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ENRICHING AND CHARACTERIZING AN AEROTOLERANT MIXED MICROBIAL COMMUNITY CAPABLE OF CELLULOSE HYDROLYSIS AND ETHANOL PRODUCTION

by

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Bachelor of Science Biology (Honours), Ryerson University 2009

A thesis

presented to Ryerson University

in partial fulfillment of the requirements for the degree of

Master of Science

in the Program of Molecular Science

Toronto, Ontario, Canada, 2011

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ABSTRACT

Cellulosic ethanol produced via consolidated bioprocessing may one day be a viable alternative to fossil fuels. However, efforts must focus on streamlining and simplifying its production in order to make this a reality. The aim of this study was to enrich a cellulolytic community and characterize its soluble end-products and bacterial diversity. The community degraded cellulose in the absence of reducing agent, and appeared to generate anaerobic conditions through oxygen-consuming aerobic respiration. Ethanol and acetate were the major fermentation products and the activity of the community was stable in aerobic and anaerobic media, as well as yeast extract-free aerobic media supplemented with other waste-based nutrient sources. Several community members showed high similarity to *Clostridium* species, suggesting the presence of some functional redundancy. Reducing agent and yeast extract both represent significant costs in the culturing of cellulolytic, ethanologenic microorganisms. The community described here exhibited this activity in the absence of both.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

AUTHOR'S DECLARATION ii

ABSTRACT iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF APPENDICES ix

LIST OF ABBREVIATIONS x

CHAPTER 1: INTRODUCTION 1

1.1 Project Significance and Motivation 1

1.2 Scope of work, Objectives, and Thesis Outline 4

1.3 Expectations 6

CHAPTER 2: LITERATURE REVIEW 7

2.1 The need for biofuels 7

2.2 Cellulose 7

2.2.1 Cellulases 9

2.2.1.1 Aerobic cellulases 10

2.2.1.2 Anaerobic cellulases 12

2.3 Fuel ethanol 14

2.3.1 Cellulosic ethanol 15

2.3.2 Consolidated bioprocessing 16

2.4 Mixed cellulolytic communities 17

2.4.1 Diversity within mixed cellulolytic communities 18

2.4.2 Mixed cellulolytic communities in the rumen 19

2.4.3 Mixed cellulolytic communities as units of evolution 20

2.4.4 Mixed communities vs. pure culture in CBP 21

2.4.5 Cellulose fermentation end-products 22

2.5 Strategies for culturing cellulolytic microorganisms 24

2.5.1 Batch vs. continuous flow culturing 24

2.5.2 Compost as a source of inoculum for cellulolytic communities.....25

2.5.3 Nutrient replacement studies involving ethanol-producing microorganisms.....26

CHAPTER 3: MANUSCRIPT 28

3.1 Introduction 28

3.2 Materials and Methods 31

3.3 Results.....38

3.4 Discussion.....55

CHAPTER 4: CONCLUSIONS AND FUTURE WORK 64

CHAPTER 5: APPENDICES.....66

CHAPTER 6: REFERENCES.....68

LIST OF TABLES

Table 3.1. Peak concentrations of minor fermentation and hydrolysis products 42

Table 6.1. Closest match between consortium members and GenBank isolates 74

LIST OF FIGURES

Fig. 2.1. The organization of cellulose molecules 8

Fig. 2.2. Structure of lignocellulose.....9

Fig. 2.3. Proposed structure of the cellulosome of *C. thermocellum*.....12

Fig. 2.4. Three strategies for the conversion of lignocellulosic material to ethanol.....16

Fig. 2.5. Conversion of pyruvate to ethanol 22

Fig. 2.6. Schematic depiction of the various fates of carbon substrates in the rumen.....23

Fig. 3.1. Cluster analysis of DGGE banding pattern in the original solid compost inoculum, the fifth enrichment culture, the consortium (enrichment 6), and the aged consortium.....39

Fig. 3.2. Ethanol and acetate production by the consortium in aerobic RM medium 41

Fig. 3.3. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days of incubation in aerobic RM medium 43

Fig. 3.4. Ethanol and acetate production by the consortium in anaerobic RM medium 45

Fig. 3.5. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days of incubation in anaerobic RM medium 46

Fig. 3.6. Ethanol and acetate production by the consortium in YE-free aerobic compost tea RM medium 48

Fig. 3.7. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days of incubation in YE-free aerobic compost tea RM medium 49

Fig. 3.8. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days incubation in YE-free aerobic wastewater RM medium.....51

Fig. 3.9. Profile of pH throughout the 14 day culturing period 52

Fig. 3.10. Phylogenetic relationship of the 8 sequenced DGGE gel bands based on 16S rRNA similarity 54

LIST OF APPENDICES

Appendix A: Regarding the end-product analysis of aerobic wastewater

RM medium samples. 66

Appendix B: Results of NCBI BLAST algorithm on consortium member sequences.....67

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CBM	Cellulose-binding motif
CBP	Consolidated bioprocessing
CDC	Cellulose-degrading consortium
CE	Cellulose-enzyme complexes
CEM	Cellulose-enzyme-microbe complexes
ddH_2O	Double distilled water
DGGE	Denaturing gradient gel electrophoresis
EPS	Extracellular polymeric substances
HPLC	High-pressure liquid chromatography
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
S_{AB}	Similarity index
SHF	Separate hydrolysis and fermentation

SSF	Simultaneous saccharification and fermentation
TAE	Tris base, acetic acid, and EDTA buffer
TCAG	The Centre for Applied Genomics
YE	Yeast extract

CHAPTER 1: INTRODUCTION

1.1 PROJECT SIGNIFICANCE AND MOTIVATION

The ability to secure a plentiful and cost effective energy source is fundamental to the survival and prosperity of any community. In the last century, the world's energy consumption has increased 17-fold and is expected to rise by an additional 54% between 2001 and 2025 (Demirbas, 2007). This has led to a massive exploitation of coal, natural gas, and other fossil fuels.

Fossil fuels are carbonaceous substances formed from the decomposition of dead organic matter and trapped within the earth for millions of years. Once extracted, they can be burned to generate energy. Although fossil fuels have in part sufficiently fulfilled the global energy requirement to date, they have two major shortcomings (Türe et al., 1997). First, they are finite resources and will be depleted in the foreseeable future (Demirbas, 2007; Rodolfi et al., 2009). Secondly, the greenhouse gases released as a result of their combustion are a leading cause of climate change (Wilkinson, 2008). As a result, there is a need for renewable, environmentally friendly alternative energy sources.

Biofuels such as bioethanol, biodiesel, and biohydrogen are defined as liquid or gaseous fuels derived from living or recently dead plant material (Wang and Chen, 2009). They are considered to have the potential to achieve carbon neutrality, meaning that the amount of carbon released during their combustion is equal to the carbon initially captured by the plant feedstock (Cheng et al., 2011). In contrast, the burning of fossil fuels releases carbon that has been sequestered deep within the earth for millions of years, resulting in an overall accumulation of carbon in the atmosphere (Berner, 2003).

Bio-ethanol is an attractive biofuel for the automotive industry because its widespread use would not necessitate a massive overhaul of the current infrastructure. It can be handled and dispensed in much the same way as gasoline, whereas gaseous biofuels such as biohydrogen would require pressurized tanks and the creation of an extensive distribution network (Schaefer and Sung, 2008). All modern cars are able to run on a blend of 5% ethanol in gasoline with no engine modification, and up to 85% with only slight alteration (Demirbas, 2007).

Currently, the vast majority of fuel ethanol comes from the fermentation of the sugars found in corn and sugarcane by yeast. This practice, however, is neither practical nor viable on a global level as the use of edible crops for fuel production puts a burden on agricultural lands and causes the costs of these foods to rise (Inderwildi and King, 2009). Ethanol can also be produced through the fermentation of the sugars found in lignocellulosic material such as corn stover, wheat or rice straw, as well as agricultural, forestry, and paper mill wastes (Lin and Tanaka, 2006). The recalcitrance of lignocellulosic biomass, however, presents a unique set of challenges and requires additional steps in comparison to corn and sugarcane ethanol production.

There are four major steps involved in the conversion of cellulosic biomass to ethanol. The first is delignification, which exposes the cellulose and renders it vulnerable to degradation. This is commonly achieved through physical means such as steam explosion, as well as chemical processes such as dilute acid treatment (Ruffell et al., 2010). This is followed by a hydrolysis step, which liberates glucose monomers as well as cellodextrins of varying length. These sugars are then fermented by live microorganisms to generate ethanol, which is subsequently separated in a distillation step (Wang and Chen, 2009).

The hydrolysis step is rate limiting and is usually achieved using cocktails of purified cellulolytic enzymes (Emsley, 2008; Wang and Chen, 2009). Recently, focus has shifted to the

prospect of using recombinant microorganisms or mixed microbial communities capable of simultaneous cellulolysis and ethanol fermentation. This would eliminate the need for purified enzymes (Gírio et al., 2010) and allow for the consolidation of the hydrolysis and fermentation steps in a strategy known as consolidated bioprocessing (CBP) (Lynd et al., 2005). Although there is currently no industrially-applied use of CBP, mixed cellulolytic communities are known to exist throughout nature in areas such as soils, rumens, and compost heaps (Wang and Chen, 2009).

The literature is extensive with regards to pure cultures of cellulose-degrading and ethanol-fermenting bacteria such as *Clostridium thermocellum* (Demain et al., 2005; Lynd et al., 2002; Xu et al., 2010). Research exploring mixed cellulolytic communities and their feasibility for use in CBP, however, is limited. This thesis describes the enrichment and characterization of a compost-derived aerotolerant mixed community capable of cellulose hydrolysis and ethanol production.

1.2 SCOPE OF WORK, OBJECTIVES, AND THESIS OUTLINE

Much attention has focused on the exploitation of pure cultures such as *C. thermocellum* for use in cellulosic ethanol production. Pure cultures, however, are often limited to a narrow range of growth conditions and are susceptible to contamination by other microorganisms, which can hinder their activity (Brenner et al., 2008). Mixed bacterial communities enriched from areas where a high level of cellulolysis is known to occur may be a viable alternative to the use of these pure culture systems. The metabolic redundancies and physiological cooperation that exists between the members of such communities may allow for efficient cellulose utilization coupled with an increased tolerance to environmental fluctuations (oxygen content, nutrient availability, pH etc.) (Kato et al., 2008; Okeke and Lu, 2011).

The scope of work was designed to address the overall objective of the project, which was to obtain and characterize a mixed, cellulolytic microbial community. This was done by accomplishing three goals: (i) Enrich an aerotolerant microbial community from compost material capable of cellulose degradation, (ii) Obtain a time-resolved profile of the soluble end-products generated by the community as a result of cellulose hydrolysis in media with differing oxygen and nutrient compositions, and (iii) using 16S rRNA PCR-denaturing gradient gel electrophoresis (DGGE) and sequencing, characterize the bacterial community diversity and monitor for changes in this profile throughout culturing in the aforementioned media.

Chapter 1 of this thesis describes the rationale and motivation behind the project as well as a summary of the objectives and expectations of the work. Chapter 2 presents a survey of the literature pertaining to cellulose hydrolysis for the purposes of bio-ethanol production by microorganisms, with a focus on mixed communities. A manuscript outlining the experimental

results and discussion are presented in chapter 3, followed by chapter 4 which presents general conclusions and future recommendations that have arisen as a result of this work.

1.3 EXPECTATIONS

Cellulosic material such as wood, leaves, and grass is efficiently degraded by microorganisms in compost (Rollins and Koenig, 2010). Thus, inoculating and enriching a compost sample in a medium containing cellulose as the major carbon source is expected to yield a cellulolytic community. The diversity within the community is expected to be considerably lower than that of the original compost inoculum.

Based on the works of Kato et al. (2004) and Wongwilaiwalin et al. (2010), which describe compost-derived cellulolytic communities, the cellulolytic consortium is expected to include a mix of anaerobic, aerobic or facultative anaerobic, cellulolytic, and non-cellulolytic members. Although the non-cellulolytic members do not directly hydrolyze cellulose, they likely enhance the overall cellulolytic activity of the community by buffering pH, providing an anaerobic environment for anaerobic cellulose degraders, and scavenging for metabolites that may otherwise cause feedback inhibition.

Since conditions within a compost pile can fluctuate significantly over time with respect to oxygen content, temperature, and nutrient availability (Young et al., 2005), it is expected that the enriched community would be robust and versatile in terms of its growth requirements. Maddipati et al. (2011), as well as Gao and Li (2011) recently demonstrated that the ethanol-producing capability of some microorganisms remains stable when they are cultured in nutrient-rich waste-products such as corn steep liquor and anaerobic digestate effluent (Gao and Li, 2011; Maddipati et al., 2011). Based on these observations, it is expected that the activity of the enrichment culture obtained in this study will persist in media in which yeast extract is replaced by other, more inexpensive and abundant nutrient sources such as compost tea (the sterile, water-soluble portion of compost), or wastewater.

CHAPTER 2: LITERATURE REVIEW

2.1 The need for biofuels

The processing and combustion of fossil fuels has led to widespread environmental damage and caused a rapid accumulation of CO_2 in the atmosphere, one of the leading causes of climate change (Wilkinson, 2008). Currently, approximately 5.4 tons of CO_2 are emitted per person per year. Considering the global population is approaching 7 billion, the amount of CO_2 entering the atmosphere each year is staggering. Analysts have suggested that the amount of CO_2 emitted annually must drop to below 2 tons per person by 2050 in order to mitigate the effects of climate change (Inderwildi and King, 2009). The use of carbon neutral plant-derived renewable biofuels may be a viable option in achieving this goal. Cellulosic ethanol in particular is an attractive biofuel due to the non-edible and abundant nature of the cellulosic feedstock.

2.2 Cellulose

Cellulose is a polymer composed of thousands of repeating D-glucose units joined by β-1-4 glycosidic bonds. It is the most abundant biopolymer on earth and exists predominantly as a structural component in the cell wall of green plants. At the site of synthesis, approximately 30 individual cellulose polymers are packaged into larger units known as protofibrils. These are then assembled into microfibrils, which are in turn organized into cellulose fibres (Fig. 2.1) (Lynd et al., 2002).

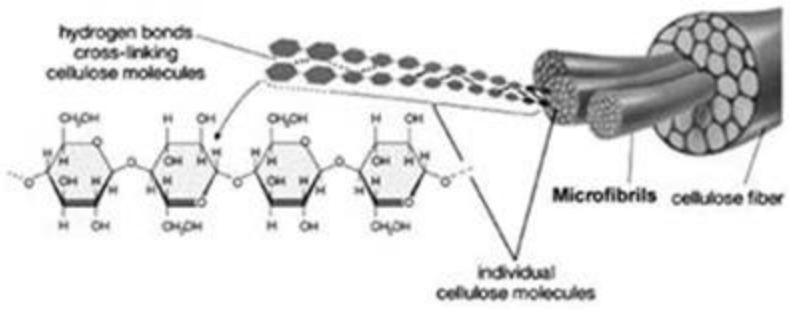

Fig. 2.1. The organization of cellulose molecules (LSU, 2006).

Cellulose is hydrophilic and insoluble in water. Unlike starch, it is a straight chain polymer and lacks branching. There is a high degree of hydrogen bonding present both within and between cellulose molecules. The location of these bonds dictates the overall crystalline structure of the complex. Independent on variations in structure, cellulose makes up 35% to 50% of plant dry weight and is considered the most abundant component of plant biomass. Cellulose usually exists in the form of lignocellulose, a complex in which cellulose fibres are encased in a network of other biopolymers, primarily hemicellulose and lignin (Fig. 2.2). Hemicellulose, which represents 20% to 30% of plant dry weight (Lynd et al., 2002), is a heteropolymer consisting of simple sugars such as xylose, mannose, galactose, and arabinose. It has a branched, amorphous structure that confers little strength to the cell wall (Emsley, 2008). Lignin, which makes up 5% to 30% of the plant dry weight (Lynd et al., 2002), refers to a group of organic polymers within which cellulose fibres are entangled. It is a major component of wood, and is more resistant to microbial degradation than cellulose and hemicellulose (Huang et al., 2010).

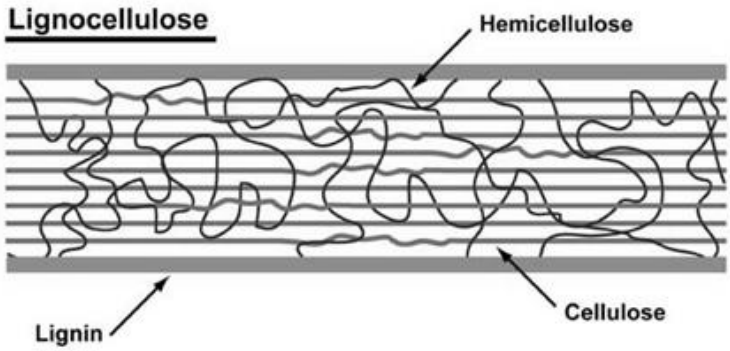

Fig. 2.2. Structure of lignocellulose (Michigan Tech Sustainable Futures Institute, 2011).

Cellulose can be hydrolyzed chemically by strong acids, or biologically by cellulase enzymes produced by microorganisms. Some organisms are able to ferment cellulose hydrolysis products to value-added end-products such as hydrogen and ethanol (Emsley, 2008).

2.2.1 Cellulases

There are several types of cellulases, each with a distinct mode of action as well as mechanism of binding. Over 500 cellulase genes are known, although very few have undergone extensive analysis regarding their functional gene products (Schwarz, 2001).

The endogluconases are a general class of cellulases that cleave β-1-4 glycosidic bonds at random points within a cellulose polymer, thereby producing new ends. Exoglucanases conversely, proceed progressively from the end of a cellulose polymer, cleaving β-1-4 glycosidic bonds and liberating cellodextrin units (Carere et al., 2008; Schwarz, 2001). Cellobiohydrolases remove cellobiose molecules from the ends of cellodextrins, while β-glycosidases liberate glucose monomers from cellobiose (Lynd et al., 2002). Interestingly, Norsker et al. (1999) have shown that effective cellulose degradation depends not only on the identity of the cellulases present, but also their ratio. This group observed that in a stable cellulose-degrading mixed community, each cellulolytic member possesses a slightly different set of cellulases, which

together are expressed in a fine balance. Thus, slight fluctuations in the ratio of the expressed cellulases can diminish the overall cellulose-degrading activity of the community (Norsker et al., 1999).

Interestingly, the ability to degrade cellulose is broadly distributed among microorganisms in a wide range of ecosystems with vastly different growth requirements (pH, oxygen, temperature, salinity etc.). Lynd et al. (2002) attribute this wide distribution to one of two evolutionary paths. First, they speculate that the ability to degrade cellulose may have been acquired by a primordial ancestor in early evolutionary development. However, the fact that cellulose biosynthesis by plants likely did not arise until much later suggests otherwise (Lynd et al., 2002). A more probable explanation therefore is that after the development of cellulose biosynthesis, the overwhelming abundance of cellulosic substrates caused a convergent evolution among microorganisms toward cellulose utilization (Lynd et al., 2002). Cellulose-degrading bacteria can be aerobic or anaerobic, although the cellulolytic strategy used by each group differs significantly.

2.2.1.1 Aerobic cellulases

Cellulolytic aerobes typically utilize cell-free cellulases which lose contact with the cell after secretion (Rabinovich et al., 2002). Within each enzyme is a non-catalytic domain known as the cellulose-binding motif (CBM) which, along with the catalytic domain is responsible for adsorption to the cellulose substrate (Schwarz, 2001). Hydrolysis products liberated from cellulose enter the bulk solution and passively diffuse through the culture medium. Wilson (2009) recently described the synergy that exists between extracellular cellulolytic enzymes,

noting that cell-free cellulases can exhibit a total activity up to fifteen times greater than the sum of their individual activities.

Wang and Chen (2009) described the existence of two distinct phenotypes with respect to extracellular cellulase production. “Labour cells” expend much of their energy and resources producing and secreting cellulases, while “lazy cells” conversely produce much less cellulase and exhibit a reduced level of cellulose hydrolysis. Interestingly, in a planktonic culture containing both lazy and labour cells, the lazy cells actually gain an advantage over the labour cells. Since hydrolysis products passively diffuse through the culture medium, lazy cells have an equal probability of encountering and absorbing them, despite expending significantly less energy in cellulase production. As a result, labour cells eventually diminish in number and lazy cells dominate the suspension (Wang and Chen, 2009). When these bacteria grow as a biofilm on a cellulosic substrate, however, the outcome is reversed. The extracellular polymeric substances (EPS) matrix of the biofilm can provide up to 90% enzyme retention. As a result, labour cells are rewarded for their efforts with a high concentration of cellulases and hydrolysis products localized just outside the cell (Wang and Chen, 2009).

Recently, a new mechanism for aerobic cellulose degradation has been proposed. The gram-negative aerobe *Cytophaga hutchinsonii* appears to possess a protein complex on its outer membrane that removes individual cellulose molecules from a cellulose fibre and transports it across the outer membrane. The molecules are then degraded by endoglucanases within the periplasmic space (Wilson, 2009).

2.2.1.2 Anaerobic cellulases

Unlike aerobic bacteria, anaerobes utilize a cellulosome, a protein complex bound to the outside of the cell wall that contains up to 11 subunits consisting of both enzymatic and non-enzymatic regions (Schwarz, 2001). The cellulosome was first observed in 1983 by Lamed et al. in *Clostridium thermocellum*, an anaerobic thermophillic bacterium that has since become one of the most extensively studied cellulolytic microorganisms (Joint Genome Institute, 2010). The enzymatic subunits of the cellulosome are joined to a single scaffoldin protein known as CipA, which is bound to the cell surface via an anchoring protein (Fig. 2.3) (Carere et al., 2008). Located along the length of the CipA are cohesion domains as well as cellulose-binding motifs. Bound to the cohesion domains are the dockerin domains of the various enzymatic components of the cellulosome (Carere et al., 2008) including cellobiohydrolase and xylanase (Schwarz, 2001). Attachment to cellulose is mediated by the cellulose-binding motifs of the scaffoldin protein. Some enzymatic subunits of the cellulosome also possess CBMs, and thus may be involved in cellulose binding as well (Carere et al., 2008).

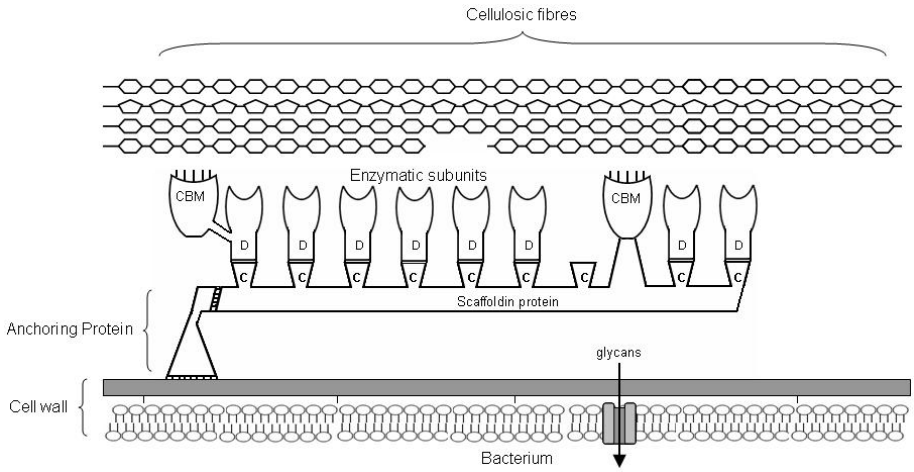

Fig. 2.3. Proposed structure of the cellulosome of *C. thermocellum* (C: cohesion domain, D: dockerin domain, CBM: cellulose-binding motif) **(**Carere et al., 2008).

Anaerobic bacteria have evolved to utilize the cellulosome because it offers several advantages over the use of cell-free enzymes. It allows the cell to directly control the ratio of enzymatic subunits being expressed (Schwarz, 2001), which has been shown to affect the rate of hydrolysis (Norsker et al., 1999). Also, since the cellulosome facilitates direct contact between the cell and the cellulosic substrate, the distance hydrolysis products must travel to be taken up and metabolized by the cell is significantly reduced (Lynd et al., 2002; Wang and Chen, 2009). Since all components of the cellulosome are docked and stationary, the enzymatic subunits can be spaced optimally to avoid non-productive adsorption and maximize hydrolysis efficiency (Schwarz, 2001).

Since aerobic cellulases each possess their own CBM, these enzymes must compete for a limited number of adsorption sites on the cellulosic substrate. This problem is mitigated through the use of a cellulosome, since the entire protein complex and all of its enzymatic subunits are joined and therefore require much fewer adsorption sites (Wilson, 2009). *C. thermocellum* exhibits the highest known rate of cellulose hydrolysis and its cellulolytic activity has been shown to be approximately fifty times greater than *Trichoderma reesei*, a well-studied aerobic cellulolytic fungus (Carere et al., 2008).

Since the production of a single cellulosome complex requires a significant input of ATP, cellulosome-utilizing anaerobic bacteria have evolved to be able to repress cellulosome synthesis when cellulose degradation is not required. For example, when *C. thermocellum* is grown in the presence of both cellulose and cellobiose, cellulose degradation is significantly reduced since one of the major hydrolysis products (cellobiose) is already present (Carere et al., 2008).

Lu et al. (2006) recently described two different complexes formed by cell-free and cell-bound cellulases. Secreted cell-free cellulases form structures known as cellulose-enzyme (CE)

complexes. Cell-bound cellulases (cellulosomes), however, form cellulose-enzyme-microbe (CEM) complexes. It has been shown that the former exhibits a much lower rate of cellulose hydrolysis than the latter. A pure culture of *C. thermocellum* is able to achieve a hydrolysis rate 2.7 to 4.7 times greater than a purified, cell-free preparation of the same enzymes (Lu et al., 2006; Wang and Chen, 2009). Although the consumption of inhibitory hydrolysis products likely accounts for some of this enhanced activity, the enzyme-microbe synergy within CEMs has yet to be fully explained.

2.3 Fuel ethanol

Ethanol is a biofuel that has garnered significant as a potential replacement for gasoline in the automotive industry. Because it is liquid, it can be handled and dispensed in much the same manner as gasoline. Schaefer and Sung (2008) describe how this key characteristic would limit the need for a complete overhaul of the current automotive fuel infrastructure, thereby reducing the economic burden of switching from a gasoline-based automotive industry. Currently the US and Brazil are the world leaders in ethanol production, with corn and sugarcane as the most common feedstocks (Dias De Olivera et al., 2005). Using food crops for fuel production, however, can generate competition over agricultural lands and serve to inflate the cost of both the food and fuel (Inderwildi and King, 2009). As a result, much research has focused on the use of non-edible lignocellulosic biomass as a feedstock for ethanol production. Sources of this biomass include agricultural, forestry, and pulp and paper mill residues (Wongwilaiwalin et al., 2010).

2.3.1 Cellulosic ethanol

The production of ethanol from lignocellulosic biomass generally involves four main steps. The first is delignification, in which lignin is removed from the lignocellulosic complex, thereby exposing the cellulose fibres and rendering them vulnerable to degradation. This is commonly achieved through physical means such as steam explosion, as well as chemical processes such as dilute acid treatment (Ruffell et al., 2010). The second step is cellulose hydrolysis, which liberates free glucose and cellobiose molecules, as well as cellodextrins of varying length. This is typically carried out using cocktails of cellulases purified from known cellulolytic bacteria and fungi. The third step involves the fermentation of the hydrolysis products (glucose, cellobiose etc.) by live microorganisms to produce ethanol. A fourth distillation step is then required in order to separate the ethanol from the culture medium (Wang and Chen, 2009).

This four step process is known as separate hydrolysis and fermentation (SHF). Since the cellulases used in the hydrolysis step are susceptible to inhibition by the accumulation of glucose and cellobiose, a new strategy has been proposed that combines hydrolysis and fermentation into a single process known as simultaneous saccharification and fermentation (SSF) (Lin and Tanaka, 2006). This ensures that hydrolysis products are quickly fermented and prevents a significant accumulation of glucose and cellobiose. Although SSF involves a simpler set-up and overcomes some of the limiting factors in SHF, it still utilizes purified cellulases, which can add to the overall cost of production (Wang and Chen, 2009).

Recently, a process known as consolidated bioprocessing (CBP) has emerged as a more attractive method of cellulosic ethanol production. In CBP, simultaneous saccharification and fermentation are achieved using a cellulolytic and ethanologenic pure culture or mixed

community. The fact that cellulose hydrolysis is performed by living cells eliminates the need for enzyme purification steps and serves to simplify cellulosic ethanol production (Carere et al., 2008; Lynd et al., 2005).

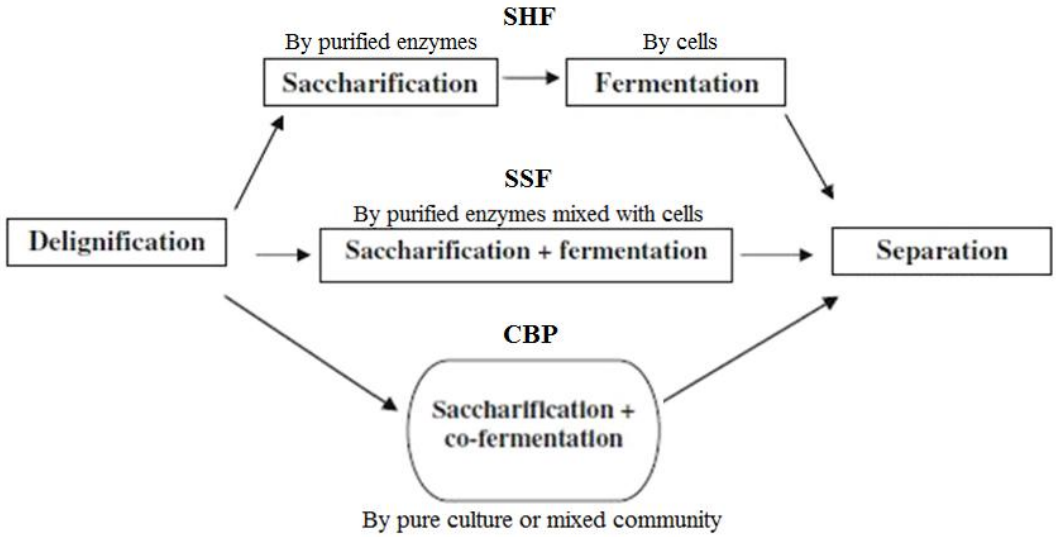

Fig. 2.4. Three strategies for the conversion of lignocellulosic material to ethanol (SHF: separate hydrolysis and fermentation; SSF: simultaneous saccharification and fermentation; CBP: consolidated bioprocessing) (modified from Wang and Chen, 2009).

2.3.2 Consolidated bioprocessing

In CBP, cellulase production, cellulose hydrolysis, and ethanol fermentation are accomplished in a single step. In comparison to SHF and SSF, CBP may simplify feedstock processing, improve conversion efficiency, and lower energy inputs (Carere et al., 2008). The latter in particular is important because it decreases the ratio of input to output energy, thereby improving the overall energy balance of the process (Carere et al., 2008).

CBP may be accomplished using pure cultures or mixed communities and research has focused on assessing and improving the performance of both. There are two general strategies that have been used to improve the performance of CBP pure cultures. The native cellulolytic strategy involves manipulating naturally occurring cellulolytic microorganisms to improve

product yield and titre. The recombinant strategy conversely, involves the introduction of cellulase genes into non-cellulolytic microorganisms that already exhibit high product yields and titres (Lynd et al., 2005).

Mixed cellulolytic communities enriched from natural populations may represent a viable alternative to the use of pure cultures in CBP. Often there are complex intrinsic synergistic interactions at play in these communities that allow for efficient cellulose utilization and product formation. While pure cultures such as *C. thermocellum* have garnered significant attention in recent years with respect to CBP, far less research has looked at mixed cellulolytic communities and assessing their feasibility for use in this application.

2.4 Mixed cellulolytic communities

Although cellulose-decomposing bacteria are ubiquitous in soils and other natural environments, they rarely exist as pure cultures, and are most often members of larger more diverse mixed communities consisting of physiologically cooperative species (Stoodley et al., 2002). Comprehensive studies exploring the structure and activities of these communities, however, have been limited (Ulrich et al., 2008).

Research has shown that the rate of hydrolysis exhibited by some cellulolytic strains in the presence of other non-cellulolytic bacteria actually exceeds the rate it achieves in pure culture. For example, a mixed community consisting of cellulose-degrading *Clostridium straminosolvens*, along with 3 non-cellulolytic strains originally isolated from the same compost sample, has been shown to degrade 8.91 mg/mL of cellulosic filter paper after 10 days. A pure culture of *C. straminosolvens*, however, degraded only 6.50 mg/mL over the same period, representing a 37% drop in cellulolytic capability (Kato et al., 2004; Soundar and Chandra,

1987). This enhanced activity is facilitated in part by the fact that when growing in a mixed community, cells are able to continuously communicate with each other through quorum sensing and by trading metabolites (Keller and Surette, 2006). This allows members to detect and respond to each other, resulting in a highly organized division of labour in which various strains perform specific tasks that improve the fitness and activity of the overall community (Keller and Surette, 2006).

2.4.1 Diversity within mixed cellulolytic communities

The ability to degrade cellulose is widely distributed among many genera in the domain *Bacteria* and in fungal groups within the domain *Eucarya*, although there are no known cellulose-degrading members of the domain *Archaea* (Lynd et al., 2002). Lynd et al. (2002) describe a high number of cellulolytic bacterial species within the aerobic order *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic order *Clostridiales* (phylum *Firmicutes*). Members of the *Clostridiaceae* family specifically are well known for their cellulose-degrading capabilities (Lu et al., 2006). Interestingly, the vast majority cellulose-degrading bacteria are either aerobic or anaerobic, but rarely both. Members of the genus *Cellulomonas* are the only reported cellulolytic facultative anaerobic bacteria (Lynd et al., 2002).

Recently, Kato et al. (2005) described a stable cellulose degrading community isolated and enriched from composting material consisting of *C. straminosolvens*, and several *Clostridium*, *Pseudoxanthomonas*, *Brevibacillus*, and *Bordatella* species (Haruta, et al., 2002; Kato et al., 2004; 2005; 2008). While only *C. straminosolvens* possesses cellulolytic capabilities, this mixed community degraded cellulose much more effectively than *C. straminosolvens* in pure culture. This suggests that non-cellulolytic bacteria are actually

important members of natural cellulolytic communities. Kato et al. (2005) proposed that the non-cellulolytic members indirectly enhance cellulose hydrolysis by consuming oxygen and buffering pH, which maintains optimal conditions for the cellulolytic members. The non-cellulolytic strains in turn depend on the sugars liberated from the cellulose.

Using fluorescence in situ hybridization, Pankratov and Dedysh (2009) recently found that bacteria from the phylogenetic class *Actinobacteria* were the dominant members (~20%) of a cellulolytic community obtained from *Sphagnum* peat bogs (Pankratov and Dedysh, 2009). Additionally, Schellenberger et al. (2010) reported that within a sample of aerated agricultural soil in which cellulose is rapidly degraded, dominant members were from the *Actinobacteria* (phylum *Actinobacteria*), *Flavobacteria* (phylum *Bacteroidetes*), as well as the *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria* (phylum *Proteobacteria*) classes. When the above community was cultivated under strict anaerobic conditions, however, members of the *Firmicutes* phylum were dominant (Schellenberger et al., 2010).

2.4.2 Mixed cellulolytic communities in the rumen

Within the rumen there exists a diverse microbial community capable of efficient and effective cellulose degradation. Miron et al. (2001) reported the concentration of bacteria in the rumen to be approximately 10^{10}- 10^{11} cells/mL, representing more than thirty different species. As well, approximately five fungal and forty protozoan species are present in the rumen at concentrations of 10^5 cells/mL and 10^5-10^7cells/mL, respectively.

Ruminal bacteria generally fall into one of five classes. Some bacteria are planktonic and float freely in the rumen fluid, while others are associated with the rumen epithelium. Some are loosely associated with food particles, while a fourth group are more firmly adhered to these

particles. Additionally, some ruminal bacteria grow on the surface of protozoa or fungal sporangia (Miron at al., 2001). In terms of cell numbers, bacteria that are loosely or firmly attached to food particles represent roughly 75% of the bacterial population. Dominant ruminal cellulolytic bacteria include *Fibrobacter succinogenes* (phylum *Fibrobacteres*) as well as two members of the *Firmicutes* phylum, *Ruminococcus flavefaciens*, and *Ruminococcus albus* (Julliand et al., 1999; Miron at al., 2001). McAllister et al. (1994) reported that the entire process of delignification, hydrolysis, and co-fermentation within the rumen occurs within 25 to 30 hours.

2.4.3 Mixed cellulolytic communities as units of evolution

Naturally-occurring mixed communities are very finely-tuned and can essentially be considered distinct units of proliferation and evolution. Adaptation within these communities can occur not only at the level of individual genes and organisms, but at the level of the entire community (Dumitrache et al., 2010).

Some members of cellulolytic communities have evolved to possess traits that do not directly benefit the organism itself, but rather improves its survival by benefiting the community as a whole. *C. thermocellum* for example, possesses genes coding for several enzymes involved in hemicellulose degradation, such as xylanase, mannase, and chitinase (Dumitrache et al., 2010). This organism, however, does not utilize the products of hemicellulose hydrolysis. This suggests that *C. thermocellum* may have evolved to produce these enzymes in order to provide hemicellulose-derived sugars to fellow community members, who in turn likely confer *C. thermocellum* with some added advantage (Dumitrache et al., 2010).

2.4.4 Mixed communities vs. pure culture in CBP

Although cellulose contains only glucose, a six carbon sugar, hemicellulose contains a variety of both five and six carbon sugars. Since hemicelluloses comprise a large portion of lignocellulosic material, effective CBP microorganisms should be able utilize both five and six carbon sugars. Brenner et al. (2008) state that engineering a single bacterial strain to enable it to utilize both five and six-carbon hydrolysis products would result in a decrease in productivity, due to an overall glucose preference. Mixed communities derived from lignocellulosic materials such as agricultural residues or forestry wastes, however, often contain both cellulolytic and hemicellulolytic species and are therefore able to utilize both five and six carbon hydrolysis products (Brenner et al., 2008).

The robustness of bacterial consortia makes them well suited for use in potential industrial-scale CBP operations. Mixed communities are better able to withstand changes in environmental conditions and defend against invading organisms than pure cultures (Brenner et al., 2008; Kato et al., 2008). In diverse microbial communities, a variety of metabolic strategies may be employed, which facilitates greater metabolite-sharing (Brenner et al., 2008; Kato et al., 2008). As a result, metabolic redundancies might exist which serve to reduce its growth medium requirements (LaPara et al., 2002).

The presence and role of fungi in naturally-occurring mixed lignocellulose-degrading communities is often overlooked. There are many known fungal strains capable of carrying out delignification (*Cerrena unicolor, Ganoderma applanatum, Ischnoderma resinosum, Poria medulla-panis*) (Blanchette at al., 1985). As a result, some have proposed that future CBP research should explore natural communities or synthetic co-cultures containing both cellulose-degrading bacteria and delignifying fungi. Such bacterial-fungal symbiosis may allow for

simultaneous delignification, hydrolysis, and fermentation, a process that occurs readily in the natural environment of the rumen (McSweeney al., 1994).

2.4.5 Cellulose fermentation end-products

Cellulose hydrolysis liberates cellulose and cellobiose monomers. These molecules are then taken up by the cell and in the absence of oxygen can be fermented to produce an array of end-products. All strict anaerobic cellulolytic species produce acetic acid and CO_2 in substantial amounts (Lynd et al., 2002). The major end-products generated by *C. thermocellum* in a flow system are ethanol and acetate in a ratio of approximately 2:1 (Lynd et al., 1989). The general scheme for ethanol production from cellulose involves the initial conversion of the hydrolysis products to pyruvate via glycolysis. Under anaerobic conditions, pyruvate is then fermented to produce ethanol through an acetaldehyde intermediate.

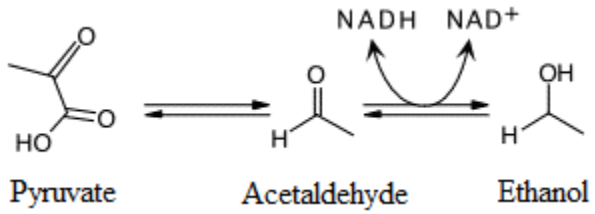

Fig. 2.5. Conversion of pyruvate to ethanol. Glucose is converted to pyruvate through the process of glycolysis.

In a cellulolytic compost enrichment culture described by Haruta et al. (2002), acetate, lactate, and ethanol were the major fermentation products, while a small amount of residual glucose was also detected in the medium. In a similar study, Zyabreva et al. (2000) found that the major end-products generated by a cellulolytic community isolated from horse feces were ethanol and acetate.

In the rumen, some species such as *F. succinogenes* and *R. flavefaciens* produce large amounts of succinate, which is subsequently converted to propionate by other bacteria (Lynd et

al., 2002). Interestingly, many ruminal species show significant stability with respect to end-product generation (Weimer, 1996). It has been shown that the ratios of fermentation end-products of the strains *F. succinogenes* S85, *R. albus* 7, and *R. flavefaciens* FD-1 show little change with fluctuations in growth rate, pH, or carbohydrate source (cellulose or cellobiose) (Lynd et al, 2002). Below is a schematic depiction of the various fates of carbon substrates in the rumen.

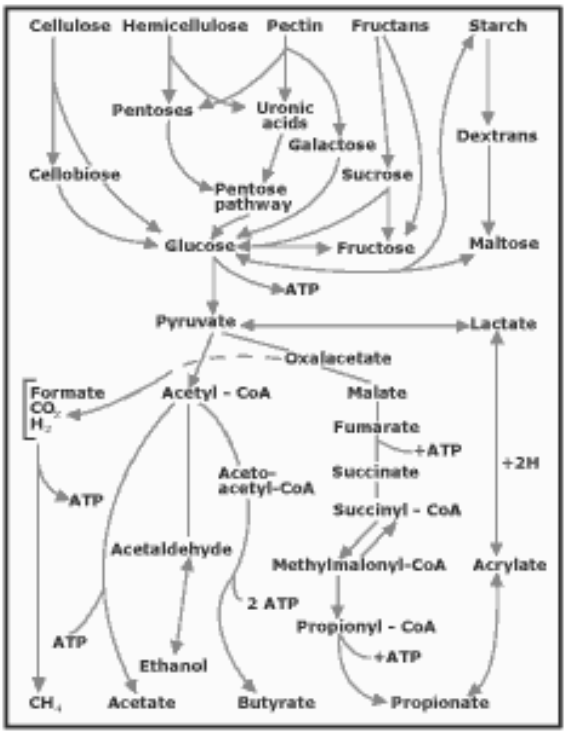

Fig. 2.6. Schematic depiction of the various fates of carbon substrates in the rumen (adapted from Comparative Nutrition Society, 2010).

Although ethanol is currently one of the most popular biofuels, it is not the only fermentation end-product with value-added properties. Methane is a natural gas that can be used in electricity generation (Steinberg, 2008), while hydrogen is a very-clean burning fuel which has been the subject of significant research (Martín and Grossmann, 2011). Short chain fatty acids such as acetic acid (acetate) can be used as a feedstock in other biofuel-producing processes, as well as precursors in a variety of manufacturing processes. Chae et al. (2008)

recently described an efficient hydrogen-producing modified microbial fuel cell using acetate as its feedstock.

2.5 Strategies for culturing cellulolytic communities

Enrichment cultures are a common way to select for and isolate microorganisms with a particular set of characteristics from larger, more diverse populations. This process involves sequentially transferring a culture in a medium that favours the growth of the microbes of interest. It has been estimated that 1 g of soil contains over 4000 different bacterial species (Entcheva et al., 2000). Thus, enrichment cultures have been used to select for cellulose-degrading communities from this diverse environment (Haruta et al., 2002; Wongwilaiwalin et al., 2010).

2.5.1 Batch vs. continuous flow culturing

There are two general strategies used to culture cellulose-degrading bacteria. In batch systems, media is replenished only at predetermined intervals while in flow systems fresh media is continuously supplied to the microorganisms. Both batch and flow systems present a unique set of advantages and disadvantages. Batch systems are simpler to operate and allow for the observation of all phases of bacterial growth. However, since one or more of the required nutrients eventually becomes limiting, exponential growth persists only for a few generations. As well, the accumulation of fermentation products like ethanol may inhibit further microbial activity (Todar, 2009). In flow systems, the growth of substrate-attached biofilms is favoured. As well, since there is a continuous supply of fresh nutrients, the time required to enrich a particular community may be reduced in comparison to batch systems. Flow systems, however,

require large amounts of media and typically involve a more complex set-up (Todar, 2009). Batch systems have been used extensively when culturing and enriching cellulolytic communities from the environment (Guevara and Zambrano, 2006; Okeke and Lu, 2011; Sizova et al., 2011; Wongwilaiwalin et al., 2010).

2.5.2 Compost as a source of cellulolytic communities

Composting refers to the process of breaking down organic matter to useful products by the action of various microorganisms. It plays an important role in the reclamation, recycling, treatment, and disposal of wastes (Young et al., 2005). The application of compost to soil has become common practice for maintaining healthy, nutrient rich soils. In soil ecosystems, the microbial community is responsible for the degradation of approximately 90% of all organic matter. The products of this decomposition can then be used by higher trophic levels, illustrating the important role these microorganisms play in nutrient cycling (Rollins and Koenig, 2010).

Soil quality is assessed based on its physical, chemical, and biological properties. The biological aspect, however, is the least studied and understood. Rollins and Koenig (2010) reported that approximately 80 to 95% of all soil microbes have not been cultured. The decomposition of organic matter during composting involves three general steps, with the substrates becoming more recalcitrant as the process progresses. First, soluble sugars are degraded, followed by cellulose and hemicelluloses, and lastly lignin. The first step is carried out by most bacteria and fungi in the community, while the second is accomplished by a far smaller group of microorganisms. The last step is carried out by an exclusive group of fungi. Rollins and Koenig (2010) suggest that the member identities and ratios within the microbial community becomes increasingly important as the process progresses, since fewer

microorganisms posses the enzymes needed to degrade more recalcitrant organic compounds. Due to its effective cellulose-degrading properties, compost has been used as source from which to enrich cellulolytic communities (Okeke & Lu, 2011; Sizova et al., 2011; Wongwilaiwalin et al., 2010).

2.5.3 Nutrient replacement studies involving ethanol-producing microorganisms

The vast majority of work involving cellulose-degrading microorganisms utilizes yeast extract (YE) as a complex nutrient source in the culture medium (Guevara and Zambrano, 2006; Haruta et al., 2002; Miyazaki et al., 2008; Pankratov and Dedysh, 2009). Yeast extract is the water-soluble portion of autolyzed yeast cells and is an excellent source of the vitamins, nitrogen, amino acids, and carbon needed sustain most microorganisms (Neogen, 2011). If bio-ethanol is to become a realistic replacement for fossil fuels, measures need to be taken to improve the cost-effectiveness of its production on a larger scale. Since the production of yeast extract represents extraneous costs and steps in the bio-ethanol production process, a less expensive and more easily attainable waste-based alternative nutrient source would be ideal.

Maddipati et al. (2011) explored the use of corn steep liquor as a replacement for YE in the medium used to culture *Clostridium* strain P11, a microorganism that produces ethanol from carbon monoxide. It was found that the microbe produced 32% more ethanol in a medium containing 20g/L corn steep liquor, as compared to a medium containing 1g/L yeast extract. They concluded that corn steep liquor is not only an inexpensive and effective replacement for YE, but it may actually improve the overall performance of the microorganism.

A similar study by Gao and Li (2011) demonstrated that the ethanol-producing activity of some yeast cells is maintained in thermophilic anaerobic digestate effluent with no added

nutrients. This result is promising not only with respect improving the cost-effectiveness of the fermentation process, but it also eliminates the need for freshwater, a valuable natural resource (Gao and Li, 2011).

CHAPTER 3: MANUSCRIPT

3.1 INTRODUCTION

Fossil fuels are unsustainable, finite resources and their production and consumption has caused widespread environmental impacts and led to rapid climate change (Wilkinson, 2008). The global energy consumption has increased so dramatically over the last century that many analysts now predict the complete depletion of fossil fuel reserves within the next fifty years (Rodolfi et al., 2009). Consequently, there is an undeniable need for better renewable energy sources.

Biofuels such as bio-hydrogen, bio-diesel, and bio-ethanol are biomass-derived renewable energy sources that have the potential to lessen our dependency on fossil fuels and increase energy security for the future. Bio-ethanol has garnered significant attention as a potential long term replacement for fossil fuels (Persson et al., 2010; Pool, 2006). It is a clean, sustainable energy source that has the potential to achieve carbon neutrality, meaning the amount of carbon emitted during its production is equal to the amount of carbon recently captured by the plant biomass feedstock. Currently, bio-ethanol is produced mainly from sugars derived from food crops such as corn and sugarcane. This practice, however, is neither practical nor viable on a global level. Using edible crops for fuel production puts a burden on agricultural lands and causes the costs of these foods to rise (Inderwildi and King, 2009). Cellulose is an attractive alternative starting material for bio-ethanol production, due to its abundance and renewable nature. It can be obtained from any non-edible plant material, such as wood, leaves, and stalks, all of which are plentiful resources even in the least economically developed regions.

Large scale cellulosic bio-ethanol production is currently limited by the recalcitrance of cellulosic biomass, which necessitates expensive and time-consuming steps required to purify

enzymes needed for its hydrolysis (Lynd et al., 2005; Okeke and Lu, 2011). Much attention has been paid to the prospect of utilizing pure cultures of anaerobic cellulose-degrading bacteria such as *C. thermocellum* to solve this problem (Demain et al., 2005; Lynd et al., 2002; Xu et al., 2010). This organism is capable of simultaneously hydrolyzing cellulose and fermenting the resulting sugars to produce ethanol. Pure cultures systems like *C. thermocellum*, however, can often only persist within a narrow range of growth conditions (pH, temperature, oxygen content) and their activity may be hindered by contamination from other microorganisms (Brenner et al., 2008).

In nature, cellulose utilization is commonly accomplished by mixed microbial communities consisting of physiologically cooperative species (Bayer et al., 2006). The diversity within such consortia allows for metabolic redundancies, leading to efficient cellulolysis coupled with increased tolerance to environmental fluctuations (Kato et al., 2008; Okeke and Lu, 2011). As a result, microbial communities isolated from environmental niches that exhibit a high level of cellulolysis may present a viable alternative to the use of pure culture systems (Guevera and Zambrano, 2006; Haruta et al., 2002; Kato et al., 2004). Sources of these communities may include pulp and paper industry wastes, forestry and agricultural residues, ruminal fluid, and compost heaps. The latter, in particular, have garnered significant attention due in part to their rich microbial diversity and considerable cellulolytic properties. Its relative abundance and ease of acquiring also make it an attractive source of inoculum.

Cellulose-degrading bacteria include both aerobic and anaerobic strains (Lynd et al., 2002). Maintaining the strict anoxic conditions required to culture anaerobic degraders, however, necessitates additional steps, resources, and costs. As a result, microbial communities that are versatile and do not require manually reduced media to carry out cellulolysis can offer an

advantage due to their overall simpler culture requirements. Compost may prove to be a good source of such communities since the microorganisms it harbours are likely well-adapted to tolerate fluctuations in oxygen content while achieving efficient degradation of cellulosic material.

Currently, the majority of industrial scale bio-ethanol production requires cocktails of purified microbial cellulases in order to achieve cellulose hydrolysis. Although the use of live, intact cellulolytic microorganisms would circumvent the need for enzyme purification steps, bacterial culturing still presents its own set of costs. As a rich source of nutrients, yeast extract is very often a major constituent of the microbiological media used to culture cellulose-degrading bacteria in aerobic media (Haruta et al., 2002; Kato et al., 2004; Miyazaki et al., 2008; Wongwilaiwalin et al., 2010). Yeast extract, however, is quite expensive and can hinder the economic viability of culture-dependent processes on an industrial scale (Maddipati et al., 2011). The ability to replace yeast extract with a low-cost and easily attainable waste-derived complex nutrient source, without hindering the cellulolytic activity of the culture, would help to circumvent a potential financial hurdle.

The objectives of this study were to: (i) enrich an aerotolerant cellulolytic mixed community from compost, (ii) obtain a time-resolved profile of its soluble end-products in aerobic and anaerobic media, as well as yeast extract-free aerobic media supplemented with either compost tea or wastewater from a municipal wastewater treatment plant, and (iii) analyze the bacterial diversity within the community and monitor for fluctuations throughout culturing in the aforementioned media.

3.2 MATERIALS AND METHODS

Community Enrichment

Inoculum

Active compost was obtained in August 2010 from an operating residential composter in Toronto, Canada, fed with common household food-scraps and yard waste. Approximately 15 g of compost was inoculated into 60 mL of modified aerobic RM medium (Ozkan et al., 2001) and incubated at 60°C. The main modification to the medium was the omission of the reducing agent cysteine. The culture was then sequentially enriched six times in the same medium. The sixth enrichment culture was considered to be a stable consortium and was used in subsequent community and end-product analyses.

Aerobic RM Media

Autoclave sterilized modified RM medium at pH 7.4 was used for all enrichments. The medium contains urea (2 g/L), KH_2PO_4 (2 g/L), K_2HPO_4 (3 g/L), yeast extract (2 g/L), and the oxygen indicator resazurin (0.002 g/L). Whatman Grade 1 Qualitative Filter Paper (Whatman, Piscataway, NJ) was used as the cellulosic substrate and was added prior to autoclave sterilization. A filter-sterilized trace minerals solution containing $MgCL_2 \cdot 6H_2O$ (20 g/L), $CaCl_2 \cdot 2H_2O$ (5 g/L), and $FeSO_4 \cdot 7H_2O$ (0.25 g/L) was added after autoclave sterilization at a ratio of 1:100 (v/v).

Analysis of End-products Generated Under Different Incubation Conditions

A time-resolved analysis of the end products generated by the culture was carried out in four different media. The media used were aerobic RM medium, anaerobic RM medium, YE-free aerobic compost tea RM medium, and YE-free aerobic wastewater RM medium.

Anaerobic RM medium

Preparation of the anaerobic RM medium was similar to aerobic RM medium, except for the addition of the reducing agent L-cysteine hydrochloride monohydrate (Sigma-Aldrich, Oakville, ON) at a final concentration of 1 g/L. Sealed serum vials were used for anaerobic culturing, with the headspace in each evacuated by vacuum and flushed with grade 4.8 nitrogen (BOC Gasses, Mississauga, ON) for alternating 30 second intervals for 12 minutes. The medium was deemed anaerobic once the resazurin had changed from pink to colourless.

YE-Free Aerobic RM Medium

Compost tea is the water-soluble portion of compost. To make YE-free aerobic RM medium supplemented with compost tea, compost was added to ddH_2O at a ratio of approximately 1:5 (w/v) and mixed for 30 minutes using a magnetic stirring-rod and stirrer. After a 2.5 hour settling period, the supernatant was gently pumped into a clean bottle using a Masterflex® Console Drive pump (Cole-Parmer, Montreal, QC). All of the aerobic RM medium components (excluding yeast extract) were dissolved in this compost tea (instead of ddH_2O) and autoclaved.

YE-free aerobic RM medium supplemented with domestic wastewater was made in a similar fashion. Primary-treated wastewater (specifically activated sludge) was obtained from the aeration tanks of the Humber Wastewater Treatment Plant in Toronto, Canada, and kept at 4°C in the dark to limit biological and light sensitive reactions. Approximately 2 L was mixed for 30 minutes using a magnetic stirring-rod and stirrer and allowed to settle for 2.5 hours. The supernatant was then gently pumped into a clean bottle using a Masterflex® Console Drive pump (Cole-Parmer, Montreal, QC). All aerobic RM medium components (excluding yeast extract) were then dissolved in this wastewater supernatant (instead of ddH_2O) and autoclaved. Although

wastewater may well be an effective replacement for other media components such as urea, KH_2PO_4, and K_2HPO_4, the focus of this treatment was to assess the feasibility of replacing yeast extract, typically the most costly component.

Experimental Setup

Three millilitres of the cellulolytic consortium in aerobic RM medium was transferred to 27 mL of the appropriate medium to achieve 1/10 inoculations. All cultures were grown in 60 mL sealed serum vials and incubated at 60°C. The mass of filter paper used in each test vial was 83 mg (±2 mg). The culture was also inoculated into cellulose-free controls of each medium. These set-ups did not contain any filter paper and were included in order to determine whether any of the observed end-products can be attributed to any non-cellulose, labile carbon sources. All cultures were set-up in triplicate.

Culture Sampling and Analysis

One and a half millilitre samples (1.5 mL) were taken from all cultures on days 1, 2, 3, 4, 6, 9, and 14. The cultures inoculated into the wastewater-supplemented media had one additional sample taken on day 5. Samples were removed using sterilized syringes and needles and placed in 1.5 mL cryovials (Cole-Parmer, Montreal, QC). The samples were immediately acidified to approximately pH 2 using 1.5 mmol HCl, and stored at 4°C for chemical analysis. Additional sub-samples were used to measure pH using a bench-top meter (Cole-Parmer, Montreal, QC).

Concentrations of ethanol, acetate, lactate, formate, butyrate, cellobiose, and glucose in the samples were analyzed by HPLC with a carbohydrate analysis column (model # HPX-87H) (BioRad Laboratories, Mississauga, ON) along with a Waters™ isocratic HPLC pump (model

#1515), autosampler module (model #2707), and refractive index detector (model #2414) (Waters, Milford, MA).

Additional 1.5 mL samples were taken from one replicate of each of the four test cultures on day 4 and 14 and stored at -20°C. These samples were used to characterize the bacterial community diversity.

End-product Calculations

Since ethanol is a value-added product, and shares a close equilibrium relationship to acetate, the ethanol:acetate ratio achieved by the consortium in each treatment was calculated by dividing the molar concentration of acetate by the molar concentration of ethanol at the point at of highest ethanol concentration. Additionally, the percent of cellulose-derived carbon bound in ethanol at the point of highest ethanol concentration was determined and reported. First, the ethanol concentration (mM) was used to determine the mass of ethanol (g) in the vial. Since ethanol is approximately 52% carbon (w/w), the total number of moles of ethanol-bound carbon was calculated by multiplying the mass of ethanol by 0.52, and then dividing by the molar mass of carbon. The total moles of carbon in the original piece of filter paper was calculated in a similar manner (given that the filter paper is 100% cellulose and carbon makes up 44.4% of cellulose (w/w)). By dividing the number of ethanol-bound carbon moles by the initial number of cellulose-bound carbon moles, the proportion of cellulose-derived carbon bound in ethanol was determined. If any ethanol was produced in the cellulose-free control culture, this value was adjusted accordingly.

Characterizing the Community Diversity

DNA Extraction and PCR Amplification

Samples were pelleted by centrifuging at 6000 x g for 3 minutes, and washed with 1 mL PBS at pH 7.4 (0.14 M NaCl, 0.002 M KCl, 0.01 M Na_2HPO_4, 0.0018 M KH_2PO_4). This was repeated two more times so that a total of three PBS-washes were carried out on each sample. DNA was extracted using a ZR Soil Microbe DNA MiniPrep™ Kit (Cedarlane, Burlington, ON). The procedure was carried out following the manufacturer's instructions. Purified DNA was stored at -20°C.

Bacteria-specific primers were used to amplify a 418 bp fragment of the 16S rRNA gene, consisting of the variable V3 and V4 regions. The forward primer used was U341F-GC (5'-CCTACGGGAGGCAGCAG-3') with a GC clamp (5'-GGCGGGGCGGGGGCACGGGGGGC GCGGCGGGCGGGGCGGGGG-3') affixed to the 5'end. The reverse primer used was U758R (5'-CTACCAGGGTATCTAATCC-3') (Muyzer et al., 1993). Both primers were synthesized by the SickKids Centre for Applied Genomics (TCAG) synthesis facility in Toronto, Canada. Each 50 µL PCR reaction contained 2 µL template DNA, 25 pmol of the forward and reverse primers, 200 µM of each dNTP (New England BioLabs, Pickering, ON), 6.875 µg BSA (New England BioLabs, Pickering, ON), 2.5 units of Taq DNA polymerase in 1x Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$) (New England BioLabs, Pickering, ON), and sterile ddH_2O.

The PCR reaction began with a 5 minute initialization step at 96°C. Each thermocycle consisted of a 1 minute denaturation step at 94°C, a 1 minute annealing step at 65°, and a 3 minute elongation step at 72°C. The annealing temperature decreased by 1°C per cycle for each

of the first 10 cycles, and then remained at 55°C for the remaining 20 cycles (Yeung et al., 2010).

Amplified PCR product was run on a 1% agarose gel with SYBR safe DNA gel stain (Invitrogen, Burlington, ON). The resulting bands were visualized under blue light using the Invitrogen Safe Imager 2.0 (Invitrogen, Burlington, ON). DNA concentration was determined by comparing to a standard curve created from a serially diluted 100 bp DNA ladder (MBI Fermentas, Amherst, NY). For each sample, a total of six PCR amplifications were performed and combined in order increase DNA concentration and reduce PCR bias. PCR products were combined and cleaned using an illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Piscataway, NJ).

Denaturing Gradient Gel Electrophoresis (DGGE)

Approximately 300 to 500 ng of DNA from each sample was run on an 8% polyacrylamide (BioRad Laboratories, Mississauga, ON) DGGE gel with a 30-70% denaturant gradient. One hundred percent denaturant was considered to be 7 M urea (BioRad Laboratories, Mississauga, ON) and 40% deionized formamide (EMD Chemicals, Mississauga, ON). The gel was cast using a gradient former (BioRad Laboratories, Mississauga, ON), and run in a DCode Universal Mutation Detection System (BioRad Laboratories, Mississauga, ON) at 80 V for 16 hours at 60°C.

Bands were visualized by staining the gel with 0.01% SYBR Gold (Invitrogen, Burlington, ON) in 1x TAE buffer. An image of the gel was captured using a Gel Logic 1500 Imaging System (Kodak, Rochester, NY) equipped with an Invitrogen Safe Imager 2.0 blue light box (Invitrogen, Burlington, ON). Changes in the consortium's DGGE profile throughout

culturing in the four treatments were analyzed and the similarity index (S_{AB}) was reported. Bands were excised from the gel and eluted in 25 µL sterile ddH_2O at 4°C for 5 days.

One micro litre of the eluted DNA was reamplified using the same primers without the added GC clamp. After a 5 minute initialization step at 96°C, each thermocycle consisted of a 1 minute denaturation step at 94°C, a 30 second annealing step at 60°C, and 1 minute elongation step at 72°C. The cycle was repeated a total of 30 times (Yeung et al., 2011).

DNA Sequencing and Phylogenetic Analysis

Sequencing of reamplified DNA was performed by the SickKids Centre for Sequencing (Toronto, Canada) using the Applied Biosystems SOLiD 3.0 System. Unreadable portions of the DNA segments were removed from the 3' and 5' ends using Chromas V2.01 software (Technelysium, Tewantin, Australia). The sequences were labelled using the form CDC ("cellulose-degrading consortium") followed by a letter designating its position on the gel.

A consensus sequence between the forward and reverse sequences was constructed using BioEdit™ Biological Sequence Alignment Editor (BioEdit, Carlsbad, CA). The NCBI BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to check the consensus sequence against a database of known 16S rRNA sequences.

Sequence alignment was carried out using ClustalW (http://www.ebi.ac.uk/clustalw/). Phylogenetic and molecular evolutionary analyses were conducted using MEGAV5.05. Gel images were analyzed and dendrogram profiles were constructed using GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium). The genotypes were visually detected based on the presence or absence of bands in each lane. A band was defined as possessing peak intensity at least 5% of the most intense band in the gel. Dendrogram patterns were clustered by the Dice method using arithmetic average groupings with a similarity coefficient matrix.

3.3 RESULTS

Culture Enrichment

An active cellulose degrading community was enriched from compost through a series of sequential enrichments in aerobic RM medium. Filter paper degradation was observed throughout each enrichment with the majority of the cellulolytic activity occurring after the resazurin in the medium had turned from pink to colourless (i.e. aerobic to anaerobic).

The bacterial diversity in the original solid compost inoculum, the fifth and sixth enrichments, as well a two week old sixth enrichment was analyzed using PCR-DGGE (Fig. 3.1). The degree of similarity between the various DGGE lanes was reported using similarity indexes (S_{AB}). This value compares two sets of data (A and B) and assigns a percentage describing their similarity. The DGGE profile found that the bacterial diversity in the original compost differed considerably compared to the later enrichments (S_{AB} = 37.7). The 5th and 6th enrichments are closer in similarity (S_{AB} = 73.7) with all the major bands (e.g. C, D, E, H) present in both. This sixth enrichment culture was considered to be a stable consortium and was used in subsequent community and end-product analyses. When the consortium (6th enrichment) was aged two weeks without the addition of new cellulose or fresh media, the community structure shifted and the similarity dropped (S_{AB} = 48.3). Band F appeared, while band D was no longer present, suggesting that the structure of this stable consortium can still vary as the available nutrients become depleted. All labelled bands were excised and sequenced and the results are summarized in Table 6.1.

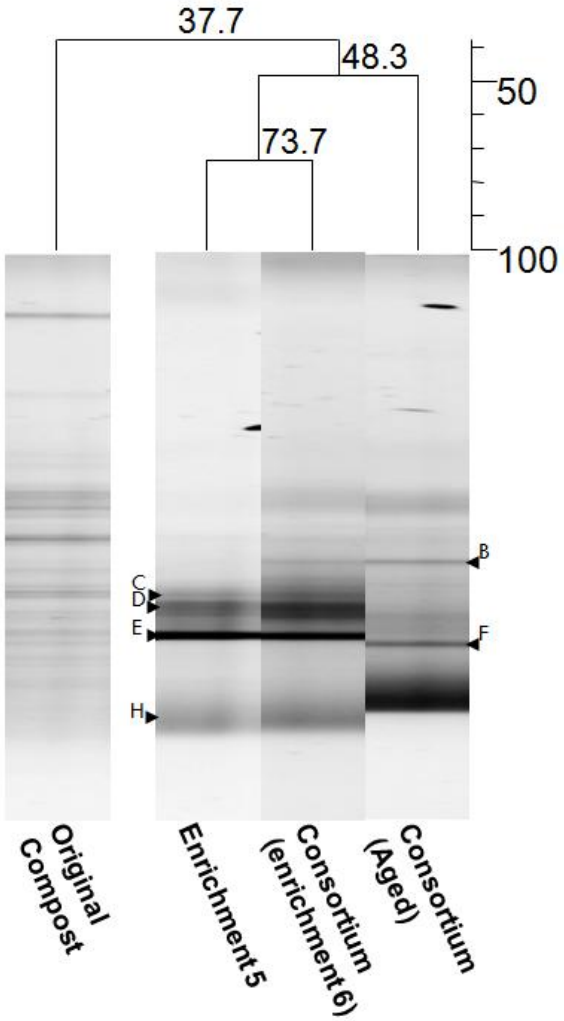

Fig. 3.1. Cluster analysis of DGGE banding pattern in the original solid compost inoculum, the fifth enrichment culture, the consortium (enrichment 6), and the aged consortium.

Analysis of Soluble End-products and Microbial Community Structure Dynamics

Due to its growing use as a biofuel, ethanol represents a value-added fermentation end-product. Therefore, measuring and monitoring its production was one of the major goals of this study. Profiles of the soluble end-products generated by the consortium were obtained from four different treatments at various time points. The four treatments were aerobic RM medium, anaerobic RM medium, YE-free aerobic compost tea RM medium, and YE-free aerobic

wastewater RM medium. Each treatment also included a cellulose-free control. Additionally, changes in the community structure were monitored during each treatment.

Aerobic RM medium

In aerobic RM medium, ethanol and acetate were the major fermentation products and reached peak values of 12.04 mM and 21.34 mM respectively (Fig. 3.2). The ratio of ethanol:acetate, taken at the point of highest ethanol concentration (day 6) was 1:1.59. The proportion of cellulose-derived carbon bound in ethanol at this point was approximately 17.8%. After day 6, the ethanol concentration decreased, while acetate levels remained fairly stable. Trace amounts of lactate, formate, and butyrate were also detected in the medium, reaching maxima of 1.57 mM, 1.97 mM, and 0.58 mM respectively by day 4 (Table 3.1). No cellobiose or glucose was detected in the medium (Table 3.1). The pH in the test culture declined from 7.4 to 6.3 by day 6, followed by a gradual increase from 6.2 to 6.4 between day 6 and 14. The pH in the cellulose-free control culture, however, remained relatively stable at approximately 7.4 throughout the incubation period (Fig. 3.9).

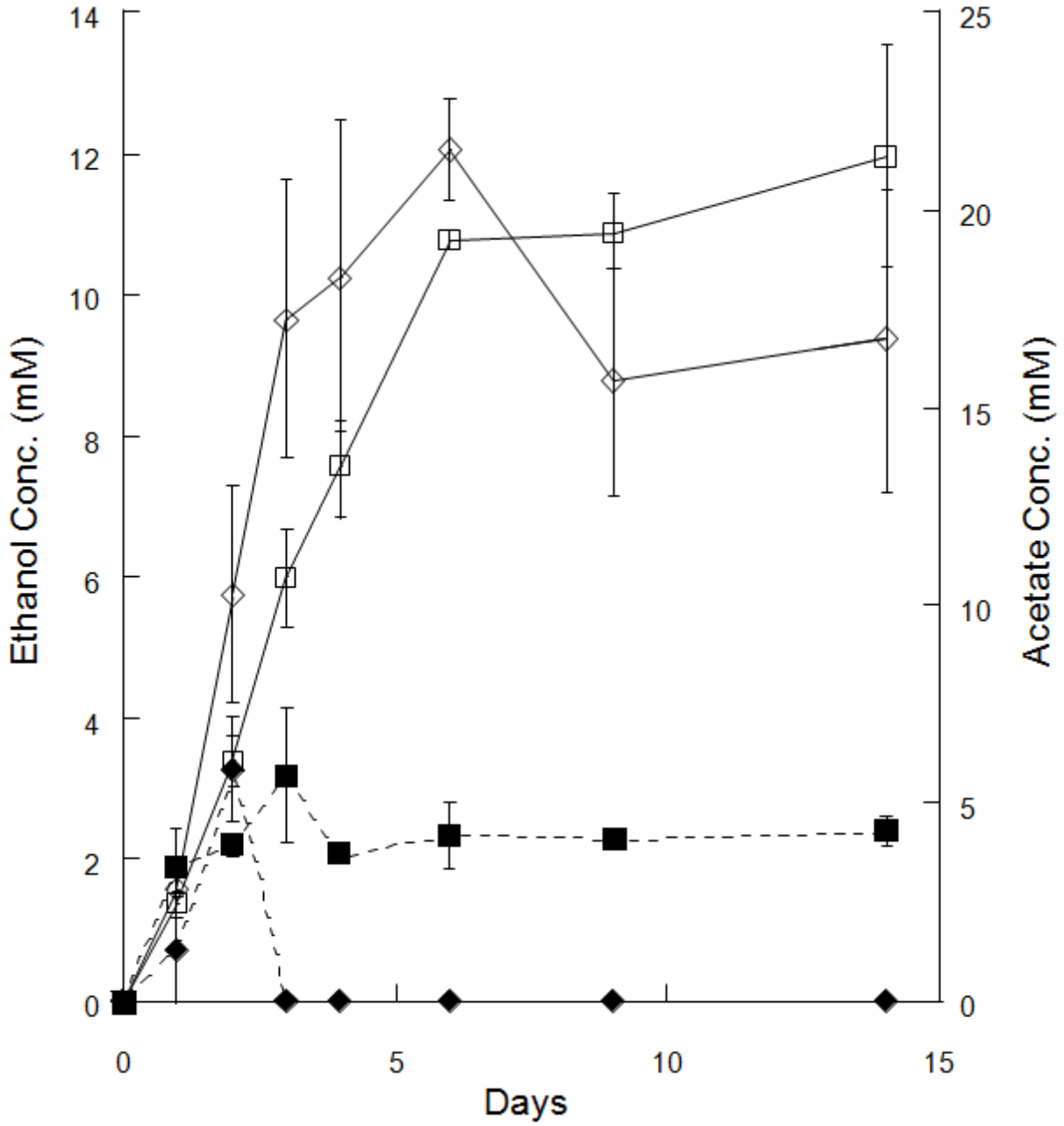

Fig. 3.2. Ethanol and acetate production by the consortium in aerobic RM medium. Ethanol production in the test culture is represented by —◇—. Ethanol production in the control culture is represented by - -◆- -. Acetate production in the test culture is represented by —□—. Acetate production in the control culture is represented by - -■- -. Error bars represent the standard error of each data point.

Table 3.1. Peak concentrations of minor fermentation and hydrolysis products.

Medium	Lactate (mM)	Formate (mM)	Butyrate (mM)	Cellobiose (mM)	Glucose (mM)
Aerobic RM	1.57	1.97	0.58	ND	ND
Anaerobic RM	ND	ND	0.65	ND	ND
Compost tea RM	2.96	1.32	0.179	ND	ND
Wastewater RM	N/A	N/A	N/A	N/A	N/A

*Peak values of all minor fermentation products occurred at day 4. ND means "not detected". N/A means "data not yet available."

Within 4 days of inoculation into aerobic RM medium, the bacterial community structure remained relatively stable (S_{AB}= 72.7) (Fig. 3.3). All of the major bands (B, C, D, E, and H) were present. Band G, however, appeared for the first time in the day 4 sample. After 14 days, the community structure shifted slightly, resulting in a slightly lower S_{AB} value of 60.2. The most notable differences were the disappearance of band D, as well as the appearance of two new (unsequenced) bands between bands B and C.

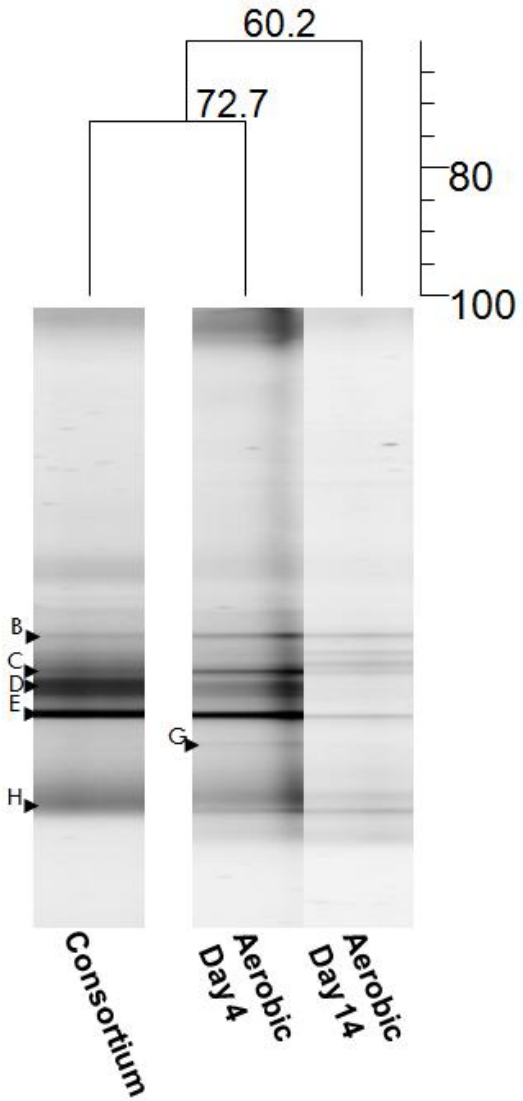

Fig. 3.3. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days of incubation in aerobic RM medium.

Anaerobic RM medium

The consortium demonstrated the ability to degrade cellulose and produce ethanol in aerobic media. Since the majority of cellulolysis occurred after the medium had turned anaerobic, and given that fermentation is an anaerobic process by definition, the consortium was inoculated into anaerobic RM medium to determine if the use of pre-reduced anaerobic media improved the overall performance of the consortium. Again, the results revealed that ethanol and acetate were the major fermentation products in this medium, although their accumulation was

markedly reduced in comparison to aerobic RM medium. The maximum ethanol and acetate concentrations achieved were 8.65 mM and 19.1 mM respectively by day 14, resulting in an ethanol:acetate ratio of 1:2.21 (Fig. 3.4). The proportion of cellulose derived carbon bound in ethanol at this point was approximately 9.9%. Some ethanol and acetate was also produced in the cellulose-free control cultures. Unlike aerobic RM medium, only butyrate was detected as a minor fermentation product and reached a maximum of 0.65 mM at day 4. No cellobiose or glucose was detected in the culture supernatant (Table 3.1). The pH in the test culture remained stable at 6.8 for the first 2 days, and subsequently decreased steadily to 5.9 by day 14 (Fig. 3.9). In the cellulose-free control culture, however, the pH decreased from 6.8 to 6.3 by day 9, and then gradually returned to 6.8 by day 14.

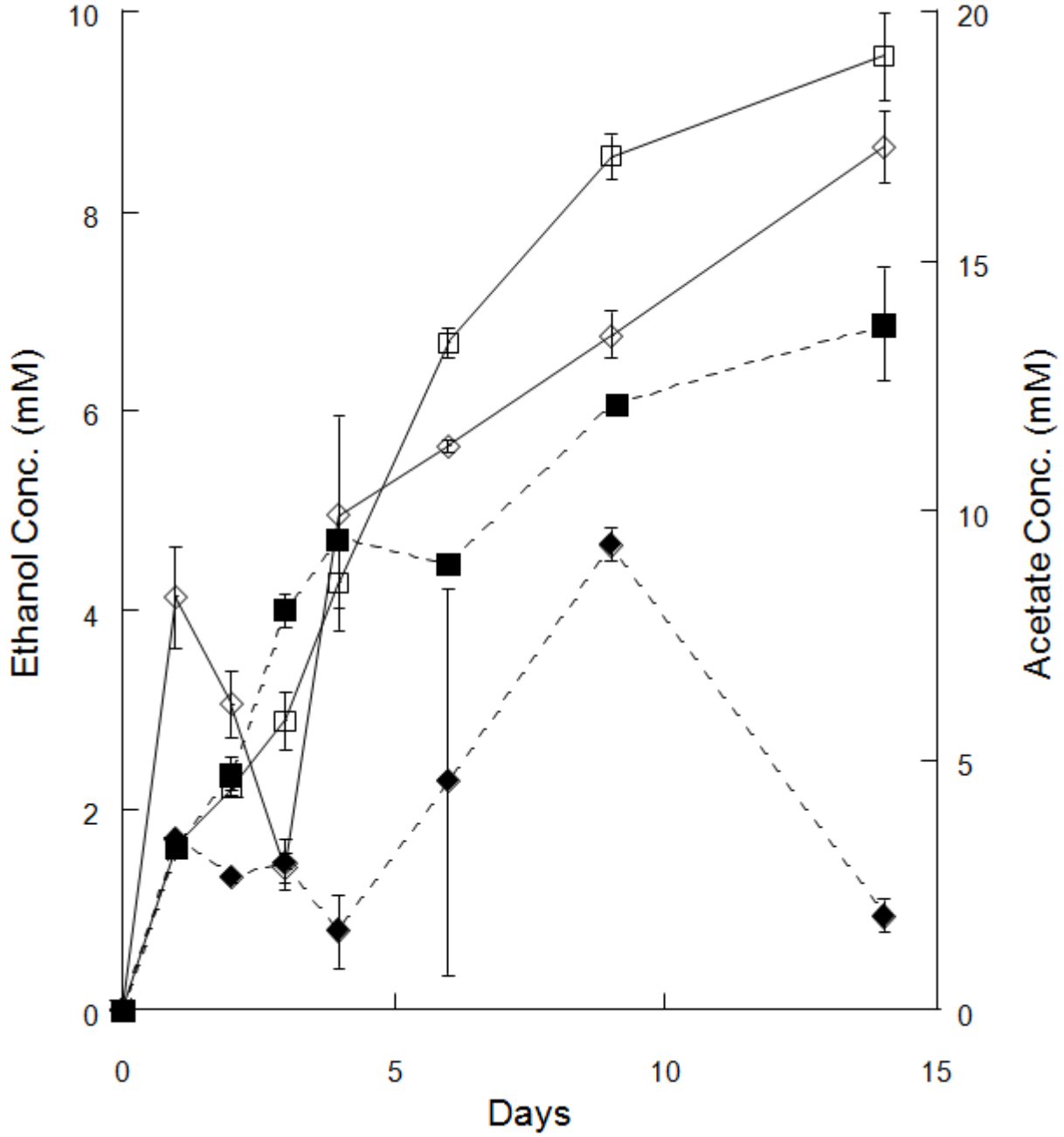

Fig. 3.4. Ethanol and acetate production by the consortium in anaerobic RM medium. Ethanol production in the test culture is represented by —◇—. Ethanol production in the control culture is represented by - -◆- -. Acetate production in the test culture is represented by —□—. Acetate production in the control culture is represented by - -■- -. Error bars represent the standard error of each data point.

The bacterial community structure in the consortium changed considerably after inoculation into anaerobic RM medium (S_{AB} = 27.2) (Fig. 3.5). At day 4, band D was no longer present. The community structure continued to shift somewhat between day 4 and 14, although

it was not to the same extent (S_{AB} = 57.1). Interestingly, band A appeared for the first time in the day 4 lane and remained until day 14. Band B conversely, persisted throughout the entire incubation period.

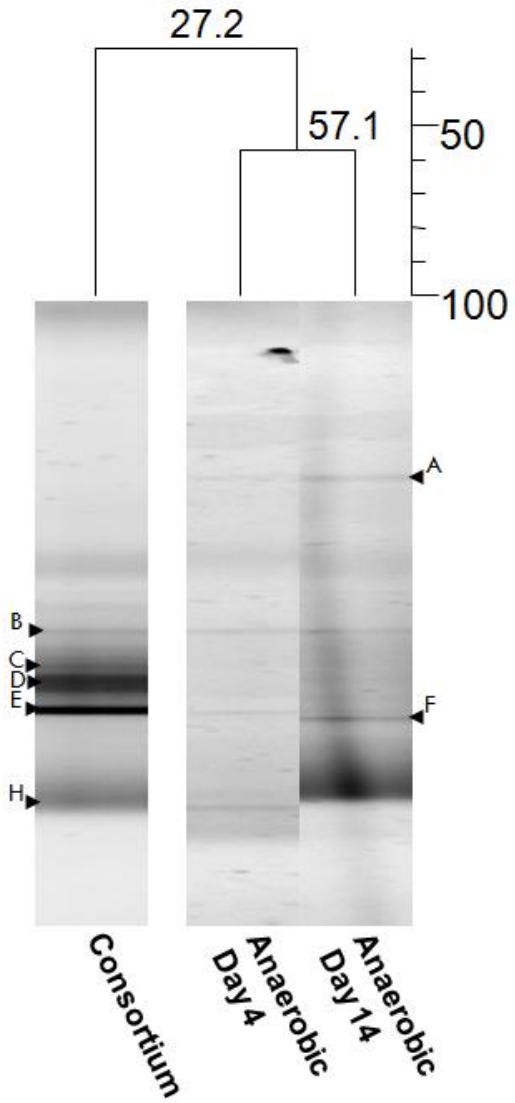

Fig. 3.5. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days of incubation in anaerobic RM medium.

YE-free aerobic compost tea RM medium

Yeast extract is a rich source of nutrients and is used extensively to culture cellulose-degrading bacteria in aerobic media. Since its use represents additional costs and resource

requirements, the next step was to assess the feasibility of replacing it with more abundant waste-derived complex nutrient sources. The consortium was inoculated into YE-free aerobic RM medium supplemented with sterile compost tea. The culture supernatant was sampled for soluble end-product analysis and the community structure on days 4 and 14 was investigated. Again, ethanol and acetate were the major fermentation products, reaching maximum concentrations of 9.88 mM by day 4, and 26.79 mM by day 14 respectively (Fig. 3.6). The ethanol:acetate ratio taken at the point of highest ethanol concentration (day 4) was 1:0.86. The proportion of cellulose-derived carbon bound in ethanol at this point was approximately 15.9%. Trace levels of lactate, formate, and butyrate were also detected, achieving maxima of 2.96 mM, 1.324 mM, and 0.179 mM respectively, all by day 4 (Table 3.1). No cellobiose or glucose was detected in the culture supernatant (Table 3.1). The pH in the test culture decreased from 7.8 to 6.5 by day 4, and levelled off between 6.3 and 6.5 for the remainder of the experiment (Fig. 3.9). In the cellulose-free control culture, however, the pH decreased initially from 7.8 to 7.3 by day 4, before levelling off between 7.3 and 7.5.

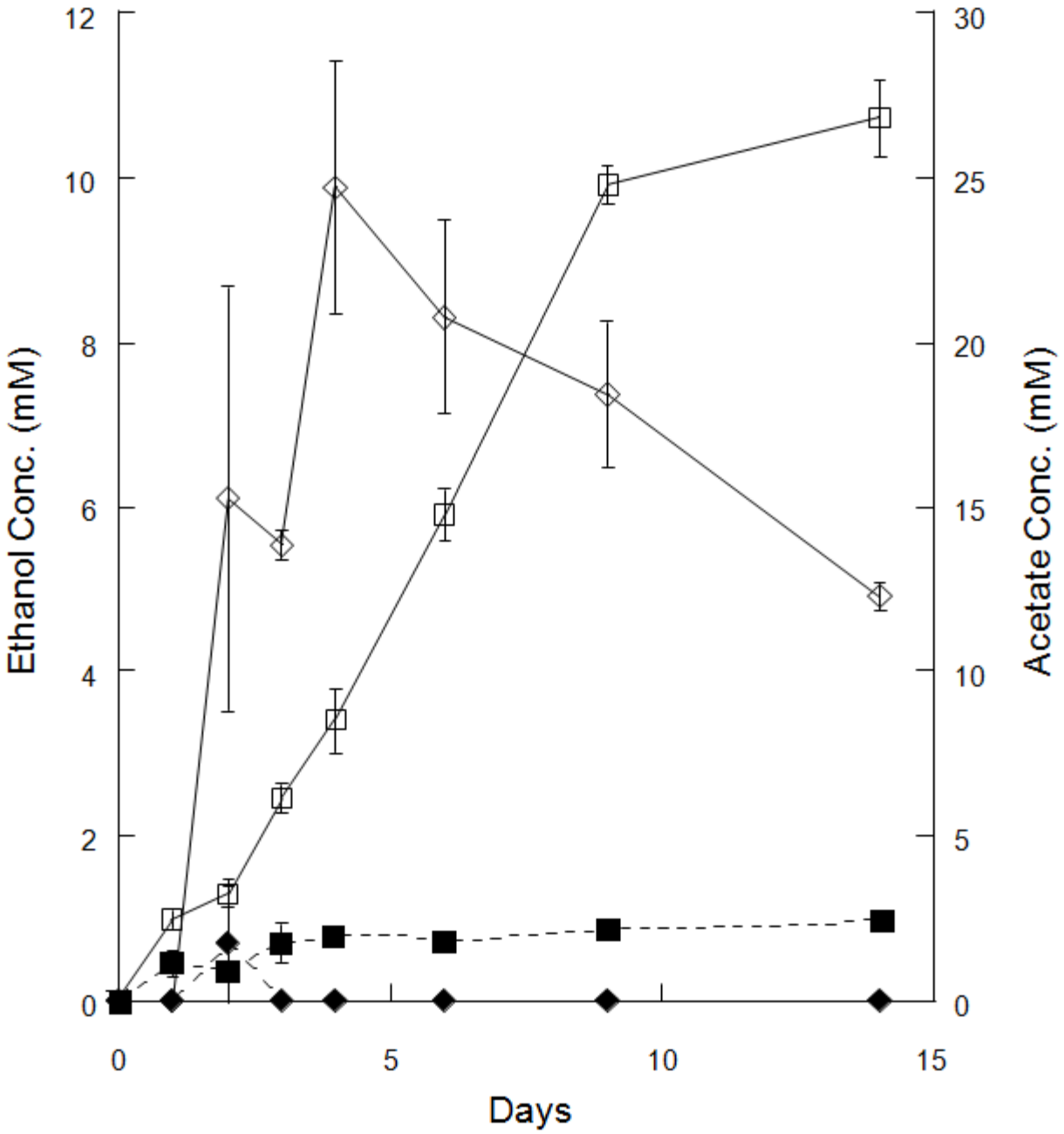

Fig. 3.6. Ethanol and acetate production by the consortium in YE-free aerobic compost tea RM medium. Ethanol production in the test culture is represented by —◇—. Ethanol production in the control culture is represented by - -◆- -. Acetate production in the test culture is represented by —□—. Acetate production in the control culture is represented by - -■- -. Error bars represent the standard error of each data point.

The bacterial community structure in the 14 day aerobic compost tea RM medium experiment remained very stable (S_{AB} =91.7) (Fig. 3.7). All of the major bands (B, C, D, E, H) in the consortium at inoculation were still present on day 4. Interestingly, band G appeared in the

day 4 lane and persisted throughout the rest of the culturing period. This band, however, did not appear in the consortium lane. Much like in aerobic RM medium, the community structure at day 14 demonstrated a slight shift, with an S_{AB} value of 59.1. Once again, band D was no longer present in the day 14 lane.

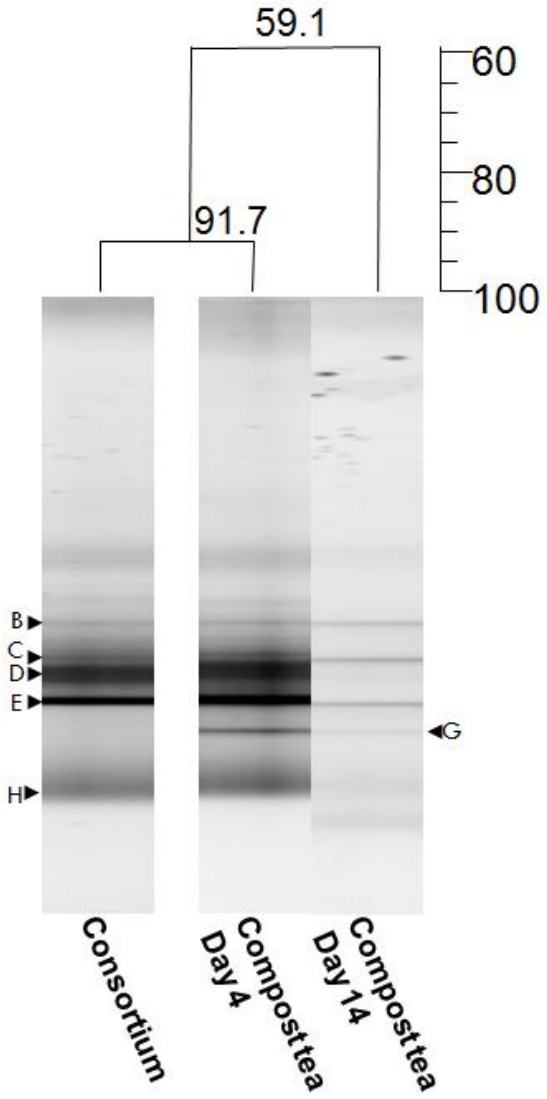

Fig. 3.7. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days of incubation in YE-free aerobic compost tea RM medium.

YE-free aerobic wastewater RM medium

Since the cellulolytic and ethanologenic activity of the consortium was maintained in media in which yeast extract had been replaced with compost tea, the next step was to assess the activity of the community in a medium in which yeast extract was replaced by wastewater, an even more abundant and inexpensive complex nutrient source. HPLC end-product analysis is currently being performed on samples of the culture supernatant (see Appendix A). In qualitative terms, the behaviour of the consortium in this medium was similar to that in the other three media. The medium turned anaerobic within the first 1 to 2 days, and the filter paper was degraded thereafter. The pH in the test culture dropped from 7.2 to 6.2 within 6 days, and subsequently increased gradually to reach 6.4 by day 14 (Fig. 3.9). The pH in the cellulose-free control culture, however, remained at 7.2 until day 9, before gradually increasing to reach 7.4 by day 14.

Changes in the community structure were analyzed after 4 and 14 days of incubation in aerobic wastewater RM medium (Fig. 3.8). The community at day 4 revealed a very high degree of similarity to the stable consortium, resulting in an S_{AB} value of 78.3. All of the major sequenced bands (B, C, D, E, H) persisted throughout the incubation period. Interestingly, between days 4 and 14, the community structure remained unchanged (S_{AB} = 100). This is contrasted to aerobic RM medium and aerobic compost tea RM medium, in which the S_{AB} at day 14 was 60.2 and 59.1 respectively.

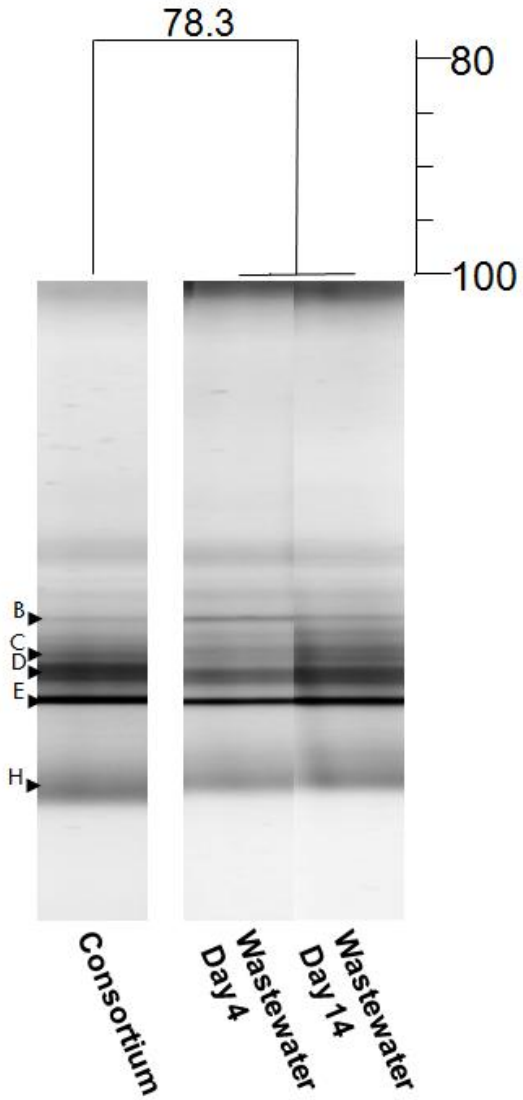

Fig. 3.8. Cluster analysis of DGGE banding pattern in the consortium at inoculation, as well as after 4 and 14 days incubation in YE-free aerobic wastewater RM medium.

pH Profiles

The pH profiles in the test cultures in all four media were characterized by a rapid decrease within the first 4 to 6 days (Fig. 3.9). Despite differences in starting pH, the final pH in the aerobic RM, aerobic compost tea RM, and aerobic wastewater RM medium test cultures was around 6.4. The test culture in anaerobic medium conversely decreased to around 5.9 by day 14. In the cellulose-free control cultures, the pH remained fairly stable throughout the incubation

period, with the final and initial pH in each respective medium being less 0.26 apart. Once again, the pH in the aerobic RM, aerobic compost tea RM, and aerobic wastewater RM control cultures at the end of the experiment were very close at approximately 7.4 to 7.5.

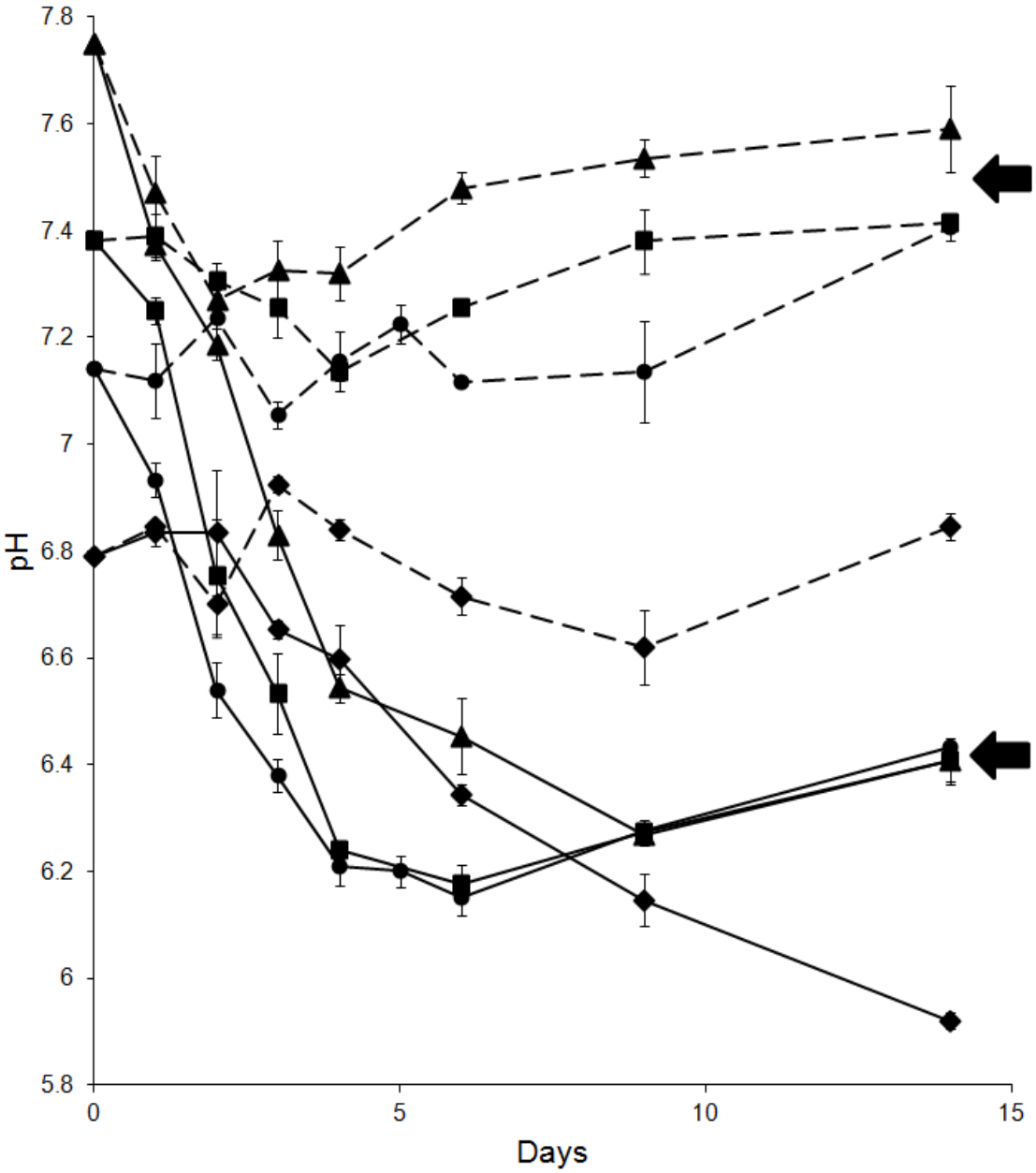

Fig. 3.9. Profile of pH throughout the 14 day culturing period. In aerobic RM medium, —■— represents the test culture, while --■-- represents the control. In anaerobic RM medium, —◆— represents the test culture, while --◆-- represents the control. In aerobic compost tea RM medium, —▲— represents the test culture, while --▲-- represents the control. In aerobic wastewater RM medium, —●— represents the test culture, while --●-- represents the control. The dark arrows indicate the close proximity of final pH achieved in the aerobic RM, compost tea RM, and wastewater RM test and control cultures.

Sequence analysis of selected DGGE bands

A total of eight DGGE bands were excised and sequenced. All sequenced bands showed at least 98% similarity to the 16S rRNA gene of known isolates from GenBank (Table 6.1). The phylogenetic relatedness of the sequenced community members are depicted in Fig. 3.10. Seven of the excised bands clustered within the *Firmicutes* phylum. One sequence (CDC-A), belonged to the *Bacteroidetes* phylum and was only present in pre-reduced anaerobic RM medium (Fig. 3.5). Several members showed a high level of relatedness to members of the *Clostridiaceae* family, specifically the *Clostridium* genus (*C. thermocellum, C. caenicola, C. cellulosi*). One sequence (CDC- G) was found to be very closely related to *Geobacillus thermoglucosidasius* and *Geobacillus stearothermophilus*, both of which are members of the *Bacilliaceae* family. Table 6.1, outlining the results of the NCBI BLAST algorithm on consortium member sequences, is included in Appendix B.

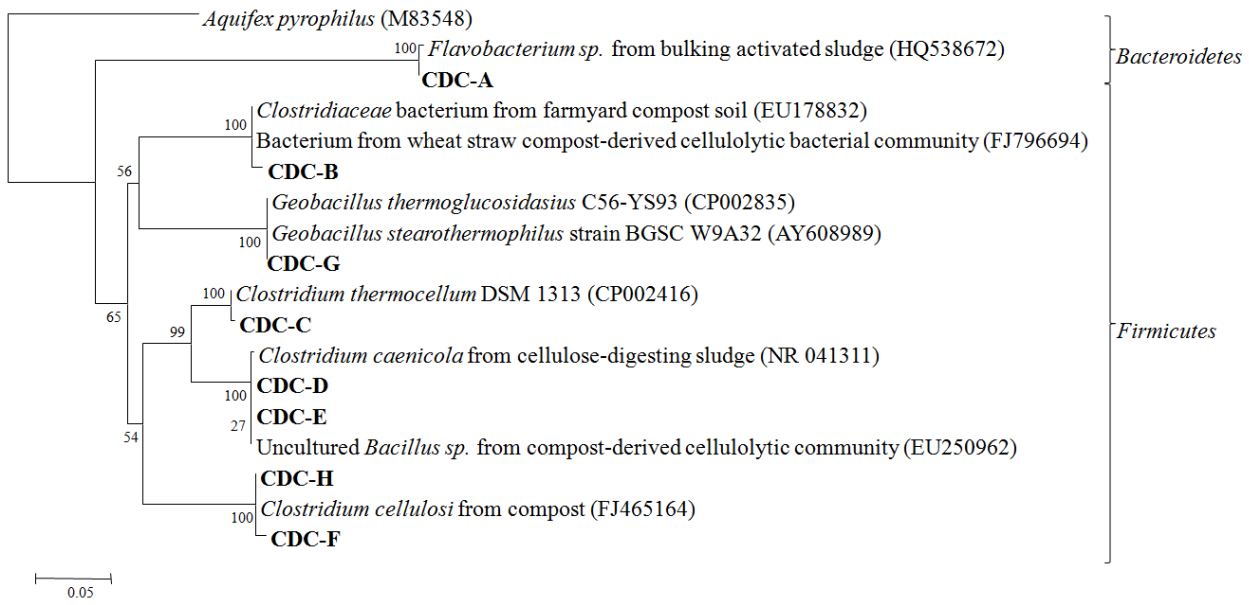

Fig. 3.10. Phylogenetic relationship of the 8 sequenced DGGE gel bands based on 16S rRNA similarity.

3.4 DISCUSSION

As fossil fuels become depleted, there is an undeniable need for alternative environmentally friendly energy sources. Currently, bio-ethanol is one of the most attractive potential long term replacements for fossil fuels. Efforts must focus on improving the cost effectiveness of its production, however, in order to make it economically competitive. Cellulosic ethanol production via consolidated bio-processing may be optimized and streamlined through the simplification of culture requirements, while maintaining stable, consistent microbial activity. In this study, we have enriched and characterized a natural bacterial consortium capable of cellulose hydrolysis and ethanol production in the absence of both reducing agent and yeast extract.

Culture enrichment is an effective means of selecting for natural bacterial consortia from larger and more diverse communities. It also substantially dilutes the nutrients present in the original inoculum, ensuring that the resulting consortium is stable and can subsist on the nutrients provided only by the medium, rather than carryover from a nutrient rich inoculum such as compost. Our DGGE results found that there was a high bacterial diversity in the original solid compost material (Fig. 3.1). The fact that it is rich in nutrients and can support a wide range of species makes it an ideal starting material from which to enrich a cellulose-degrading community (Entcheva et al., 2000; Rollins and Koenig, 2010). Through enrichment, this diversity was markedly reduced and species involved in cellulose hydrolysis became the major DGGE bands. As the consortium aged and nutrients became depleted, there was a shift in diversity, with some members likely entering a dormant phase. Likewise, a similar shift was observed by day 14 in both the aerobic RM (Fig. 3.3) and compost tea RM (Fig. 3.7) treatments.

Cellulose degradation by the consortium occurred only after the medium had turned from aerobic to anaerobic, suggesting the co-existence of aerobic or facultative anaerobic, as well as anaerobic community members. Similarly, results from Kato et al. (2004) and Wongwilaiwalin et al. (2010) suggested that efficient cellulose utilization by compost-derived mixed communities in aerobic media was accomplished through interactions between non-cellulolytic facultative anaerobes and cellulolytic anaerobes. Generally, the proposed explanation is that the facultative anaerobes respire in the initial stages and generate the anaerobic environment needed by the anaerobic cellulose-degraders. A similar interaction is likely responsible for the cellulose degrading activity of our stable consortium.

Once it was determined that the consortium demonstrated effective and consistent cellulose-degrading activity, a profile of its soluble end-products was established by inoculating it into aerobic RM medium and sampling the culture supernatant. Ethanol and acetate were the major fermentation products (Fig. 3.2), along with trace amounts of lactate, formate, and butyrate (Table 3.1). The fact that glucose and cellobiose was not detected in the culture supernatant (Table 3.1) suggests that the consortium was efficiently utilizing the hydrolysis products as they were being produced. Recently, Wang and Chen (2009) described that a lack of hydrolysate accumulation benefits the overall cellulolytic activity of the community because it negates cellulase inhibition.

Kato et al. (2004) saw a similar result when they compared the activity of a cellulolytic *Clostridium* in pure culture and co-culture with several aerobic and facultative anaerobic strains in aerobic media. They found that after ten days, the cellooligosaccharide concentration in the supernatant was approximately 40% lower in the co-culture as compared to the pure culture. This implies that the non-cellulolytic members of these cellulose-degrading communities may

actually be rewarded for their oxygen scavenging in the initial stages by gaining access to the cellulose-hydrolysis products in the later stages.

In aerobic RM medium, at the point of highest ethanol concentration (day 6), the ethanol:acetate ratio was approximately 1:1.59. *C. thermocellum* in batch culture has been shown to produce a ratio of 1:0.43 (Lynd et al., 1989). An important difference, however, is that *C. thermocellum* in pure culture requires strict anaerobic conditions, while this consortium was active in non-reduced aerobic media. Carere et al. (2008) indicated that the ethanol:acetate ratio of the consortium could be enhanced by adding acetate to the culture medium, shifting the metabolism of members away from acetate formation and toward ethanol production. Acetate does represent a value-added product in itself, however, as it is a common precursor used in many household and industrial products and materials.

Since fermentation is an anaerobic process, and the cellulolytic activity of the consortium was most prevalent after the medium had turned anaerobic, the performance of the community was assessed in pre-reduced anaerobic conditions. Again, our results showed that ethanol and acetate were the major soluble fermentation products (Fig. 3.4), although their accumulation was reduced compared the levels observed in aerobic media (Fig. 3.2). By day 14, ethanol had reached a concentration of only 8.6 mM, resulting in an ethanol:acetate ratio of 1:2.21. In the aerobic treatment, however, 12.04 mM of ethanol was reached after just 6 days, resulting in a ratio of 1:1.59. This result suggests that a pre-reduced environment did not improve the overall performance of the consortium, and that culturing in non-reduced aerobic media might actually enhance its cellulose-degrading and ethanol-producing activities. This interesting property may have arisen as a result of the enrichment process. Since all enrichments were carried out under initially aerobic conditions, the consortium may have been conditioned to tolerate fluctuating

oxygen levels, such that it has adapted to actually perform better in aerobic media. Additionally, this result also suggests that in aerobic media, the contribution of aerobic or facultative anaerobic members likely goes beyond simply providing an anaerobic environment. This notion is supported by the fact that there was a considerable shift in community structure within the first 4 days of culturing, with several bands from the consortium no longer present (Fig. 3.5). The aerobic and facultative anaerobic members likely indirectly enhance the cellulolytic activity of the consortium by providing growth factors, consuming inhibitory metabolites, or neutralizing pH (Kato et al., 2004; Wongwilaiwalin et al., 2010).

Interestingly, substantial ethanol and acetate production was detected in the cellulose-free control cultures in anaerobic media (Fig. 3.4), while very little accumulation was observed in aerobic media (Fig. 3.2). It therefore appears that in non-reduced aerobic media, the carbon sources within yeast extract are required by the aerobes and facultative anaerobes in order to respire and generate anaerobic conditions. In pre-reduced anaerobic media, however, yeast extract is not needed for this aerobic metabolism, and is thus subject to fermentation by anaerobic community members.

Yeast extract is a rich source of carbon, nitrogen, amino acids, and vitamins, and is almost unanimously the complex nutrient source used in the cultivation of cellulolytic bacteria in aerobic media (Haruta et al., 2002; Kato et al., 2004; Miyazaki et al., 2008; Wongwilaiwalin et al., 2010). Its use in industrial scale consolidated bioprocessing may be unfeasible and uneconomical, however, as its preparation represents extraneous steps, resources, and costs (Maddipati et al., 2011). Since one of the aims of consolidated bioprocessing is to progress away from yeast-based fermentation (Lynd et al., 2005), the continued use of yeast extract to culture CBP microorganisms may be counter-productive.

The consortium was cultured in media lacking yeast extract, but supplemented with other more inexpensive and abundant waste-derived complex nutrient sources. Since compost was the initial source of inoculum, it was expected that the nutrients within it would effectively sustain the community members in the absence of yeast extract. Compost tea (the water-soluble portion of the compost) contains the soluble nutrients found within compost and is commonly used by gardeners as a means of providing plant nourishment. In the compost tea medium experiment, the consortium again produced ethanol and acetate as the major fermentation products (Fig 3.6). The maximum ethanol concentration (9.88 mM) was slightly lower than that peak value in regular aerobic RM medium (12.04 mM), although it actually occurred two days earlier. The ethanol:acetate ratio on day 4 was approximately 1:0.86, suggesting that the consortium was actually producing more ethanol than acetate at this point. This ratio is higher than the one achieved in aerobic RM medium (1:1.59) and anaerobic RM medium (1:2.21), and is closer to the ratio reportedly achieved by *C. thermocellum* in batch culture, which was 1:0.43 (Lynd et al., 1989).

Although an effective replacement for yeast extract, compost tea is not entirely readily available and its preparation still requires some energy-consuming steps. Therefore, it was important to explore the use of an even more abundant, inexpensive, and simple to prepare nutrient source. Wastewater is a plentiful resource and its use in the production of value-added products such as bio-ethanol would be particularly beneficial. In aerobic wastewater RM medium, the structure of the consortium remained almost unchanged throughout the 14 day culturing period. The fact that no bands disappeared between days 4 and 14 implies a rich nutrient composition in the medium. While some members were no longer present by day 14 in aerobic RM (Fig. 3.3) and aerobic compost tea RM media (Fig. 3.7), no such drop in community

diversity was observed in this medium. Assuming the ethanol-producing activity of the consortium persisted as expected (data pending- see Appendix A), wastewater appears to be a good candidate for use as a complex nutrient source in large scale CBP bio-ethanol production.

The pH profile in the test cultures in all three aerobic media (RM, compost tea, wastewater) was strikingly similar (Fig. 3.9). Despite starting at different initial pHs, each test culture reached a minimum of 6.1 to 6.2 within 6 to 9 days, and ended the experiment at 6.4. Interestingly, this is very similar to the pH profile of *C. thermocellum* in batch culture (Freier et al., 1988). This result, coupled with the end-product data speaks to the versatility of the consortium. Whether yeast extract, compost tea, or wastewater was used as the complex nutrient source in aerobic media, the end-product and pH profiles remained consistent.

Although a seemingly simple idea, few studies have explored the feasibility of replacing yeast extract with more abundant, inexpensive, and economical complex nutrient sources for the production of bio-ethanol. Gao and Li (2011) demonstrated that anaerobic digestate effluent can be used as a complex nutrient source for a strain of ethanol-producing yeast. Similarly, Maddipati et al. (2011) revealed that corn steep liquor is an effective replacement for yeast extract in syngas fermentation using *Clostridium* strain P11. These results, along with our data, suggest that the activity of ethanol-producing microorganisms is not significantly affected when yeast extract is replaced by other more abundant waste-derived complex nutrient sources. This is significant since it may circumvent the need for yeast extract in large scale bio-ethanol production, thereby simplifying the industrial process and reducing process-associated costs.

Several bands from the stable consortium were excised and sequenced (Fig. 3.10). CDC-B showed 99% sequence similarity to a *Clostridiaceae* bacterium initially isolated from farmyard compost soil (Kim et al., 2008). Since agricultural residues are a rich source of lignocellulosic

biomass, this member likely contributed to the cellulolytic activity of the consortium in some way, although an exhaustive analysis of this bacterium has not been performed.

The member CDC-C showed 99% similarity to a strain of *C. thermocellum*, one of the most widely studied cellulolytic bacteria. This organism utilizes cellulose and produces ethanol, acetate, and hydrogen as its major fermentation products. The fact that *C. thermocellum* is a cellulose hydrolysis specialist, exhibiting one of the fastest known rates of cellulose utilization (Lu et al., 2006), suggests that the member designated CDC-C is likely one of the key cellulose-degraders within the consortium.

CDC-D and CDC-E both exhibited a high similarity to *Clostridium caenicola*, a flagellated, spore-forming anaerobe initially isolated from the anaerobic sludge of a cellulose-degrading methanogenic bioreactor (Shiratori et al., 2009). Although non-cellulolytic, this microorganism can use the main products of cellulose hydrolysis (glucose and cellobiose) to produce hydrogen, carbon dioxide, acetate, lactate, and ethanol (Shiratori et al., 2009).

The members labelled CDC-H and CDC-F both exhibited a high similarity to *Clostridium cellulosi*, an anaerobic cellulolytic bacterium initially isolated by Yanling et al. (1991) from cow manure compost. Aside from cellulose itself, *C. cellulosi* can utilize a wide range of carbon sources, including cellobiose, glucose, and xylose. Its major fermentation products are hydrogen, carbon dioxide, acetate, and ethanol (Yanling et al., 1991).

CDC-A showed a high level of similarity to a *Flavobacterium* species (phylum *Bacteroidetes*) initially isolated from bulking activated sludge. Interestingly, this member was most prevalent in the anaerobic treatment and was the only sequenced consortium member closely related to a gram negative species.

The presence of band G was most pronounced at day 4 in aerobic RM and aerobic compost-tea RM media. This member was found to belong to the *Geobacillus* genus, and is very closely related to the facultative anaerobes *G. thermoglucosidasius* and *G. stearothermophilus*. Although more research is needed to fully elucidate the roles played by the various members of the consortium, the member CDC-G is likely one of the key oxygen scavengers, since it respires in the presence of oxygen. This would account for its pronounced presence in the earlier stages of culturing in aerobic media.

Although *G. thermoglucosidasius* and *G. stearothermophilus* are non-cellulolytic, they can utilize the main products of cellulose hydrolysis (cellobiose and glucose) to produce lactate, formate, acetate, and ethanol (Banat et al., 2004; Cripps et al., 2009; Fong et al., 2006). *G. thermoglucosidasius* specifically has garnered attention as a good candidate for use in bio-ethanol production due to its high ethanol tolerance, which is approximately 10% (Taylor et al., 2009). Cripps et al. (2009) recently reported three genetically modified strains of *G. thermoglucosidasius* that were optimized to achieve ethanol yields of 90% theoretical.

Seven of the eight sequenced consortium members fell within the *Firmicutes* phylum (Fig. 3.10). Six members showed a high degree of similarity to members of the *Clostridiaceae* family, specifically the *Clostridium* genus. This implies a certain level of functional redundancy within the consortium. Based on the sequencing data, there were at least two members closely related to species with cellulose-degrading properties (*C. cellulosi* and *C. thermocellum*), while at least six members showed high similarity to known ethanol producers (Shiratori et al., 2009, Yanling et al., 1991 Cripps et al., 2009; Carere et al., 2008). This most likely contributed to the observed versatility and robustness of the consortium. Its cellulolytic and ethanologenic

properties were maintained in media with varying oxygen and nutrient compositions, despite minor shifts in community structure.

Conclusions

As fossil fuel reserves become depleted, there is a growing need for alternative, environmentally friendly energy sources. Biofuels such as bio-ethanol may well be one of the most promising solutions. If it is to become a viable alternative to petroleum-based fuels, however, efforts must focus on consolidating, streamlining, and simplifying its production in order to maximize its cost effectiveness. Currently, reducing agents and yeast extract are two of the most costly media components used to culture ethanol-producing microorganisms (Maddipati et al., 2011). In this study, we have enriched and characterized a robust and versatile mixed bacterial consortium capable of cellulosic ethanol production in yeast extract-free, non-reduced aerobic media, using compost tea or wastewater as the complex nutrient source.

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

In this study, results suggested that effective cellulose utilization by mixed communities in aerobic environments appears to be largely achieved through interactions between non-cellulolytic, cellulolytic, aerobic, and anaerobic species. In aerobic media, a complex nutrient source seems to be required in order to generate anaerobic conditions, although results indicated that this does not necessarily need to be yeast extract. According to the data, the use of other nutrient-rich, waste-derived substances is not only a viable alternative to yeast extract, it may even enhance the ethanol:acetate ratio achieved by the consortium. Despite minor changes in community structure, the consortium was found to be fairly robust and versatile in terms of its activity in the various growth conditions. The fact that several members appeared belong to the same genus, might suggest that a certain functional redundancy exists within the consortium, a property not often found in pure culture systems.

Several of the sequenced community members showed a high level of relatedness to known hydrogen producers (*C. thermocellum*, *C. cellulosi*, *C. caenicola*), suggesting the consortium may be capable of hydrogen production. Future work therefore should focus on analyzing its gaseous end-products in order to confirm this hypothesis. Like ethanol, hydrogen is a promising biofuel and its presence would add to the overall value of the consortium.

Cellulose is most often found in nature in the form of lignocellulose, which contains hemicellulose, lignin, as well as cellulose. Therefore, future work should include assessing the consortium's ability to utilize hemicellulose, as well as pretreated lignocellulosic material such as wood. It would also be useful to repeat some of the experiments using non-sterilized cellulosic substrates and complex nutrient sources. This would illustrate if, and to what extent, the activity of the consortium is affected by the presence of competing contaminating

microorganisms. Since sterilization of large amounts of lignocellulosic biomass may not be feasible on a large scale, the ability to effectively utilize non-sterilized substrates would improve the industrial readiness of consortium.

It would also be very interesting to examine the activity of a synthetic culture constructed from community isolates or purchased ATCC™ strains matching the sequenced consortium members. Assuming the cellulolytic and ethanologenic activity of the consortium persists, co-cultures of the various members can be performed in order to begin characterizing the role(s) played by each. Such work could eventually lead to the construction of a "designer consortium" in which the composition and proportion of the members can be optimized in order to maximize ethanol production.

The ultimate success of biofuels depends heavily on their ability to compete economically with fossil fuel-based energy sources. Therefore, in biofuel research, it is crucial to continuously assess the feasibility of scaling-up the system, method, or process in question in order to ensure only the most simplified, streamlined, and cost-efficient avenues are pursued.

CHAPTER 5: APPENDICES

Appendix A

Regarding the end-product analysis of aerobic wastewater RM medium samples

The stable consortium was inoculated into aerobic wastewater RM medium and sampled over two weeks. Analysis of the soluble end-products is currently being performed by our collaborators at the University of Manitoba in the lab of Dr. Richard Sparling. At the time of final printing, however, this analysis had not yet been completed.

Appendix B

Results of NCBI BLAST algorithm on consortium member sequences

Table 6.1. Closest match between consortium members and GenBank isolates.

Name	Sequence	Closest isolate	%	Phylum
CDC-A	cctacgggaggcagcagtgaggaatattggtcaatggtcgcaagactgaaccagccatgccgc gtgcaggaagacggtcctatggattgtaaactgcttttatacaggaagaaaccctcccacgtgtgg gagcttgacggtactgtaggaataaggatcggctaactccgtgccagcagccgcggtaatacgg aggatgcaagcgttatccggaatcattggntttaaagggtccgtaggcggtcttataagtcagtggt gaaagcccatcgctcaacgatggaactgccattgatactgtaagacttgaatgcttaggaagtaac tagaatatgtagtgtagcggtgaaatgcttagatattacatggaataccaattgcgaaggcaggtta ctactaagtgattgacgctgatggacgaaagcgtggggagcgaacaggattagataccctggta g	*Flavobacterium* sp. bk_54 16S ribosomal RNA gene, partial sequence	99	*Bacteroidetes*
CDC-B	cctacgggaggcagcagtgggggatattgcacaatgggggaaaccctgatgcagcgacgccg cgtgagcgaagaaggccttcgggtcgtaaagctctgtcctaagggaagataatgacggtacctta ggaggaagccccggctaactacgtgccagcagccgcggtaaaacgtagggggcgagcgttgt ccggaattactgggcgtaaagggtgcgtaggcggctatttaagtcagatgtaaaaggctccgggt caaccgtagtgagaatttgaaactgaatagcttgagtcaaggagagggaagtggaattcctggtgt agcggtgaaatgcgtagatatcaggaggaataccagtggcgaaggcgacttcctggccttagact gacgctgaggcacgaaagcgtggggagcaaacaggattagataccctggtag	*Clostridiaceae* bacterium Aso3-CS341 16S ribosomal RNA gene, partial sequence	99	*Firmicutes*
CDC-C	cctacgggaggcagcagtggggaatattgcgcaatgggggaaaccctgacgcagcaacgccg cgtgaaggaagaaggccttcgggttgtaaacttctttnattggggacgaaggaagtgacggtacc caaagaacaagccacggctaactacgtgccagcagccgcggtaatacctaggtggcgagcgtt gtccggaattactgggtgtaaagggcgcgtaggcggggatgcaagtcagatgtgaaattccggg gcttaaccccggcgctgcatctgaaactgtatctcttgagtgctggagaggaaagcggaattccta gtgtagcggtgaaatgcgtagatattaggaggaacaccagtggcgaaggcggctttctggacagt aactgacgctgaggcgcgaaagcgtggggagcaaacaggattagataccctggtag	*Clostridium thermocellum* DSM 1313, complete genome	99	*Firmicutes*
CDC-D	cctacgggaggcagcagtggggaatattgcgcaatgggggaaaccctgacgcagcgacgccg cgtgaaggaagaaggccttcgggttgtaaacttctttgatcggggacgaaggaagacggtaccc gaggaacaagccacggctaactacgtgccagcagccgcggtaatacgtaggtggcgagcgttg tccggaattactgggcgtaaagggcgtgtaggcgggggggacaagtcagatntgaaataccgca gcttaactgcggggctgcatctgaaactnttcttcttgagtgtcggagaggaaagnggaattccca gtgtagcggtgaaatgcgtagagattgggaggaacaccagtggcgaaggcggctttctggacga caactgacgctgaggcgcgaaagcgtggggagcaaacaggattagataccctggtag	*Clostridium caenicola* strain EBR596 16S ribosomal RNA, partial sequence	99	*Firmicutes*
CDC-E	cctacgggaggcagcagtggggaatattgcgcaatgggggaaaccctgacgcagcgacgccg cgtgaaggaagaaggccttcgggttgtaaacttctttgatcggggacgaaggaagacggtaccc gaggaacaagccacggctaactacgtgccagcagccgcggtaatacgtaggtggcgagcgttg tccggaattactgggcgtaaagggcgtgtaggcgggggggacaagtcagatgtgaaataccgca gcttaactgcggggctgcatctgaaactgttcttcttgagtgtcggagaggaaagtggaattccca gtgtagcggtgaaatgcgtagagattgggaggaacaccagtggcgaaggcggctttctggacga caactgacgctgaggcgcgaaagcgtggggagcaaacaggattagataccctggtag	*Clostridium caenicola* strain EBR596 16S ribosomal RNA, partial sequence	98	*Firmicutes*
CDC-F	cctacgggaggcagcagtgggggatattgcacaatggaggaaactctgatgcagcgacgccgc gtgagggaagaaggtcttcggattgtaaacctctgtctttcgggacgaaggaagtgacggtaccg aaagaggaagccacggctaactacgggccagcagccgcggtaatacctaggtggcgagcgttg tccggaattactgggtgtaaagggggcgtangcgggttgtcaagttggatgtgaaatctctgggct taactcagaggttgcattcaaaactggcgatcttgagtgaggtagaggcaggcggaattcccggt gtagcggtgaaatgcgtagatatcgggaggaacaccagtggcgaaggcggcctgctgggcctt aactgacgctgaggcacgaaagcatggggagcaaacaggattagataccctggtag	*Clostridium cellulosi* strain D3 16S ribosomal RNA gene, partial sequence	99	*Firmicutes*
CDC-G	cctacgggaggcagcagtagggaatcttccgcaatggacgaaagtctgacggagcgacgccgc gtgagcgaagaaggtcttcggatcgtaaagctctgttgttagggaagaagaagtgccgttcgaac agggcggcgcggtgacggtacctaacgagaaagccccggctaactacgtgccagcagccgcg gtaatacgtagggggcgagcgttgtccggaattattgggcgtaaagcgcgcgcaggcggtccct taagtctgatgtgaaagcccacggcttaaccgtggagggtcattggaaactgggggacttgagtg cagaagaggagagcggaattccacgtgtagcggtgaaatgcgtagagatgtggaggaacacca gtggcgaaggcggctctctggtctgtaactgacgctgaggcgcgaaagcgtggggagcaaaca ggattagataccctggtag	*Geobacillus thermoglucosidasius* C56-YS93, complete genome	100	*Firmicutes*
CDC-H	cctacgggaggcagcagtgggggatattgcacaatggaggaaactctgatgcagcgacgccgc gtgagggaagaaggtcttcggattgtaaacctctgtctttcgggacgaaggaagtgacggtaccg aaagaggaagccacggctaactacgtgccagcagccgcggtaatacgtaggtggcgagcgttg tccggaattactgggtgtaaagggtgcgtaggcgggttgtcaagttggatgtgaaatctctgggctt aactcagaggttgcattcaaaactggcgatcttgagtgaggtagaggcaggcggaattcccggtg tagcggtgaaatgcgtagatatcgggaggaacaccagtggcgaaggcggcctgctgggcctta actgacgctgaggcacgaaagcatggggagcaaacaggattagataccctggtag	*Clostridium cellulosi* strain D3 16S ribosomal RNA gene, partial sequence	100	*Firmicutes*

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