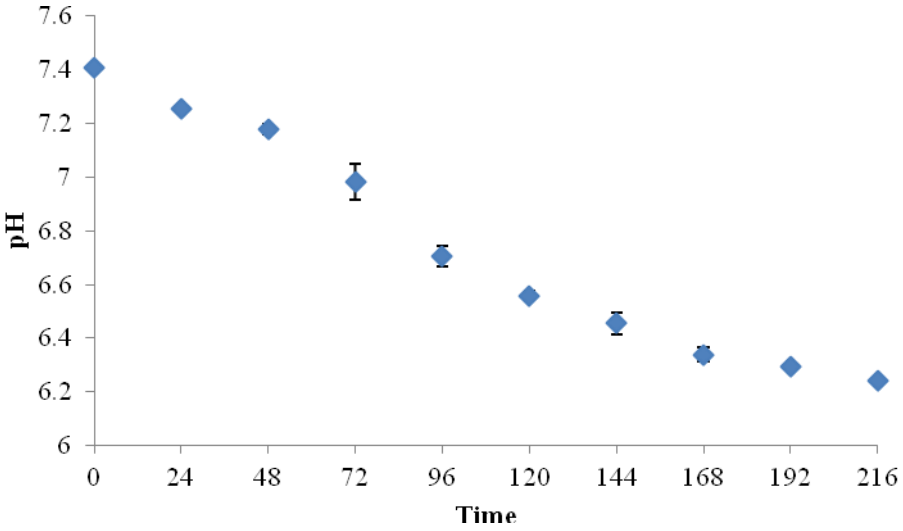
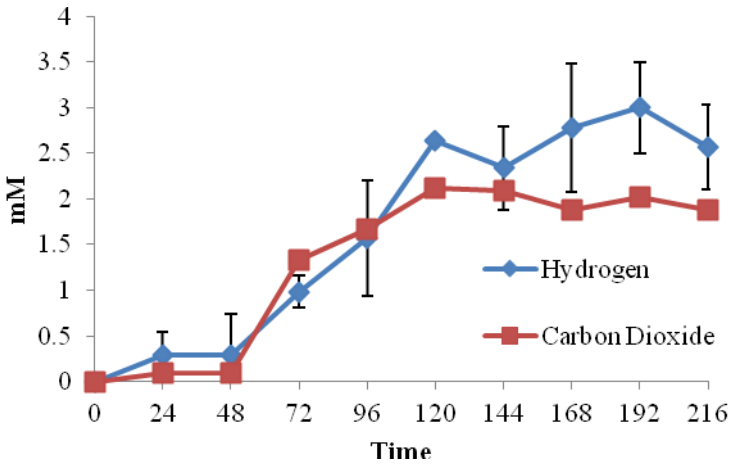
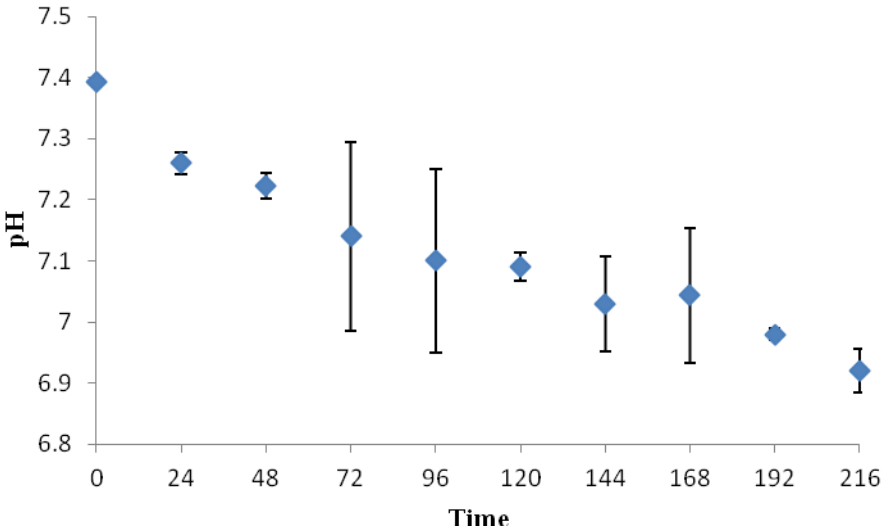


Figure 4.4 A) Gas production by *C. termitidis* on 2 g/L Glenn cultivar ground wheat straw. B) Change in pH during the growth of *C. termitidis* on 2 g/L Glenn cultivar ground wheat straw. Error bars represent the standard deviation of the biological replicates.

A)



B)



4.4 Discussion and Conclusions

The results from the end-point and time point experiment indicated that *C. termitidis* has poor growth (approximately 50% less end products) on both raw and super heated steam treated wheat straw when compared to α-cellulose. It was also observed that there was no significant difference in growth between ground wheat straw and super heated steam treated wheat straw. This may be due to the unavailability of consumable cellulose in ground straw. In case of the super heated steam treated wheat straw, the pre-treatment (by boiling and super-heated steam) might have also caused some structural modifications to ligno-cellulose, which reduced the accessibility of cellulose chains to *C. termitidis* or cellulases. In the co-culture of *C. termitidis* and *C. intestinale*, the cellulolytic *C. termitidis* supplies the soluble sugars for *C. intestinale* to grow actively. As *C. termitidis* showed significant growth on wheat straw, the co-culture can also be tested for its growth on the same substrate. The next studies will focus on finding the natural ratio established between *C. termitidis* and *C. intestinale* on α-cellulose and wheat straw.

Chapter 5

Continuous Passaging of the Co-culture to Find the Natural Ratio on cellulose and wheat straw

5.1 Introduction

In the previous chapters, substrate utilization, end-product synthesis, and changes in populations of *C. termitidis* and *C. intestinale* in co-cultures generated from different initial inoculation ratios were analysed. Although statistically significant differences were observed in the biomass production and substrate utilization, this was not reflected in the end product synthesis. It was also observed that the maximum cell density achieved by growth of *C. intestinale* was limited, regardless of the initial inoculum ratio. Hence, it was important to understand the natural ratio established between *C. termitidis* and *C. intestinale* when the two bacteria were co-cultured over an extended period of time. The previous chapter provided strong evidence for the considerable growth of *C. termitidis* on grounded and super heated steam wheat straw. So these substrates were also included in this study.

In their natural habitats, bacterial species are engaged in numerous interactions with other species through various mechanisms, such as commensalism, mutualism, competition, or parasitism (Jagman et al., 2012). *C. intestinale* was isolated and identified as a contaminant from a cellobiose stock bottle with *C. termitidis* (Ramachndran et al., 2011). So it is expected that these individual species will also establish a stable co-culture over long-term sub-culturing.

5.2 Materials and Methods

The microorganisms and medium preparation used for this study were described in Chapter 2, Section 2.1.The experiment was performed in Balch tubes containing medium 1191 plus 2 g/L α-cellulose and wheat straw (un-treated and pre-treated) derived from the Glen cultivar. Raw wheat straw was ground by using a coffee grinder and sieved using a

0.355 mm sieve (un-treated). Then a few grams of the sample was added to water and boiled in a pressure cooker for 30 minutes. The samples were retrieved and placed in a mesh cage and exposed to 200 ^{o}C superheated steam, flowing over it at a constant flow rate for 15 minutes (pre-treated) as per the protocol developed by Dr. Cenkowski's group in the Department of Biosystems Engineering at the University of Manitoba. This experiment was performed to understand the natural ratio established by these bacteria on three different substrates (α-cellulose, ground wheat straw and boiled/super-heated steam treated wheat straw) upon repeated sub-culturing.

The pre-cultures of *C. termitidis* and *C. intestinale* were grown on 2 g/L α-cellulose and cellobiose, respectively. At the mid-log phase, the cultures were mixed in a 1:1 ratio (v/v) to prepare a master mix of the inoculum and were then inoculated into the experimental tubes with different substrates. After inoculation, the tubes were incubated at 37 ^{o}C for 168 hours (h). At 168 hours post-inoculation (h pi), 1 mL (10%) of the co-culture was transferred into fresh tubes containing the same substrate. The procedure was repeated for 6 passages on each individual substrate. Samples were collected at the end of each passage for pH measurement, end-product analysis, and quantification of genome copy number for each species by qPCR. Liquid samples were analysed as described in Chapter 2, Section 2.2.2. Genome copy numbers of each bacterial species in the co-culture were determined as described in Chapter 2, Section 2.3.

5.3 Results

5.3.1 Growth Characteristics

At the end of each passage (168 h pi), pH was measured and taken as a direct indicator of bacterial growth, due to production or organic acids (Figure 5.1). Among the substrates, the co-culture showed higher growth on α-cellulose followed by the un-treated wheat straw. Poor growth of the co-culture was observed for the boiled/steam treated wheat

straw. This may be due to structural changes in the lignocellulosic material resulting from steam pre-treatment, which may have reduced the accessibility of the cellulose chains to *C. termitidis* and/or cellulase enzymes secreted by *C. termitidis* in the co-culture.

5.3.2 Liquid End-products

At the end of each passage (168 h pi) liquid samples were collected for acetate and butyrate quantification (Figure 5.2). Acetate synthesis was taken as an indicator of *C. termitidis* growth, while butyrate synthesis was considered as an indicator of *C. intestinale* growth in the co-culture. Similar to the results of the pH measurements, the co-culture on α-cellulose showed maximum acetate production followed by the un-treated wheat straw. These data demonstrate that *C. termitidis* grew actively on α-cellulose and un-treated wheat straw. Although butyrate concentrations produced by the co-cultures were statistically different on the individual substrates, the differences were not as high as those observed for acetate. These data clearly show that the growth of *C. intestinale* was completely dependent on the growth of *C. termitidis* (Figure 5.2).

5.3.3 Quantification of individual bacteria at the end of each passage

Changes in numbers of *C. termitidis* and *C. intestinale* cells at the end of each passage, as indicated by genome copy number, were quantified by qPCR. The ratio established among the individual bacteria in the co-culture was calculated by dividing the genome copy number of *C. termitidis* by that of the *C. intestinale*. The ratio of the genome copy numbers of *C. termitidis* to the genome copy number of *C. intestinale* in the inoculum was found to be 13:1. This may be converted to a simple ratio of *C. termitidis*: *C. intestinale* of approximately 3:1. As hypothesized, the co-culture developed a stable ratio of individual species on each substrate (Table 5.1).

Figure 5.1 pH readings of the co-culture on 2 g/L cellulose, pre-treated wheat straw, and un-treated wheat straw at the end of each passage. Error bars represent standard deviation about the means of three biological replicates.

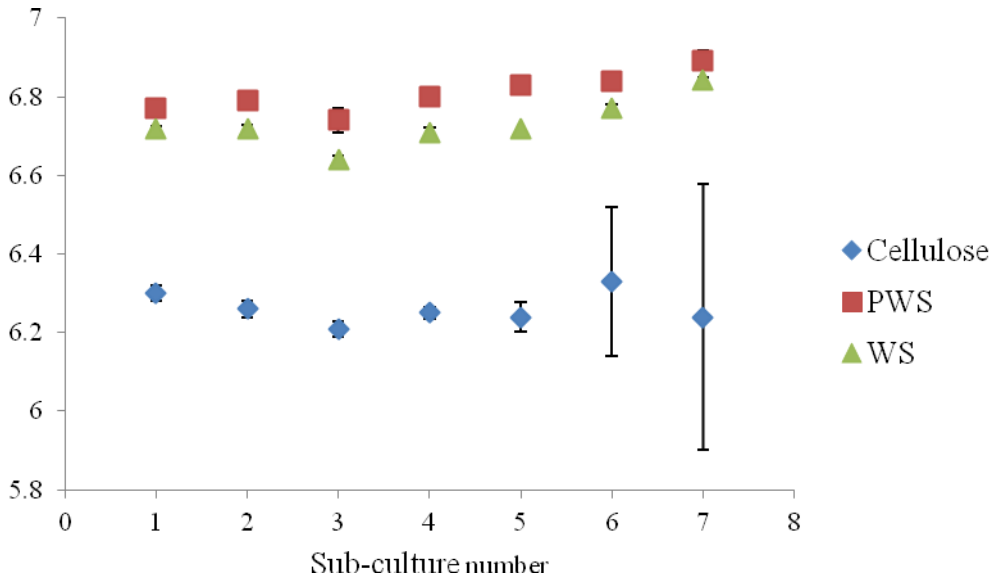
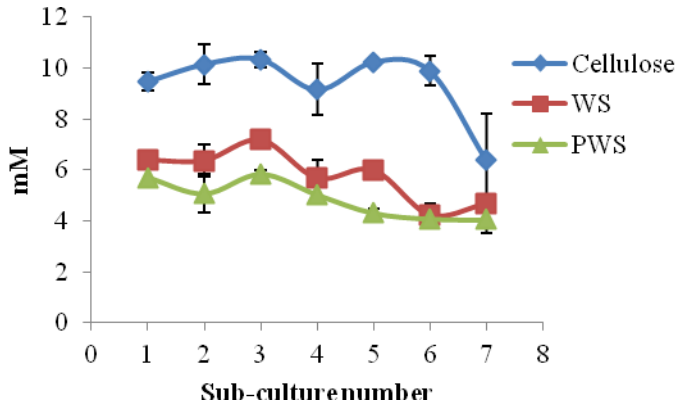


Figure 5.2 A) Acetate and B) Butyrate produced by the co-culture at the end of each passage. Error bars represent standard deviation about the means of three biological replicates.

A)



B)

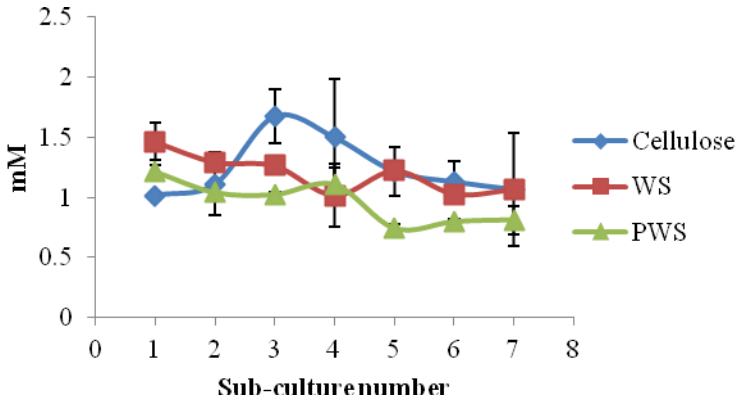
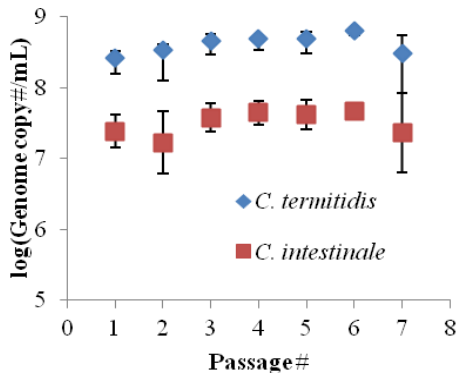
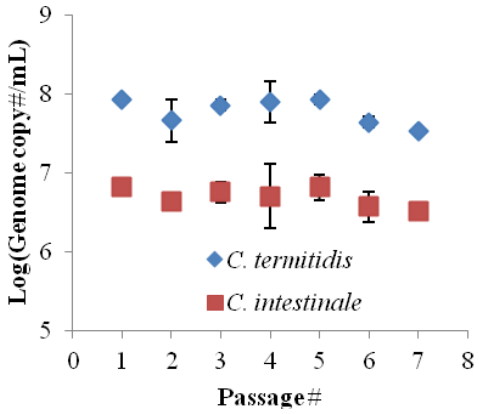


Figure 5.3 Quantification of *C. termitidis* and *C. intestinale* genome copy numbers at the end of each passage in co-cultures grown on A) α-cellulose, B) un-treated wheat straw, and C) pre-treated wheat straw. Error bars represent standard deviation about the means of three biological replicates.

A)



B)



C)

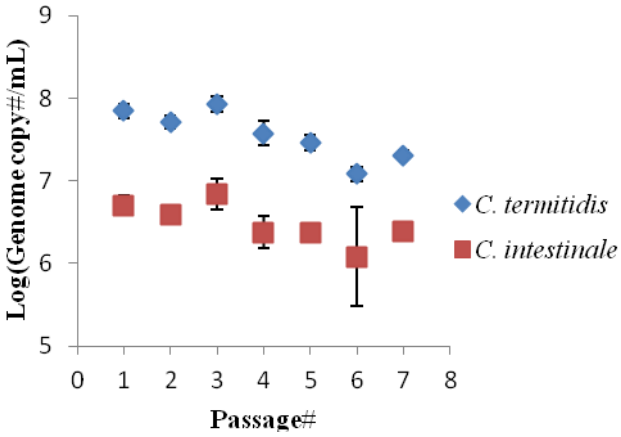


Table 5.1 Ratios of *C. termitidis* : *C. intestinale* cells, as indicated by genome copy number, at the end of each passage.

Passage Number	**Genome copy # *C. termitidis*/ µL : Genome copy # *C. intestinale*/ µL**		
	α-Cellulose	**Un-treated wheat straw**	**Pre-treated wheat straw**
1	10.9	12.9	13.8
2	20.5	10.7	13.2
3	12.2	12.8	12.5
4	11.1	15.6	15.8
5	11.7	13.0	12.4
6	13.7	11.9	9.9
7	13.2	10.1	8.3
Average	13.3 ± 3.3	12.4 ± 1.7	12.3 ± 2.47

5.4 Discussion and Conclusions

Microbial species in nature interact with other species and establish a balance among them to carry on their biological functions. This study was conducted with the view of identifying the natural balance of microbial cells which may be established among *C. termitidis* and *C. intestinale* on α-cellulose, un-treated wheat straw, and pre-treated wheat straw. The data from the study supported this hypothesis, as we observed the establishment of stable co-cultures on each substrate, after continuous passaging (Table 5.1). Though the co-culture ratio on α-cellulose had stable values and higher cell numbers (Figure 5.3), the co-culture ratio on wheat straw substrates showed low cell numbers, indicating poor growth of *C. intestinale* one those substrates. This may be due to the poor growth of *C. termitidis* on these substrates due to the unavailability of consumable cellulose from these substrates and that in turn affected the growth of *C. intestinale*. The pre-treatment (by boiling and steam) might have also caused some structural modifications to lignocellulose, which reduced the accessibility of cellulose chains to *C. termitidis* or cellulases. This also gives us a clear indication that *C. intestinale* cannot grow independently on wheat straw. The next chapter will describe studies of co-cultures initiated with a *C. termitidis*: *C. intestinale* ratio close to the natural ratio established, 13:1, with analyses of substrate utilization and end-product synthesis, relative to the *C. termitidis* monoculture. Time points will be selected based on the gas production for the ‘Omics analysis and the changes at molecular levels will be characterized at the molecular levels and compared to the respective monocultures to find changes in the expression of genes.

Chapter 6

‘Omics analysis of the co-culture of *C. termitidis* and *C. intestinale*: A time point study

6.1 Introduction

In Chapter 3, different inoculum ratios of *C. termitidis* and *C. intestinale* were investigated to determine if co-cultures of *C. termitidis* and *C. intestinale* resulted greater substrate conversion and increased end-product concentrations compared to *C. termitidis* monoculture. The experiments had only two sampling times (100 h pi and 168 h pi). Statistical differences were observed in the substrate utilization and total biomass production in cultures generated from initial inoculation the ratios 1:1 and 1:0.2, but no differences were observed in the concentrations of end-product synthesized at the two time points sampled. None of the co-culture initial inoculum ratios showed a greater increase in end-product synthesis or substrate utilization relative to the monoculture of *C. termitidis* as in other co-culture systems discussed in literature.

In Chapter 5, repeated passaging of the co-culture (1:1 v/v) on different substrates (α- cellulose, un-treated wheat straw, and pre-treated wheat straw) was conducted to determine if an equilibrium would be established between the populations of *C. termitidis* and *C. intestinale* in the co-culture at the end of growth (168 h pi). As expected, an equilibrium was established between the two species on individual substrates which was approximately 13:1.

In this chapter, data resulting from a time point study of the *C. termitidis* and *C. intestinale* co-culture (1:25) was analysed to understand the growth patterns of each species in the co-culture over time, and further to characterize the differences in the transcriptome and proteome of the co-culture compared to the monocultures. In addition to this, the ‘Omics data can also provide evidences on how one species in the co-culture can sense the presence

of the other partner by the identification of signalling molecules involved in quorum sensing circuits.

6.2. Materials and Methods

The micro-organisms and medium preparation used for this study were described in Chapter 2, Section 2.1.The experiment was performed in Balch tubes containing medium 1191 plus 2 g/L α-cellulose. As in previous studies, *C. termitidis* and *C. intestinale* for the inoculum were cultured on 2 g/L α-cellulose and 2 g/L cellobiose respectively. Mid-log phase *C. termitidis* and *C. intestinale* cells taken from their respective monocultures were mixed in a ratio of 5:1 (based on protein estimation) and inoculated into the experimental tubes. The control tubes were inoculated with cells derived from *C. termitidis* monocultures and the tubes were incubated at 37 ^{o}C. This particular ratio was chosen for this study because this ratio was easy to handle and to avoid additional processing that could have resulted in contamination, and generated robust co-cultures. Quantification of the individual bacteria in the inoculum by qPCR showed that the ratio of the genome copy number of *C. termitidis* to genome copy number of *C. intestinale* was equal to 1:25. Though this co-culture has increased number of *C. intestinale* than *C. termitidis* (unlike previous studies*)*, the data can still be used to characterize the co-culture in detail with the help of 'Omics tools.

Three co-culture and three monoculture tubes were sacrificed every 24 hours to measure the gas production, to collect samples for liquid end-product analysis, and for DNA extraction. The composition of head space gas (H_2 and CO_2) was analysed by as described in Chapter 2, Section 2.2.2. The sampling time points for transcriptomics and proteomic analysis were selected on the basis of H_2 gas production by the co-cultures. Previous studies have established that H_2 production by *C. termitidis* very closely follows cell growth (Ramachandran et al. 2008).

DNA was extracted from cells and processed for qPCR analysis of the *cpn60* gene as described in Chapter 2, Section 2.3. RNA and protein samples for 'Omics analyses were prepared as described in Chapter 2, Section 2.4. Data from transcriptomic (RNA seq) and proteomic analyses obtained from the co-cultures were compared to those of *C. termitidis* and *C. intestinale* monocultures to identify changes in gene and/or gene product expression in each species.

6.3 Results and Discussion

6.3.1 Growth Characteristics

Co-culture and monoculture sample tubes (in triplicates) were collected at every 24 hours. The growth patterns of the co-cultures and the monocultures were measured by estimating their biomass content by extraction of total proteins. No significant differences were observed in the total biomass produced by the co-cultures versus the monocultures. By 24 h pi, cell mass production by the co-cultures increased significantly compared to the monocultures, and then stabilized (Figure 6.1A). This may be due to the fast growth of the co-culture on cellobiose carried over from the inoculum.

6.3.2 Gas Production

H_2 and CO_2 concentrations in the head space of the culture tubes was analysed by the method as described in Chapter 2, Section 2.2.2. Although the starting inoculum ratio was different from the previous experiments, the H_2 and CO_2 concentrations produced by the co-cultures were not statistically different from the concentrations synthesized by the monocultures. These data were very important to understand the growth pattern of the co-culture in detail and to select sampling points for further analysis. Based on the H_2 production pattern, exponential (log) phase growth of the co-culture occurred between 74 and 144 h pi, while stationary phase occurred from approximately 144 h pi to the end of the experiment.

6.3.3 Liquid End-Product Analysis

Liquid samples were collected at the end of every 24 hours and analysed as described in Chapter 2, Section 2.2.2. Acetate synthesis was taken as a direct indicator of *C. termitidis* growth, while butyrate synthesis was taken as a direct indicator for *C. intestinale* growth in the co-culture. Although there was no significant difference in butyrate synthesis between the co-culture and the monoculture, the ethanol production in the monoculture was higher when compared to the co-culture. Also it was observed that the ethanol concentration in the co-culture at the end of growth dropped, indicating that the ethanol produced might have been consumed by *C. intestinale* after 120 h pi (Figure 6.3).

6.3.4 Quantification of Individual species in the Co-culture

Population of individual bacteria in the co-culture was quantified as described in Chapter 2, Section 2.3. There were no significant differences in the growth patterns of *C. termitidis* in the co-culture compared with the monoculture. This result suggested that the growth of the individual species in the co-culture was not affected by the presence of the other species. The qPCR data were consistent with the growth patterns of the individual species determined by the end-product synthesis patterns: acetate for *C. termitidis* and butyrate for *C. intestinale* (Figures 6.4 and 6.5).

6.3.5 Substrate utilization by the co-culture and the monoculture

The amount of residual cellulose in each experimental tube was determined at the end of every 24 hours. Substrate utilization by the co-cultures was not statistically different from those of the monocultures (Figure 6.6).

6.3.6 Analysis of Transcriptomics and Proteomics Data

As indicated above, transcriptomic and proteomic analyses were performed with extracts from co-cultured *C. termitidis* and *C. intestinale* cells harvested at 120 h pi and 192 h pi. The samples and the data were processed by the method described in Chapter 2 Section

2.4. The data from the co-culture was compared with that of the monocultures of *C. termitidis* and *C. intestinale* (from different experiments). Cells harvested at 120 h pi were assumed to be in mid- to late log phase, based on end-product (H_2 and organic acid) synthesis curves, while cells harvested are 192 h pi were clearly in stationary phase. However, quantification of *C. termitidis* and *C. intestinale* genome copy number by qPCR revealed that both species were in the late-log phase of growth at 120 h pi (Figure 6.4B and 6.5B) and only these samples are taken for comparison.

The data obtained from the 'omics analyses were "filtered" to reveal changes the expression levels (significant up- or down-regulation) of genes specific to each species in the co-culture. The 1D LC/MS/MS proteomics analyses yielded very low coverage of the expression data for most of the genes that more than 50 % of the gene products were not detected in the proteome. However, the transcriptomics data had excellent coverage (almost 100% of the genes in the genome), and was therefore used for the comparison of gene expression levels, while the proteomics data were used as supplementary data to confirm the changes in gene expression observed.

The expression data obtained from the transcriptomics and proteomics showed very good correlation, and the expression of genes from *C. termitidis* and *C. intestinale* indicates that both species were metabolically active in the co-culture (Figure 6.7A). RNA and protein expression levels did not change significantly in *C. termitidis* between late-log and stationary phase. However, the total number of *C. intestinale* genes transcribed was significantly greater in the stationary phase of the co-culture compared to late-log phase, while protein expression levels decreased in the stationary phase compared to the late-log phase (Figure 6.7 A and 6.7 B).

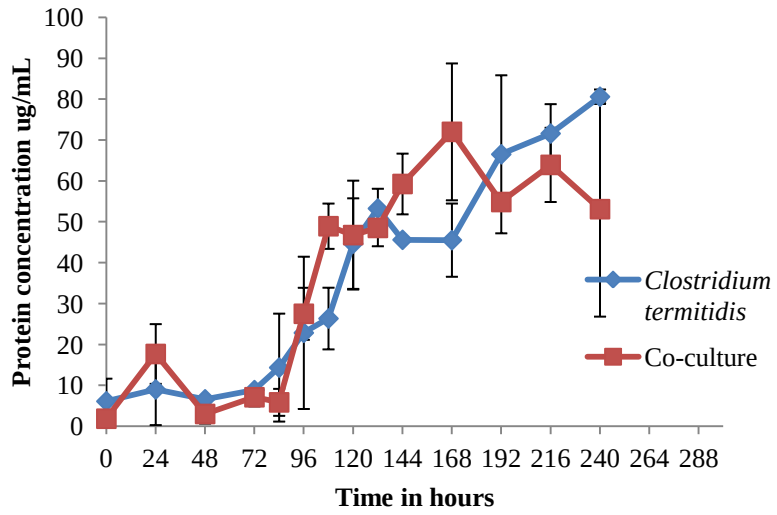
6.3.6.1 Comparison of the genes and gene products involved in substrate utilization and end-product synthesis

6.3.6.1.1 Changes in gene and gene product expression levels (CAZymes and Core Metabolic Genes) in *Clostridium termitidis* in co-culture versus *C. termitidis* monoculture during late-log phase (120 h pi)

C. termitidis encodes many carbohydrate active enzymes (CAZymes) which function in the efficient degradation of cellulose and associated polysaccharides (Munir et al., 2014). This group includes: 1) endo-β-glucanases, which breaks the internal regions of the cellulose chain into soluble oligosaccharides; 2) exo-β-glucanases, which act on the reducing or non-reducing ends of cellulose chains to release cellodextrins; and 3) β-glucosidases, which convert cellodextrins into glucose. The *C. termitidis* genome encodes a total of 355 CAZyme domain sequences, which is a much greater number of CAZyme domains than is found in other cellulolytic Clostridia. Of all the CAZymes domains found, glycosyl hydrolases and cellulose binding domains (CBMs) were most abundant (Munir et al., 2014).

Figure 6.1 A) Total biomass produced by co-cultures and monocultures on 2 g/L α-cellulose. B) Change in pH during growth of the co-cultures and monocultures. No statistically significant differences were observed in either biomass production or pH change. Error bars represent standard deviation about the means of three biological replicates.

A)



B)

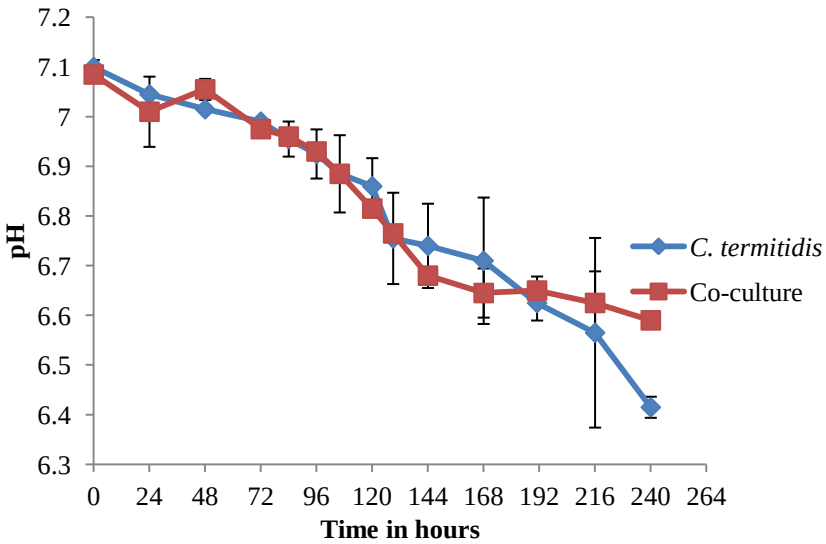


Figure 6.2 Hydrogen (H_2) production by co-cultures and monocultures. Based on the H_2 production curve, log phase occurred from approximately 72 to 144 h pi, with mid- to late-log at approximately 120 h pi, while stationary phase occurred from 144 h pi onward

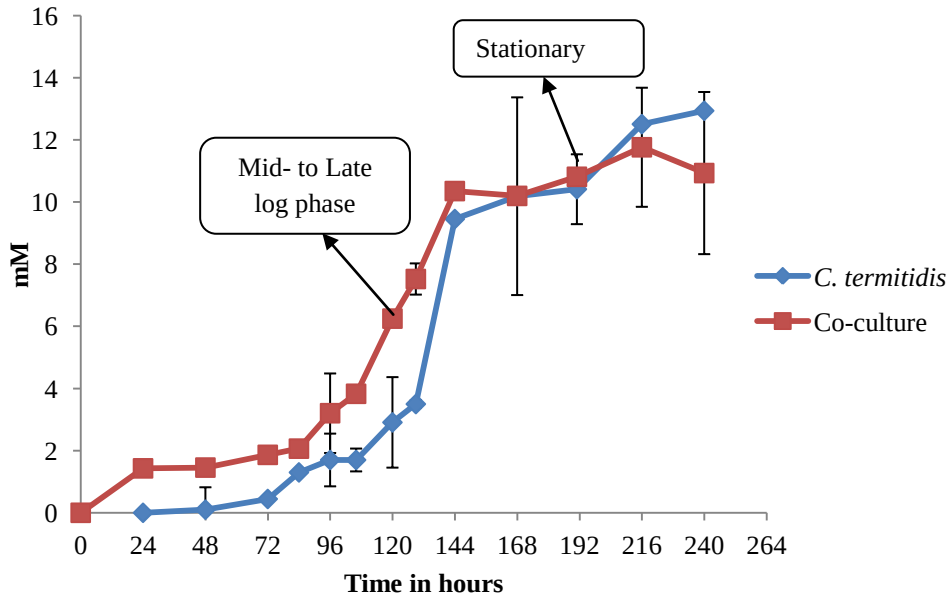
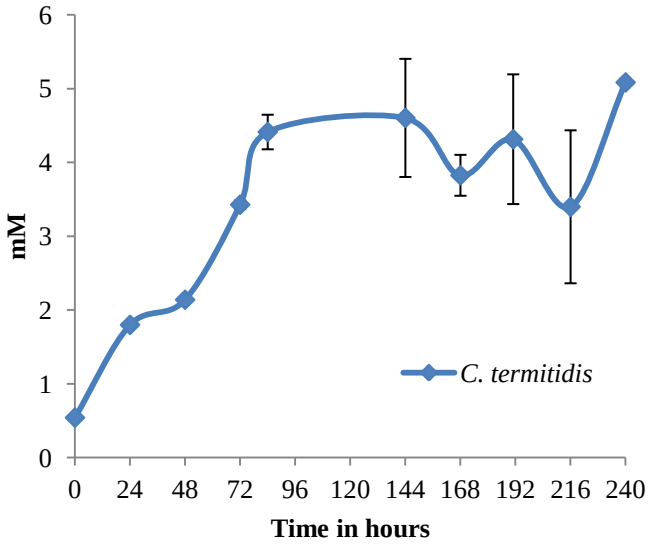


Figure 6.3 A) Ethanol Synthesis by the monoculture of *C. termitidis* and B) Co-culture. In the co-culture close to the end of growth a drop in ethanol concentration was observed and this may be due to the concumption of ethanol by *C. intestinale* in the co-culture.

A)



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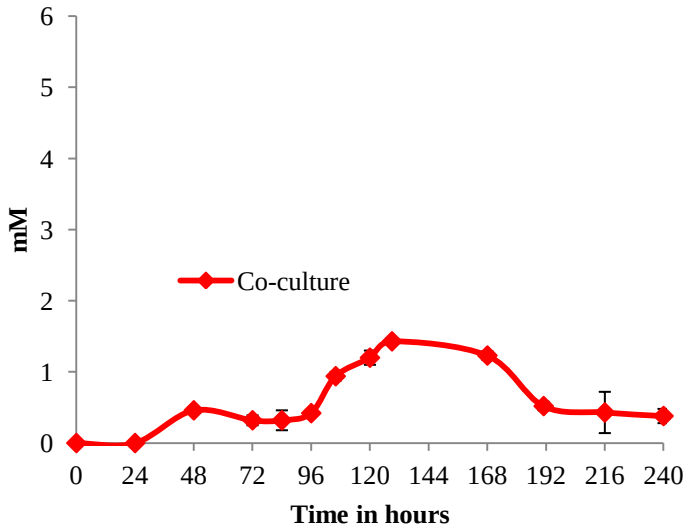
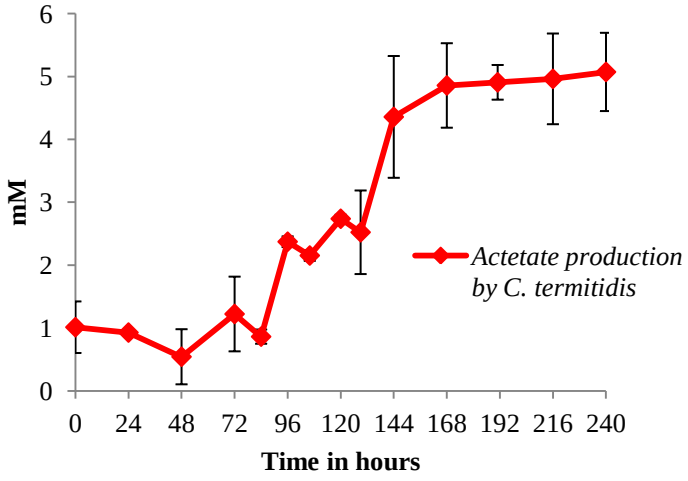


Figure 6.4 A) Acetate synthesis by *C. termitidis* in the co-culture. Acetate synthesis was indicative of *C. termitidis* growth, as *C. intestinale* synthesizes only minimal amounts of acetate; and B) the increase in *C. termitidis* genome copy number (cell growth) quantified by qPCR. Quantification of genome copy number by qPCR revealed that *C. termitidis* cells were in late-log phase in the co-culture at 120 h pi.

A)



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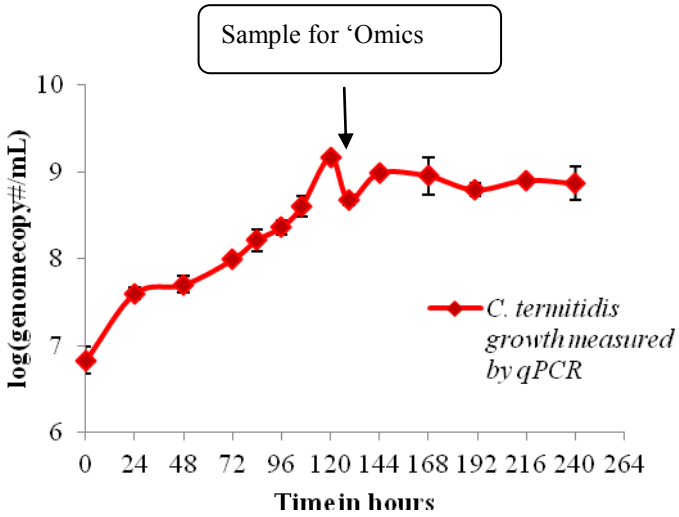
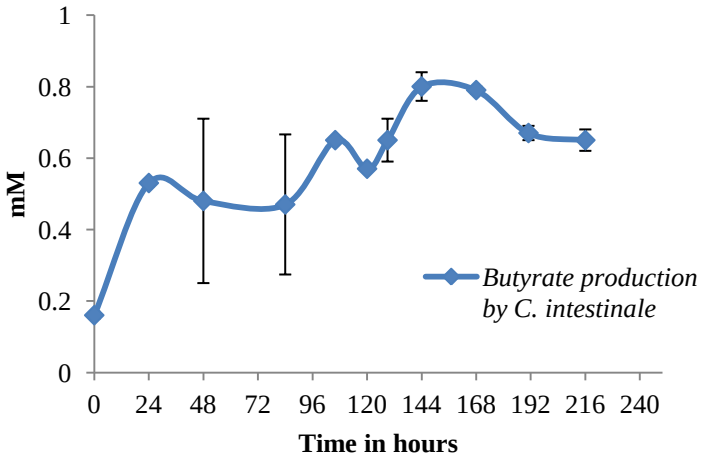


Figure 6.5 A) Butyrate synthesis by *C. intestinale* in the co-culture. Butyrate synthesis was indicative of *C. intestinale* growth, as *C. termitidis* does not synthesize butyrate; and B) the increase in *C. intestinale* genome copy number (cell growth) quantified by qPCR. Quantification of genome copy number by qPCR revealed that *C. termitidis* cells were in late-log phase in the co-culture at 120 h pi.

A)



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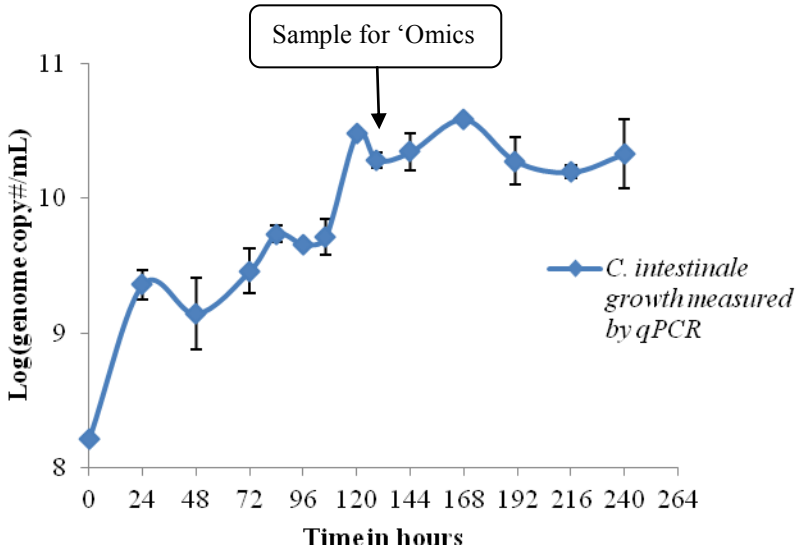
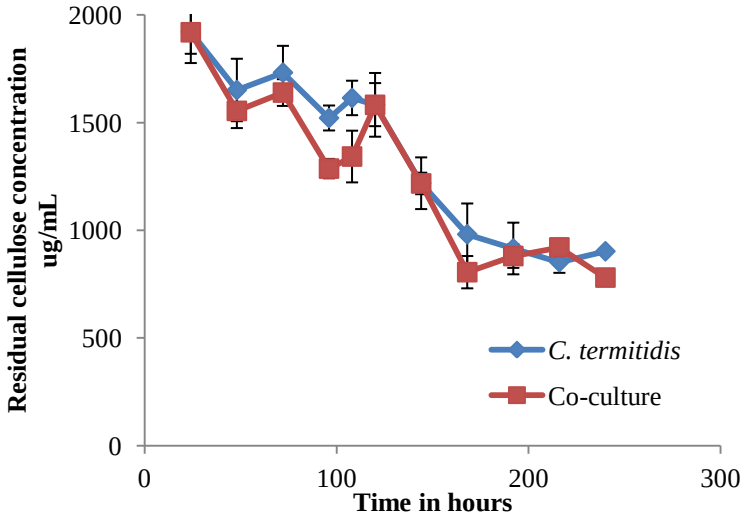


Figure 6.6 A) Substrate consumption (residual cellulose concentrations) in co-cultures and *C. termitidis* monocultures. B) Residual cellobiose left in the monoculture of *C. termitidis* at different time points. These sugars were not detected in the co-culture samples indicating that they were completely utilised.

A)



B)

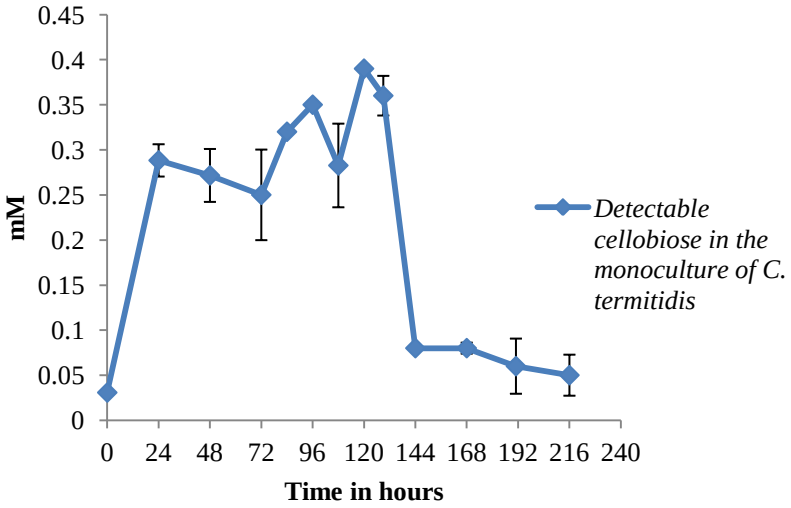
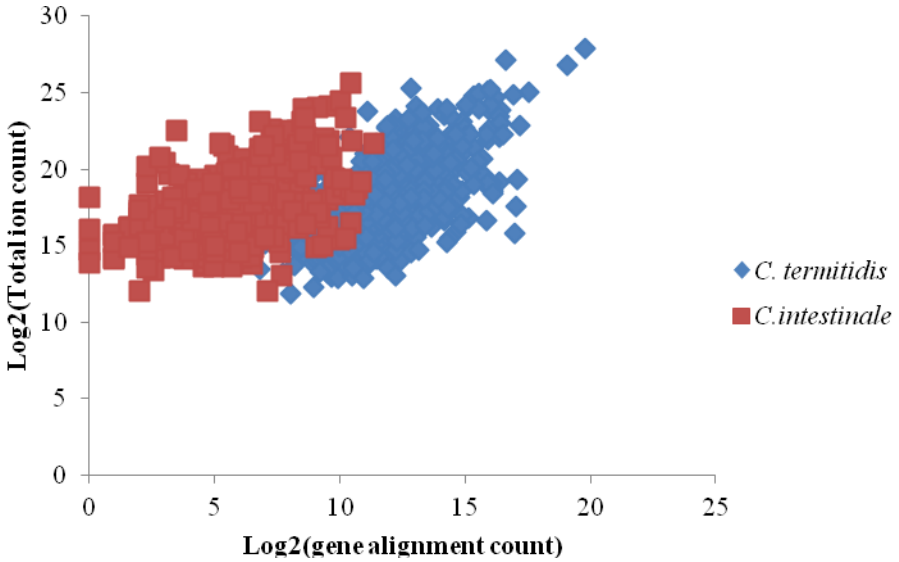
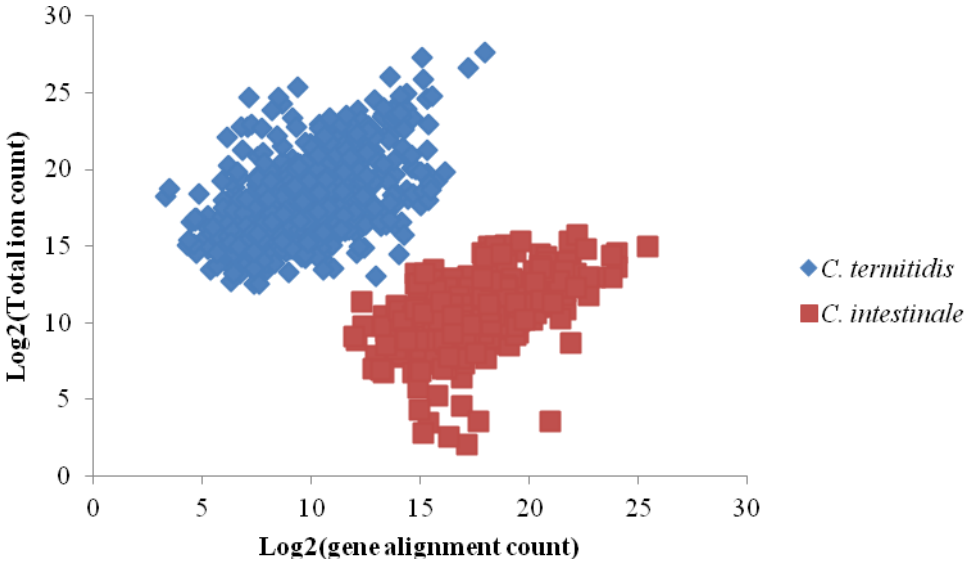


Figure 6.7 Correlation between transcriptomic and the proteomic expression data for the *C. termitidis* and *C. intestinale* co-culture during: A) late-log phase (sampled at 120 h pi of the co-culture); and B) stationary phase (sampled at 192 h pi from the co-culture).

A)



B)



In the co-culture, *C. termitidis* breaks down cellulose to release the soluble sugars (cellobiose and glucose), which are consumed by the *C. intestinale*. It was hypothesized that *C. intestinale* would consume the residual cellobiose and glucose at a rate that would create a "soluble sugar deficit environment". It was expected that the demand for the soluble sugars in the co-culture would positively stimulate expression of *C. termitidis* genes and gene products involved in cellulose attachment and degradation (endo- and exoglucanases). Therefore, our initial analysis focused on expression of these genes and gene products in the co-culture versus the *C. termitidis* monoculture, to determine if significant changes (up- or down-regulation) could be detected. This analysis revealed that the expression patterns of *C. termitidis* glycosyl hydrolases in the co-culture and monoculture were very similar, and no significant differences were observed (Table 6.1).

From previous experiments, it was observed that *C. termitidis* monocultures degrade cellulose and that cellobiose continued incubation results in an accumulation of cellobiose to approximately 0.5 mM (Figure 6.6B). However, no free cellobiose was detected in the co-cultures, suggesting that it was consumed by *C. intestinale* for growth. Moreover, it was previously reported (in Chapters 3 and 4), that *C. intestinale* cells proliferate to a fixed cell density in the co-culture, regardless of the initial inoculation ratio of *C. termitidis* and *C. intestinale* cells, suggesting that higher cell densities of *C. intestinale* did not increase the demand for soluble sugars, and thus did not influence the synthesis of *C. termitidis* glycosyl hydrolases to increase cellulose hydrolysis. Similarly, no significant up- or down-regulation was observed in the genes involved in *C. termitidis* fermentation end-product (H_2, acetate, or ethanol) synthesis pathways. These data are consistent with the *C. termitidis* co-culture and monoculture end-product synthesis patterns observed. This clearly indicates that the presence of *C. intestinale* in the co-culture did not influence the expression of *C. termitidis* core metabolism pathway genes and gene products (Table 6.1, 6.2 and Figure 6.8, 6.9).

6.3.6.1.2 *C. intestinale* in co-culture and monoculture

Comparison of the butyrate concentrations synthesized by *C. intestinale* in the co-culture (Figure 6.4A) with those of the monoculture revealed that *C. intestinale* did not grow significantly faster in the co-culture, as hypothesized. The concentrations of butyrate in the samples collected for 'Omics analyses at 120 h pi (late-log phase) and 192 h pi (stationary phase) were 2 mM and 6 mM, respectively.

'Omics analyses compared expression levels of genes and gene products involved in core metabolism, substrate utilization, and end-product synthesis in *C. intestinale* cells in the co-culture to those of the *C. intestinale* monoculture (Table 6.2). As with *C. termitidis*, no significant changes in expression levels (up- down-regulation) were observed between *C. intestinale* in the co-culture versus the monoculture (Tables 6.1, 6.2, 6.3 and Figure 6.8,6.9). These data support the conclusion that the presence of *C. termitidis* in the co-culture did not influence the core metabolic pathways, substrate consumption, or the end product synthesis pathways of *C. intestinale*. However, the growth of *C. intestinale* in the co-culture was significantly lower (by comparing the amount of butyrate synthesized from Ramachandran et al., 2011) than in the monoculture (on 2 g/L cellobiose), which is most likely a result of low substrate (cellobiose) concentrations generated by the slow hydrolysis of cellulose by *C. termitidis* in the co-culture.

6.3.6.2 Changes observed in the transcriptomes and proteomes of co-cultures versus monocultures during late-log phase (120 h pi)

Although no significant changes in expression levels (up- or down-regulation) were observed in the core metabolism, substrate utilisation, or end-product synthesis pathway genes or gene products of *C. termitidis* and *C. intestinale* in co-cultures versus their respective monocultures (Tables 6.1, 6.2, 6.3 and Figure 6.7,6.8), comparison of the *C. intestinale* co-culture and monoculture transcriptomes revealed changes in transcription

levels of the genes related to bacterial biopolymer synthesis, virulence factors, and vitamin synthesis as discussed below.

Table 6.1 Comparison of transcriptomics and proteomics expression data of CAZymes of *C. termitidis* in the co-culture and the monoculture.

Locus Tag	Gene Product Encoded	Transcriptomics Expression – log2 (Gene Alignment Count)[a]		Proteomics Expression –log2TIC	
		Monoculture	Co-culture	Monoculture	Co-culture
Cter_0526	Cellulose binding domain-containing protein	11.2	13.2	20.8	24.1
Cter_1801	Dockerin type I cellulosome protein	9.4	9.5	13.3	Not detected
Cter_1802	Dockerin type I cellulosome protein	8.7	8.6	14.2	Not detected
Cter_5022	Dockerin type I cellulosome protein	10.3	11.1	15.2	16.6
Cter_0514	Dockerin type I cellulosome protein	10.6	11.8	18.0	19.3
Cter_0517	Dockerin type I cellulosome protein	9.8	11.5	19.0	22.0
Cter_0525	Cellulosome anchoring protein cohesin subunit	12.6	14.3	21.2	23.0
Cter_0272	Cellulose binding domain/Glycosyl hydrolase	10.6	11.1	16.2	16.6
Cter_0518	Glycoside hydrolase 9 family protein	10.9	12.0	20.6	22.6
Cter_0522	Glycoside hydrolase 9 family protein	11.4	12.9	21.2	22.5
Cter_2830	Glycoside hydrolase 9 family protein	16.2	14.3	16.1	18.3
Cter_2831	Glycoside hydrolase 9 family protein	17.8	14.5	17.7	20.1
Cter_0521	Glycoside hydrolase 9 family protein	13.5	14.0	22.3	23.9
Cter_2867	O-Glycosyl hydrolase	8.6	9.8	10.6	14.9

[a] Gene Alignment Count and TIC values rounded up or down to one significant digit.

Table 6.2 Comparison of *C. termitidis* core metabolism gene expression levels (Transcriptomics and Proteomics expression Data) in co-cultures and monocultures.

Locus Tag	Gene Product Encoded	Transcriptomics Expression – log2 (Gene Alignment Count)[a]		Proteomics Expression –log2TIC	
		Monoculture	Co-culture	Monoculture	Co-culture
Cter_2546	Cellobiose phosphorylase	10.8	12.1	16.4	17.6
Cter_2734	Cellobiose phosphorylase	11.7	12.9	17.6	17.2
Cter_0036	Cellobiose phosphorylase	8.5	7.4	Not detected	Not detected
Cter_4899	Cellobiose phosphorylase	2.1	5.0*	Not detected	Not detected
Cter_4494	Cellobiose phosphorylase	11.6	13.2	20.0	20.9
Cter_3440	Cellobiose phosphorylase	4.7	6.8	8.1	Not detected
Cter_4298	Cellobiose phosphorylase	3.6	5.6	Not detected	Not detected
Cter_1604	Glucose-6-P isomerase	3.39	4.43	Not detected	Not detected
Cter_3865	Glucose-6-P isomerase	10.3	12.0	19.7	20.0
Cter_0067	6-Phosphofructokinase	8.5	11.6	18.1	18.5
Cter_4719	6-Phosphofructokinase	12.7	14.6	21.2	22.2
Cter_5379	6-Phosphofructokinase	1.6	2.3	Not detected	Not detected
Cter_1264	Fructose-1,6-bisphosphate aldolase	12.3	13.5	20.1	19.9
Cter_5375	Triosephosphate isomerase	2.6	3.6	Not detected	Not detected
Cter_1845	Triosephosphate isomerase	1.9	4.8*	Not detected	Not detected
Cter_1092	Glyceraldehyde-3-phosphate dehydrogenase, type I	5.0	6.2	10.9	Not detected
Cter_4809	Glyceraldehyde-3-phosphate dehydrogenase, type I	16.5	17.0	22.8	24.9

Cter_4786	Bifunctional phosphoglycerate kinase/ isomerase	13.0	15.5	22.5	24.2
Cter_2329	Phosphoglycerate mutase	7.7	9.6	12.0	Not detected
Cter_4779	Enolase	15.4	17.0	19.2	19.3
Cter_2504	L-lactate dehydrogenase	13.4	14.0	15.6	Not detected
Cter_1187	Pyruvate-formate lyase	4.8	6.6	Not detected	Not detected
Cter_4126	Pyruvate-formate lyase	0	1.66*	Not detected	Not detected
Cter_4127	Pyruvate-formate lyase	4.5	5.9	Not detected	Not detected
Cter_3262	Pyruvate-formate lyase	4.8	6.2	Not detected	Not detected
Cter_0232	Pyruvate-formate lyase	7.1	6.9	Not detected	Not detected
Cter_4518	Acetate Kinase	12.6	10.5	20.6	18.6
Cter_2745	Transketolase	11.7	12.9	20.0	20.4
Cter_4373	Transketolase	2.9	6.4	Not detected	Not detected
Cter_5477	Iron containing alcohol dehydrogenase	2.4	4.0	Not detected	Not detected
Cter_3280	Iron containing alcohol dehydrogenase	14.7	15.9	22.7	24.2
Cter_1650	Iron containing alcohol dehydrogenase	5.5	6.4	11.2	Not detected
Cter_2586	Iron-containing alcohol dehydrogenase	12.9	12.8	19.4	19.4
Cter_4786	Bifunctional phosphoglycerate kinase/ isomerase	13.0	15.1	22.5	24.2
Cter_0649	Pyruvate Kinase	12.5	14.1	18.1	17.4
Cter_1022	Pyruvate Ferredoxin Oxidoreductase -beta	14.4	15.4	20.3	21.9
Cter_0199	Pyruvate Ferredoxin Oxidoreductase - alpha	9.5	10.8	16.0	Not detected
Cter_0412	Malate Dehydrogenase	9.5	11.5	18.0	19.5

Cter_0411	Malic Enzyme	13.5	14.9	21.8	22.2
Cter_4519	Phosphate acetyltransferase	9.4	8.4	18.6	16.8
Cter_0809	Pyruvate, phosphate dikinase	11.2	12.4	20.8	21.6

[a] Gene Alignment Count values, TIC rounded up or down to one significant digit.
*- Significantly upregulated in the co-culture

Figure 6.8 Core metabolic genes (glycolysis) detected from the co-culture transcriptome. Genes specific to each orgasnisms are denoted by their locus tag. G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6-bisphosphate; DHA-P, Dihydroxyacetone phosphate; GA-3-P, glyceraldehyde-3-phosphate; PG, phosphoglycerate; PEP, phosphoenolpyruvate (Rydzak et al., 2012).

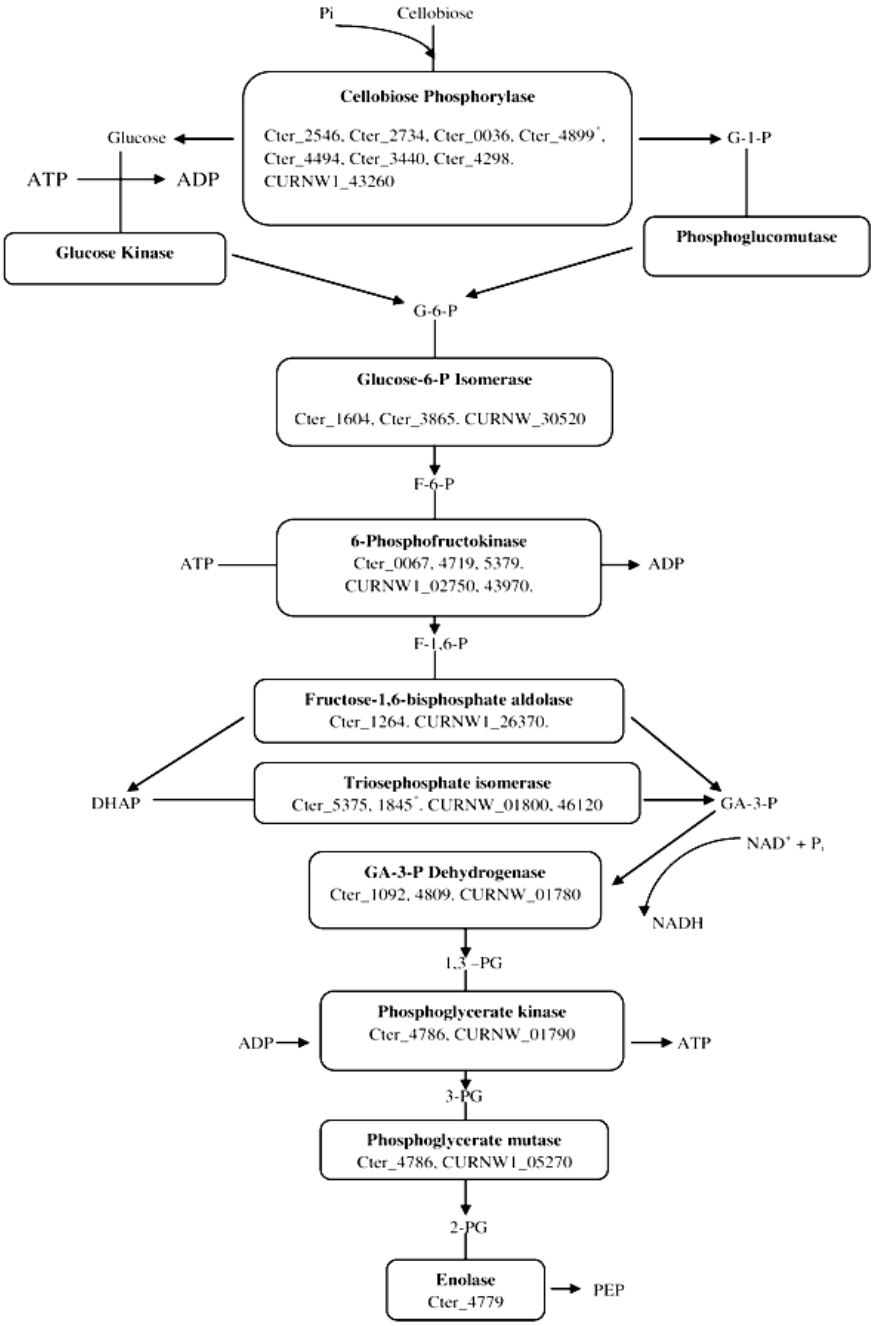


Figure 6.9 Genes related to end-products synthesis detected in the co-culture transcriptome. Some of the genes were not detected as they are improperly annotated or did not make into the final sequence of the contigs. ATP- Adenosine triphosphate; ADP-Adenosine diphosphate (Carere et al., 2008).

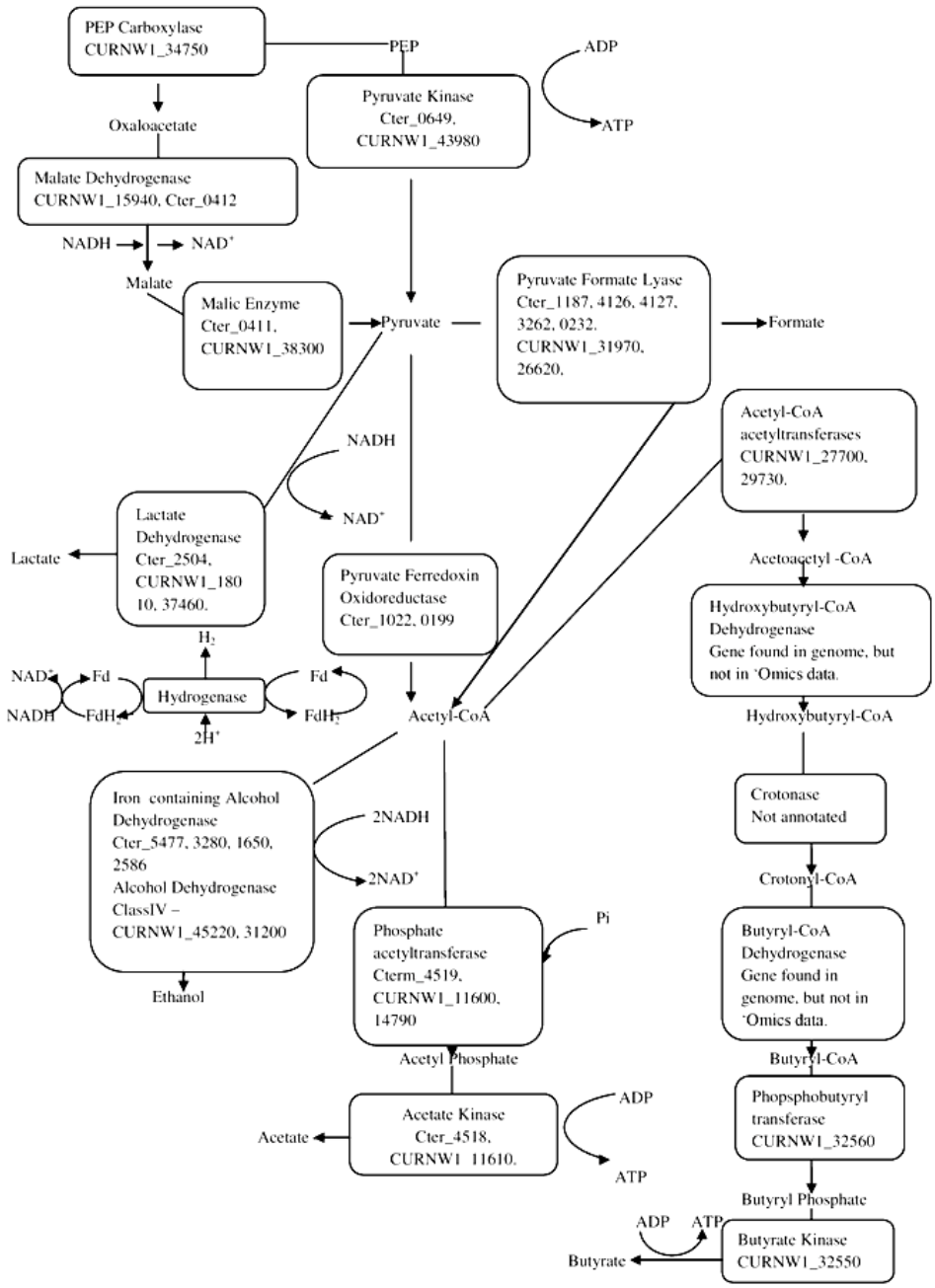


Table 6.3 Comparison of *C. intestinale* core metabolism gene expression levels in co-cultures and the monocultures.

Locus Tag	Gene	Transcriptomics Expression - log2 (Gene Alignment Count)[a]		Proteomics Expression – log2 TIC	
		Monoculture	Co-culture	Monoculture	Co-culture
CURNW1_43260	Cellobiose phosphorylase	8.3	5.0	Not detected	Not detected
CURNW1_02750	6-phosphofructokinase	7.8	9.3	16.3	Not detected
CURNW1_43970	6-phosphofructokinase	12.4	10.2	22	20.6
CURNW1_01800	Triosephosphate isomerase	15.4	12.7	24.4	19.8
CURNW1_46120	Triosephosphate isomerase	2.3	1*	Not detected	Not detected
CURNW1_01780	Glyceraldehyde-3-phosphate dehydrogenase, type I	17.7	14.4	26.3	21.8
CURNW1_11610	Acetate kinase	12.8	10.1	20.0	18.5
CURNW1_32550	Butyrate kinase	14.1	11.4	24.6	22.7
CURNW1_28180	Pyruvate Kinase	12.2	10.7	19.8	14.0
CURNW1_04270	Pyruvate Kinase	7.2	4.8	Not detected	Not detected
CURNW1_43980	Pyruvate Kinase	15.3	12.2	23.9	22.1
CURNW1_31970	Pyruvate Formate Lyase	8.6	6.3	Not detected	Not detected
CURNW1_45220	Alcohol dehydrogenase class IV	8.9	7.2	Not detected	Not detected
CURNW1_31200	Alcohol dehydrogenase, class IV	13.9	10.6	21.8	18.4
CURNW1_18010	L-lactate dehydrogenase (FMN-dependent) and	8.5	6.0	Not detected	Not detected
CURNW1_37460	Lactate dehydrogenase and related dehydrogenases	10.6	8.8	19.0	16.7
CURNW1_26620	Pyruvate-formate lyase	4.5	2.3	Not detected	Not detected
CURNW1_30520	Glucose-6-phosphate isomerase	14.6	11.7	23.6	16.3
CURNW1_26370	Fructose bisphosphate aldolase	10.0	11.3	18.4	Not detected
CURNW1_01790	3-phosphoglycerate kinase	15.8	12.8	25.1	19.0
CURNW1_05270	Phosphoglycerate mutase	15.7	12.2	21.3	Not detected
CURNW1_43980	Pyruvate Kinase	15.3	12.2	23.9	22.1
CURNW1_34750	Phosphoenolpyruvate	12.4	10.1	17.9	Not detected

	carboxylase, archaeal type				
CURNW1_15940	Malate Dehydrogenase	10.9	7.1	16.5	Not detected
CURNW1_38300	Malic Enzyme	11.0	9.8	19.6	16.7
CURNW1_11600	Phosphate acetyltransferase	12.8	9.7	17.8	16.8
CURNW1_14790	Phosphate acetyltransferase	7.7	4.5	Not detected	Not detected
CURNW1_27700	Acetyl-CoA acetyltransferases	6.2	4	19.5	15.8
CURNW1_29730	Acetyl-CoA acetyltransferases	16.2	13.0	25.9	20.9
CURNW1_32560	Phosphate butyryltransferase	13.2	10.9	23.9	19.2

[a] Gene Alignment Count,TIC values rounded up or down to one significant digit;

* Significantly up-regulated in the co-culture

6.3.6.2.1 Changes in expression levels of *C. termitidis* genes in co-cultures versus monocultures in stationary phase

Unlike in *C. intestinale*, the *C. termitidis* genes involved in vitamin biosynthesis (Thiamine monophosphate synthase and Thiamine biosynthesis protein ThiS) were expressed at significantly higher levels in the co-culture compared to the monoculture (Table 6.4). The genes involved in sporulation (Spo0E-like sporulation regulatory protein, Stage III sporulation protein AE, Sporulation protein YhbH, and Stage V sporulation protein B) were also highly transcribed in co-cultures compared with monocultures, indicating significant levels of sporulation of *C. termitidis*. Transcription levels of gene that encodes methyl-accepting chemotaxis proteins (MCPs) was also very high in co-cultures compared to monocultures. These proteins are membrane proteins which undergo reversible methylation when bacterial cells adapt to changes in environmental conditions (induced by chemo-attractants or repellents) by altering their motility (swimming) behaviour. *C. termitidis* genes involved in the synthesis of RNA Polymerase Sigma factors, associated with initiating RNA synthesis (Lonetto et al., 1994) were expressed at higher levels in the co-culture compared to the monoculture. In contrast, *C. termitidis* genes involved in fatty acid biosynthesis pathways were expressed at much lower levels in the co-culture compared with the monocultures.

6.3.6.2.2 Changes in transcription levels of *C. intestinale* genes in co-cultures versus monocultures

In monocultures of *C. intestinale*, genes related to polymer biosynthesis and bio-film formation were transcribed at very high levels (Rehm, 2010). However, transcripts of these genes were not detected in the co-culture or detected at significantly low levels (Table 6.5). These data suggest that in the co-culture, *C. intestinale* has less ability to synthesize proteins related to the formation of bio-films. Similar observations were noted in the transcription of genes related to bacterial pathogenicity or virulence factors (Ethanolamine utilization protein,

Tfp pilus assembly protein PilO, and Antimicrobial peptide transport system genes). While these genes were transcribed at high levels in the monoculture, they were transcribed at very low levels in the co-culture. The genes related to vitamin synthesis (Thiamine biosynthesis protein ThiS, Thiamine monophosphate synthase, Thiamine biosynthesis protein ThiC, Cobalamin biosynthesis protein CobD, Cobalamin biosynthesis protein CbiM) were also transcribed at low levels in the co-culture compared with the monoculture. Na^+/H^+ antiporters are essential in maintaining the intracellular pH of the cells. This is essential in coupling electrochemical gradients of ions and organic solutes to drive transport reactions (Padana et al. 1994). The transcription of these genes in *C. intestinale* was significantly lower in the co-culture indicating that the pH maintenance and toxin removal system (Na^+ at higher concentrations) of the bacteria was severely affected in the co-culture. This may be a main reason for the lack of activity observed in the bacteria in the co-culture as anticipated. In contrast, *C. intestinale* genes related to riboflavin or Vitamin B_2 synthesis (riboflavin biosynthesis protein, riboflavin biosynthesis protein, and Acyl-CoA dehydrogenases) were expressed at significantly higher levels in the co-culture compared to the monoculture.

6.4 Conclusions

The present study has provided a clear understanding of the growth of *C. termitidis* and *C. intestinale* in co-cultures versus their respective monocultures by quantification of fermentation end-products and genome copy numbers. No differences in substrate utilization or end-product concentrations synthesized by each species in co-cultures versus monocultures were observed. However, the ethanol synthesized by the co-culture was very less than that of the monoculture. This may due to the consumption of ethanol by *C. intestinale* in the co-culture, after the residual cellobiose is utilised. The qPCR method used provided an excellent way to track the growth of individual species at different time points in the co-culture and compare them to the growth patterns/rates of the respective monocultures.

It was expected that *C. termitidis* would be stimulated by the presence of *C. intestinale* to increase the hydrolyse cellulose and consequently release more cellobiose, as *C. intestinale* solely depends on the hydrolysed sugars released by *C. termitidis*. However, this pattern was not observed, and no increase in *C. termitidis* CAZyme (cellulose degrading enzymes) genes and gene products were detected by the transcriptomic and proteomic analyses. Therefore, it was concluded that *C. intestinale* grew on the cellobiose released by the cellulose hydrolysis, but did not induce *C. termitidis* to hydrolyse cellulose at a faster rate nor to stimulate increased cellobiose synthesis. This is also supported by the fact that both the organisms did not show significant changes in selected core metabolic genes when compared to the monocultures, and by the expression of *C. termitidis* sporulating genes in the co-culture – an indication of conditions that were unfavourable to growth.

It can be concluded that the co-culture of *C. termitidis* and *C. intestinale* may be considered as an example of a cellulose degrading, mesophillic co-culture that does not display significant changes in substrate utilization or hydrogen production compared to the monocultures. This disproves our hypothesis that co-cultures can always be used to bring a change in substrate utilization or end-product synthesis compared to their monocultures. However, varying the inoculation methods (inoculating the monocultures at different times) may produce different results and require further research to develop this co-culture as a successful candidate for biofuel production.

Table 6.4 Changes observed in the transcriptome and proteome of *C. termitidis* as a monoculture and in the co-culture.

Locus Tag	Gene	Transcriptomics Expression - log2 (Gene Alignment Count)[a]		Proteomics Expression – log2TIC	
		Monoculture	Co-culture	Monoculture	Co-culture
Cter_1814	Thiamine monophosphate synthase	2.5	6.5	Not detected	Not detected
Cter_1818	Thiamine biosynthesis protein ThiS	0.0	3.7	Not detected	Not detected
Cter_5177	Spo0E like sporulation regulatory protein	1.0	6.5	Not detected	Not detected
Cter_5056	Stage V sporulation protein AE	2.6	6.8	Not detected	Not detected
Cter_1701	Spore coat associated protein JA (CotJA)	2.0	6.8	Not detected	Not detected
Cter_2151	Stage III sporulation protein AE	2.9	6.8	Not detected	Not detected
Cter_3426	Stage V sporulation protein B	3.7	7.5	Not detected	Not detected
Cter_5510	Methyl-accepting chemotaxis protein	3.9	10.4	Not detected	14.2
Cter_5183	4'-phosphopantetheinyl transferase	11.0	5.6	Not detected	Not detected
Cter_1141	RNA polymerase sigma factor, sigma-70 family	1.0	3.9	Not detected	Not detected
Cter_1387	RNA polymerase sigma factor, sigma-70 family	3.6	6.4	Not detected	Not detected
Cter_3032	RNA polymerase sigma factor, sigma-70 family	6.7	9.3	12.2	Not detected

[a] Gene Alignment Count values rounded up or down to one significant digit.

Table 6.5 Changes observed in the transcriptome and proteome of *C. intestinale* as a monoculture and in the co-culture.

Locus Tag	Gene	Transcriptomics Expression - log2 (Gene Alignment Count)[a]		Proteomics Expression –log2 TIC	
		Monoculture	Co-culture	Monoculture	Co-culture
CURNW1_19650	Bacterial polymer biosynthesis proteins	12.3	0	14.6	Not detected
CURNW1_28980	Bacterial polymer biosynthesis proteins,	12.0	9.8	16.9	16.1
CURNW1_26660	Ethanolamine utilization protein	4.2	1	Not detected	Not detected
CURNW1_30900	Tfp pilus assembly protein PilO	4.5	2.8	Not detected	Not detected
CURNW1_15040	ABC-type multidrug transport system, ATPase	6.8	4.8	Not detected	Not detected
CURNW1_03080	Thiamine monophosphate synthase	12.9	4.9	Not detected	Not detected
CURNW1_03070	Thiamine biosynthesis protein ThiC	14.4	6.6	23.7	16.3
CURNW1_03040	Thiamine biosynthesis protein ThiS	10.6	3.1	Not detected	Not detected
CURNW1_00490	cobalamin biosynthesis protein CobD	8.8	5	Not detected	Not detected
CURNW1_25640	cobalamin biosynthesis protein CbiM	4.9	3.5	Not detected	Not detected
CURNW1_14020	Na+/H+ antiporter, bacterial form	5.8	1	Not detected	Not detected
CURNW1_36350	Riboflavin biosynthesis protein	4.9	9.9	20.8	Not detected
CURNW1_36340	Riboflavin synthase, alpha subunit	4	8.4	Not detected	Not detected

[a] Gene Alignment Count values rounded up or down to one significant digit.

Chapter 7

General Discussion and Conclusions

The underpinning hypothesis of this thesis was that co-cultures of cellulolytic bacterium, *C. termitidis*, and non-celluloytic bacterium, *C. intestinale* would display synergistic effects resulting in significant increases in substrate utilization and concentrations of end-products synthesized (H_2, CO_2, organic acids, ethanol) compared to the *C. termitidis* monoculture, when cultured on 2 g/L cellulose. As *C. intestinale* is non-cellulolytic, it was expected that *C. termitidis* would hydrolyse cellulose and release soluble sugars, which would be in part, consumed by *C. intestinale*. It was further expected that the increased demand for soluble sugars in the co-culture would stimulate the rate of cellulose hydrolysis by *C. termitidis,* which in turn could result in an increase in the concentrations of end-products synthesized, and that these changes in metabolism would be observed at the level of gene and/or gene product expression.

In Chapter 3, different initial inoculum ratios of *C. termitidis* and *C. intestinale* were evaluated for changes in cellulose consumption and concentrations of end-product synthesized at 168 h pi. . Of all the ratios tested, only ratios 1:1 and 1:0.2 showed a statistically significant difference in substrate utilization and total cell mass production. Although these differences were statistically significant, they were not very large. Moreover, no significant differences were observed in these cultures for gas or liquid end-product synthesis. Finally, none of the other initial co-culture inoculum ratios showed differences in either substrate utilization or end-product synthesis, as reported in literature.

In Chapter 4, *C. termitidis* was cultured on pre-treated Glenn cultivar wheat straw (milled, boiled, and super-heated steam-treated) and raw wheat straw (milled only) to evaluate not only the growth of *C. termitidis* on these substrates, but also to determine the effect of pre-treatment on increasing access of the bacterium and its hydrolytic enzymes to

the cellulose within the biomass. Pre-treatment of milled wheat straw by boiling followed by super-heated steam did not result in greater cell growth and end-product concentrations in either the *C. termitidis* and *C. intestinale* co-culture, or the *C. termitidis* monoculture.

In chapter 5, a stable co-culture inoculum ratio (1:1 v/v) was prepared and the co-culture was passaged repeatedly on different substrates (α-cellulose, milled and pre-treated wheat straw, and milled only wheat straw) to determine the natural equilibrium/ratio established between *C. termitidis* and *C. intestinale* in co-culture grown on these substrates (Table 5.1). In chapter 6, a time point study was performed with an initial inoculum ratio of *C. termitidis* : *C. intestinale* = 1:25 to determine time points suitable for transcriptomic and proteomic analyses. As described above, one of the objectives of the thesis was to determine if changes in metabolism of *C. termitidis* and/or *C. intestinale* could be observed at the level of gene and/or gene product expression in co-culture versus *C. termitidis* or *C. intestinale* monocultures. A multiplex qPCR method targeting the *cpn60* sequence of the individual species in the co-culture was developed to quantify the growth of individual species in the co-culture versus monocultures at different time points for all the experiments.

The results from the experiments indicated clearly that no significant differences in substrate hydrolysis or end-product concentrations were observed in the *C. termitidis* and/or *C. intestinale* co-culture compared to the *C. termitidis* or *C. intestinale* monocultures. On 2 g/L cellobiose, quantification of the *C. termitidis* or *C. intestinale* genome copy numbers by qPCR showed that both the species grew in the co-culture and that the growth rate of *C. termitidis* was not affected by the presence of *C. intestinale* as compared to its monoculture. However, growth and end-product concentrations synthesized by *C. intestinale* in the co-culture on 2 g/L α-cellulose were limited compared to the *C. intestinale* monoculture on 2 g/L cellobiose. This may be due to the slower growth of *C. termitidis* on α-cellulose: the doubling-time of *C. termitidis* as a monoculture on 2 g/L α-cellulose was 19.5 hours, while

the doubling-time of *C. intestinale* monocultures on 2 g/L cellobiose was 4.5 hours (Ramachnadran et al., 2008 and 2011)

It was also observed that in *C. termitidis* monocultures, α-cellulose hydrolyzed by *C. termitidis* cellulases resulted in the release of soluble cellobiose that accumulated (approximately 0.2 - 0.4 mM), and was detectable, in the culture medium in excess. However, no soluble cellobiose was detected in the co-culture medium, suggesting that the soluble cellobiose was consumed completely by *C. intestinale* (Figure 6.6B). From these results, it was inferred that *C. intestinale* grows on the excess cellobiose released by hydrolysis of α-cellulose by *C. termitidis* cellulases, but the presence of *C. intestinale* in the co-culture did not stimulate *C. termitidis* to hydrolyse cellulose at a faster rate. Although the gas production and most of the end-products synthesized were similar between the monoculture and the co-culture, the ethanol synthesized by the monoculture was significantly higher compared to the co-culture. Also it was evident that the ethanol synthesized in the co-culture dropped at the end of growth indicating ethanol concumption (Figures 6.3A and 6.3B). It can be concluded that *C. intestinale* after consuming the cellobiose at 168 h pi, has switched towards using ethanol as its substrate. Similar behaviour was also observed in *C. kluyveri* which could consume ethanol and acetate to produce butyrate.

Transcriptomic and proteomic analyses of *C. termitidis* and *C. intestinale* in the co-culture versus the *C. termitidis* monoculture confirmed that expression levels of *C. termitidis* CAZyme genes and gene products was not changed by the presence of *C. intestinale* in the co-culture. Furthermore, no significant changes were observed in the core metabolic genes of *C. termitidis* or *C. intestinale* in the co-culture compared to their respective monocultures.

There are numerous reports in the literature on the effective fermentation of cellulosic materials by defined co-cultures (Chen et al., 2008; Liu et al., 2008,;Lu et al., 2013; Li et al., 2011), which result in increased cell mass production, increased substrate

consumption, and/or greater concentrations of one or more fermentation end-products compared to monocultures of the co-culture species. In most of these co-cultures, one of the co-culture partners does not consume, or only partially utilizes a hydrolysis by-product, while the other partner utilised this substrate as its sole carbon source (Liu et al., 2008). However in the co-culture of *C. termitidis* and *C. intestinale*, both the species compete for the cellobiose released by cellulose hydrolysis, and this may be the reason we did not observe the expected synergistic interactions described in other co-culture reports. In the current co-culture studies, the use of sophisticated tools such as multiplex qPCR and 'Omics analyses, enabled detailed characterization of the changes in each species in the co-culture versus their respective monocultures.

In conclusion, the hypothesis that synergistic interactions between species in co-cultures result in significant increases in substrate utilization and end-product synthesis compared to their monocultures was not supported by the data. Unlike other co-cultures reported in the literature, co-cultures of *C. termitidis* and *C. intestinale* did not show significant increases in substrate utilization or end- product concentrations synthesized when cultured on 2 g/L α-cellulose. Advanced tools such as qPCR and 'Omics tools provided strong evidence in support of this conclusion by quantifying cell growth and gene and gene product expression in each species in the co-culture versus their respective monocultures at the molecular level. In addition, the 'Omics data showed that there were regulations of genes involved the vitamin biosynthesis pathways, biofilm formation and pathogenicity indicating that the individual species in the co-culture sense each other's presence.

7.1 Future Studies

The current studies clearly indicates that the co-culture of *C. termitidis* and C. *intestinale* was not able to increased substrate utilization and end-product concentrations, as described for other co-cultures, mainly because of the following reasons:

- The slower growth of *C. termitidis* on α-cellulose, which is the main contributor to the growth of *C. intestinale* in the co-culture.
- Both the partners in the *C. termitidis* - *C. intestinale* co-culture utilized the same soluble substrate, which may have resulted in competitive rather than synergistic interactions between the two species.

The continuous passage experiments revealed that two species developed a stable population ratio/equilibrium over time. It would be very interesting if the inoculum from the final passage could be taken as an inoculum for a time point analysis of this co-culture towards substrate utilization and end-product synthesis. In addition, future studies could be conducted in a way to inoculate *C. termitidis* first and allow it to accumulate soluble sugars before addition of *C. intestinale* to the bioreactor. The sugars already produced and accumulated may be an additional source of substrate for *C. intestinale* growth and end-product synthesis. Although no changes in core metabolism genes and gene products were observed in *C. termitidis* and *C. intestinale* in the co-culture, some changes were observed in the genes related to pathogenicity and biofilm formation, which may be related to quorum sensing. Further studies could be directed towards analysing these data sets in detail to understand how the individual species communicate/sense each other in the co-culture.

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