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The Enrichment And Characterization Of Compost And Wastewater-Derived Microbial Cellulolytic Consortia For Biofuel Production

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THE ENRICHMENT AND CHARACTERIZATION OF COMPOST AND WASTEWATER-DERIVED MICROBIAL CELLULOLYTIC CONSORTIA FOR BIOFUEL PRODUCTION

by

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ABSTRACT

Over the last decade, a rise in energy demand and diminishing fuel resources have created a challenge for finding an alternative solution that could supplement our current energy sources. This study demonstrated that ethanol and other useful end-products can be produced from the fermentative activity of microbial consortia derived from cellulose-rich waste environments. Compost and wastewater were used as inoculum sources to enrich cellulolytic cultures at incubation temperatures 50 ºC and 60ºC. A chemically defined medium was used without complex nutrients such as yeast extract. Four cellulolytic cultures were obtained and their end-products were monitored over an active cellulose degrading period. The compost culture incubated at 50ºC produced the highest concentration of butyrate while the wastewater-derived culture incubated at 60ºC produced the highest ethanol concentration. Optimization of DNA extraction and purification from complex environmental samples such as the compost and wastewater cultures used in this study was also discussed.

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Dziękuje Wam

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CBD	Cellulose binding domain
CBP	Consolidated bioprocessing
CFU	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
GC	Gas chromatography
HPLC	High-performance liquid chromatography
MOPS	3-(N-morpholino) propanesulfonic acid
MTBE	Methyl tertiary-butyl ether
NAD+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
SLH	Surface layer homology
SHF	Separate hydrolysis and fermentation
SSF	Simultaneous saccharification and fermentation
TAE	Tris base, acetic acid, and EDTA buffer
TCAG	The Centre for Applied Genomics

CHAPTER ONE: INTRODUCTION

1.1 THESIS SIGNIFICANCE AND MOTIVATIONS

One of the challenges in today's society is living in a world that has grown dependent on industrial processes that bring about potential negative health and environmental effects. The fossil fuel industry in Canada is one example of an industry that exploits the terrestrial and aquatic environments for the development of a product that has been extensively used to satisfy global energy demand (Martin and Tisworth, 2010). Fossil fuels are formed from the decomposition of dead organic matter that is trapped within the earth for millions of years. Once they are extracted from the earth and refined, they are burned to generate energy; however, there are two major concerns related to this process (Türe *et al*, 1997). Firstly, it is predicted that fossil fuels will be depleted in the foreseeable future. Secondly, the greenhouse gases released from the combustion process are a leading cause of climate change (Wilkinson, 2008).

In Canada, petroleum products refined from crude oil power about 33% of the country's total energy needs (Canadian Centre for Energy, 2010). From that amount, more than 70% is refined into transportation fuels which include gasoline and diesel for vehicles, kerosene for jets and fuel oil for ships (Martin and Tisworth, 2010). On a global level, the transportation sector is responsible for 60% of the world oil consumption and it accounts for more than 70% of global CO and 19% of global CO_2 emissions (Clarke, 2008)[1]. Thus, an alternative sustainable solution must soon be implemented for meeting the vastly growing oil demand and changing climate.

[1] Values are based on the year 2007

1.1.1 First and Second Generation Biofuel

Biofuel, derived from sustainable biological sources such as agricultural crops, waste vegetable oils, and woody biomass, is thought by many researchers to be a potential substitute for petroleum-derived fuels such as gasoline and diesel (Mabee, 2007). The use of biofuel, such as bioethanol, biodiesel and biohydrogen, is associated with lower greenhouse gas emissions and improved energy balance when compared to petroleum-based fuels (Lynd *et al*., 2005). This is because the burning of biofuels, in other words the release of carbon, is equivalent to its uptake by the plant feedstock. In contrast, the combustion of fossil fuels results in an accumulation of carbon in the atmosphere which has been stored within the earth for millions of years (Berner, 2003).

The current biofuel industry is largely based on first generation biofuels, those derived from sugar and starch-based crops. These biofuels are believed to cause a burden on agricultural lands and cause the price of food to rise (Pimentel *et al.*, 2008). Another burden of first generation biofuels is the use of agriculture products for transportation needs and not as food sources. As the World Health Organization (2011) reports, more than 3.7 billion people in the world are malnourished and the growing of crops for fuels takes up land, water and energy that could otherwise be used for the production of food (Pimental *et al*., 2008). Alternatively, biofuels can be derived from lignocellulosic materials which include abundant sources such as agricultural, forest and paper mill wastes. These are known as second generation biofuels (Brethauer and Wyman, 2010).

1.1.2 The Challenges of Second Generation Biofuel Production

Despite the knowledge that biofuel can be derived from cellulosic materials, there are limitations in their industrial applications that must be first addressed before they can be used on a greater scale. Unlike the simpler biochemical conversion of sugar crops to ethanol, processing ethanol from cellulosic biomass requires two extra initial steps which have been found to be energy and cost- expensive (Lynd *et al*., 2005). The first is delignification, a chemical process, which separates cellulose from lignocellulose, a complex cross-linked polymeric molecule, and therefore exposes cellulose for further degradation. The second step is hydrolysis, a biochemical process that liberates glucose monomers from cellulose and prepares the feedstock for the next step of the process, fermentation (Hess *et al*., 2007).

To date, research has focused on modifying the hydrolysis step, which is the rate-limiting step because the pure cultures of microorganisms that break down cellulose cannot hydrolyze all of its components and tolerate high levels of end-products that arise from the process (Lynd *et al*., 2005). Thus great focus has been spent on genetically engineering microorganisms that are capable of meeting these conditions. The achievements and limitations of this research are further discussed in section 2.2 of this paper.

A relatively new approach in addressing the limitation of the hydrolysis and fermentation steps in biotechnology is the study of diverse microbial individuals within cellulolytic microbial cultures. Although microbial communities that degrade cellulose are known to exist in nature, research on exploring their ability to produce biofuel is limited. This study aims to address this gap in current research.

1.2 SCOPE OF WORK AND OBJECTIVES

As discussed previously, potential biotechnology exists that can convert abundant cellulosic materials to valuable end-products such as ethanol and hydrogen; however, a microbial consortium that is capable of cellulose degradation and ethanol fermentation on an industrial scale has not yet been identified (Martin and Tisworth, 2010). Thus, the scope of this study is to contribute to the current knowledge of second generation biofuel development with an emphasis on mixed-cellulolytic microbial cultures derived from natural cellulosic sources.

The objectives were to enrich and characterize mixed cellulolytic microbial consortia that produce favorable end-products and to optimize DNA extraction and purification protocols required for further characterization of community members within the cultures. The first objective was accomplished by four goals: i) enriching mixed microbial cultures derived from compost and municipal wastewater; ii) utilizing a chemically-defined medium in batch for the maintenance of culture activity; iii) utilizing and comparing the activity of cultures under two thermophilic incubation temperatures of 50ºC and 60ºC; and iv) obtaining a time-resolved profile of the soluble end-products that were produced by the consortia from cellulose degradation. The second objective was met with the goal of optimizing DNA extraction by modifying the extraction protocol and purifying the DNA by ethanol precipitation to obtain PCR products.

The first chapter in this work describes the motivations and rationale behind the study while Chapter two contains a literature review with a focus on cellulose hydrolysis and mixed microbial consortia. The chapters addressing the objectives are Chapter three which pertains to the enrichment of cultures with an end-product analysis and Chapter four which includes the DNA analysis. Lastly, Chapter five contains the conclusions and future work that can arise from this study.

1.3 EXPECTATIONS

Based on the limited studies on mixed cellulolytic microbial cultures, the main expectation is that a cellulolytic microbial consortium will be enriched from the inoculum sources chosen. It is expected that two distinct communities will be obtained, one from compost and another from wastewater with the ability of producing valuable products. Haruta *et al*. (2002) have identified aerobic and anaerobic members within their mixed culture that originated from an inoculum of animal waste. The culture was enriched with a complex medium, it was incubated at a temperature of 50°C and resulted in ethanol, acetate and lactate production. Further studies showed that cellulolytic communities are expected to include anaerobic, aerobic or facultative anaerobic, cellulolytic, and non-cellulolytic microorganisms. Details of these studies will be discussed in section 2.3 of this thesis.

Aside from its inoculation sources, this study differs from previous studies by using a chemically defined medium without the use of yeast extract (Johnson *et al*., 1983). As a result, upon successful characterization of a mixed microbial culture, this study will utilize only the essential ingredients necessary for sustaining a mixed culture, potentially giving industry an ability to reduce the cost of expensive ingredients otherwise needed in a complex medium.

In addition, the incubation temperature of 60°C is expected to yield greater cellulose degradation of the microbial communities derived from compost (Ronan 2011). Preliminary experiments at 75°C did not yield cellulose degradation. Furthermore, past experiments with the same chemically-defined medium used to culture communities at 37°C have shown little to no cellulose degradation and end-product formation. Thus, thermophilic temperatures of 50°C (Haruta *et al*., 2002) and 60°C (Ronan, 2011) are expected to yield high microbial activity and similarly to Ronan (2011), yield a quantifiable amount of valuable end-products.

CHAPTER TWO: LITERATURE REVIEW

2.1 A HISTORY OF BIOFUEL

The process of converting biological materials into useful end-products such as ethanol reaches as far back as 6000 B.C. when ancient Egyptians produced ethanol by naturally fermenting plant-based materials (Demirbas, 2009). However, it was only in 1826 that ethanol was first prepared synthetically by Henry Hennel in the United Kingdom (Demirbas, 2009).

Since the industrial revolution, mankind has been developing and modifying technology to improve the standard of living. The modern engine, for example, has been fed a series of materials derived from biological sources before it ran exclusively on petroleum-derived products (Pousa *et al*., 2007). In the early 20th century, the development of the ignition engine by Nikolaus August Otto was sponsored by the sugar factory of Eugen Langen and ethanol derived from sugar was used as a fuel source (Antoni *et al*., 2007). However because of its low cost and availability, petroleum-derived fuel became the dominant source of energy for engines throughout the 21st century (Solomon *et al*., 2007).

In the 1970s, the demand for petroleum was quickly rising on a global level and the world grew conscious of a changing climate as a result of greenhouse gas emissions. However, it was the high oil prices of the OPEC (Organization of Petroleum Exporting Countries) oil embargo in 1973 that motivated many countries to search for alternative fuel sources (Solomon *et al*., 2007). In 1975, Brazil was one of the first countries to develop a "Proalcoo" program based on ethanol derived from sugar cane. It passed regulations for a bioethanol-gasoline blend of 22% and since automobile manufacturers produce vehicles accepting blends of 10% ethanol (E10), vehicles with ethanol-tolerant engines were introduced on the market. However, as time

progressed and the petroleum prices dropped again, adopting biologically renewable fuels was unlikely around the world (Solomon *et al*., 2007).

Since the late 1980s, world oil prices fluctuated and have been the centre of international political disputes. American geologist Marion King Hubert was the first to calculate that worldwide conventional oil reserves would peak, reaching the maximum rate of oil extraction, around the year 2000 (Clarke, 2008). This caused intense oil exploration activity and the development of technologically-challenging and costly methods of oil extraction from unconventional oil reserves such as the Alberta tar sands. During this time, there was a call for new developments that focused on improving the fermentation of biomass to yield greater amounts of desirable biofuel products, the first generation biofuel (Clarke, 2008).

2.1.1 The Current State of Biofuel

The beginning of the bioethanol industry in North America was triggered by the staggering prices of fuel in the 1970's. To this day many countries around the world have been focusing on the potential of crop-derived biomass as an alternative energy source for reducing the need for foreign gas and oil imports (Rastogi *et al*., 2008). Among them, Canada has devised a renewable fuels strategy under Bill C-33 of the Canadian Environmental Protection Act with a goal of reducing greenhouse gas emissions resulting from fuel use, encouraging greater production of biofuels and accelerating the commercialization of new biofuel technologies (Demirbas, 2009; Balat, 2010). More specifically, the renewable energy strategy mandated a 5% renewable content in gasoline by 2010, which has already been implemented, and a 2% renewable content in diesel fuel and heating oil by 2012 (Demirbas, 2009). Although these regulations are promising, increasing the ethanol-gasoline ratio will be impractical for Canada's current biofuel feedstock, corn and wheat, to supply. In comparison to the U.S. and Brazil, which

produce over 5 billion gal/year, Canada has the ability to produce 200 million gal/year; thus, for this and other reasons, there is a need for yet another biofuel feedstock[2] (USDA, 2010).

2.1.2 The Need for Second Generation Biofuel

Approximately 95% of the world's current bioethanol production is in Brazil and the U.S. with sugar cane and corn as the main feedstock sources (Antoni *et al*., 2007). It is known that crop-derived biofuels are heavily criticized for competing with food prices and food production (Pousa *et al*., 2007). However studies also show that the full life cycle assessment of crop-based biofuels including energy balances, greenhouse gas emissions and other air contaminants are not significantly different from fossil fuels. Wang *et al*. (2007) show that first generation biofuels only reduce greenhouse gas emissions by 20% when compared to the fossil fuel industry. A 52% reduction is predicted if lignocellulosic sources were used instead (Wang *et al*., 2007).

An attractive alternative for biofuel production is the use of cellulosic waste materials known as second generation biofuel. Materials such as decaying straw, woodchip piles or municipal waste and wastewaters derived from agriculture, forestry and municipal facilities can be used to produce favorable end-products. These non-edible materials have been recognized as the world's most abundant and renewable resources and are the least expensive biofuel feedstock available (Lynd *et al*., 2002). Thus, there is a strong belief among researchers that second generation biofuel will play a strong role in the near future.

[2] Values in U.S. gallons

2.2 THE HYDROLYSIS OF CELLULOSE

2.2.1 The Structure of Cellulose and Cellulosic Environments

Cellulose, found in the cell walls of plants, is the greatest component of plant biomass (Lynd *et al*., 2002). It can be found in 35-50% of plant dry weight but can occur in its pure form in cotton balls or filter paper (Lynd *et al*., 2002; Kato *et al*., 2004). Cellulose fibers consist of thousands of linear chains of glucose units. These are known as microfibrils and are found embedded in a complex crystalline structure within hemicelluloses, complex cross-linked polymeric molecules which are embedded in lignin, a coarse material making up plant cell walls (Figure 2.1) (Hess *et al*., 2007). In nature, cellulose is almost always found within this complex structure, referred to as lignocellulose (Lynd *et al*., 2005)

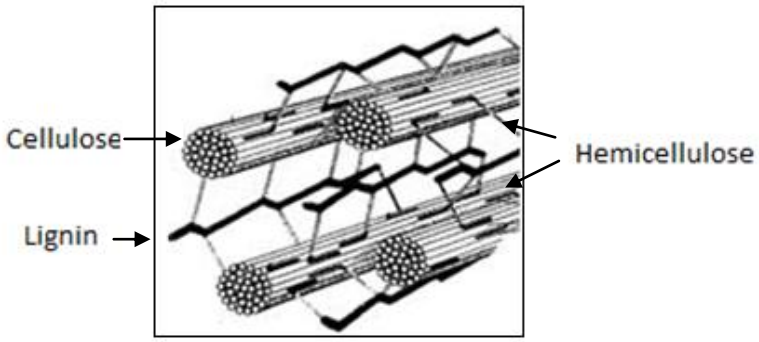

Figure 2.1 The organization of cellulose molecules within lignocelluloses (Adapted from: UC, 2011)

It is estimated that 5-10% of cellulosic materials are degraded under anoxic conditions. In naturally occurring cellulosic environments such as soils and freshwater hot springs, anaerobic activity begins close to the surface. In freshwater swamps and estuaries, cellulosic materials and cellulolytic activity are found to occur in the sediments (Leschine, 1995). Cellulolytic activity also occurs in the rumen of animals that feed on cellulose-rich materials such as in the rumen of cows (Balows and Jennison, 1949). Although in the natural environment many organisms exist, it has been found that bacterial activity is the quickest and most dominant for cellulose

degradation. In comparison, some fungi with the ability to degrade cellulose are slow growing and produce substances that are poisonous for humans. Their end-product formation is also limited (Lynd *et al.,* 2002).

2.2.2 Cellulose Degradation

The complete pathway of cellulose degradation is shown in Figure 2.2. This pathway is based on anaerobic cellulose degradation by cellulolytic bacteria (Lynd *et al.,* 2002). Cellulose is broken down into glucose monomers. Not shown is the breakdown of cellulose to cellobiose and cellodextrins prior to glucose, a hexose sugar. In contrast, hemicelluloses are broken down into pentose sugars that include xylose and arabinose. They are then broken down to glucose 6-phosphate (Lynd *et al.,* 2002). The glucose derived from cellulose undergoes the Embden-Meyerhoff pathway which involves ten reactions that form it into pyruvate. Under anoxic conditions, the two moles of pyruvate are then reduced to lactate or Acetyl-CoA (Leschine,1995).

The conversion of pyruvate to Acetyl-CoA also yields, CO_2 and hydrogen, which can be converted to methane gas by methanogens (Leschine, 1995). Acetyl-CoA can further be converted to ethanol through acetaldehyde and acetate through Acetyl-p (Desvaux *et al.,* 2001). Approximately the same conditions are required for acetate and butyrate production. During acetate production, 4 mols of ATP are formed and during butyrate production, 3 mols of ATP are formed (Zigova *et al.,* 2000). At high growth rates, more ATP is needed by the cells and acetate production is dominant. Butyrate and lactate are detected under very slow growth conditions; however, the production of a certain product is ultimately dependent on the presence of enzymes involved in a particular pathway. For example, for the butyrate pathway, the enzymes involved in the pathway are influenced by ATP concentrations and NADH:NAD+ ratios. Under low ATP and high NADH:NAD+, butyrate production will cease (Zigova *et al.,* 2000).

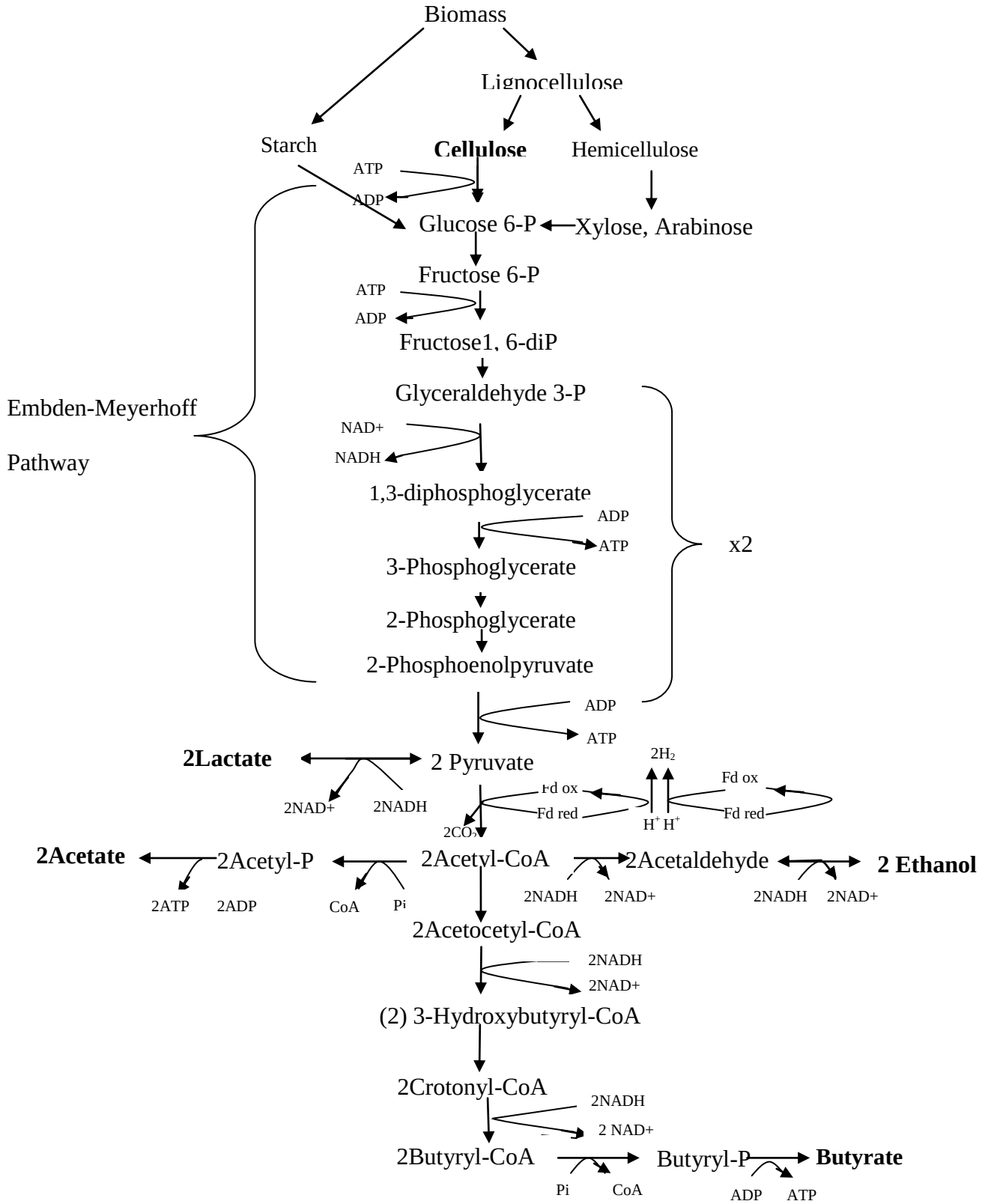

Figure 2.2 A diagram of cellulose degradation into acetate, lactate, butyrate and ethanol byproducts (adapted from Leschine, 1995; Desvaux *et al.*, 2001; Kumar and Gayen, 2010)

2.2.3 Desirable End-Products Produced by Cellulose Hydrolysis

2.2.3.1 Ethanol

The most desirable end-products derived from cellulose hydrolysis are ethanol and hydrogen gas. Ethanol is a highly favored renewable resource because it can be used in a gasoline blend and it can be used within an existing fuel infrastructure (Lynd *et al.*, 2005). It is used as an oxygenated fuel additive because it reduces emissions of carbon monoxide, nitrogen oxides, and hydrocarbons (Wheals *et al.*, 1999). As an additive, ethanol competes with methyl tertiary-butyl ether (MTBE) which is preferred by the petroleum industry; however, MTBE is believed to be toxic and carcinogenic. Ethanol also has a higher octane rating than petroleum fuels. This allows combustion engines to run at a higher compression ratio and gives it superior net performance (Wheals *et al.*, 1999; Carere *et al.*, 2008). Among these reasons, ethanol is the most widely used liquid biofuel and its demand is expected to increase as more ethanolic motor-fuel formulations are being developed (Carere *et al.*, 2008).

2.2.3.2 Hydrogen Gas

Hydrogen is a clean fuel and does not contribute to greenhouse gas emissions into the atmosphere upon combustion (Mizuno *et al.*, 2000). Hydrogen, which is costly to synthesize by chemical processes, is valuable to the technological developments of hydrogen-based fuel cell vehicles. Despite the fact that these vehicles exist as prototypes, it is expected that they will be practical in the next two decades and innovative ways of attaining hydrogen gas are being sought out (Solomon *et al.*, 2007). Hydrogen is currently generated by fossil fuel processing or by electrolysis using solar power which is energy intensive and expensive. Thus, biological production of hydrogen is more desirable especially if it were derived from abundant raw materials (Mizuno *et al.*, 2000).

2.2.3.3 Butyrate

Another desirable end-product of cellulose hydrolysis is butyrate. Butyrate and its derivatives have many applications in the chemical, food and pharmaceutical industries. In the chemical industry, butyrate is used to form a thermoplastic called cellulose acetate butyrate. In the food industries, it is used for food flavors and its esters are used for fruit fragrances (Zigova *et al.*, 2000). It has also been found that butyrate has a therapeutic nature for the treatment of hemoglobinopathies, cancer and gastrointestinal diseases (Zhu, 2003; Pouillart, 1998). Butyrate is currently produced commercially by petrochemical routes; however, it would be more favorable for its production by fermentation routes. This is significantly favored for the food and pharmaceutical industries; however, biotechnological production of butyrate is not commercially competitive due to low productivity and low concentrations produced by this process (Zhu, 2003; Zigova *et al.*, 2000). Thus, production of butyrate by fermentation processes would have a great industrial potential.

2.2.4 Bacterial Mechanisms for Degrading Cellulosic Materials

Due to the diversity of cellulosic environments, different mechanisms for microorganisms to degrade cellulosic materials exist. Habitat characteristics such as water and nutrient availability, oxygen availability, redox potential, and temperature variability have allowed microorganisms to develop unique strategies for cellulose hydrolysis. For example, in the presence of oxygen, lignin components can be completely decomposed to CO_2 and H_2O by a single species of white rot fungi. However under anoxic conditions degradation often requires the participation of mixed bacterial populations to degrade that same material (Leschine, 1995). Both scenarios of cellulose decomposition have been and are currently actively studied. It is

known that under both oxic and anoxic conditions, cellulose degrading microorganisms secrete extracellular enzymes; however they differ in the complexity of the cellulase system and the cells ability to adhere to the substrate (Lynd *et al.*, 2002).

2.2.4.1 The Mechanism of Cellulolysis by Aerobic Microorganisms

The cellulase systems of aerobic microorganisms are known to be less complex than those of anaerobic microorganisms. These microorganisms have been found to secrete extracellular enzymes without the need for cell adhesion to the substrate. Thus, the aerobic system is known as the free-state. The free enzymes are made up of a single polypeptide chain which contains a cellulose binding domain (CBD). At the onset of the activity of cellulolysis, the enzymes bind to the substrate and break it down into smaller components which are available in the environment for cellular metabolism (Bayer *et al.,* 2008).

2.2.4.2 The Mechanism of Cellulolysis by Anaerobic Microorganisms

For anaerobic bacterial species, adhesion to the substrate is a requirement for rapid and efficient cellulose hydrolysis. This system is known as being cell-associated (Lynd *et al.,* 2002). Unlike aerobic microorganisms, this cellulase system consists of a multienzyme complex known as the cellulosome (Demain *et al.,* 2005). The subunits within the cellulosome are highly organized in a chain-like array and because of this, the products of cellulolysis are able to travel down the fibrous structure towards the cell without being free in the environment (Demain *et al.,* 2005). The subunits within the cellulosome contain a set of interacting functional modules. The CBD is one type of module that is selective for binding to the substrate and the catalytic modules are specialized for the hydrolysis of the cellulose chain. Two other families of modules, the cohesins and dockerins join the subunits to the complex (Bayer *et al.,* 2006). Furthermore, Type

I cohesins join together to form a subunit called scaffoldin which hold the dockerin units. The dockerin units then hold specific enzymes for cellulolysis (Bayer *et al.*, 2006).

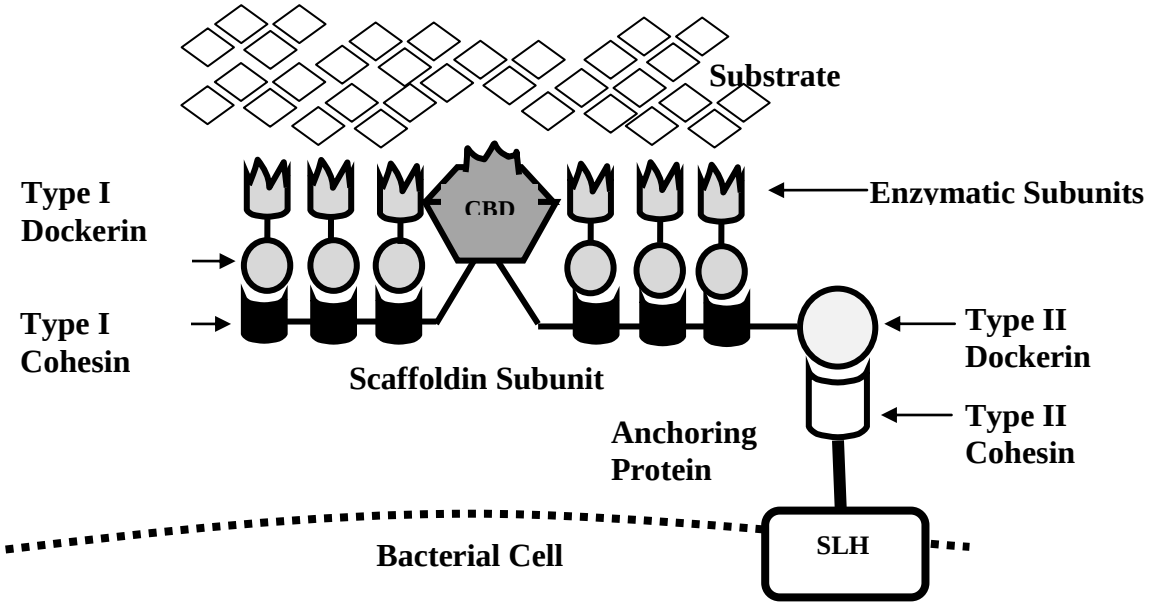

Figure 2.3 A diagram of the cellulosome in anaerobic cellulolytic microorganisms (adapted from Lynd *et al.*, 2002*;* Gilbert, 2007)

2.2.4.3 The Main Types of Extracellular Cellulases

Despite the differences in aerobic and anaerobic mechanisms, there are three major enzymes that partake in cellulose decomposition. They are endoglucanases such as 1,4-D-glucan-4-glucanohydrolases, exoglucanases such as 1,4-β-D-glucan glucanohydrolases, and β-glucosidases (Demain *et al.*, 2005). Endoglucanases cut at random amorphous sites in the cellulose chain and reduce it to shorter chains of cellulose. Next are exoglucanases which act on the reducing or non-reducing ends of cellulosic chains to produce cellobiose or cellodextrins. Lastly, β-glucosidases hydrolyze cellobiose to glucose (Lynd *et al.*, 2002).

2.2.5 Biofuel Production Processes

Given its complex intertwined structure, lignocellulose is more resistant to microbial degradation than cellulose and hemicellulose alone (Huang *et al*., 2010). Thus in a large scale biofuel process, raw cellulosic materials must be subjected to a pretreatment, or delignification step. Costly materials such as strong acids or biological components such as cellulase enzymes produced by cellulolytic microorganisms are needed for this separation of substrate (Lynd *et al*., 2005).

Once the cellulose is isolated from hemicelluloses and lignin, it undergoes hydrolysis into glucose. Here, specialized cellulolytic microorganisms, such as those that belong to the genus *Clostridium* are used (Lynd *et al*., 2002). In conventional industrial processes, hemicelluloses that are isolated from the pretreatment step are also hydrolyzed into their simpler components, pentose sugars, as they can also yield biofuels. The hydrolysis of these five-carbon sugars such as fructose requires a set of different specialized microorganisms such as the yeast *Saccharomyces cerevisiae (S. cerevisiae)* because *Clostridium* bacteria do not have the ability to degrade pentose sugars (Lynd *et al*., 2002). In contrast, *S. cerevisiae,* which is used in the fermentation processes of first generation biofuel, does not have the ability to degrade hexose sugars such as glucose from cellulose. Thus in conventional industrial processes, these steps occur in separate bioreactors which hold different conditions to maintain the growth of the desired microorganisms (different levels of pH, oxygen, temperature, salinity etc.). Due to this, the conventional processes are energy intensive and expensive (Lynd *et al*., 2005).

Over the past decade researchers have been focusing on modifying the cellulose-degradation processes. A leading researcher studying microbial cellulose utilization, Lee Rybeck Lynd, has contributed significantly to the development of consolidated bioprocessing (CBP). CBP is a

process that combines the four conventional biological transformations into a single step, as shown in Figure 2 (Lynd *et al.*, 2005). As a result of combining the delignification, hydrolysis of pentose and hexose sugars, as well as the fermentation steps, the costs of the production of ethanol significantly fell. Lynd has found that in CBP the costs that occur from lost yield, utilities, raw materials, and capital together comprise 4.23 ¢/gal ethanol. In contrast, conventional four step reactors yield a total cost of 18.9 ¢/gal ethanol (Lynd *et al.*, 2005).

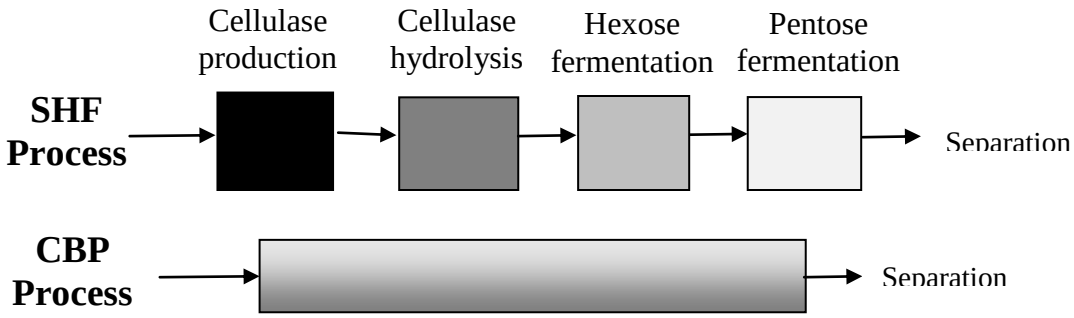

Figure 2.4 The conventional saccharification hydrolyzing fermenting (SHF) technology and consolidated bioprocessing (CBP) (Adapted from: Lynd *et al.*, 2002)

In the separate hydrolysis fermentation (SHF) process described earlier, each bioreactor operates at different temperatures. The preferred temperature for cellulose hydrolysis is 50°C and approximately 32°C is often best for the fermentations to meet the conditions of available microorganisms (Brethauer and Whyman, 2010). It would be more efficient to operate a single bioreactor with a set temperature. Higher temperatures are also more effective for avoiding contamination by mesophilic microorganisms. Despite the benefits of operating a single step fermentation process at higher temperatures, doing this with currently available microorganisms has been found to increase their membrane fluidity which then decreases their ethanol tolerance (Lynd *et al.*, 2002).

Although this process appears favorable, the problem with CBP is that no microorganisms have been identified to perform all four steps continuously. Thus, many researchers, including Lynd, have been focusing on genetically engineering a single strain that has the desired traits to simplify the conventional industrial processes (Lynd *et al*., 2005; Carere *et al*., 2008).

2.3 MICROORGANISMS FOR BIOFUEL PRODUCTION

2.3.1 Pure Cultures of Cellulolytic Bacteria

Since the 1980s, academics and governments have pursued the study of specialized cellulolytic bacteria, yeast and fungi. The cellulolytic microorganisms studied include those in the aerobic order *Actinomycetales* and the anaerobic order *Clostridiales.* Members of the *Clostridiacea* family, specifically in the *Clostridium* genus are well known for their cellulose-degrading capabilities (Lu *et al*., 2006; Lynd *et al*., 2002). Until 2004, it was believed that cellulose degradation occurs by organisms that are either strictly aerobic or anaerobic, but not both. Until that time, only members of the *Cellulomonas* genus were reported to be facultative anaerobic cellulose degraders contributing to hydrogen but not ethanol production (Baganara *et al*., 1987). In addition, focus that in the past has been on mesophilic microorganisms is currently shifting to include thermophilic microorganisms because of the industrial advantages of operating bioreactors are high temperatures.

To address the needs for CBP technology, research has primarily focused on using known cellulose degraders and genetically engineering them to tolerate pretreatment conditions which require an acidic environment, anaerobic conditions and high temperatures at which fermentation for the production of biofuel occurs (Lynd *et al*., 2005). Another favorable trait of cellulose degraders is high end-product tolerance which most pure cultures do not have. Current efforts in

this area include using non-cellulolytic microorganisms capable of tolerating high ethanol concentrations, which can otherwise be inhibitory for the process of cellulose degradation. For example, genes encoding enzymes needed for cellulose degradation from organisms such as *C. thermocellum* are added are introduced into *S. cerevisiae* and *Escherichia coli (E. coli*) which are tolerant to high ethanol concentrations (Junji *et al*, 2004; Lynd *et al*., 2005; Carrere *et al*., 2008). Although a large amount of research has focused on these possibilities, to date anaerobic growth of these modified microorganisms on cellulose substrates such as filter paper have been unsuccessful. However, a recent study found that a recombinant strain of *S. cerevisiaw* was able to ferment raw starch and xylan, by hydrolyzing it to the five-carbon sugar xylose, resulting in a yield of 0.3 to 0.4 g ethanol/g substrate (Junji *et al*., 2004). A microbial culture that yields an amount of ethanol equal or greater to the theoretical yield of 0.51 g ethanol/ g glucose or 0.58 ethanol/ g xylan is the target for industrial application (Dowe and Macmillan, 2008). Thus, a search for an ideal microorganism that is able to convert cellulose to favorable end-products is still underway.

2.3.2 Mixed-Cellulolytic Microbial Cultures

Despite a focus on a single microorganism to carry out cellulolysis resulting in favorable end-product formation, using a mixed microbial community that can carry out similar functions can be more advantageous for large scale biofuel production. It is known that mixed microbial populations can carry out functions that can be difficult for a single species to do alone (Brenner *et al.,* 2008). Thus, it has been suggested that different populations can be optimized to function in different steps in the cellulose hydrolysis pathway. The benefit of a microbial community also allows it to adapt better to environmental changes. Its ability to withstand periods of nutrient limitations, for example, can be because of the diversity of metabolic modes that exist within the

diversity of species present. The minor population can become most active within the community if it has the metabolic activity on which the whole consortium can depend (Brenner *et al.,* 2008).

Many microbial species that are highly specialized in utilizing cellulose are known to reside in cellulosic waste materials. However, mixed-microbial communities found in these cellulolytic environments have been seldom studied and understood. A study conducted by Odom and Wall (1982) showed that, when facultative anaerobic cellulose species, *Cellulomonas sp.,* and one anaerobic non-cellulose degrading microorganism, *Rhudopseudomonas capsulata*, were introduced together, 3 to 4 times more hydrogen was produced in this co-culture than in their respective pure cultures. This indicated that symbiotic relationships between species that have different environmental requirements are capable of occurring in cellulosic environments.

Haruta *et al.* published a study in 2002 in which a mixed-microbial culture was characterized from farm animal feces by enrichment with a complex, high-nutrient liquid medium at 50°C (Haruta *et al*., 2002). The enrichment- culture technique has been extensively used for selecting and isolating certain bacteria; however, characterizing whole microbial communities from cellulosic environments has not been done prior to this study. Currently-available DNA sequencing techniques indicated that the majority of the mixed community members were the non cellulolytic aerobic and facultatively anaerobic *Bordatella* sp., strictly aerobic *Brevibacillus* sp., and the cellulolytic anaerobic *Clostridium* sp. (Haruta *et al*., 2002). This mixed culture produced ethanol, lactate and acetate at 1.6, 0.12, 0.18 g/L, respectively.

A continuation of this study in 2005 showed that pure cultures of the above microorganisms also showed these favorable properties when combined. The interaction of the members was studied by "knocking-out'' a species from the mixed culture at a time. It was found that the aerobic bacteria *Brevibacillus* and *Pseudoxanthomonas* introduced anaerobic

conditions to the batch culture while the cellulolytic bacterium *C. straminosolvens* supplied acetate and glucose. It was also found that the production of excess acetate by the *Clostridium* sp. inhibited cellulose degradation and suppressed the growth of the *Bordatella* sp. in the culture (Kato *et al.,* 2005). This study contributed to a better understanding of the interactions within one mixed culture that degrades cellulose.

Another study by Kato *et al.* (2004) has shown that a culture of a cellulolytic anaerobic microorganism and an aerobic non-cellulolytic microorganism degrades cellulose more effectively than a pure culture of the anaerobic cellulolytic microorganism, *Clostridium straminisolvens (C. straminisolves).* After 10 days, 6.50 mg ml^{-1} of filter paper was degraded by a *C. straminisolvens* pure culture and 8.91 mg ml^{-1} by the mixed culture (Kato *et al*., 2004). Although the bacteria used in this study were purchased strains from pure cultures, the fact that these microorganisms produced a higher ethanol content than a specialized cellulose degrader indicates that a mixed-microbial culture that is obtained from a natural cellulosic environment can potentially be capable of producing a greater amount of ethanol than a pure culture.

Since the above articles have been published, few findings have been published on characterizing individual members from naturally derived microbial communities. Rastogi *et al*., (2009) published an article on isolating a mixed culture of cellulose-degrading bacteria from a deep subsurface gold mine; however, its ability to produce biofuels such as ethanol or hydrogen has not been measured. Thus, the present study is crucial in not only characterizing a mixed-microbial community from cellulosic environments not studied before but also in quantifying the end-products that can be potentially produced from the symbiotic interactions of the microbial members.

2.4 COMPOST AND WASTEWATER INOCULUM SOURCES

As previous studies suggest, agricultural waste, organic municipal solid waste as well as wastewater hold a diverse community of microorganisms that participate in the degradation of cellulosic materials (Lynd *et al*., 2002). Composting is the actively managed process of organic matter brought about by the growth of microorganisms (Ministry of Agriculture and Food, 1996). Composting stabilizes organic matter such as the carbon found in cellulose and reduces the volume of the compost by 20 to 60 percent. End-products include humus, the organic fraction of soil, as well as carbon dioxide, heat, water, and other minor gases (Ministry of Agriculture and Food, 1996). Furthermore, Rollins and Koening (2010) reported that 80-95% of soil microbes have not been cultured. This makes compost a favorable inoculum source for characterizing a novel mixed-microbial community.

Microorganisms such as bacteria, fungi and actinomycetes account for the decomposition of products and the rise in temperature within the process. Among these microorganisms, the most important are the aerobic bacteria, the initiators of decomposition, and thermophilic anaerobic bacteria, the major degraders (Rollins and Koening, 2010). The process of compost begins with psychrotolerant bacteria that work during conditions lower than 21°C. Their activity generates a small amount of heat and changes the environment favorable for mesophilic bacteria (10- 45°C). Lastly, as more heat is released, thermophilic bacteria (above 50°C) thrive; however when these bacteria die, the temperature decreases and dormant mesophilic bacteria regain activity. The pH varies from 6-7 at the beginning of the process and levels off to a pH of about 7.5 (Ministry of Agriculture and Food, 1996). This pattern can be seen in Figure 2.5.

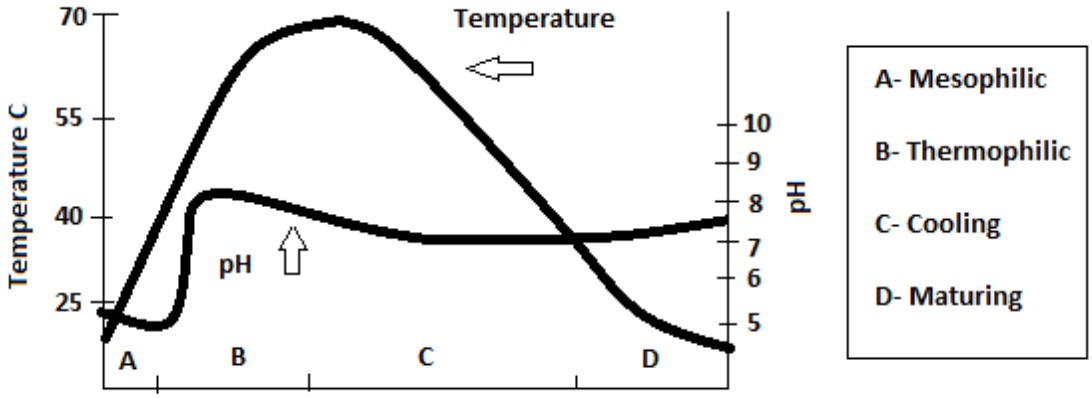

Figure 2.5 Temperature and pH of composting processes (Adapted from: Ministry of Agriculture and Food, 1996).

The exact composition of microorganisms used within the treatment processes of municipal wastewater facilities is often unknown (Norweco, 2011). It is generally understood that the secondary treatment step contains many aerobic and facultative anaerobic bacteria that are native to the location of the facility and obtained from natural mixed cultures (Smith, 2011). In addition, current regulations only require *E. coli* and coliform control in wastewater effluent. The presence of *E. coli*, which is a fecal coliform, is a strong indicator of recent fecal pollution. Total coliform which includes a family of bacteria called Enterics, grow under the same conditions as other disease-causing bacteria; thus, the presence of total coliform in sewage signifies inadequate treatment and suggests the presence of potential pathogenic microorganisms (Nathanson, 2008; Region of Peel, 2011). The certificate of approval for the Ashbridges Bay wastewater facility requires zero coliform in the effluent discharged into Lake Ontario (City of Toronto, 2010). Due to the limited knowledge of microorganism composition within wastewater, characterizing a mixed community from this source is of interest to this study.

2.5 MEDIA FOR CELLULOSE DEGRADING BACTERIA

The most studied cellulolytic bacteria are *Clostridium thermocellum* which were first isolated from manure. Medium requirements for their cultivation include a basal solution, a mineral solution and vitamin solutions. Different carbon sources can also be added. These include avicell or cellobiose as well as a strip of filter paper, which is a pure form of cellulose. An example of a medium that is commonly used for cellulolytic bacteria is M medium. The basal solution usually includes buffers KH_2PO_4 and K_2HPO_4 as well as a nitrogen source such as NH_4Cl, a redox potential indicator such as resazurin and yeast extract. The mineral solution composition contains $FeCl_2$, $CaCl_2$, $MnCl_2$, or $CuSO_4$. Lastly, the vitamin solution contains biotin, folic acid, riboflavin, Vitamin B12, p-aminobenzoic acid, and lipoic acid.

Many researchers have also found that adding yeast extract, a complex nutrient source, into the medium achieves a higher growth rate and end-product formation. While testing the microorganism's adhesion to various substrates, Demain *et al.,* (2005) found that at a high rate of yeast extract *C. thermocellum* bound to glucose and when this rate was lowered, it had the ability to bind to cellulose and cellobiose. Despite this favorable characteristic, yeast extract remains an expensive product if introduced on a larger scale (Carere *et al.,* 2008).

Interestingly, Ronan (2011) enriched a cellulolytic community from compost and tested its cellulolytic activity under different variations of the RM medium. Ethanol and acetate were the major fermentation products under both aerobic and anaerobic media. The author also supplemented yeast extract with complex waste-based nutrient sources. It was found that cellulolytic activity persisted in yeast extract-free, non-reduced aerobic media, using compost tea or wastewater as the complex nutrient source. Thus, the activity of ethanol-producing

microorganisms was not significantly affected when yeast extract was replaced with other, cost efficient, complex nutrient sources (Ronan, 2011).

In the past, pure cultures of *Clostridium* sp. were subjected to a chemically defined medium to control pH conditions in the cultures or to study the bacterial kinetics and metabolism of cellulose degradation (Lynd and Ahn, 1996; Desvaux *et al.*, 2001). In this investigation, yeast extract or other complex nutrient sources were not utilized. Instead, the focus was on the use of a chemically defined medium to obtain mixed microbial consortia with the ability to ferment cellulose into desirable end-products.

CHAPTER THREE: MICROBIAL CELLULOLYTIC CONSORTIA

3.1 INTRODUCTION

Since the dependency on fossil fuels is consistently growing, resources to meet this need are diminishing and alternative means for meeting fuel consumption are being vastly explored. The potential of using biomass to produce fuel, known as biofuel, is an attractive alternative due to its potential to reduce greenhouse gas emissions without added changes to current biofuel infrastructure (Carrere *et al*., 2008). Organic materials such as sugar cane, corn, and cellulosic waste materials can be used as feedstock for producing biofuel in a liquid state (ethanol or butanol) and in a gaseous state (hydrogen or methane) (Forge, 2007).

As the most abundant biopolymer on earth, cellulose is found to be the most cost efficient and most readily available feedstock for large scale biofuel production but because of its complex crystalline structure, many microorganisms cannot degrade it (Solomon *et al*,, 2007; Lynd *et al,* 2002). In the last couple of decades, researchers have focused on isolating cellulolytic microorganisms, perfecting their media requirements and genetically engineering them for maximum biofuel production (Lynd *et al.,* 2002). Despite these efforts there are shortcomings that do not meet the criteria for large scale biofuel production.

The technology of consolidated bioprocessing (CBP) involves the elimination of the expensive enzyme producing stage that is commonly found in current biofuel technologies. The saccharification step and fermentation steps are also combined for lower biofuel production costs, energy inputs and maintenance costs (Carere *et al.,* 2008). A single microorganism or consortium of microorganisms that can utilize a combination of substrates, be active under anaerobic thermophilic conditions, yield high hydrolysis rates, yield high product concentrations

and produce little or no organic acids are still sought out for this technology (Lynd *et al.,* 2002; Lynd *et al.,* 2005). The model cellulolytic bacterium, *Clostridium thermocellum*, has been given much attention to solve this problem; however it does not have the ability to degrade pentose sugars to ethanol and its growth is inhibited by high ethanol concentrations (Ng, 1981; Lynd *et al.,* 2002).

In cellulosic environments, cellulose materials are degraded by the activity of many microorganisms that coexist and interact with one another (Haruta *et al.*, 2002). It has been reported that mixed cultures of cellulolytic and non-cellulolytic bacteria ferment raw cellulosic materials to produce ethanol and hydrogen with byproducts of acetate and lactate at an incubation temperature of 50ºC. In 2002 Haruta *et al.* reported a mixed microbial consortium isolated from compost waste materials with the ability to degrade rice straw (Haruta *et al.,*2002). The cellulolytic anaerobic bacterium *Clostridium straminosolvens* and non cellulolytic aerobic bacteria such as a *Brevibacillus* sp., *Pseudocanthomonas* sp., and *Bordatella* sp. were then tested on their cellulose-degrading abilities in a defined mixed culture (Kato *et al.,* 2005). Synergistic relationships were observed, function was constant, and the interactions occurring between the biota were essential for a stable coexistence within the culture. The amount of ethanol produced was 80 mg/g of filter paper degradation with acetate:ethanol ratio of 1:0.5 (Kato *et al.,* 2005). Thus, mixed microbial communities may have a significant potential for the CBP process.

Cellulosic environments are immensely diverse in their physical and chemical properties (pH, temperature, nutrients) (Lynd *et al*., 2002). Thus, it is expected that using different sources of inoculum will yield diverse mixed microbial consortia. Local composting materials have already been proven a good source of diverse microorganisms with the ability to produce

valuable end-products (Kato *et al.*, 2002; Kato *et al.*, 2008). Wastewater sludge has been used for obtaining enrichments of mixed hydrogen producing bacteria (Chan *et al.*, 2001; Liu *et al.*, 2003). The soluble end-products from this inoculum have been seldom studied.

Another desirable property for CBP is utilizing a practical growth medium for maintaining the cultures (Lynd *et al.*, 2002). Of importance are the buffering capacities within the medium that keep the pH near a value of 6, under which cellulose hydrolysis occurs. It is also believed that an abundance of salts may lead to growth inhibition and yield little needed byproducts. This has yet to be observed (Lynd *et al.*, 2002). Many researchers have also used complex media that includes either peptone or yeast extract to culture cellulolytic microorganisms (Haruta *et al.*, 2002; Kato *et al.*, 2008; Miyazaki *et al.*, 2008). Although rich in nutrients, these products are expensive and can hinder the economic feasibility of the CBP process (Madipatti *et al.*, 2011).

The focus of this study was to utilize two cellulosic-rich environments as inoculum sources to obtain two potentially mixed cellulolytic consortia. Municipal organic waste (compost) and wastewater sludge were used at two incubation temperatures (50ºC and 60ºC) to establish cellulose-degrading communities by the enrichment technique. A defined medium that contained no yeast extract was used and the soluble end-products that were produced over an active filter paper degradation period were quantified.

3.2 MATERIALS AND METHODS

3.2.1 Inoculum and Enrichment Setup

3.2.1.1 Experimental Setup for the Compost Enrichment

A sample of compost was collected from an operating residential composter in Toronto, Ontario, which was fed food-scraps and yard waste (Ronan, 2011). Compost in the amount of 5g was added to 20 mL of a chemically defined liquid medium (MJ medium) as a starter culture for successive enrichments. The medium was composed of 1.5 g KH_2PO_4, 2.9g K_2HPO_4, 2.1 g NH_4Cl, 10 g MOPS, 3 g sodium citrate $2H_2O$, resazurin, 1 mL of a vitamin solution (pyridoxamine hydrochloride, biotin, *p*- Aminobenzoic acid, Vitamin B_{12}) and 10 mL of a mineral solution ($MgCl_2$ $6H_2O$, $CaCl_2$ $2H_2O$, $FeSO_4$ $6H_2O$) in 1 L MQ H_2O (Johnson *et al.*, 1981). Modifications in the medium were made in substituting cellobiose, a powder aggregate of cellulose, with a sterile 1x4 cm piece of 100% cellulose Whatman No. 1 filter paper (Whatman, Piscataway, NJ).

Subsequent enrichments were made by inserting a fresh piece of filter paper into a 100 mL anaerobic vial containing the 10 mL of the preceding culture and 30 mL of the medium. The vial was capped with a rubber stopper and a metal clamp. Two experiments were set up this way and incubated at 50ºC and 60°C. The degradation of filter paper was observed daily and complete filter paper degradation was noted in both cultures. After obtaining stable consortia, which were observed for consistent filter paper degradation when compared to previous enrichments, an experiment was set up in triplicate for a time-point analysis for the measurement of soluble substrates within the consortia.

3.2.1.2 Experimental Setup for the Wastewater Enrichment

Wastewater was collected from the secondary treatment step of a wastewater treatment facility in Mississauga, Ontario. A piece of filter paper and approximately 20mL of the wastewater was added to 20mL of the modified MJ medium. Subsequent enrichments were made differently for the 50ºC and 60ºC experiments. Ten mL of culture were added to 30 mL of the medium, incubated at 50ºC and 20 mL of culture and 20 mL of medium were added to be incubated at 60ºC. Enrichments were continued and the filter paper was observed for degradation (Haruta *et al*, 2002; Ronan, 2011). Similarly as to the compost enrichment, once two stable consortia were obtained (one from each incubation temperature), an experiment in triplicate was setup for soluble end-product analysis.

3.2.2 Time Point Analysis Experiment

Once stable communities were obtained for both incubation temperatures and inoculum sources, they were transferred into 15 mL vials and were set up with the same culture: medium ratios as obtained by their enrichments. For each incubation time and inoculum source, three vials with fresh filter paper were set up. Approximately 1mL of the stable community was taken for soluble end-product analysis and acidified to a pH of 2 using HCl (37% wt./wt.). Samples were taken at d1 of inoculation, at the first observation of filter paper degradation, after complete filter paper degradation, and once all residual material disappeared. Fresh filter paper and medium were not added between the time points. Samples for end-product analysis were stored at -20°C until ready for analysis (Ronan, 2011). The pH was monitored with a bench-top pH meter (Cole-Parmer, Montreal).

3.2.3 End-Product Analysis

3.2.3.1 High Performance Liquid Chromatography

Samples taken for end-product analysis were thawed and centrifuged for 5 minutes at 10,000 rpm. Approximately 700 µL of the supernatant was removed and placed into 1 mL glass HPLC vials (Cole-Parmer, Montreal, QC). Concentrations of ethanol, acetate, lactate, butyrate and cellobiose were analyzed by HPLC with a carbohydrate analysis column (Bio-Rad HPX-87H) (Ronan, 2011). The mobile phase used was 5mM of H_2SO_4 and was injected through the column at a rate of 0.6mL/ min. The column was kept at a constant temperature of 60ºC. Each sample was run for 26 minutes to allow for the detection of all soluble constituents in the culture. The data were analyzed by selecting the report format on the Totalchrom program. A standard curve was created for the mixtures of standards at 1, 2, 5, 10 and 20 mM concentrations. The standards were run individually at a concentration of 10mM to determine their retention times.

3.2.3.2 Gas Chromatography

A qualitative analysis of the gas composition within the compost-derived and wastewater-dervied cultures incubated at 60ºC was completed at one time point, after final degradation of filter paper by the stable cultures. The cultures were set up with fresh medium and fresh filter paper in 15mL glass screw top vials with PTFE/Silicone septa (Supelco, Bellefonte, PA) that allowed for GC handling. The samples were analyzed by the ANALEST Laboratory at the University of Toronto. The gases CO_2, CH_4, and CO were used as standards with Helium as the carrier gas.

3.2.4 End-Product Calculations

The acetate:ethanol ratio was calculated for all cultures at the highest point of ethanol concentration since ethanol and acetate share a close equilibrium relationship. The molar concentration of acetate was divided by the molar concentration of ethanol. The acetate:butyrate ratio was also calculated in a similar manner for cultures that produced butyrate. The percent of cellulose-derived carbon bound in ethanol at the highest point of ethanol concentration was also determined. This was done in three steps. First, the mass of ethanol (g) in the vial was determined from the ethanol concentration (mM) at the highest concentration. The mass of ethanol was then multiplied by 0.52 (since ethanol is approximately 52% carbon (wt./wt.)) and divided by the molar mass of carbon to determine the total number of moles of ethanol-bound carbon. The second step required the calculation of the total moles of carbon in the original filter paper. This was done by weighing the filter paper prior to inoculation and multiplying by 0.44 (since carbon makes up 44.4% of cellulose (wt./wt.) and filter paper is 100% cellulose). Lastly, the proportion of cellulose-derived carbon bound in ethanol was determined by dividing the number of ethanol-bound carbon moles by the initial number of cellulose-bound carbon moles (Ronan, 2011).

3.3 RESULTS

3.3.1 Culture Enrichment

Four filter paper degrading cultures were enriched using the chemically defined MJ medium. Two stable cultures were obtained from an inoculum source of wastewater, one incubated at a temperature of 50ºC and the other at an incubation temperature of 60ºC. Cultures were declared stable when cellulolytic activity persisted upon repeated enrichment transfer. Similarly, two stable cultures were obtained from the compost inoculum source at an incubation temperature of 50ºC and another at 60ºC. The filter paper degradation and the colour of the media were observed on a daily basis to determine the differences that occurred between the four cultures (Table 3.1).

Table 3.1 Observations of the stable compost and wastewater cultures incubated at both 50 and 60ºC temperatures during filter paper degradation

Observations	Compost Culture 50ºC	Compost Culture 60ºC	Wastewater Culture 50ºC	Wastewater Culture 60ºC
Time (d) for visible filter paper degradation	20	7	14	12
Resazurin colour one day after inoculation	Pink	Pink	Pink	Pink
Resazurin colour at the onset of filter paper degradation	Beige	Beige	Beige	Beige
Observations of filter paper at the onset of degradation	Holes formed over filter paper	Visible isolated strands	Cloudy mass formed	Visible isolated strands
Presence of gas at the end of filter paper degradation	No	Yes	No	Yes

The time that filter paper degraded differed for each of the cultures. Overall degradation occurred at a slower rate when incubated at 50ºC than at 60ºC. A significant difference was seen in the compost-derived cultures at 50ºC (d20) and 60ºC (d7). There are also differences in gas

production. Gas was noticeable in both compost-derived and wastewater-derived cultures incubated at 60ºC but not in cultures incubated at 50ºC at the end of filter paper degradation.

The filter paper appearance during filter paper degradation differed in the 50ºC cultures (Figure 3.1). The active microbial community members form visible holes in the compost culture at 50ºC whereas those present in the 60ºC culture degraded the filter paper by isolating its strands, which were visible in the liquid medium. A cloudy mass formed at the bottom of the vial in the wastewater culture at 50ºC, whereas visibly separated strands were present at the higher temperature. All four cultures at the first day of inoculation appeared pink. During filter paper degradation and once the filter paper degraded, all cultures appeared light yellow. The control at 50 and 60ºC consisting of medium with filter paper but no inoculum remained pinkish-purple.

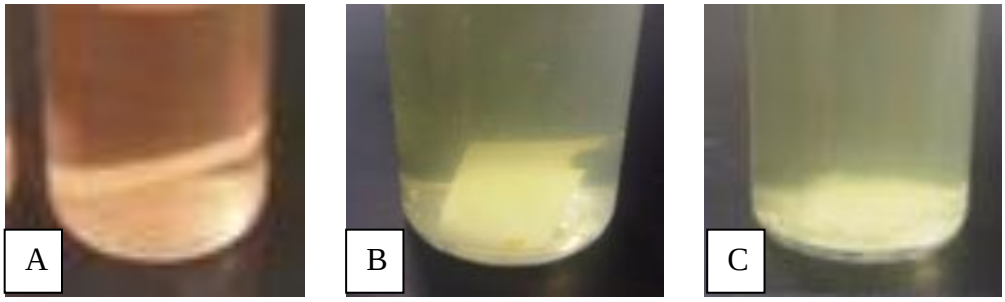

Figure 3.1. Images showing the characteristics before and during the degradation of filter paper in selected cultures. A) Pink medium that is present during the first day of inoculation, here the wastewater at 60ºC, B) Visible holes in the filter paper from the 50ºC compost culture, C) Cloudy mass on filter paper characteristic of the 50ºC wastewater culture.

3.3.2 Soluble End-Product Analysis of the Compost-Derived Cultures

3.3.2.1 The Compost –Derived Culture at 50 ºC

In the culture that was incubated at 50ºC, the major end-product produced was butyrate (Figure 3.2). The concentration of butyrate was 1.1 mM at the time of inoculation and rose to 10.5 mM by d11, which was the point of visible filter paper degradation. As the filter paper was completely degraded (d20), the amount of butyrate declined to an amount of 7.3 mM and

eventually to 5.5 mM by d 26. At the highest production of butyrate (d11), the acetate concentration was at 2.7 mM resulting in an acetate:butyrate ratio of 1:3.9.

Ethanol was detected from d1 to d26 but did not rise above 2.7 mM. The acetate to ethanol ratio at the highest point of ethanol production was 1:1. The carbon in ethanol derived from cellulose was 5%. Also observable in this culture's activity was the high lactate concentration at the beginning of the experiment (11.3 mM) which fell below 1 mM after filter paper degradation (d20).

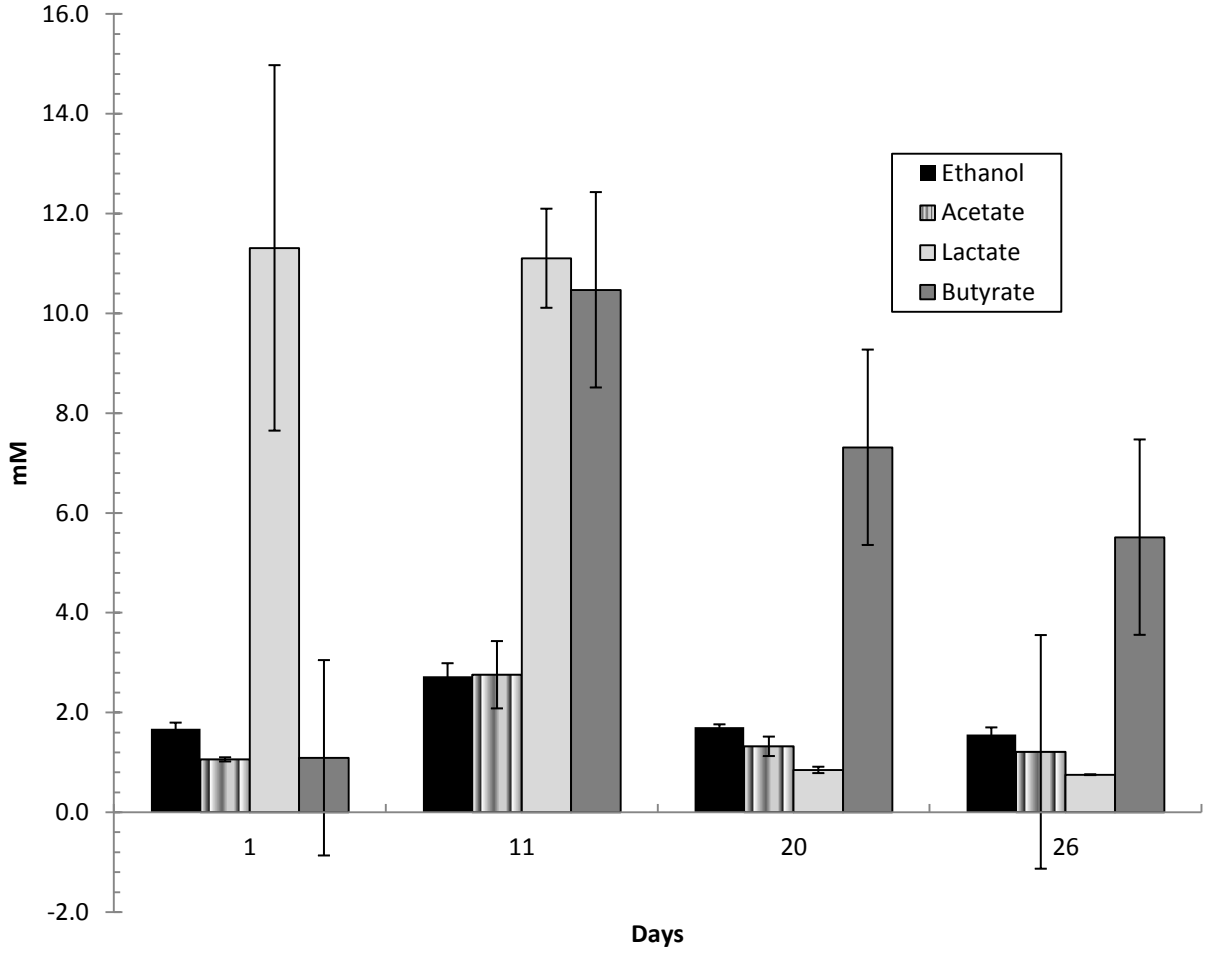

Figure 3.2 The soluble end-product analysis from the 50ºC compost culture over a period of 26 days.

3.3.2.2 The Compost –Derived Culture at 60ºC

The culture derived from compost and incubated at 60ºC produced significant amounts of acetate and lactate with low amounts of ethanol (Figure 3.3). The maximum point of ethanol production was 10.1 mM with acetate concentration at 19.0 mM (d7). The acetate to ethanol ratio was calculated to be 1:0.52, higher than that of the culture at 50ºC despite the quicker time of filter paper degradation at 60ºC. The pH at this time was 6.05 as shown in Figure 3.5. In contrast to the culture incubated at 50ºC, the amount of butyrate did not vary over time.

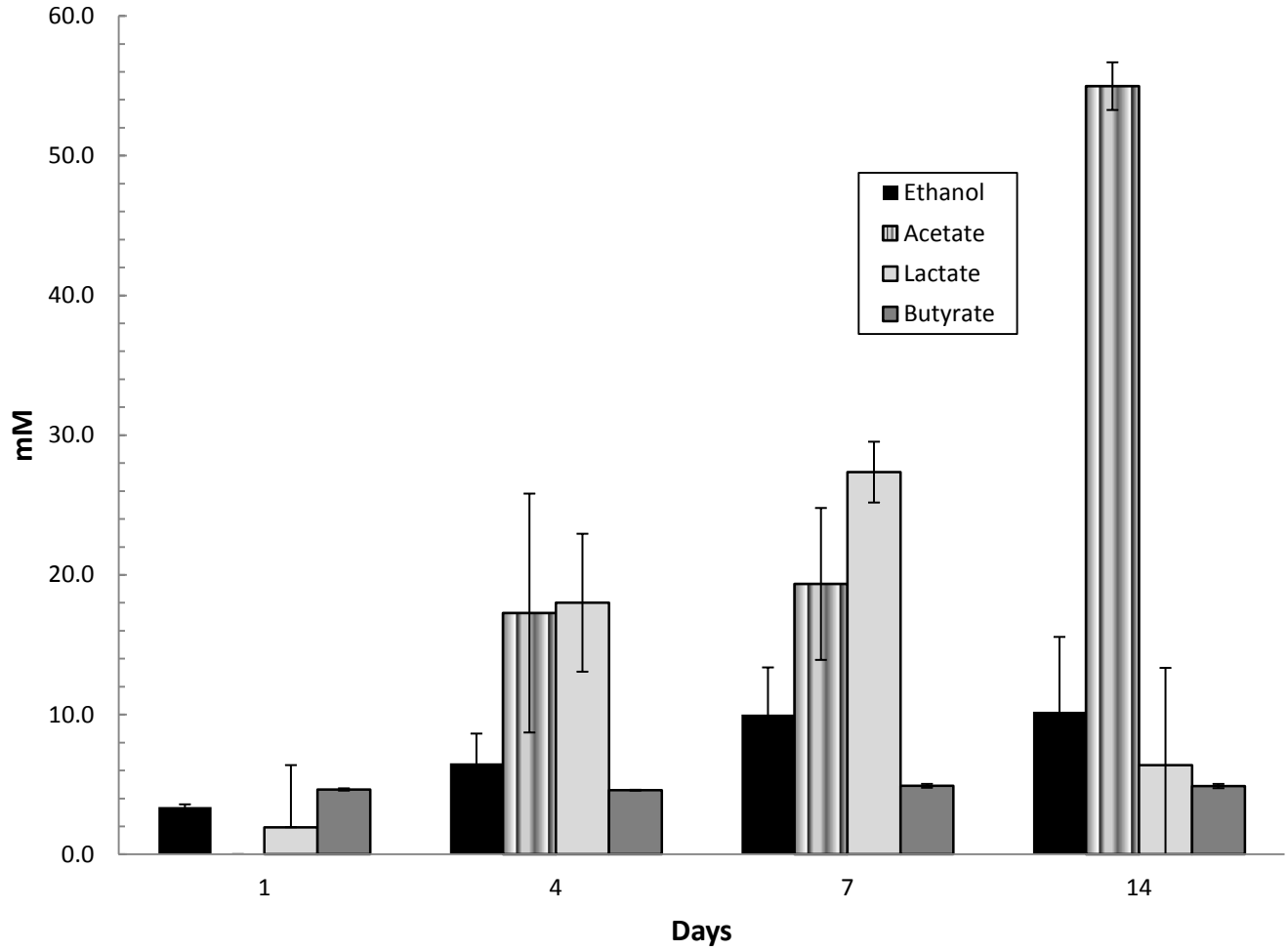

Figure 3.3 The soluble end-product analysis from the 60ºC compost culture over a period of 14 days.

3.3.3 Cultures Derived from a Wastewater Inoculum

3.3.3.1 The Wastewater-Derived Culture at 50ºC

The major end-products produced by the wastewater-derived culture at 50ºC were acetate at 21.0 mM maximum (d14) and ethanol at 4.9 mM maximum (d14). The acetate to ethanol ratio was 1:0.23 and the pH was 6.6 as shown in Figure 3.6. The calculated cellulose-derived carbon in ethanol from was 6.1%. Also observable was the decrease of lactate from 2.1 mM to 0.8 mM (d1 to d14) as well as the constant presence of butyrate over time at 1.0 mM.

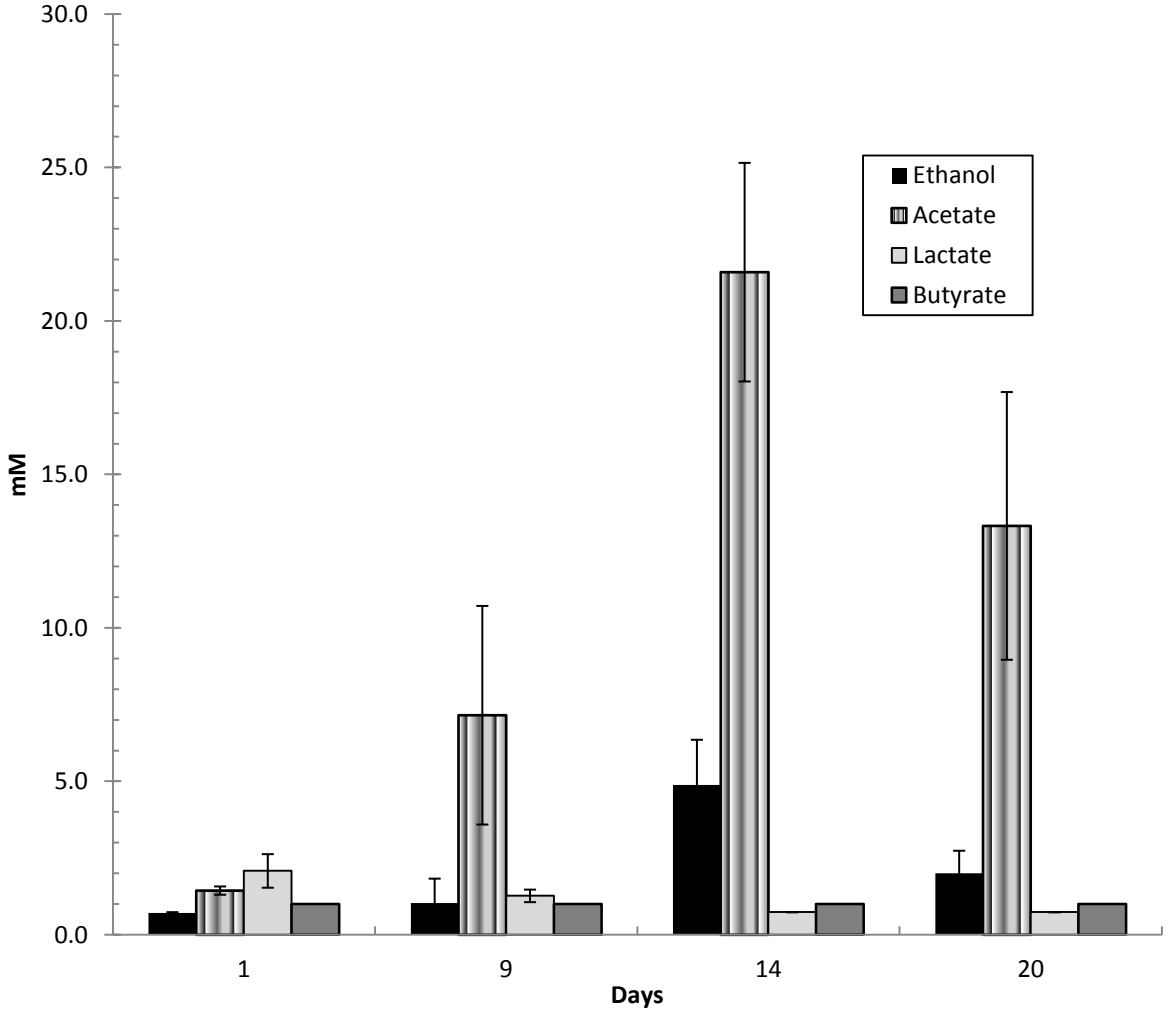

Figure 3.4 The soluble end-product analysis from the 50ºC wastewater culture over a period of 20 days.

3.3.3.2 The Wastewater-Derived Culture at 60ºC

The major end-products in this culture were ethanol and acetate. On d12, ethanol reached its highest concentration at 35.3 mM. At this time point, the acetate concentration was 7.6 mM, resulting in an acetate: ethanol ratio of 1:4.6. The cellulose-derived carbon bound in ethanol was 55.4%. The pH at this time was 6.4 (Figure 3.6). The concentration of lactate increased once both ethanol and acetate decrease over time (Figure 3.5).

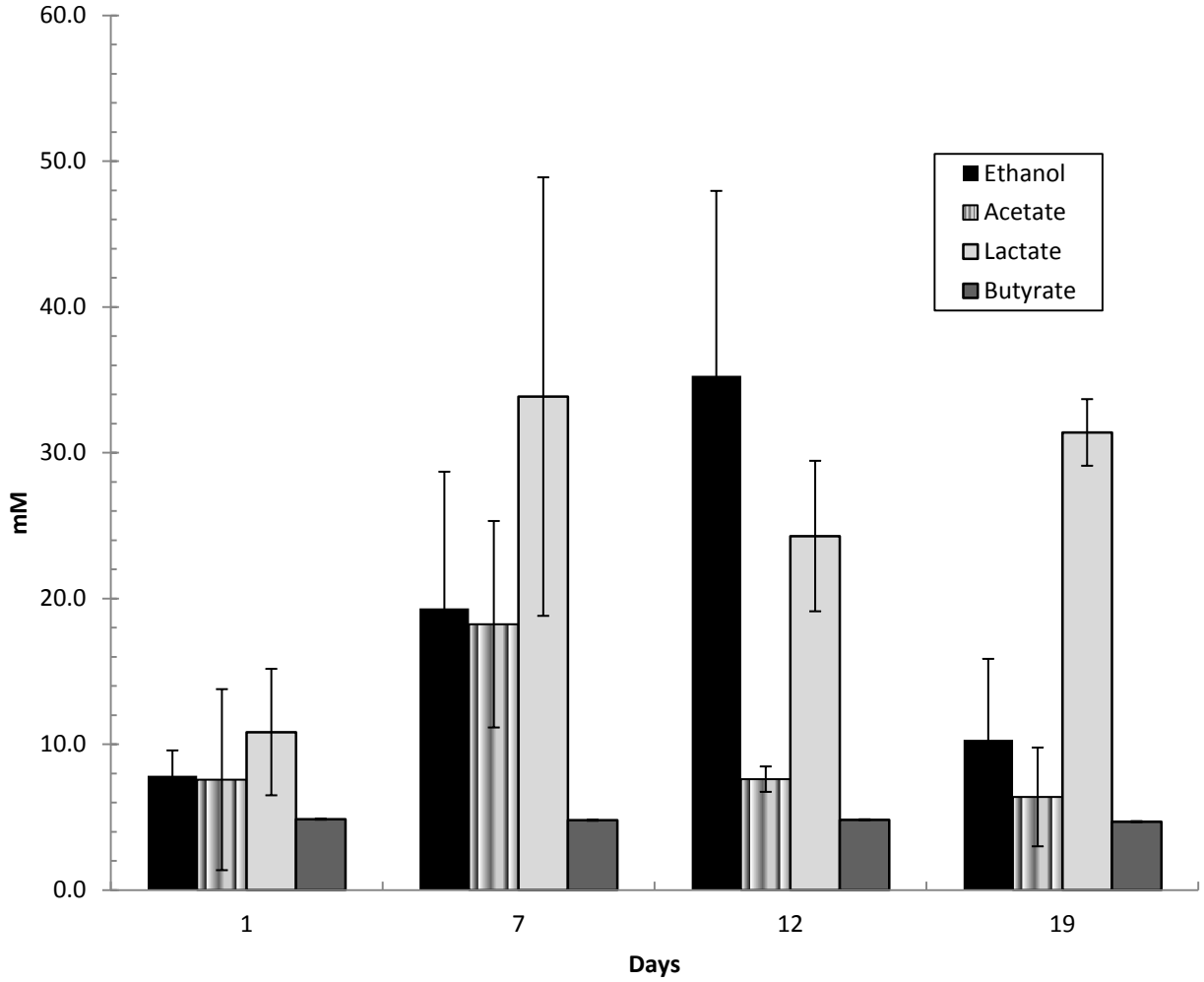

Figure 3.5 The soluble end-product analysis from the 60ºC wastewater culture over a period of 19 days.

3.3.4 The pH of all Cultures at 50 and 60°C Incubation Temperatures

The pH levels of all four cultures were found within the range of pH 6 and 7. The control line includes medium with filter paper incubated at 50ºC and 60ºC. In all cultures the pH was the lowest at the third time point, that when filter paper was degraded completely, except for in the compost 60ºC culture where the lowest pH 6.06 was at the fourth time point. Both cultures at 50ºC displayed a higher pH range than both cultures at 60ºC. In addition, at both incubation temperatures, the wastewater cultures have a pH range higher than their compost neighbors.

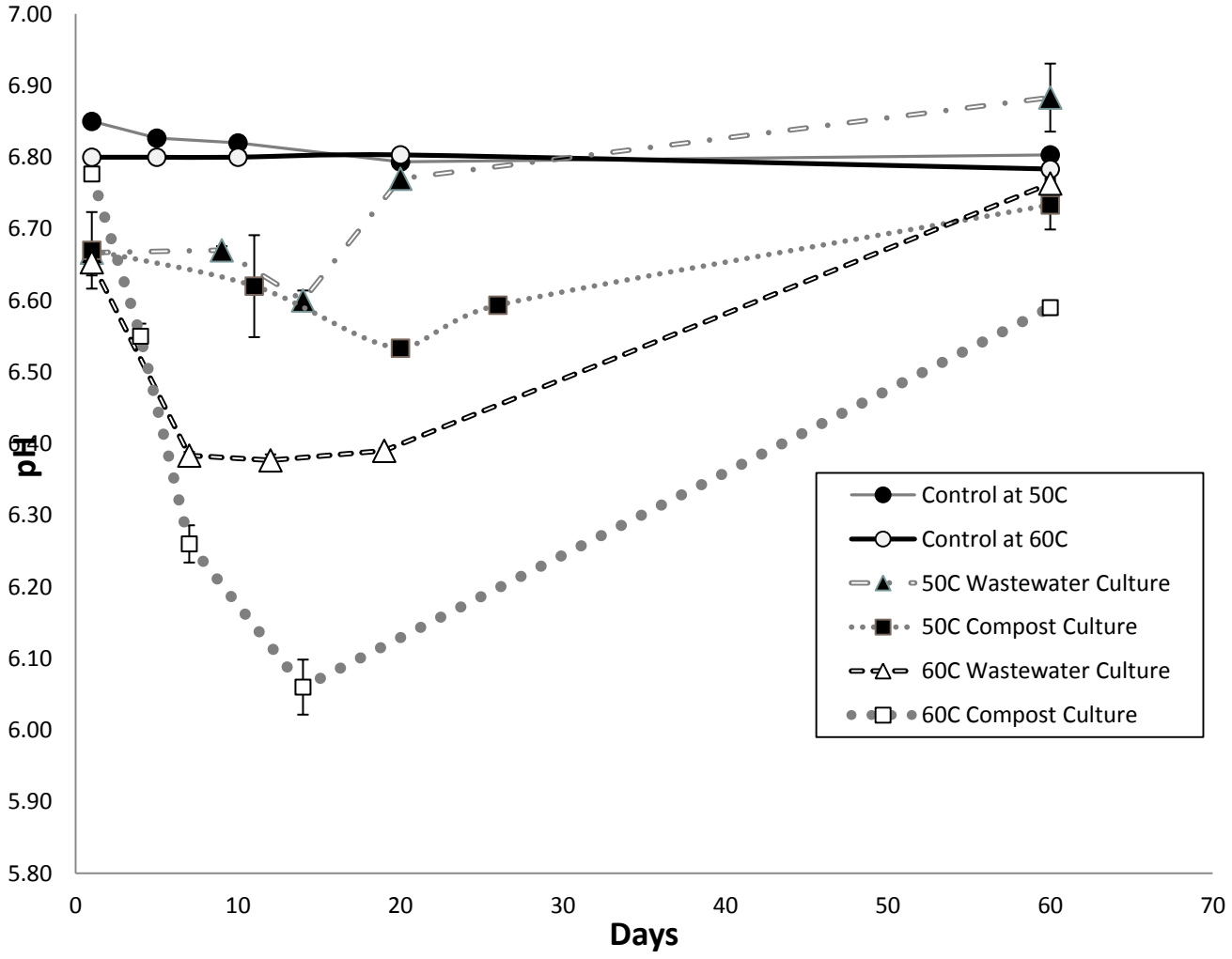

Figure 3.6 The pH values among the four time points of noticeable culture activity in all four cultures.

The wastewater culture incubated at 50ºCstarted with a pH of 6.67at Day 1 and dropped to a pH of 6.60 at Day 14, when the filter paper was completely degraded. After a significant amount of time, the pH rose to 6.88. In the wastewater culture at 60ºC, the pH began on Day 1 at 6.65 and fell to 6.39 when filter paper has degraded. In compost, the 50ºC culture 6.67 and fell to 6.53 when the filter paper has been noticeably degraded. The 60ºC culture began at 6.76 and fell to 6.06 by Day 14 when the filter paper was degraded. After this drop in pH, it rose to 6.59 when the culture was left until Day 60 of the experiment. Similarly to this culture, at Day 60, the other three cultures changed in pH values that were above their starting points. The wastewater 50ºC culture for example began with a pH of 6.67 and at Day 60 it was 6.88.

3.3.5 Qualitative Gas Analysis of the 60ºC Cultures

As depicted in Table 3.1, the cultures that were incubated at 50ºC did not produce evidence of gas. Both 60ºC cultures, during their period of filter paper degrading activity, produced visible signs of gas when the cultures were handled. A qualitative analysis was conducted to determine the gases produced by the cultures.

3.3.5.1 The 60ºC Compost Culture

In the compost culture, both CO_2 and CH_4 were produced. Hydrogen gas was not detected. As seen in Figure 3.7, CH_4 was detected at time 0.72 at a percentage of 55%. CO_2 was also detected at 1.1 minutes at a percentage of 50%. No other gas peaks were visible on the chromatograph, signifying no other detectable gases.

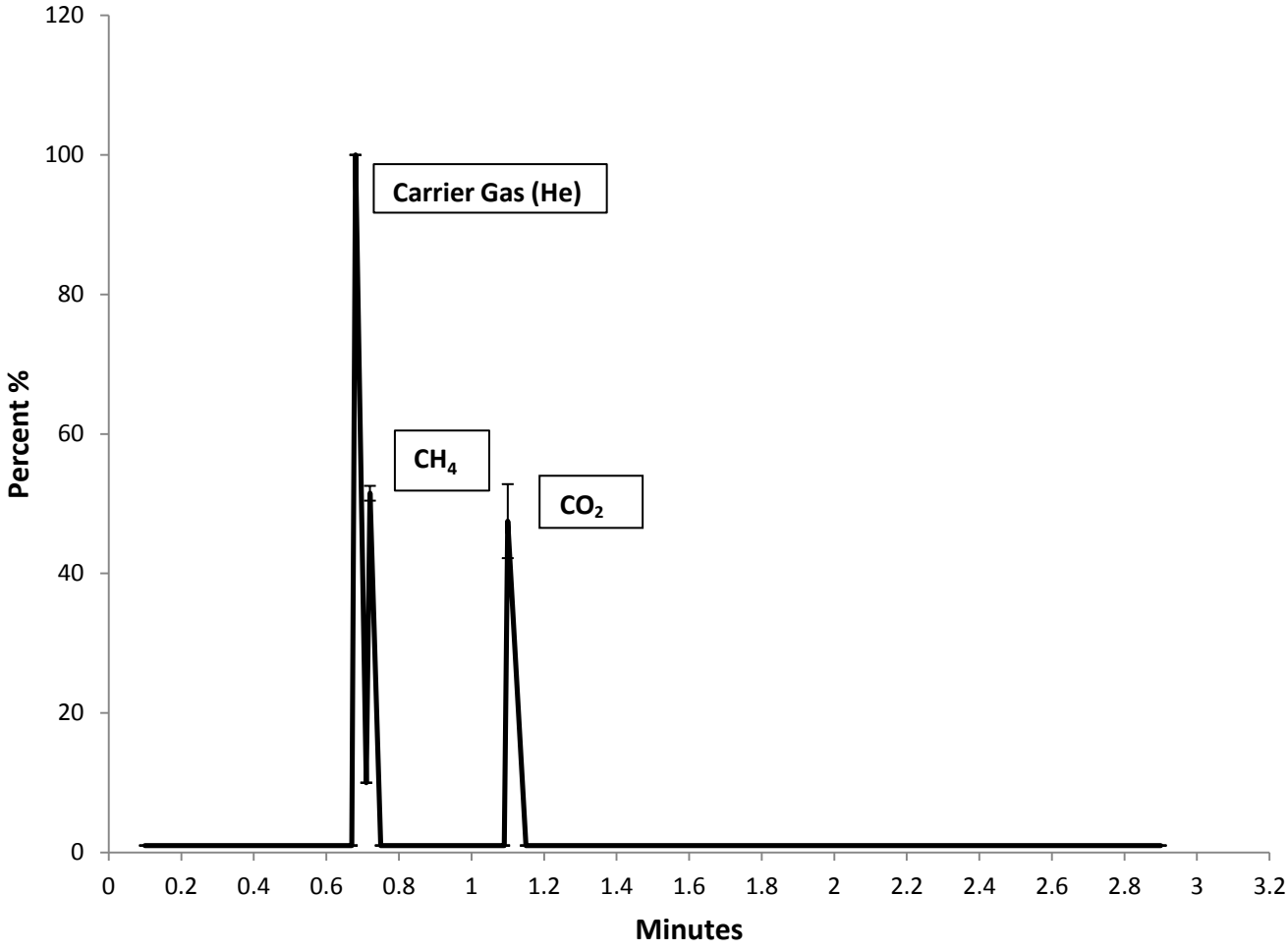

Figure 3.7 The qualitative gas analysis of the compost culture at 60ºC, Methane gas is detected at time 0.72 and CO_2 is detected at 1.07. On average the amount of gas that was detected was 55% methane and 50% CO_2.

3.3.5.2 The 60ºC Wastewater Culture

The gases detected in the wastewater culture incubated at 60ºC were CO_2 which was detected at a time of 1.07. No other gases, outside of the carrier gas were detected. The chromatograph is presented in Figure 3.8 which shows the CO_2 detected at 45%.

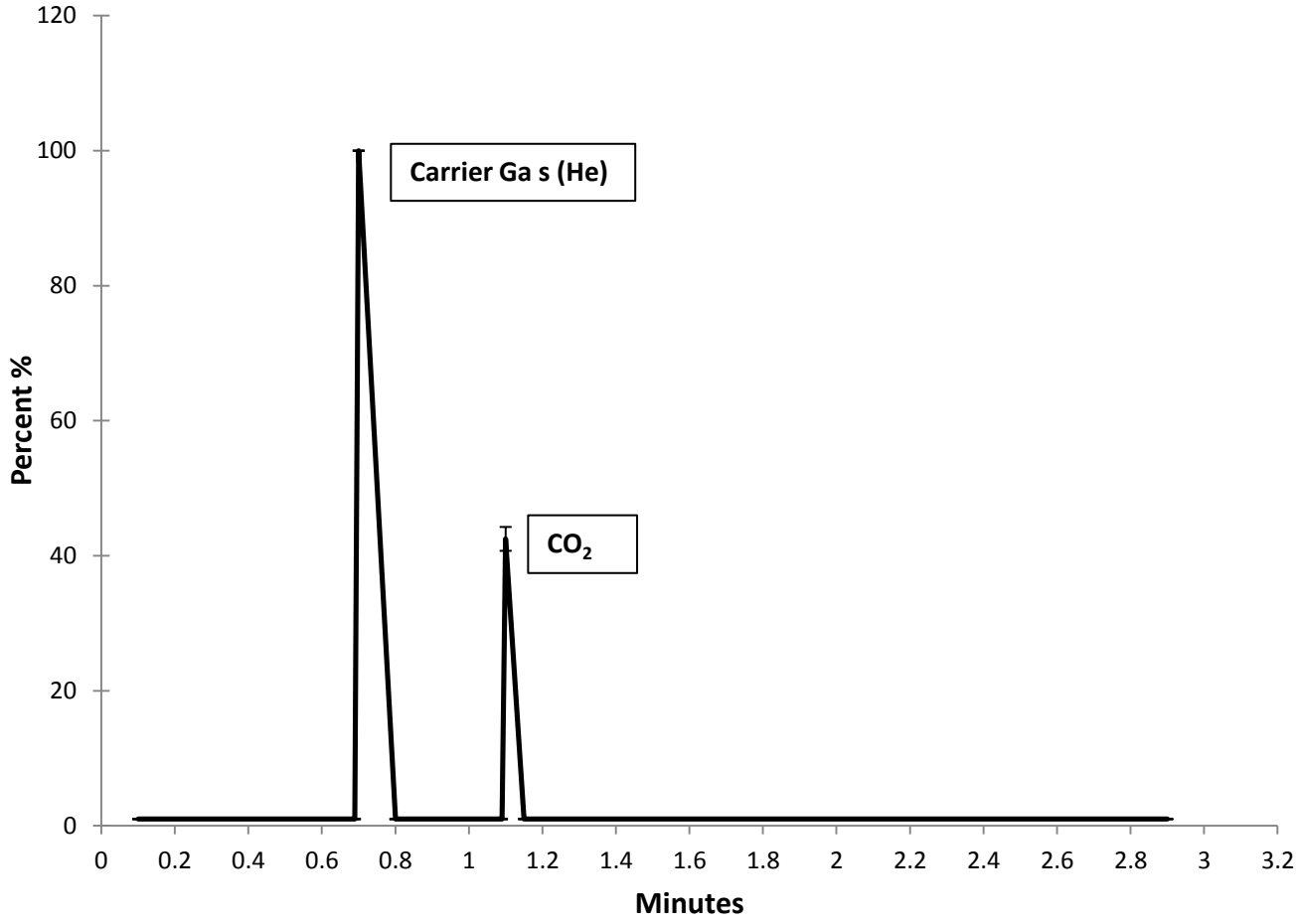

Figure 3.8 The qualitative gas analysis of the wastewater culture at 60ºC. Methane gas is not detected; however, CO_2 is detected at 1.07.

3.4 DISCUSSION

With the prediction that energy demand will double by the year 2050, renewable technologies that are carbon-neutral and have the potential to suppress rising greenhouse gases are needed (Carere *et al.,* 2008). The conversion of cellulosic materials to valuable end-products such as ethanol, butyrate, or hydrogen gas is one of the most promising alternative means of using renewable resources to displace fossil fuels. In order for consolidated bioprocessing to meet large scale demand and be competitive with petrol-based fuels it must first fit its design to be cost efficient. A key missing ingredient in the process is a microbial population, consisting of an assemblage of the same microorganisms, or a community, consisting of an assemblage of different populations, that produce significant amounts of desired end products such as ethanol when it degrades complex materials such as raw cellulosic materials (Prescott *et al.,* 2005). Ideally, this community must have the ability to do so in one step without the addition of cellulases which are expensive to produce and sustain. The medium must also provide stability for the microorganisms and include ingredients that do not further hinder the costs of the process (Lynd *et al.,* 2002).

3.4.1 Culture Characteristics

In this study two inoculum sources of compost and wastewater were used with a chemically defined medium to enrich diverse cellulolytic communities that produce valuable end-products. The use of two incubation temperatures (50ºC and 60ºC) resulted in four cultures that vary significantly in the end-products that were formed after filter paper degradation. All cultures degraded filter paper over different periods of time ranging from 7 to 20 days. Filter paper in the 50ºC cultures was observed to degrade over a longer time period (14 to 20 days)

than the 60ºC cultures (7 to 12 days) in both compost and wastewater-derived cultures. This was significantly observed in the compost-derived cultures which at 50ºC broke down the filter paper completely by d20 and at 60ºC broke it down by d7. This can be explained by the fact that all microorganisms react differently when subjected to different environmental changes such as temperature. These environmental changes may favor the growth of specific microorganisms over others. Thus the microbial communities that adapted to these conditions are predicted to have developed cellulose utilization strategies that differ from each other in their end-product formation as well as cellulolytic and non cellulolytic microbial interactions (Lynd *et al.*, 2002). This is yet to be determined by applied molecular techniques such as PCR-DGGE which will show a profile of bacterial community members over the selected time points of end-product analysis.

A similarity in all four cultures was the colour of the medium throughout filter paper degradation which was indicated by the redox indicator resazurin. At d1 of inoculation the cultures were pink in color, indicating aerobiosis, but during and after filter paper degradation the color was beige, indicating anaerobiosis. It can be predicted that aerobic microorganisms consume the oxygen present in the medium and generate anoxic conditions in which anaerobic bacteria hydrolyze filter paper (Ronan, 2011). Compared to other studies, this finding is novel because anoxic growth conditions were not used to grow and maintain the cultures. The possibility of all four cultures to exist in oxic conditions indicates their potential ability to degrade more complex materials such as lignin, which is degraded by aerobic microorganisms within the environment (Ahmad *et al.*, 2010). This is favorable to larger scale biofuel processes which can potentially reduce the energy and cost expensive pretreatment steps that separate lignin and hemicelluloses from pure cellulose.

As seen in Figure 3.1, the filter paper appeared to be degraded differently by the cellulolytic consortia. In the compost-derived culture at 50ºC, holes on the filter paper are easily seen while in the wastewater-derived culture at the same temperature, the filter paper turned into a pulp-like material. In an article about mesophilic cellulolytic microorganisms, the authors described each new isolate by the morphology of the filter paper when it was degraded. The aerobic *Pseudomonas erythra*, for example, created brown spots on the filter paper, followed by the development of a red hue (Fuller and Norman, 1943). While observations like these are seldomly reported in current literature, recently Wang *et al*. (2011), described characteristics of filter paper degradation and biofilm formation with the use of the Syto 9 stain and confocal laser scanning microscopy. The cellulosome-producing *Clostridium thermocellum* created crater-like depressions on cellulose which were produced from the attachment of the microorganism to the substrate surface (Wang *et al.*, 2011). Although in the current study the characteristics seen on the filter paper at the onset of degradation cannot infer about the type of microorganism that is actively degrading it; the differences in its appearance suggest that different microorganisms are present within each culture.

3.4.2 Compost-Derived Culture at 50ºC

The compost-derived culture at 50ºC produced a noticeably large amount of butyrate when compared to the other end-products (Figure 3.2). As butyrate production increased from d1 to d10, lactate production decreased suggesting that the culture utilized lactate for butyrate production. In the metabolic pathway for butyrate formation, lactate is converted back to pyruvate which is converted to acetyl-CoA (Zhu, 2003). The butyrate formation and acetate formation branches both emerge from acetyl-CoA. The butyrate formation branch begins with

the conversion of acetyl-CoA to butyryl-CoA and eventually butyrate while the acetate formation branch begins with the acetyl-CoA conversion to acetyl-P and later acetate (Zigova and Sturdik, 2000). Thus, in theory, with the production of butyrate there will always be a production of acetate. Although the fermentation pathway of acetate is more favorable because it produces more ATP (4 mols), the production of butyrate is required by cellular metabolism to maintain glycolysis under anaerobic conditions (Zhu, 2003). This is the prediction for this culture.

The most studied anaerobic microorganisms that produce butyrate belong to the genera *Clostridium, Butyribacterium,* and *Butyrivibrio (*Shen *et al.,* 1996). *Clostridium* sp. utilize cellulose, starch, and lactose as carbon sources, *Butyribacterium* sp. utilize hexose, lactate, and pyruvate while *Butyrivibrio* sp. utilize glucose (Zigova and Sturdik, 2000). A *Clostridium* species, *C. thermobutyricum,* which was cultured in an immobilized cell reactor at optimal temperature of 55ºC, has been found to have an acetate: butyrate ratio of 1:1.6. The carbon source used were glucose and yeast extract was also used in the medium (Li *et al.,* 2011). Studies using mutants of the same bacterium resistant to glycerol have also shown acetate: butyrate ratios ranging from 0.6 to 1.1 (Shen *et al.,* 1996). In the compost-derived culture incubated at 50ºC, the acetate: butyrate ratio was calculated at 1:3.9 at the highest butyrate production (Figure 3.2).

The acetate:ethanol ratio for this culture was 1:1 based on the highest point of ethanol produced which occurred at d11 (Figure 3.2). Of interest to industry would be the ethanol concentration produced between d1 and d11 since it was between this time that an optimum level of ethanol may have bee recorded. A further analysis of end-products produced could be done with stricter time points.

3.4.3 Compost-Derived Culture at 60ºC

The butyrate and acetate concentrations, when comparing the compost-derived cultures at 50 ºC and 60ºC are significantly different. In the HPLC results for the 60ºC culture, the acetate: butyrate ratio is 1:0.2 (Figure 3.3) compared to the 50ºC culture (1:3.9). This can be explained by the difference in the observed time of the degradation of filter paper between the two cultures. It was found that in cultures of high metabolism, cells have a higher energetic demand and need more ATP; thus, more acetate is produced. For slower growing and metabolically active communities, butyrate production is more dominant (Zigova and Sturdik, 2000).

The acetate: ethanol ratio in the compost-derived 60ºC culture was 1:0.52. The highest concentration of ethanol was measured at d7 of the study. When compared to a batch culture of *C. thermocellum*, this ratio is slightly lower, since the bacterium alone yields an acetate:ethanol ratio of 1:0.43 (Lynd *et al.,* 1989). An analysis of stricter time points may allow for a greater ethanol yield to be monitored. In Figure 3.3, it can also be seen that acetate production rose continuously from d 1 to d 14. Lactate also rose in concentration but began to decline after d7, after visible filter paper degradation. The production of lactate from cellulose signifies the need for producing NAD^+ to maintain cellulolysis; however, as the amount of filter paper visibly decreased through degradation, the lactate within the culture decreased. The rise in acetate suggests that it is produced from lactate (via pyruvate) and the presence of CO_2 and methane as seen in Figure 3.7 is a further indicator that hydrogen may have been produced but was quickly utilized by methanogens to be converted to methane within the culture (Soubes *et al.,* 1988).

3.4.4 Wastewater-Derived Cultures

The wastewater-derived culture at 50ºC demonstrated a decline in lactate production from d1 to d20 and the butyrate concentration detected remained constant throughout filter paper degradation. The only rises in concentrations within this culture were that of acetate and ethanol. Both increased significantly from d 9 to d 14 from 7.2 mM to 21.6 mM and 1.0 mM to 4.9 mM, and then fell by d20 to 13.3 mM and 2.0mM, for acetate and ethanol, respectively. In the cellulolytic pathway, this suggests that the acetate degradation was favored for ATP production while ethanol was produced as a byproduct of the pathway. The acetate: ethanol ratio was 1:0.23 with the pH at 6.6.

The acetate:ethanol ratio of the wastewater-derived culture incubated at 60ºC was the lowest of all four cultures (1:4.6). From d1 to d7 of culture activity, acetate, ethanol and lactate concentrations increased. There was more lactate produced than acetate or ethanol at this time suggesting the culture favored the conversion of pyruvate to lactate for NAD^+ production. After d7 to d12, both lactate and acetate concentrations decreased while ethanol continued to increase. This suggests that lactate and acetate were being utilized by the culture; the lactate to produce ethanol and the acetate to produce CO_2 (Figure 2.2). Kato *et al. (*2005) found that non cellulolytic microorganisms, a *Pseudoxanthomona* sp. and a *Brevibacillus* sp., presented a similar relationship of ethanol and CO_2 production from lactate and acetate. In the wastewater-derived 60ºC culture, the ethanol concentrations decreased while lactate concentration increased once more after observed filter paper degradation. The pH was 6.65 at d1 and remained at pH 6.4 from d7 to d 12.

3.4.5 pH in cultures derived from compost and wastewater

The pH, in addition to temperature and the redox potential, is an important factor that affects the rate of cellulolysis and extent of cellulose utilization by a microorganism (Lynd *et al.*, 2002). Most bacteria degrade cellulose at a narrow range of pH (~pH 6.0 to 7.0). It has been found that cellulose hydrolysis is inhibited by a pH below 6.0 except of those in the rumen of animals capable of degrading cellulose (Russell *et al*., 2008). Values above 7.0 have also been found with an increased production of acetate (Kato *et al.*, 2005). In Figure 3.6, all four cultures began with a pH in the range of 6.6-6.8 and dropped as filter paper degradation was observed. After filter paper disappearance in the cultures, the pH rose and either lactate or acetate concentrations increased. This is true for both compost-derived and wastewater-derived cultures at 60ºC which appear to have a faster growth rate than the 50ºC cultures. After a significant amount of time after filter paper degradation (d60) the pH rose passing 6.7 in the 50ºC cultures as well.

3.5 CONCLUSIONS

In this study, four cellulolytic cultures were obtained from two inoculum sources and grown aerobically in a chemically defined medium. Two different inoculum temperatures (50ºC and 60ºC) were used which promoted varying quantities of end-products produced within the cultures. Thermophilic conditions as well as the use of a chemically defined medium are favorable characteristics for large scale biofuel production for energy conservation and maintenance costs. The ability of the cultures to grow under oxic conditions, which change to anoxic as cellulolysis began suggests that raw lignocellulosic materials may potentially be degraded by microbial members within the cultures.

The compost-derived culture incubated at 50ºC produced a low acetate: butyrate ratio at 1:3.9 which has not been previously observed from mixed cellulolytic cultures. The wastewater-derived culture at 60ºC produced ethanol with an acetate:ethanol ratio of 1:4.6. The detection of methane in the compost-derived culture at 60ºC suggests that there may have been H_2 produced before it was quickly consumed by possible methanogens within the culture. Further continuation of this study can include using stricter time points allowing for the optimal detection of end-products. Knowledge of the end-product cycle that occurs by the mixed cultures would be favorable to industry for controling the conditions required to obtain maximum yields. Overall, these findings show that mixed microbial consortia have favorable application to industry for biofuel production. Identifying the key members active in these cultures is the next step that would further improve the application of mixed cultures in large scale processes. Knowledge of the interactions between identified bacterial species could improve industrial maintenance and control of the hydrolysis and fermentation reactions that occur within the cultures.

CHAPTER FOUR: MOLECULAR ANALYSES OF SELECTED CULTURES

4.1 INTRODUCTION

Extracting DNA from samples derived from cellulosic environments can be of benefit when developing community profiles for mixed cellulolytic cultures that produce favorable end-products. However, the application of DNA-based techniques, such as PCR amplification of the 16S rRNA gene, depend on the efficacy of nucleic acid extraction and further purification methods (Dineen *et al.*, 2010). In this study, obtaining PCR product was shown to be difficult for samples that have been enriched from cellulosic environments and obtaining low concentrations of PCR product further deterred the application of molecular fingerprinting techniques such as DGGE.

Cellulosic environments include soil, compost and wastewater sludge which are complex in chemical and nutrient composition. The hydrolysis of cellulose also produces a variety of end-products that can add onto the already complex composition of humic acids. These substances can be originally transferred from the environment when developing enrichments of mixed cellulolytic cultures. It has been reported that humic substances and other soil components such as iron, polysaccharides and urea inhibit the very sensitive procedure of PCR (Lakay *et al.*, 2006). It is believed that humic acids are difficult to remove from soil DNA because of their similar molecular structure and similar solubility properties: both are long chain molecules and carry a negative charge (Lakay *et al.*, 2006, Dong *et al.*, 2006). It has been found that the inhibiting substances bind to the restriction endonucleases and *Taq* polymerase, both essential components for PCR. Furthermore, primer annealing occurs reducing the sensitivity of DNA

detection (Sutlovic *et al.,* 2005). In a recent study Wang and Fuji (2011) found that as little as 5 ng/ µL of humic acids inhibited enzyme activity significantly.

The step that must be improved revolves around the extraction of pure DNA. Methods of purifying DNA include fervent washes with buffers differing in EDTA concentration, the use of hydroxyapatite columns, or cesium chloride-ethidium bromide centrifugations; however, they are time consuming, involve working with highly toxic chemicals and have not been found to be consistent in obtaining purity (Watson and Blackwell, 2000; Li *et al,* 2010). Dilutions of the samples may also be created, which can attenuate the contaminants within the sample but reduce the detection limits of DNA (Tsai and Olson, 1992).

A common method for concentrating and purifying DNA is precipitation with absolute ethanol. This method requires the addition of sodium acetate buffer to equalize ion concentrations, the addition of cold 100% ethanol for a pre-determined amount of time which precipitates the DNA from the rest of the solution, and centrifugation which separates the DNA from the solution. This procedure has been tested to work in the past- significantly for samples with low amounts of DNA and inhibiting substances which may be found in environmentally-derived samples (Fregel *et al.,* 2010).

The intention of this investigation was to provide conditions in which DNA could be precipitated from complex environmental samples that contain impurities preventing PCR amplification. The amplified products could eventually be used in community profiling by DGGE or other DNA-based approaches such as qPCR. The samples used for DNA extraction and purification were collected from mixed cellulolytic cultures which were derived from

wastewater and compost inocula in this study. Samples were purified by the optimized DNA precipitation protocol and DGGE was performed.

4.2 MATERIALS AND METHODS

4.2.1 Extracting DNA

DNA was extracted from two cellulose-degrading cultures that were enriched at 60ºC from compost and wastewater inocula, respectively. Both cultures contained approximately 0.1% of their original inoculum sources. A 1mL sample of DNA from each culture was taken at the visible point of filter paper degradation. Samples were washed twice with 1 mL PBS and DNA was extracted from the sample using the ZR Soil Microbe DNA MiniPrep™ Kit (Dedarlane, Burlington, ON). The procedure was slightly modified by repeating the filtering step (removing humic acids) twice. The extracted DNA was stored at -20°C until further use.

4.2.2 Optimized Ethanol DNA Precipitation

The extracted DNA was subjected to ethanol precipitation which required the preparation of sodium acetate (3M, pH 5.2). The volume of the DNA sample was measured and 1/10 volume of sodium acetate was added and mixed. Two to 2.5 volumes of cold 100% ethanol (calculated after salt addition) were added, mixed, and stored on ice or at -20ºC for 20-25 minutes. The optimized method extends the cold storage to -20 ºC overnight and -80ºC for 2 hours. The sample was spun at maximum speed in a centrifuge for 15 minutes. The optimized method extends the time of centrifugation to 15 minutes and 30 minutes and tests for centrifuge temperatures of 4ºC and 20ºC. The supernatant was carefully decanted. Modifications to the standard protocol include not adding 70% ethanol to the pellet, which is the next step. This step was omitted not to lose any more of the already low quantities of DNA in the sample. The pellet

was dried at room temperature and resuspended in TE buffer or water before it was used for PCR.

4.2.3 Polymerase Chain Reaction

The PCR reaction mix was used to amplify a 417 bp fragment of the bacteria-specific 16S rRNA gene. It contained 2uL of sample DNA, 25pmol of the forward and reverse primers, 200uM of each dNTP (New England BioLabs, Pickering, ON), 6.875ug BSA (New England BioLabs, Pickering, ON), 2.5 units of *Taq* DNA polymerase in 1x *Taq* buffer (New England BioLabs, Pickering, ON), and sterile ddH_2O. The forward primer used was U341F-GC (5`-CCTACGGGAGGCAGCAG-3`) with a GC clamp affixed to the 5`end and the reverse primer used was U758R (5`-CTACCAGGGTATCTAATCC-3`) (Muyzer *et al.*. 1993). Both primers were synthesized by the SickKids Centre for Applied Genomic (TCAG) synthesis facility in Toronto, ON. The PCR cycle used began with a five minute initialization step at 96°C, followed by 20 cycles of denaturation at 94°C for one minute, annealing at 65°C for one minute, and elongation at 72°C for three minutes. For each of the first 10 cycles, the annealing temperature decreased by 1°C and then remained at 55°C for the remaining cycles. After this, the samples were kept at 4°C until ready for use (Yeung *et al.*, 2010).

4.2.4 Quantification of PCR Product

To quantify the extracted PCR product, 1, 2, 5 and 7 uL of the products were run on a 0.8, 1 and 2% agarose gel, visualized under the BioDoc-It™ Imaging System (UVP, Upland, CA). The resulting bands were quantified by the ChemiImager 4400 ™ software (Alpha Innotech, Santa Clara, CA), with the use of a standard curve from a serial dilution of the 100 bp

DNA ladder (MBI Fermentas, Amherst, NY) (Ronan, 2011). The amount of DNA in the PCR product was quantified using a Nanophotomoter (Pearl , MBI, Montreal, QC).

4.2.5 DGGE

Before running a DGGE gel, the PCR products were cleaned using an illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Piscataway, NJ). To obtain significant amounts of DNA for DGGE, the extracted DNA of a particular sample was amplified a minimum of five times and the total volume of each PCR product was run in one well on a 1% agarose gel. The bands that resulted were visualized under the Safe Imager 2.0 Blue-Light Transilluminator (Invitrogen, Grand Island, NY) and excised using a sterile knife and forceps. The three amplified replicates of each sample were combined into one 1.5 mL centrifuge tube. The DNA within the agarose bands was extracted with the use of the Gel/PCR DNA Fragments Extraction Kit (IBI Scientific, Peosta, IA).

An amount of 500 ng of the amplified DNA was run on an 8% polyacrylamide DGGE gel with a denaturing gradient of 30-70% (where 7 M urea and 40% deionized formamide was considered 100% denaturant). The gradient was cast with a gradient former (BioRad Laboratories, Mississauga, ON). The gel was run at 80V for 16 hours at 60°C. The bands were visualized by staining the gel for 30 minutes with 0.01% SYBR Gold (Invitrogen, Burlington, ON), excised from the gel, and DNA was eluted in 25uL of sterile ddH_2O at 4°C for 5 days (Berthiaume, 2011; Ronan, 2011).

The eluted DNA was reamplified by the TD20 PCR cycle using the same primers. Sequencing was performed by the SickKids Centre for Sequencing (Toronto, ON). A consensus sequence between the forward and reverse primers was constructed using BioEdit Biological

Sequence Alignment Editor and the NCBI BLAST algorithm was used to check the consensus sequence against a 16SrRNA sequence database (Berthiaume, 2011).

4.3 RESULTS

4.3.1 DNA Extraction Optimization

Two samples from cellulose-degrading cultures were chosen for this analysis a stable 60ºC compost-derived culture (DNA concentration of 11.8 ng/µL) and a stable 60ºC wastewater-derived culture (DNA concentration of 9.75 ng/µL). When 1, 2, 5 and 7 µL of the DNA samples were run on a 1% agarose gel, the resulting bands were very faint and lightly smeared (data not shown). Upon optimization of DNA volumes, storage times and temperatures, as well as centrifuge times and temperatures, a noticeable difference in the brightness of bands was observed. For both samples, using a starting volume of 20 µL, a storage temperature (temperature required for DNA precipitation) of -80ºC for 2 hours followed by a centrifuge temperature of 4ºC for 30 minutes at 15,000 rpm yielded the brightest bands. The complete observations are tabulated in Table 4.1.

Table 4. 1. A comparison of DNA precipitation storage and centrifuge temperatures and times to optimize the PCR reaction for samples from cellulolytic cultures

Item	Volume (uL)	Storage (°C)	Storage Time*	Centrifuge Temperature	Centrifuge Time (min)	PCR band appearance
1	50	-80	2hrs	4	15	None
2	50	-20	O/N**	4	15	None
3	20	-80	2hrs	4	15	Faint
4	20	-20	O/N	4	15	Faint
5	20	4	O/N	4	30	Faint
6	20	4	O/N	20	30	Faint
7	20	-20	O/N	20	30	Faint
8	**20**	**-80**	**2hrs**	**4**	**30**	**Good**

*Temperature and time required for DNA precipitation **O/N signifies over night

4.3.2 Electrophoresis of PCR-amplified DNA

Optimization of changes to the original protocol included the length of storage time for DNA precipitation, and centrifuge temperature and duration. Once the DNA was amplified by PCR, a 1% agarose gel was run containing the PCR products; however, a 0.8% agarose gel displayed the bands more clearly. Figure 4.1 shows a 0.8% agarose gel with lanes 2 and 3 representing the optimized conditions that were highlighted in Table 4.1.

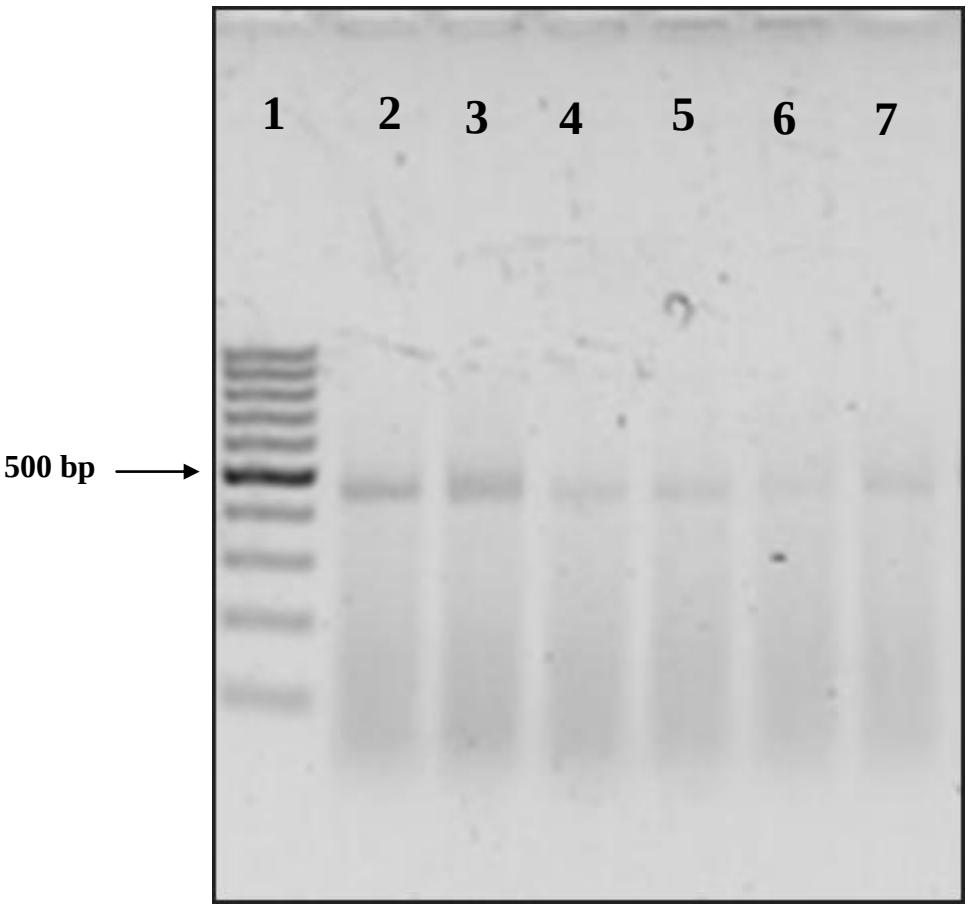

Figure 4.1. A comparison of the compost (C) and wastewater (W) samples (5 µL/lane) under different optimized conditions. Lane 1 contains a molecular ladder with the 500bp mark shown, lane 2 contains C under -80ºC storage and 4ºC centrifugation, lane 3 contains W under the same conditions. Lane 4 contains C under 4ºC and 4ºC, lane 5 contains W under -20ºC O/N and 20ºC. Lane 6 contains W under 4ºC and 4ºC. Lastly, lane 7 contains -20ºC O/N and 20ºC centrifugation. The size of the expected PCR product was 417 bp.

4.3.3 DGGE

Once a sufficient amount of pooled PCR product (500ng) was obtained, the amplified DNA was subjected to a DNA cleanup procedure as described in section 4.3.5. The results are shown in Figure 4.2 for the compost culture and in Figure 4.3 for the wastewater culture.

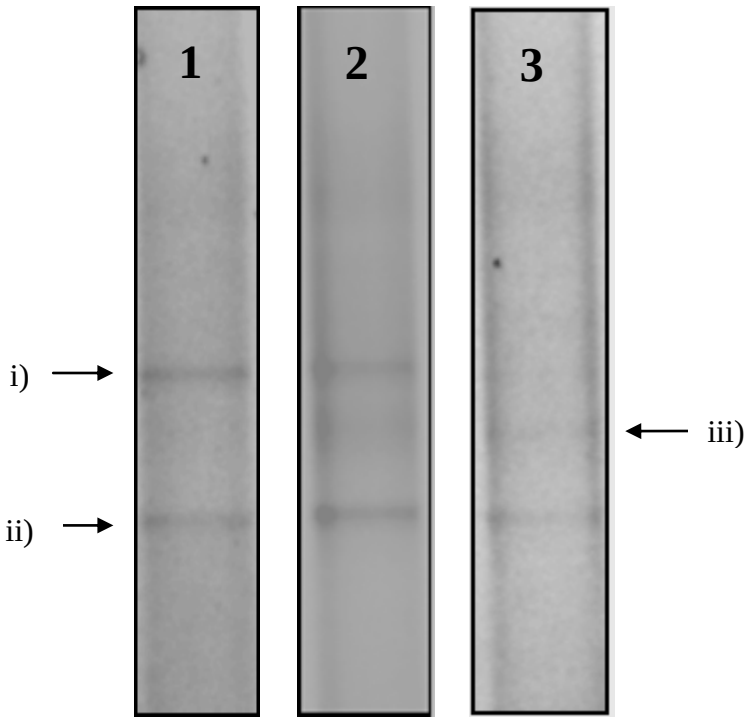

Figure 4.2. DGGE gel lanes containing samples from the compost-derived culture at 60ºC. Lane 1 shows the compost culture profile at d 1 after inoculation, lane 2 shows the profile of the same culture at d 4 at the onset of degradation and lane 3 shows the same culture profile on d 7, where filter paper was visibly degraded. Numerals i through iii denote DNA bands which were excised and prepared for DNA sequencing.

The bands that resulted in the DGGE polyacrylamide gel from samples taken in the compost culture at d1, d4, and d7 are similar. At d 1 and d 4 (lanes 1 and 2) two dominant bands (i and ii) are visible. On d4 (lane 2) a faint third band (iii) can be observed which is more obvious on d 7 (lane 3).

In Figure 4.3 the wastewater culture at d1, d7, and d12 of cellulose degradation are shown in lanes 1, 2, and 3, respectively. In lane 1, two bands (i and ii) are visible which are also seen in lane 2. In lane 3, bands i and ii are fainter; however, there is a band visible near the top of the gel (iii) and another band appearing between bands i and ii (band iv).

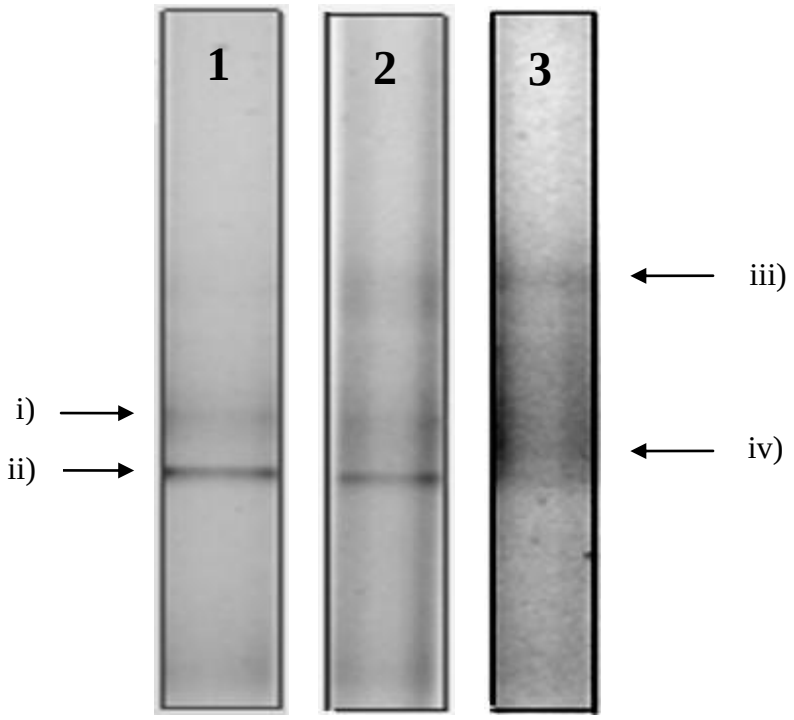

Figure 4.3. DGGE gel lanes containing samples from the wastewater-derived culture at 60ºC. Lane 1 shows the culture at d 1 after inoculation, lane 2 is the same culture at d 7 at the onset of degradation and lane 3 contains DNA from d12 where filter paper was visibly degraded. Numerals i through iv denote DNA bands which were excised and prepared for DNA sequencing.

For both compost and wastewater cultures the later time points show less defined bands and smearing. This was seen for all time points after filter paper degradation (not shown). Preparation of DNA extracted from the bands for subsequent sequencing analysis is in progress.

4.4 DISCUSSION

Two samples from cellulose-degrading cultures derived from different inoculum sources were chosen for this analysis because they are predicted to contain diverse cellulolytic communities. When the DNA from the samples was extracted conventionally and the PCR product was quantified, no bands appeared near the 500 bp molecular marker which would signify the amplification of the 417 bp 16SrRNA gene. The same was true for when PCR product was pooled and quantified by electrophoresis. The samples used had a DNA concentrations of 11.8 ng/µL and 9.75 ng/µL for the compost and wastewater-derived samples respectively which were determined by the nanophotometer.

The conditions of DNA precipitation that allowed for the appearance of bands was that of using 20 µL of sample and storing at -80ºC for 2 hours. Once this has occurred the centrifuge temperature was preset at 4ºC and spun for 30 minutes, not the recommended 15 minutes. In an article by Zeugin and Hartley (1985), the conditions of DNA precipitation were also tested in examining the effects of incubation temperature and centrifuge times. The DNA was that of herring sperm and concentrations of 2.4 ng/mL, 40 ng/mL, and 400 ng/mL were subjected to incubation temperatures of 0ºC, -20ºC, and -70ºC. The investigation found that temperature played a minor role in precipitation but that increasing the centrifugation time after incubation resulted in significant precipitation amounts (Zeugin and Hartley, 1985). In this investigation, it was found that the incubating temperature of -80ºC as well as the centrifugation time and temperature had an effect on DNA precipitation. After incubation at such a low temperature, the transition to a cooled centrifuge set at 30 minutes of centrifugation resulted in clearer bands than with any other conditions (Figure 4.1).

In addition to the precipitation conditions, when the percent of agarose was changed and the samples were run, the bands were clearer. Using different concentrations of agarose in the gel affects the migration of DNA within the gel. Higher percentages slow the rate that the DNA moves from the negative electrode towards the positive electrode. This is due to the gel providing greater frictional drag in larger molecules. In addition, the pores of the gel are decreased and as a result the movement of the larger moleculaes through the gel is impeded (Duncan, 1993).

Preparing samples for DGGE included combining the PCR product and cleaning it to remove impurities that result around the band on an agarose gel in the form of smearing. The cleaner the product, the more defined the bands will appear on the DGGE polyacrylamide gel. To retain more PCR product, the total volume of the PCR products (rather than 5 *u*L of sample in one lane) was run by electrophoresis on an agarose gel. It was seen that cutting out the single bands within the agarose gel and extracting the DNA from within prevented the inclusion of the surrounding impurities and resulted in little product lost. The amount of DNA in the sample was then confirmed nanophotometrically and prepared for DGGE.

The DGGE gel that was run (Figures 4.2 and 4.3) contained PCR products from both compost and wastewater samples at different time points of filter paper degradation. The samples were obtained from the applied DNA precipitation methods. The DGGE method involves the use of a polyacrylamide gel which contains chemical denaturants of increasingly higher concentrations. As the PCR product migrates through a polyacrylamide gel it begins to denature when it reaches a threshold denaturant concentration. This results in a pattern of bands, each in theory representing a different bacterial population (Muyzer and Smala, 1996).

As can be seen in Figure 4.2, several bands are visible throughout the lanes found from the compost-derived culture. Two bands (i and ii) are present at d 1 and d 4 of observed cellulose degradation. A third band (iii) appeared when degradation was complete and the first band (i) is faint. In Figure 4.3, representing samples from a wastewater-derived culture, two bands are visible. The same bands are also visible through the second time point of the study at d 7. A third and fourth band appeared in the last time point. Both were not as bright in the other lanes. In the DGGE samples that contain bands that were fainter in some time points over those in the other lanes were seen for both compost and wastewater cultures at later time points (e.g. d 1 and d7 in Figure 4.2, and d1 and d12 in Figure 4.3).

Since the samples represent different time points of filter paper degradation activity it is possible that the abundance of end-products produced, interfered with the extraction of purified DNA or those certain bacterial populations declined in the accumulation of products formed within the culture. For example, an excess of acetate production in a cellulolytic community inhibited cellulose degradation by *Clostridium straminisolvens* (Kato *et al.,* 2005). In addition, limitations of the PCR methods such as improper primer binding or formation of chimeric molecules can alter the result on a DGGE gel by overestimating the number of bands produced (Muyzer and Smalla, 1998).

Both samples used in this study were derived from environmental sources. In one gram of soil there can be found 10^9 cells with a minimum of 4000-7000 different bacterial genomes (Ranjard *et al.,* 2000). The profiles that were obtained in this study by DGGE do not show more than three bands. This is a limitation of DGGE, that it does not always illustrate the considerable diversity within the extracted DNA sample. In a study by Inceoglu *et al.* (2010) , different DNA

extraction kits were applied to one sample of soil. Each extraction kit revealed a different molecular profile on the DGGE gel. There were some bands that appeared in the same positions; however, majority of bands were method specific. All methods showed from 20-45 bands on the DGGE gel (Inceoglu *et al.,* 2010). Past studies all found that despite sequence variation within methane-oxidizing bacteria, their 16SrRNA fragments could not be resolved by DGGE. The use of different regions of the 16SrRNA gene may also result in different resolution or separation (Muyzer and Smalla, 1998).

In addition to this limitation is the possibility of some DNA sequences migrating to the same position on the gel due to their similarities in sequences. This makes it difficult to observe and excise bands for further amplification and sequencing. It must also be noted that DGGE is not a quantitative method. Conjectures made about species abundance based on band intensity are of speculative nature due to the above limitations and the belief that only microorganisms present in a culture above 1% can potentially be identified by culture-independent methods (Sekiguchi *et al*. 2001; Li *et al.,* 2011). Although culture-independent methods were chosen for this molecular analysis, culture-dependent methods of isolation should not be ignored and further considered for the complete characterization of microbial individuals within the cultures.

4.5 CONCLUSION

It is known by microbiologists that only a small fraction of bacteria in the world have been isolated and characterized. The application of PCR, DGGE and culture-based isolation techniques can be used together to fingerprint different community members within a microbial community. Further sequencing of the samples derived from these isolation techniques would then allow for the identification of the main microbial members active in the cultures. Before

these methods can be applied, however, DNA must be extracted and quantified from the cultures. It is known that interfering environmental substances, such as humic acids, impede PCR which further prevents for an adequate community profile from DGGE. In this investigation, it was found that the DNA from samples derived from cellulosic environments was better amplified by PCR after purification by an optimized ethanol precipitation protocol. The conditions were altered in the incubation temperature and incubation time as well as centrifugation temperature and time. Most favorable results were obtained under conditions of DNA precipitation at -80ºC for 2 hours followed by centrifugation at 4ºC for 30 minutes.

CHAPTER FIVE: CONCLUSIONS AND FUTURE WORK

This study investigated the soluble end-products produced from the fermentation of cellulose by consortia enriched from cellulosic waste environments and grown in a chemically defined medium. Two incubation temperatures, 50ºC and 60ºC, and two inoculum sources, compost and wastewater resulted in four cellulolytic cultures that produced different concentrations of ethanol, acetate, lactate and butyrate. Samples taken over four points of observed filter paper degradation resulted in patterns of end-product formation that differed in all four cultures; however, conducting an analysis with the use of stricter time points would provide a more accurate interpretation of the end-product cycle which could be useful for industrial applications. The compost-derived culture incubated at 50ºC produced the most butyrate while the wastewater-derived culture at 60ºC produced the most ethanol. The cultures at 60ºC also produced detectable amounts of either CO_2 or CH_4 gas.

The molecular work of selected samples obtained from this study included the optimization of DNA extraction and purification. The samples contained inhibiting factors that prevented amplification of the 16SrRNA gene by PCR. This resulted in molecular profiles on a DGGE gel; however, this technique cannot replace culture-based techniques for the isolation of all community members within the cultures. The compost-derived culture at 60ºC and the wastewater-derived culture at 60ºC showed the presence of bands on the DGGE gel that were more prominent in earlier time points and disappeared at later time points. Sequencing of these bands will provide information on the identity of the bacterial species.

In addressing future studies that can arise from this study, pure strains of the identified members can be purchased from known laboratories to develop a synthetic culture. The cellulose-degrading ability and end-product formation of this defined synthetic culture can be compared to the enrichment-derived consortia. Furthermore, the role of each microorganism in

hydrolysis and fermentation can be studied by creating modifications of the synthetic culture. Thus, if the consortia yielded four different individuals and cellulolytic activity is efficient, they can be taken out one by one until their roles are further understood. For industrial purposes having a synthetic microbial community would simplify maintenance of the bioreactors. Knowing the roles of microbial individuals in hydrolysis or fermentation can allow technicians to optimize bioreactor conditions or vary proportions of microorganisms used to maximize biofuel yield.

The microbial cultures enriched in this study contained the ability of degrading cellulose in the form of filter paper; however, in nature, cellulose is often found in the form of a highly complex molecule, lignocellulose. In future work, these consortia can be tested on the ability to break down raw cellulosic materials such as agricultural or forest waste. The filter paper used in this study was sterile; however, in a large scale industrial process, sterilizing large amounts of feedstock materials can be impractical. Thus this future work can also compare the activity of consortia when the raw feedstock is sterile and non-sterile.

In conclusion, this thesis project addresses many levels of interest. To a scientist, the results obtained will further the knowledge of the complexity of interactions that take place within the formed micro-niches of cellulolytic environments. To environmentally-concerned citizens, the implications of this research hold the potential to decrease greenhouse gas emissions that are largely emitted by use of petroleum-derived products. Cellulosic waste is also readily available as it is one of the cheapest and most abundant feedstock materials in the world. This gives cellulosic biofuel the potential to lower prices of production and diminish any competition with food sources. Lastly, progressing towards bioethanol-gasoline blends on a regulatory level and advancing the research of CBP biotechnology can be a contributing factor to phasing out the current dependency on fossil fuels.

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