1-1-2011

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CULTIVATION OF A CELLULOLYTIC MICROBIAL COMMUNITY UNDER CONTINUOUS FLOW CONDITIONS

By

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Bachelor of Science
Biology
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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Cultivation of a cellulolytic microbial community under continuous flow conditions

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ABSTRACT

A stable microbial consortium capable of cellulose hydrolysis under initial aerobic conditions has been enriched in a continuous flow system. Anaerobic conditions were created and maintained within the flow system using only microbial metabolism. A DGGE community profile of samples taken throughout the enrichment process indicates that the community stabilized upon entry into the continuous flow system, and maintained function and members throughout. The effect of flow rate on cellulose degradation and end product production was assessed. The cellulose degradation rate was found to be notably faster at a flow rate of 0.1 ml/min (*D* 0.75/hr) than a flow rate of 0.2 ml/min (*D* 1.50/hr). Furthermore, an ethanol to acetate ratio nearly 8 times higher than previously reported values in the literature was also observed at 0.1 ml/min (*D* 0.75/hr). With reduced flow rate and high ethanol to acetate ratios, the enriched community may be well suited for consolidate bioprocessing.

ACKNOWLEDGEMENTS

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First and foremost I would like to thank my supervisor, Dr. Gideon Wolfaardt, for giving me continued guidance and support as well as the opportunity to complete my thesis under his supervision. As well, I give special thanks to Dr. William Yeung, whose expertise and patience greatly aided in the completion of my thesis. Furthermore, I would like to thank my committee members, Dr. Martina Hausner and Dr. Kimberly Gilbride, whose input was insightful and much appreciated. In addition, the contribution of Dr. Richard Sparling and Dr. John Schellenberg for their help with end product analysis was much appreciated. I would like to extend thanks to all the Wolfaardt and Hausner lab members for all the help they provided to me over the years. And finally, I give thanks to my family and friends, for their unwavering support.

This research was supported by the contributions from the Agricultural Bioproducts Innovation Program, the Canada Research Chair Program, Genome Canada, Mascoma Corp, and NSERC.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CBP	Consolidated bioprocessing
CE	Cellulose-enzyme complex
CEM	Cellulose-enzyme-microbe complex
DGGE	Denaturing gradient gel electrophoresis
EPS	Extracellular polymeric substance
GFP	Green fluorescent protein
HPLC	High performance liquid chromatography
PCR	Polymerase chain reaction
SHF	Separate hydrolysis and fermentation
SSCF	Simultaneous saccharification and co fermentation
TAE	Tris base acetic acid ethylenediaminetetraacetic acid

CHAPTER 1: INTRODUCTION

Research to produce sustainable ethanol has been in progress for several decades, and ethanol has been established as a biofuel. However, until gasoline has been completely depleted, it seems as if there will still be reluctance to make the jump to renewable biofuel for several reasons (Lynd et al., 2009). For one, gasoline contains more energy than its renewable competitors such as hydrogen, bio-diesel, and bio-ethanol, and has an extensive infrastructure in place. As a result, for a renewable alternative to be a viable option it must be able to be produced at a price that is competitive with gasoline, and ideally, be compatible with the current infrastructure. The negative environmental impacts of burning gasoline have been noticed globally, and have helped the shift towards environmentally friendly carbon neutral fuels such as bio-ethanol.

Bio-ethanol derived from plants has traditionally been produced from food crops such as sugar cane and corn; however, the industry is undergoing a shift towards using non food crops such as switchgrass, and agricultural waste such as corn stover (Inderwildi and King, 2008; Schmer et al., 2008). Both of these potential feed stocks are cost effective, environmentally friendly to produce, and abundant (Lynd et al., 2005; Lynd et al., 2008). Bio-ethanol offers several advantages over other potential bio-fuels, such as being compatible with the current gasoline infrastructure and the automotive industry. It can also be mixed (up to 10%) with gasoline in current automobiles, and has even prompted the production of 'E85' vehicles, which can run with up to 85% ethanol (Smith & Workman, 2004).

Conventionally, industrial production of ethanol has been a four step process of pre-treatment (where cellulose is separated from the un-usable lignin), hydrolysis (where the cellulose is broken down into simpler sugars using purified enzymes), fermentation (where the

sugars are converted into desirable end products by fermentative organisms such as yeast), and distillation (where the end products are purified and separated)(Schwarz, 2001; Leschine, 1995; Wang & Chen, 2009; Moiser et al., 2004). However research by Lynd (1996) focuses on consolidating the hydrolysis and fermentation steps to be more efficient, resulting a process called *consolidated bioprocessing* (CBP). In CBP, enzymes and yeast are replaced with a bacterial pure culture or a mixed community, capable of hydrolysis and fermentation (Lynd et al., 2005).

Cellulose degradation can be performed by a number of microorganisms either in pure culture or mixed communities. Pure cultures, such as *Clostritium thermocellum* (an anaerobic thermophile) have been the focus of many research papers because of their ability to efficiently hydrolyse and ferment cellulose to value added end products such as ethanol, making them an excellent candidate for CBP (Lovitt et al., 1984; Lynd et al., 2007). Bioengineering strains of *C. thermocellum* to have higher ethanol tolerance, or increased end product yield has also been the focus of recent publications (Lynd et al., 2005). However, pure culture anaerobes are often limited to specific growth conditions and any deviation can result in a population crash (Brenner et al., 2008). Furthermore, there are often added costs to create and maintain anaerobic conditions necessary for anaerobic pure culture growth. Mixed communities on the other hand have not been as well characterized as pure cultures, but recent findings by Kato et al. (2004) have revealed that they may be able to hydrolyze cellulose as efficiently as pure culture anaerobes without the associated costs. Mixed communities are also generally more robust and able to tolerate fluctuating environmental conditions (Brenner et al., 2008). With this in mind, it appears as if the scenario for cost effective ethanol production may be to achieve anaerobic

hydrolysis (like that of *C. thermocellum*) in an initially aerobic medium using a mixed community.

The current study (i) investigated the ability of a mixed community enriched from compost, to hydrolyse cellulose in a continuous flow system under initially aerobic conditions, and (ii) evaluated the end products being produced.

1.1 Hypothesis and Objectives

Consolidated bio-processing using pure culture anaerobes, such as *C. thermocellum*, may require costs for creating an anaerobic environment, and may result in low end product yield due to end product inhibition. It was hypothesized that an enriched cellulolytic consortium, capable of creating anaerobic pockets facilitating attached anaerobic hydrolysis under initial aerobic conditions can be enriched and maintained with the use of exogenous O_2 scavengers.

The study focused on three objectives:

1. To conceptualize and design a continuous flow system which would be used to enrich for an attached phase cellulolytic mixed consortium capable of producing anaerobic conditions from aerobic medium, and be able to hydrolyse cellulose.
2. To use the flow system to enrich and cultivate a stable cellulolytic consortia.
3. To identify if desirable end products such as ethanol are being produced.

CHAPTER 2: LITERATURE REVIEW

2.1 – Alternative Fuels

With the supply of gasoline steadily declining, a search for environmentally friendly and sustainable alternative fuels has been underway for more than 25 years (Lynd et al., 2005). There are several biofuels that demonstrate potential, such as hydrogen, biodiesel, and bioethanol derived from cellulosic biomass, which is the focus of this study. Cellulosic biomass is abundant as it only requires the sun's energy to grow, while it does not form a major part of human diet. It is a major component of agricultural waste and has limited industrial uses (Lynd et al., 2002; Wood & Ingram., 1992). The idea of converting plants into ethanol has been around for over 100 years, and over this period the literature surrounding the topic has grown. There is no worldwide standard in the industrial conversion of cellulosic biomass into ethanol (Lynd et al., 2002). This is due primarily to the costly delignification and distillation steps, as well as the general recalcitrance of cellulose (Lynd et al., 2002; Wang & Chen, 2009; Moiser et al., 2005). The ideal long term goal is to efficiently convert cellulosic waste, which for the most part is discarded, into a renewable sustainable fuel source (Lynd et al., 2005; Eveleigh et al., 1983; Hill., 2007). Cellulosic ethanol as an alternative fuel would not just be greener to manufacture, but also greener to combust, with lower levels of carbon monoxide, carbon dioxide, and green house gas emissions being produced (US Department of Energy, N.D).

2.2 – Cellulose

Cellulose is one of the most abundant organic compounds on earth, and each year over 10^{11} tonnes are naturally produced (Leschine, 1995). At its core, it is comprised of glucose molecules

joined by β 1 – 4 bonds, and grouped in pairs is called cellobiose. Chains larger than two glucose molecules are called cellulose microfibrils, and groups of microfibrils are called macrofibrils (Fig. 2.1). Since it is environmental friendly to produce, and is a major waste product, lignocellulosic biomass presents itself as an ideal candidate as feedstock for alternative fuel production (Lynd et al., 2005).

Cellulosic biomass is comprised of three main components; lignin, cellulose, and hemicellulose. Hemicellulose is composed of several different five and six carbon sugars such as xylose and arabinose, which are more susceptible to hydrolysis. Cellulose on the other hand is only comprised of the hexose sugar glucose, and is arranged in a linear fashion. Cellulose is also much stronger and resistant to hydrolysis than hemicellulose (contributing to its recalcitrance). Lignin is the protective sheath which surrounds both cellulose and hemicellulose, and is difficult to degrade (Leschine, 1995; Mosier et al., 2005). Once cellulose has been liberated from lignin however, its hydrolysis releases simple sugars which can be fermented into desirable end products such as ethanol and acetate (Moiser et al., 2005).

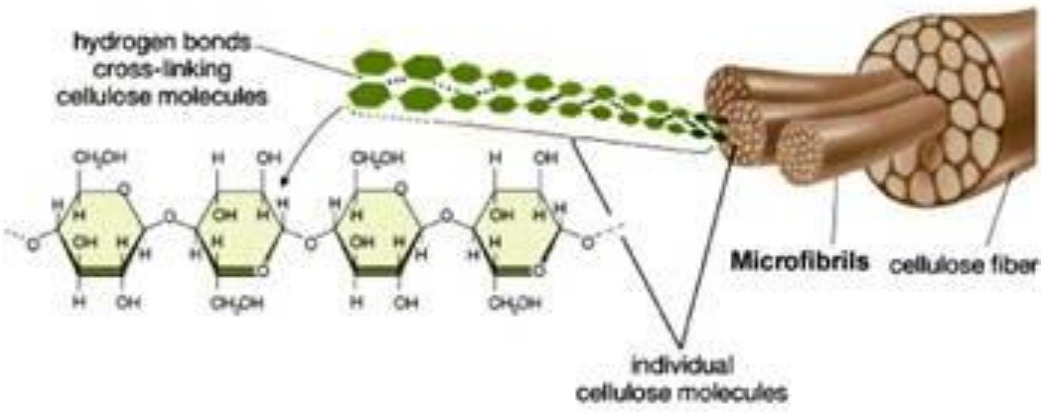


Figure 2.1: Cellulose structure (LSU, 2006)

2.3 – Bioprocessing

Bioprocessing is a method of energy production, utilizing readily available cellulose sources such as plant biomass (Lynd et al., 2005). Though this is not a new idea, novel steps are being taken to optimize the process using biotechnology (Lynd et al., 2005). Bioprocessing of lingnocellulosic biomass is conventionally composed of four steps: delignification, hydrolysis, fermentation, and distillation. Delignification is the removal of lignin, which is an organic polymer that surrounds and protects cellulose. Its removal is usually facilitated by acidification or steam-explosion, which are often costly processes (Fig. 2.2) (Moiser et al., 2004; Datar et al., 2007; Alvira et al., 2009).

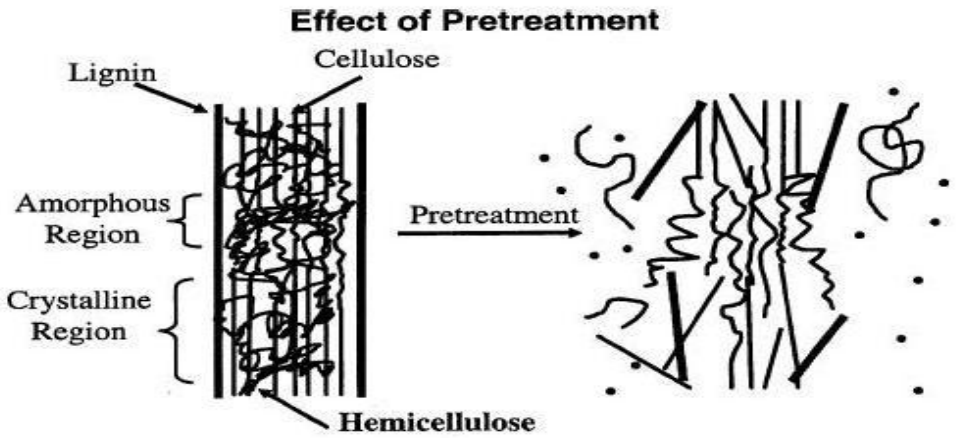


Figure 2.2: Effect of Pre-treatment (Moiser et al., 2005)

Cellulose hydrolysis is the liberation of five or six carbon simple sugars from plant biomass and is traditionally done using celluloytic enzymes, which can be either extracellular or cell associated (Schwarz, 2001; Leschine, 1995). Fermentation refers to the conversion of glucose molecules by microorganisms into the desired products such as ethanol or hydrogen. Distillation refers to the separation and purification of desired products, and is a costly step (Kato et al., 2004). Currently there are several different methods of bioprocessing that all have the same end results. Examples include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP)(Fig. 2.3).

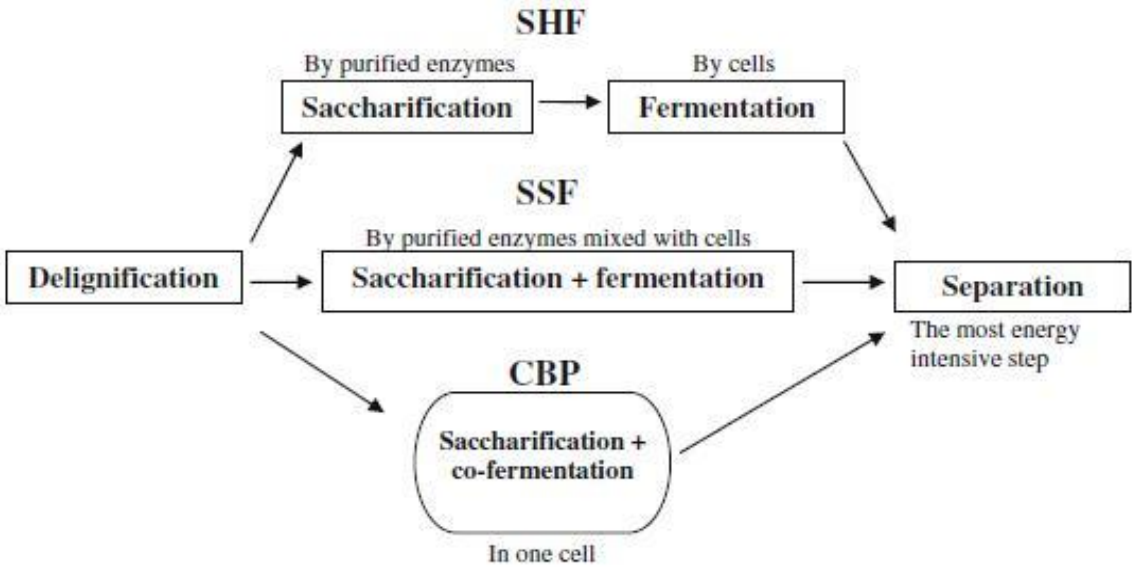


Figure 2.3: Multiple Bioprocessing Procedures(Wang & Chen, 2009)

In a separate hydrolysis and fermentation (SHF) setup, all four steps mentioned above are done separately, each in a within its own reactor (Moiser et al., 2004). Purified enzymes are used to hydrolyze the cellulose, and fermentative organisms are used to ferment the hydrolysis products into desired end products. Simultaneous saccharification and fermentation (SSF) is similar to SHF, but the saccharificaiton (hydrolysis) and fermentation steps are combined into a single reactor (Lynd et al., 2005; Kato et al., 2004; Moiser et al., 2004). Purified enzymes are added with fermentative microorganisms, reducing reactor costs, and increasing efficiency (Lynd et al., 2005). A study by Öhgren et al. (2007) indicates that SSF gives a 13% higher ethanol yield when compared to SHF.

With simultaneous saccharification and co-fermentation (SSCF) both cellulose and hemicellulose are hydrolysed. This process also includes the fermentation of both glucose and

xylose (Moiser et al., 2004). This can be carried out by genetically modified microorganisms that are able to ferment both hexose and pentose sugars (Moiser et al., 2004).

Consolidated bioprocessing (CBP) presents itself as an attractive option for ethanol production as it is simpler, more efficient, and more cost effective than both SHF and SSF (Lynd et al., 2005). CBP combines the production of cellulases, substrate hydrolysis, and fermentation into one step (Carere et al., 2008; Lynd et al., 2005). Combining all these steps eliminates the need for purified enzymes, as well as the heating and sterilizing of separate reactors (Moiser et al., 2004). Furthermore, when cellulose degrading microorganisms interact directly with cellulose, they create a cellulose-enzyme-microbe (CEM) complex which is more efficient at hydrolyzing cellulose than purified enzymes (Lynd et al., 2005). Although CBP has been projected to be nearly twice as efficient than other processes in terms of raw material utilization, cost, and equipment requirements, it has not yet been implemented on an industrial scale.

2.3.1 – Continuous flow and batch systems

Flowcells are lab scale continuous flow systems that allow for growth and cultivation of microorganisms (Fig 2.4). Flowcells have several advantages over growing cultures in batch, which is generally used for microbial growth and cultivation. The continuous flow of medium provides a constant source of nutrients, which allows for prolonged periods of sustained growth. In batch systems conversely, nutrients can become completely depleted. Furthermore, the accumulation of potentially harmful end products that can lead to cell death may be avoided (Lynd et al., 2005). Another advantage of continuous flow systems is the potential to select for

attached phase organisms which are not washed out with planktonic cells. With that said, flowcell preparation and setup can often require larger amounts of medium, additional time for sterilization and rinsing, and are often susceptible to tubing leaks, which can results in lost time. Batch cultures however are not affected by these parameters, and can allow for testing of a large numbers of variables in a considerably shorter time. In addition, batch culture allows for a full growth cycle of microorganisms to occur as nutrient levels are depleted over time. A disadvantage of batch systems, however, is the accumulation of inhibitory metabolites.

A chemostat is another example of a continuous flow system. They generally contain larger volumes than flowcells (>10 ml), and may provide stirring/agitation by propeller/impeller or by bubbling, which is not possible in a flowcell (Fig 2.5)(Waites et al., 2001). These systems, however, are generally much more complicated than flowcells, and may require trouble shooting to optimize the process.

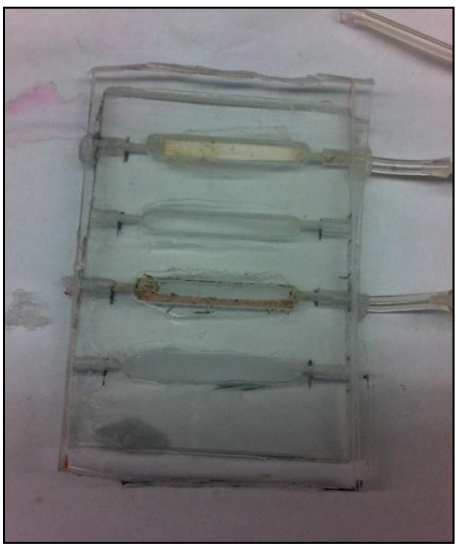


Figure 2.4: Flowcell

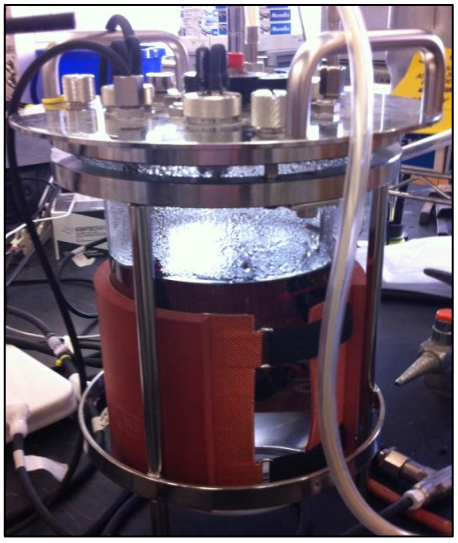


Figure 2.5: Bioreactor

2.4 – Celluloytic Bacteria

Naturally occurring cellulolytic microorganisms are capable of hydrolyzing crystalline cellulose into simple sugars, which can then be fermented into desirable end products such as ethanol. In this process, long glucose chains called microfibrils are broken down into cellobiose and glucose, which are then fermented into end products (Buchana et al., 2000). Cellulose, however, cannot be hydrolyzed by a single enzyme, but rather requires several enzymes working together (Schwarz, 2001).

2.4.1 – Aerobic Hydrolysis

Aerobic cellulolytic microorganisms have been found to produce all the enzymes required to break down cellulose, but secrete them extracellularly at high amounts (Schwarz, 2001). This form of breakdown can be inefficient, as the enzymes are produced in bulk, and are not as effective as cell-bound enzymes (Lynd et al., 2005; Schwarz, 2001).

2.4.2 – Anaerobic Hydrolysis and the Cellulosome

Some anaerobic microorganisms do not secrete their cellulolytic enzymes, but rather have evolved to possess efficient cell bound enzyme complexes known as cellulosomes (Fig 2.6). The cellulosome is comprised of a complex grouping of cell-bound proteins, which work together to efficiently degrade cellulose, and are usually found on the cell surface of anaerobic microorganisms capable of cellulose hydrolysis (Schwarz, 2001). These extracellular enzymes facilitate tight binding to the target substrate, forming cellulose-enzyme-microbe (CEM) complexes. This reduces the amount of cellulolytic enzymes that need to be produced, as the

substrate and enzymes are in much closer contact (Schwarz, 2001). This process is more efficient than the extracellular secretion of each enzyme (Schwarz, 2001; Lynd et al., 2005). Lu et al. (2006) have demonstrated that cellulose degradation using a cellulosome is 2.7 - 4.7 times more efficient when compared to purified enzymes. This is further supported by Lynd et al. (2005), who also described the superiority of CEM complexes over CE complexes.

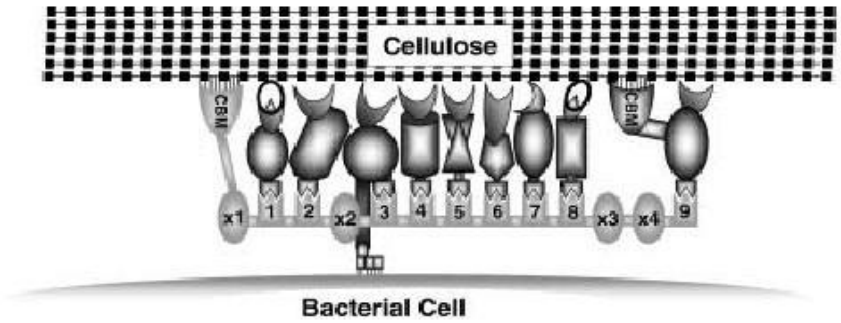


Figure 2.6: The cellulosome (Schwarz, 2001)

Anaerobic organisms are generally very specific with regards to carbon source preferences (Lynd et al., 2002). Some anaerobic organisms can only utilize one or two substrates, while others can utilize close to one hundred (Lynd et al., 2002). It is because of this specificity that anaerobic cellulolytic organism are considered promising candidates to be used in CBP.

2.4.3 – Thermophiles

Thermophiles are microorganisms which demonstrate optimal growth at temperatures ranging from 45°C to 80°C, and some, such as *Clostridia sp.* have been found to be very efficient at hydrolyzing cellulose (Leschine, 1995). Others, such as *Zymomonas sp.* have been found to have

high ethanol resistance (Zaldivar et al., 2001). Thermophilic saccharide fermenting microorganisms are of particular interest because they can ferment biopolymers directly into ethanol, have high metabolic activity, and can utilize cellulosic biomass (Lovitt et al., 1984; Lynd et al., 2007).

2.4.3.1 – Clostridia

Clostridia are a genus of anaerobic, cellulolytic, Gram positive bacteria that have the highest know rate of cellulose degradation, and have been isolated from a number of sources including soils, compost, and animal feces (Carrere et al., 2008). They demonstrated a very high growth rate on crystalline cellulose, and ferment cellodextrins into desirable end products such as ethanol (Lovitt et al., 1984; Alejandro et al., 1980; Ng et al., 1981; Lynd et al., 2007). Furthermore, Strobel et al. (1995) stated that *C. thermocellum* has separate pumps for glucose, cellobiose, and cellodextrins, which may explain the efficiency of cellulose utilization by *Clostridium* species. Two species which have been thoroughly studied, and are of industrial interest, include *C. thermocellum,* and *C. thermohydrosulfuricum.* Both *C. thermocellum* and *C. thermohydrosulfuricum* can survive in the range of 26 g/L to 60 g/L ethanol, thus making them ideal candidates for increased ethanol production (Lynd et al., 2005). *C thermohydrosulfuricum* is of interest because it ferments a wide range of saccharides, like xylose and glucose into ethanol, while *C. thermocellum* prefers only cellobiose (Lovitt et al., 1984; Lynd et al., 2002).

2.4.3.2 – Zymomonas

Other notable ethanol fermenting species include *Zymomonas mobilis*, which is tolerant to ethanol at concentrations up to 150 g/L (O'Mullan et al., 1995; Scopes & Griffiths-Smith, 1985).

A limiting aspect of *Z. mobilis*, however, is that it can only use simple sugars such as glucose, sucrose, and fructose, and not cellulosic biomass (Wood & Ingram, 1992; Eveleigh et al., 1983). This limitation of nutrient sources, combined with its high ethanol resistance has spurred research exploring the genetic modification of other microorganisms with *Z. mobilis'* high ethanol tolerance genes. Wood and Ingram (1992) have done work to genetically modify *E. coli* and *Klebsiella* with *Zymomonas* genes, however these strains were no more efficient than wild type *C. thermocellum*.

2.4.4 - Genetically modified organisms

An alternative approach to achieving the perfect organism for CBP is genetic modification. One method would be to modify an organism with cellulolytic abilities, such as *C. thermocellum*, to improve the yield of end products. A considerable limitation with this approach has been the lack of suitable gene-transfer techniques, which can require specialized protocols and equipment (Lynd et al., 2005). Another approach would be to use organisms that are capable of producing high end product yields, such as *S. cerevisiae*, and modifying them to be able to utilize cellulose (Lynd et al., 2005; Shaw et al., 2008). A hurdle of this approach has been to expressing sufficient cellulase enzyme to allow for anaerobic growth on cellulose.

2.5 – Biofilms

A biofilm is generally described as a community of microorganisms living on a given surface that is enveloped by extracellular polymeric substances (EPS) (Blenkinsopp & Costerton, 1991). The composition of biofilms can be diverse, as they can contain a variety of microorganisms.

The organization of a biofilm plays an important role in determining the composition of organisms found within it. Compared to planktonic cells of the same species, biofilms are generally more resistant to dehydration and toxic compounds (Blenkinsopp & Costerton, 1991). Figure 2.7 depicts a traditional example of biofilm development, where dispersal of planktonic cells only occurs once a biofilm reaches maturity. Recent research by Bester et al. (2005) however, demonstrated that planktonic cell yield is not limited to mature biofilms, but rather occurs within hours of initial biofilm formation.

As biofilms grow they begin to develop a variety of chemical gradients. Some examples of gradients that can occur include a pH and oxygen. This is due to aerobic organisms colonizing the outermost layers and utilizing the majority of the oxygen present, creating anaerobic pockets closer to the site of attachment (Blenkinsopp & Costerton, 1991). These anaerobic zones create localized niches for anaerobic bacteria, which often work synergistically with aerobic organisms (Brenner et al., 2008). With a variety of bacteria growing together, biofilms are capable of utilizing a variety of different nutrient sources. Synergistic and mutualistic relationships can develop, and biofilms are often observed thriving in oligotrophic environments (Blenkinsopp & Costerton, 1991).

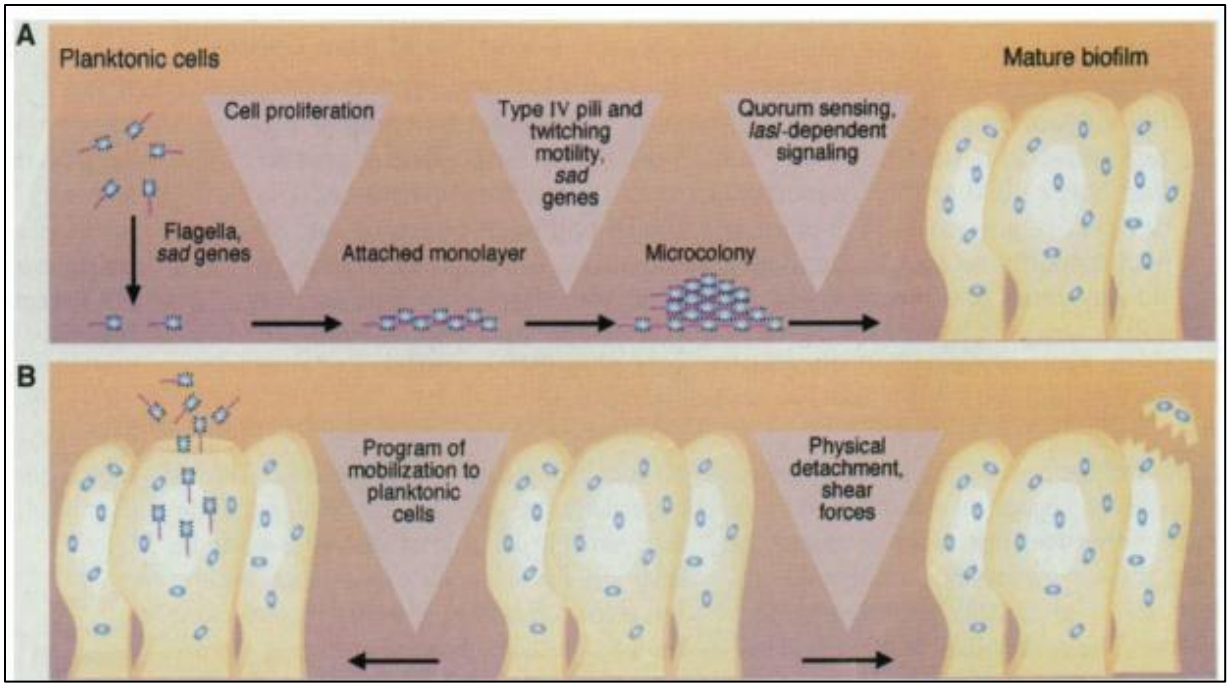


Figure 2.7: Traditional biofilm life cycle (Costerton et al., 1999) A: Development of biofilm B: Dispersal of biofilm

2.5.1 – Mixed community biofilms and cellulose hydrolysis

Cellulose hydrolysis is most efficiently done by anaerobic organisms due to the presence of the cellulosome (Schwarz, 2001). Kato et al. (2004) found cellulose degradation of filter paper was efficiently carried out using a mixed culture consisting of aerobes and one cellulolytic anaerobe. Hydrolysis by the combined community was more efficient than the anaerobe in pure culture under optimal growth conditions. Furthermore, this test was carried out under static aerobic conditions, which would typically inhibit the growth of the anaerobic cellulose degrader. It was concluded that the aerobic members were required in order to generate a reduced anaerobic environment. As biofilms mature and become thicker, it has been determined that they form anaerobic pockets on the site of attachment, which are thought to house anaerobic bacteria (Blenkinsopp & Costerton, 1991).

2.5.2 –Biofilms synergistic relationships

Biofilm physiology plays an important synergistic role in the hydrolysis of cellulose. Blenkinsopp and Costerton (1991) have demonstrated that as biofilms grow, an oxygen gradient develops at the site of attachment, which may provide ideal conditions for anaerobic cellulolytic bacteria to proliferate. Since the majority of biofilms are grown on inert surfaces, cells closer to the point of attachment must rely on nutrient diffusion. Cellulolytic biofilms conversely, represent a niche group in which the point of attachment is also the substrate, and therefore do not rely on nutrient diffusion (Dumitrache et al., 2010). Kato et al. (2005) described an increased efficiency in cellulose degradation when anaerobes are grown together in an aerobic environment. With the knowledge of biofilm physiology, it is hypothesized that the aerobes present in a mixed cellulolytic community consume oxygen and create anaerobic zones at the substrate interface, thereby facilitating anaerobic hydrolysis of cellulose. Kato et al. (2005) also described an increased synergistic relationship when certain aerobes were removed from the biofilm, suggesting that not all organisms in the biofilm contribute a positive effect. Furthermore, mixed communities are capable of performing complex multi-step processes by communicating and dividing the workload. Mixed community biofilms have been found to be more resilient in low nutrient conditions, due to the presence of members with diverse metabolic capabilities. This provides an obvious advantage over pure culture systems (Brenner et al., 2008. Kato et al., 2004).

2.5.3 – Cellulose-enzyme-microbe and Enzyme-microbe Complexes

A study by Lynd et al. (2005) on *C. thermocellum* demonstrated a several-fold increase in the

efficiency of cellulose degradation when the organism was attached to the target substrate, forming a cellulose-enzyme-microbe (CEM). This is in contrast to enzymatic hydrolysis by cellulolytic enzymes that are not attached to a cell, forming a cellulose-enzyme (CE) complex. The CEM is thought to allow for increase hydrolysis efficiency by providing direct access to cellulose, as well as the concentration of specified enzymes.

Connective Text

As outlined in the literature review, the majority of research performed to date has focused on optimization and genetic modification of pure cultures to allow for efficient hydrolysis and fermentation of cellulosic biomass, resulting in the production value added end products, specifically ethanol. The use of mixed communities however, has not been as well studied, and recent advancements have indicated that mixed communities may have the potential to simplify the overall process by being able to tolerate a range of growth conditions, and benefit from potential synergistic relationships. The present study focuses on the cultivation and enrichment of a mixed community capable of cellulose hydrolysis and fermentation in a continuous flow system, and the assessment of value added end products, specifically ethanol and acetate. Furthermore, the ability to hydrolyze cotton and produce end products was tested at various flow rates.

CHAPTER 3: CULTIVATION OF A CELLULOLYTIC MICROBIAL COMMUNITY UNDER CONTINUOUS FLOW CONDITIONS

3.1. Abstract

A stable microbial consortium capable of cellulose hydrolysis under initial aerobic conditions has been enriched in a continuous flow system. Anaerobic conditions were created and maintained within the flow system using only microbial metabolism. Upon inoculation into the sampling flow system, three distinct zones were observed (aerobic aqueous, anaerobic aqueous, and yellow active) and separately assessed for cellulolytic capabilities. A DGGE community profile of samples taken throughout the enrichment process indicated that the community stabilized upon entry into the continuous flow system, and maintained function and member composition throughout. The effect of flow rate on cellulose degradation and end product production was assessed. The cellulose degradation rate was found to be notably faster at a flow rate of 0.1 ml/min (*D* 0.75/hr) than a flow rate of 0.2 ml/min (*D* 1.50/hr). Furthermore, an ethanol to acetate ratio of 8.5:1 was also observed at 0.1 ml/min (*D* 0.75/hr). With reduced flow rate and high ethanol to acetate ratios (8.5:1), the enriched community may be well suited for consolidate bioprocessing.

*This chapter has been prepared to be submitted for publication according to the word count limitation, pending the arrival of HPLC results of ethanol and acetate levels at a flow rate of 0.1 ml/min and 0.2 ml/min (*D* 0.75/hr and 1.50/hr) by external collaborators.

3.2. Introduction

As most of the earth's fossil fuel resources are projected to be depleted within one human generation, the need for an environmentally friendly renewable fuel source has shifted from being an ideal to a necessity (Rodolfi et al., 2009). With recent advancement in environmentally friendly renewable fuel sources such as bio-diesel, bio-hydrogen, and bio-ethanol, it may be possible to reduce the worlds' dependency on fossil fuels.

Traditionally bio-ethanol has been derived from food crops such as corn and sugarcane; however, this situation is not ideal, and has been criticized for using food to produce fuel (Inderwildi and King, 2008). As a result, there has been a shift towards the use of non-food crops such as switchgrass, and agricultural waste such as corn stover. These are both cost effective and abundant sources of cellulose which can be converted into fermentable sugars for biofuel production (Zaldivar et al., 2001; Lynd et al., 2005).

Cellulosic bio-ethanol production has generally been a four-step process including pre-treatment, hydrolysis, fermentation, and distillation. Simultaneous saccharification and co-fermentation (SSCF) is a process which combines saccharification, where both cellulose and hemicelluloses are hydrolyzed, and fermentation, where both glucose and xylose are fermented, together. Consolidated bioprocessing (CBP) combines the hydrolysis and fermentation steps by using microorganisms such as *Clostridium* (Lynd et al., 2007), *Bacillus* (Romero et al., 2007), *Zymomonas* (O'Mullan et al., 1995), *Saccharomyces* (Peng et al., 2011) and other species, rather than using purified enzymes for hydrolysis, and yeast for fermentation. It has been suggested that CBP can potentially increase the yields of bio-ethanol by 13%, compared to systems using all

four steps separately (Lynd et al., 2008).

C. thermocellum (a thermophilic anaerobe capable of both hydrolyzing and fermenting cellulose) has been a suitable microorganism to evaluate the potential for bio-ethanol production because it attaches directly to the substrate, forming a cellulose enzyme complex, which results in more efficient hydrolysis compared to using purified enzymes (Lynd et al., 2005). Though anaerobic attached phase hydrolysis is more efficient than planktonic phase free enzyme hydrolysis and the use of purified enzymes, pure cultures are often limited to specific growth conditions, and any deviation can result in a population collapse (Brenner et al., 2008). When working with pure cultures, the creation and maintenance of specific growth conditions, such as a strictly anaerobic environment required for anaerobic hydrolysis, often require specialized equipment and higher operational cost, which may render such an approach less attractive for industrial application. It would therefore be advantageous if processes are developed in which microbial metabolism create the desired conditions without the added costs (Lu et al., 2006; Lynd et al., 2005).

Kato et al. (2004) demonstrated that mixed cultures with both aerobic and anaerobic community members are capable of creating anaerobic conditions from initially aerobic medium, to subsequently facilitate anaerobic cellulose hydrolysis. Furthermore, hydrolysis was shown to be more efficient when the aerobic and anaerobic members were combined, in comparison to the isolated cellulose degraders in monoculture. In contrast to pure cultures cultivated in suspension, mixed community biofilms have been shown to be generally more robust, tolerant to fluctuating environmental conditions (Brenner et al., 2008), able to utilize both pentose and hexose sugars,

as well as benefit from synergistic relationships (Weibel, 2008; Georgieva et al., 2007). Through the creation of an anaerobic microenvironment, these mixed communities have the potential to perform both anaerobic hydrolysis and fermentation (CBP) in an otherwise aerobic bulk environment.

Cellulose hydrolysis is typically tested in static batch systems, which do not select for attached phase organisms, however, it can also be tested under continuous flow, which can select for attached phase communities (Zaldivar et al., 2001). The use of a flow system that allows a steady state synergism where aerobic metabolism maintains an environment conducive for anaerobic cellulolytic activity should be a valuable tool to evaluate the potential of cellulolytic biofilm communities, and assess the production of value added end products such as ethanol, acetate, and hydrogen. Furthermore, biofilms developed under continuous flow conditions may facilitate increased cell dispersion. Advantages of this include increased colonization of the desired substrate, potentially leading to increased hydrolysis, as well as being able to grow in a range of flow rates without concern of cell washout (Kroukamp et al., 2010).

The present study considered the premise that attached cellulolytic communities could be stably maintained over extended periods of time under flow conditions. The objectives of the present study were to enrich for an attached cellulolytic degradation consortium that can produce desirable products, to develop a flow system that allows for the synergistic cooperation between aerobic and anaerobic cellulolytic community members, and to characterize both aerobic and anaerobic cellulolytic communities. Using this enriched attached consortium, cellulose degradation rate, and end product formation was assessed at various flow rates.

3.3. Materials and methods

Enrichment of Cellulose Degrading Consortia

A 31.164 cm^3 plexiglass flowcell (10.6 cm x 4.2 cm x 0.7 cm) with plexiglass lid (12.8 cm x 6 cm x 0.5 cm) (Fig. 3.1) was used to enrich a mixed community. The lid was equipped with 3 sampling ports (0.8 cm x 1.6 cm; Fig. 1) for both solid and aqueous samplings in different parts of the flowcell without disrupting the flow. As a cellulosic substrate for attachment, 0.62g of cotton was added to the flow chamber. A peristaltic pump (Rabbit, Rainin Instruments, Oakland, Califorina, USA.) was used to deliver RM medium (Ozkan et al., 2001) (2 g/L KH_2PO_4, 3 g/L K_2HPO_4, 2 g/L Urea, 0.5 g/L yeast extract, 0.002 g/L resazurin, and 1 mL of trace element mix ((0.9836 mM $MgCl_2$, 0.34 $CaCl_2$, 0.00899 mM $FeSO_4$) was added after autoclave sterilization)) at a flow rate of 0.2 mL/min into the flowcell (Displacement rate (D) = 1.50/hr). The flow system was initially inoculated with 2 mL of a 1:4 mix of fresh active compost suspended in sterile RM medium by direct injection through a butyl rubber inoculation port (Fig. 3.1 B) with a sterile syringe and 16 G needle. The flow was turned off during inoculation and kept off for 1 hour to allow for cell attachment to the substrate. The system was incubated at 60°C and run until cotton degradation reached sampling port D, at which point solid samples and 10 mL of liquid samples were taken for DNA analysis and DGGE community profiling. Also, a sample (~0.01 g) of the actively degrading cotton was taken for the subsequent enrichment by inoculating through sampling port E (Fig. 3.1). All subsequent enrichments and experiment inoculation was done using cotton that is actively being degraded, and has been stained yellow by what appears to be a yellow affinity substance secreted by the mixed community (Schwarz, 2001). Once a stable consortium was established, samples of the actively degrading yellow cotton were cryopreserved with 40% glycerol for future experiments.

Identification of Attached Cellulolytic Community Members

The sampling flowcell was run until cotton was roughly degraded to sampling port D, at which point 10 mL samples of the anaerobic aqueous liquid (clear/colourless), aerobic aqueous liquid (pink/purple), and roughly 0.1 g of yellow actively degrading cotton were taken using sampling ports E, C, and D, respectively (Fig. 3.2 C). Samples were inoculated into vials containing 40 mL of RM medium, 0.1 g of cotton, and incubated at 60°C.

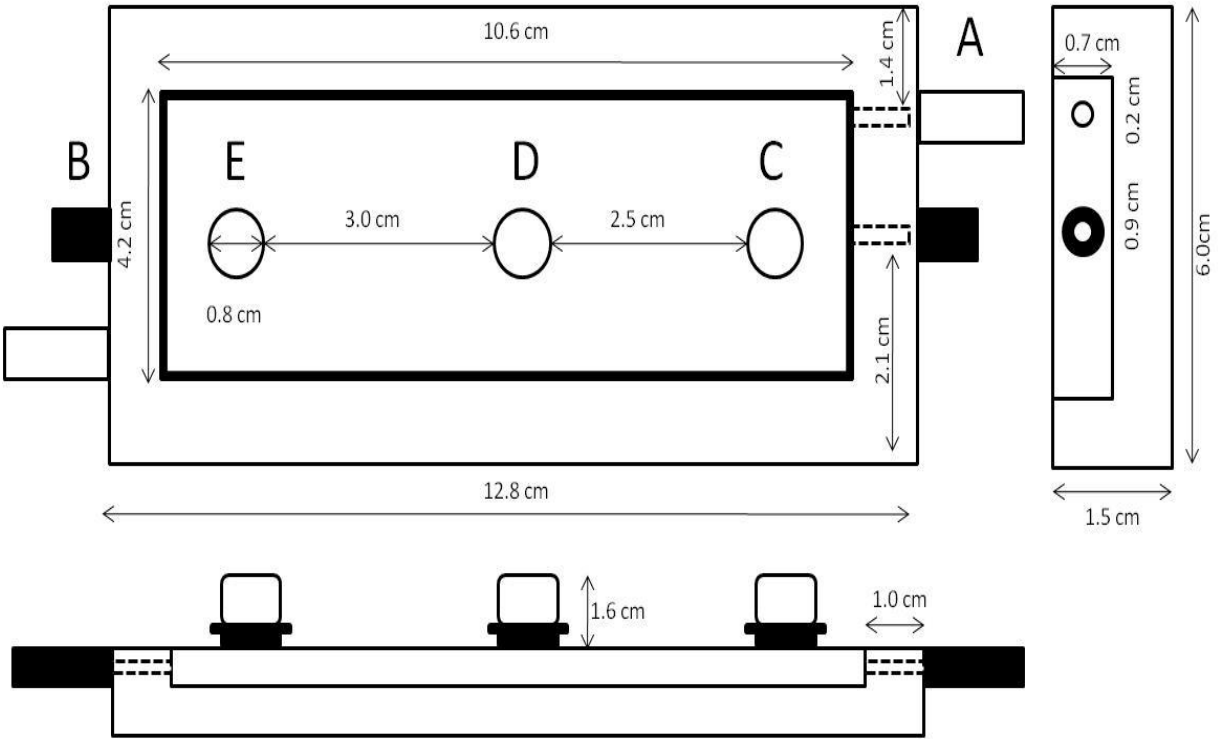
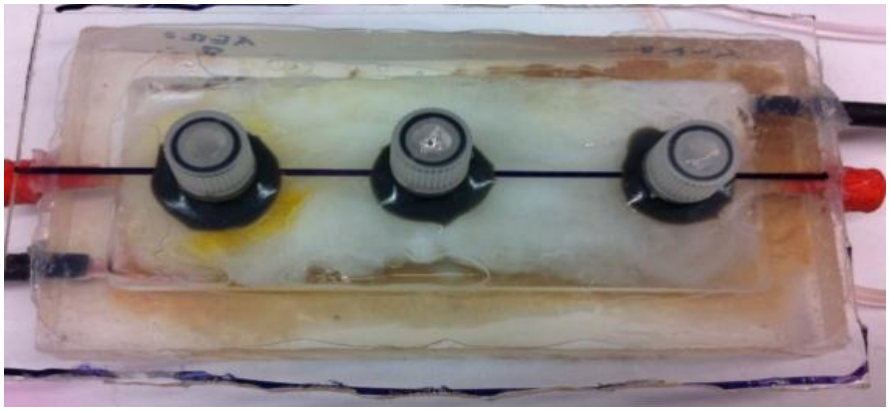


Figure 3.1: Sampling flowcell. A) Medium in flow. B) Inoculation port. C) Aerobic zone sampling port. D) Active cotton sampling port. E) Anaerobic zone sampling port.

A)

B)



C)

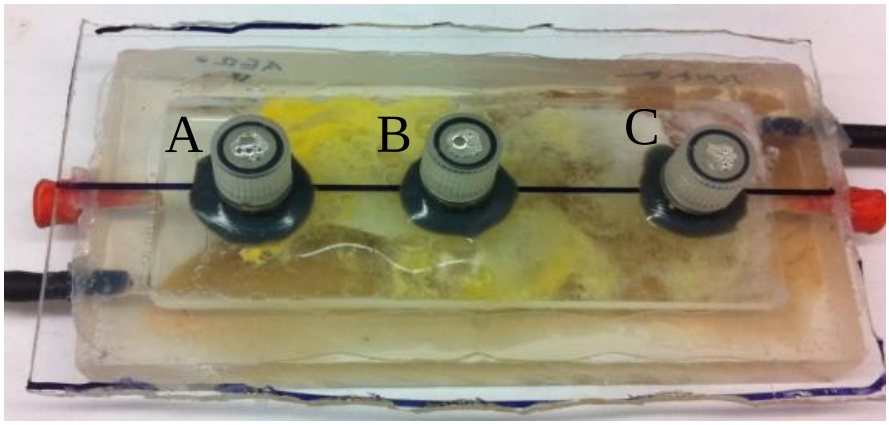


Figure 3.2: Cellulose breakdown within the sampling continuous flow system. A) Aerobic and anaerobic zones are visible. B) Flow system is completely anaerobic, yellow colouring appears. C) Cellulose breakdown with anaerobic aqueous zone (A), active cotton (B), aerobic aqueous (pink) zone (C) visible.

Cellulose Degradation Rate

A total of eight 8 mL vial flowcells (Fig. 3.3) were filled with ~0.13 g of sterile cotton, supplied with RM medium at a flow rate of 0.1 ml/min (D = 0.75/hr), and 0.2 ml/min (D = 1.50/hr) using a peristaltic pump (Rabbit, Rainin Instruments, Oakland, Califorina, USA.), and inoculated with a piece of active cotton (~0.01 g) from the enriched community. As the community began to hydrolyzed cellulose, the remaining substrate was quantified by sacrificing 2 of the 8 vial flowcells at each time point, without replacement. The flowcells were opened by removing the stopper, and the remaining cotton was removed. The inside of the vial was also rinsed with roughly 200 ml of sterile water, and both solid and liquid samples were filtered through a pre-weighed 1.5 µm glass microfiber Whatman filter (Maidstone, Kent, United Kingdom) and subjected to vacuum until there was no visible water remaining. The filter was then dried at 120°C for 3 hours. Samples were then weighed and the amount of cotton degradation was calculated (Watanabe et al., 2011). At a flow rate of 0.1 ml/min (D = 0.75/hr), samples were taken at day 4, 6, 7, and 8, while at a flow rate of 0.2 ml/min (D = 1.50/hr) samples were taken at day 4, 6, 8 and 10.

End Products

Two 8 mL vial flowcells (Fig. 3.3) were supplied with RM medium using a peristaltic pump (Rabbit, Rainin Instruments, Oakland, Califorina, USA.), and a peristaltic pump (205S, Watson Marlow, Wilmington, Massachuttes, USA) at a flow rate of 0.1 mL/min (D = 0.75/hr) and 0.05 mL/min (D = 0.375/hr). A total of 0.05 g of cotton was added to each flowcell as a substrate and a point of attachment, and they were inoculated with ~0.01 g of actively degrading cotton. Samples were collected at 8 point over 5 days by first sterilizing the outer surface of the effluent

tubes with 70% ethanol, and then collecting 1 mL in a 2ml Eppendorf micro centrifuge tube on ice. Samples were then acidified to pH 2 by adding 1.5 mM of HCl and sent to the University of Manitoba to be analyzed for acetate and ethanol using a Waters, model #1515 isocratic HPLC pump, and a Biorad column, model #HPX-87H.

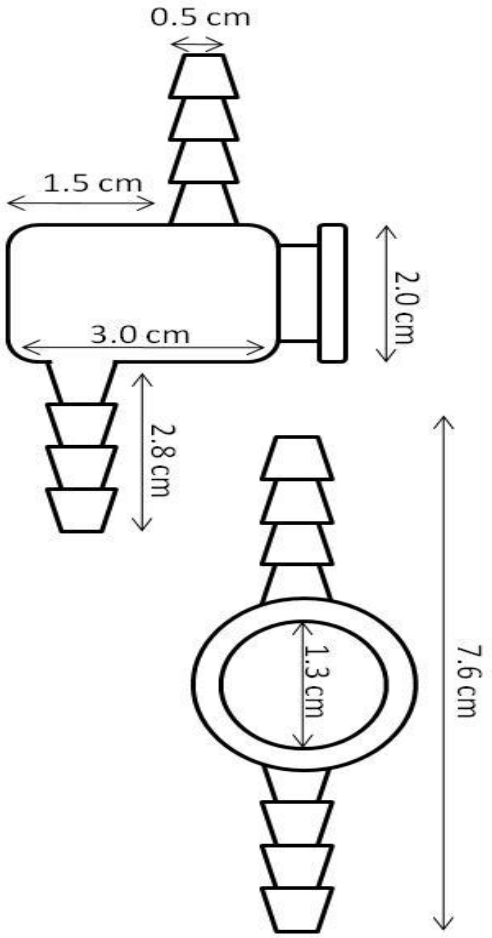


Figure 3.3: Vial flowcell

DNA extraction and PCR

DNA extraction of all enrichment samples was performed using a ZR Soil Microbe Kit™ (Zymo® Research, Orange, CA, USA) as per the manufacturers instruction. Presence of DNA

was then confirmed using a 1% agarose gel.

The reverse and forward primer used for PCR amplification were U758R (5'-CTACCAGGGTATCTAATCC-3'), and U341F-GC (5'-CCTACGGGAGGCAGCAG-3') which contained a GC clamp (5'-GGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGCGGGGG-3') at the 5' end for further downstream use with DGGE (Muyzer et al., 1993). This set of primers was designed to amplify the V3 and V4 region of 16S *Escherichia coli* and produces a 418bp fragment.

Each 50 µl reaction consisted of 1 µl template DNA, 200 µM of each dNTP (New England BioLabs, Pickering, ON), 25 pmol of forward and reverse primer, 10x BSA (New England BioLabs, Pickering, ON), 2.5 units of Taq polymerase (New England BioLabs, Pickering, ON), 1x Taq buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$). The PCR protocol was carried out as follows: a 5 minute initialization step at 96°C, and followed by a 1 minute denaturation step at 94°C. Primer annealing was done at 65°C for 20 cycles, with a 1°C temperature reduction each cycle, followed by an elongation time of 3 minutes at 72°C. This was followed by 20 further cycles with an annealing temperature of 55°C (Yeung et al., 2011). Three separate reactions were performed on each DNA sample to reduce PCR bias.

Samples were run on a 1% agarose gel using SYBsR safe as a gel stain (Invitrogen, Burlington, ON), visualized using a Invitrogen Safe Imager 2.0 (Invitrogen Canada, Burlington, ON), and quantified using 1, 2, and 4 µl of a 100bp molecular weight ladder as a standard curve (MBI Fermentas, Amherst, NY).

Denaturing Gradient Gel Electrophoresis (DGGE)

Extracted DNA samples prepared for DGGE were amplified using the same PCR protocol mentioned above, however the forward primer U341F contained a GC clamp attached the 5' end (5' - GGCGGGGCGGGGGCACGGGGGGCGCGGCGGGCGGGGCGGGGG-3') (Muyzer et al., 1993). PCR products were purified using the illustra GFX PCR purification kid (GE Healthcare), and triplicate PCR reactions were combined to prevent PCR bias. Purified samples were then run on a 8% polyacrylamide gel with a denaturing gradient of 30-70% (7 M urea and 40% deionized formamide were considered to be 100% denaturant). Gels were cast with a gradient former (BioRad Laboratories, Mississauga, ON), and approximately 500 ng of the purfied 16S rRNA sample was loaded into each well of the DGGE gel. Electrophoresis was run at constant 80 V for 16 hrs at 60°C in 1 X TAE running buffer, using a DCode Universal Mutation Detection System (BioRad Laboratories, Mississauga, ON). Nuclealic acids in the resulting gel were stained for 30 minutes in SYBR Gold (Invitrogen, Burlington, ON), followed by de-staining in 1X TAE. Desired bands were excised using a sterile scalpel and placed in 20 µl of sterile ddH_2O, and left at 4°C for 72 hrs prior to re-amplification.

3.4. Results

Continuous flow sampling system

The compost and medium mixture were inoculated into the inoculation port of the sampling flowcell (Fig. 3.1 B), and resulted in two occurrences: the consumption of oxygen creating an anaerobic environment, and the hydrolysis of cellulose. After inoculation, the resazurin containing medium around the cotton changed from pink to clear, suggesting that the oxygen is being consumed, resulting in anoxic conditions (Fig. 3.2 A, B). Once an anaerobic environment was achieved, a distinct yellow colouring appeared on the substrate as seen in Figure 3.2 B, and quickly resulted in cellulose degradation (Fig 3.2 C).

Identification of cellulolytic zone

Once the hydrolysis of cellulose began occurring within the sampling flow system, three distinct zones were observed: an aerobic aqueous phase, actively degrading yellow cotton, and an anaerobic aqueous phase (Figure 3.2 C). Cellulose degradation and consumption of oxygen, creating anaerobic conditions, were only observed when inoculated with solid actively degrading cotton, and not when inoculated with the aqueous liquid from either the aerobic or anaerobic sides (Table 3.1).

Table 3.1: Identification of zones responsible for cellulose hydrolysis based on figure 3.2 C

	Anaerobic Aquesous Phase	Actively Degrading Cotton	Aerobic Aqueous Phase
Day 3	Aerobic (pink) No Cellulose Breakdown	Anaerobic (clear/colourless) Cellulose Breakdown (yellow colouring)	Aerobic (pink) No Cellulose Breakdown

Enrichment under continuous flow

As the attached phase members of the community were identified as being responsible for cellulose hydrolysis, successive enrichments of the attached phase community using active cotton, were carried out using the sampling flowcell. Enrichment 1 (cultured in batch, and consisted of a mix of compost and medium) was used as an initial inoculum for the continuous flow enrichment, and the total number of DGGE bands sees a immediate reduction from that seen in compost (Fig 3.4), resulting in a S_{AB} of 48.5. Enrichments 2 - 6 were done under continuous flow conditions, using yellow actively degrading cotton as an inoculum for each enrichment. The total number of bands is further decreased from compost and enrichment 1 to enrichment 2 - 6, with a S_{AB} of 42.3. However enrichment 2 & 3, and 4 - 6, which are all under continuous flow, see a S_{AB} of 93.3, indicating that the community is relatively stable

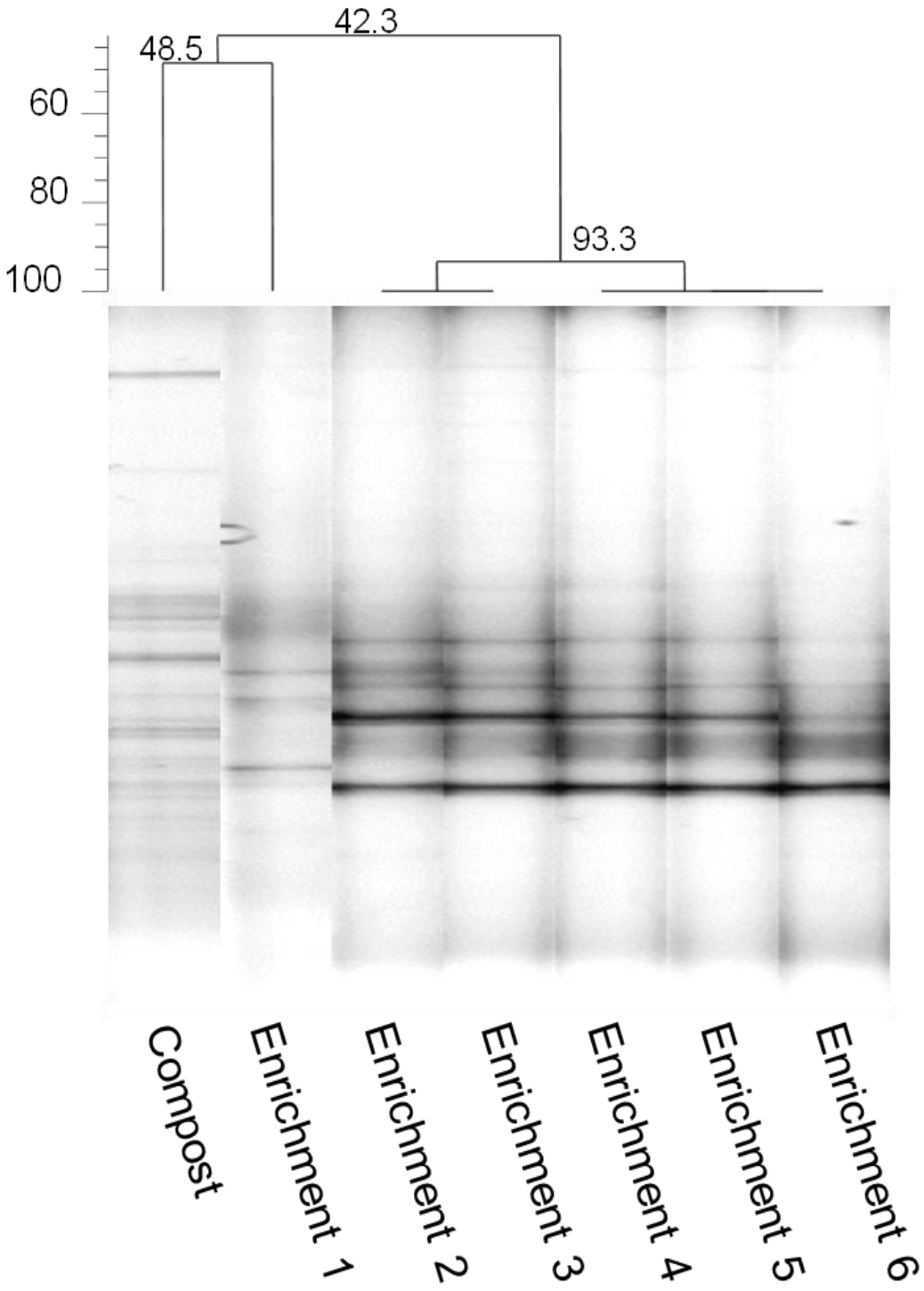


Figure 3.4: Similarity index of mixed community and DGGE profile of enrichments.

Cellulose degradation at a flow rate of 0.1 ml/min and 0.2 ml/min (*D* 0.75/hr and 1.50/hr, respectively)

Results indicated that a flow rate of 0.1 ml/min resulted in cellulose degradation at a faster rate than a flow rate of 0.2 ml/min (Fig. 3.5). Breakdown occurs almost immediately with a flow rate of 0.1 ml/min, while there was a5 day lag period was before hydrolysis occurs was observed with a flow rate of 0.2 ml/min.

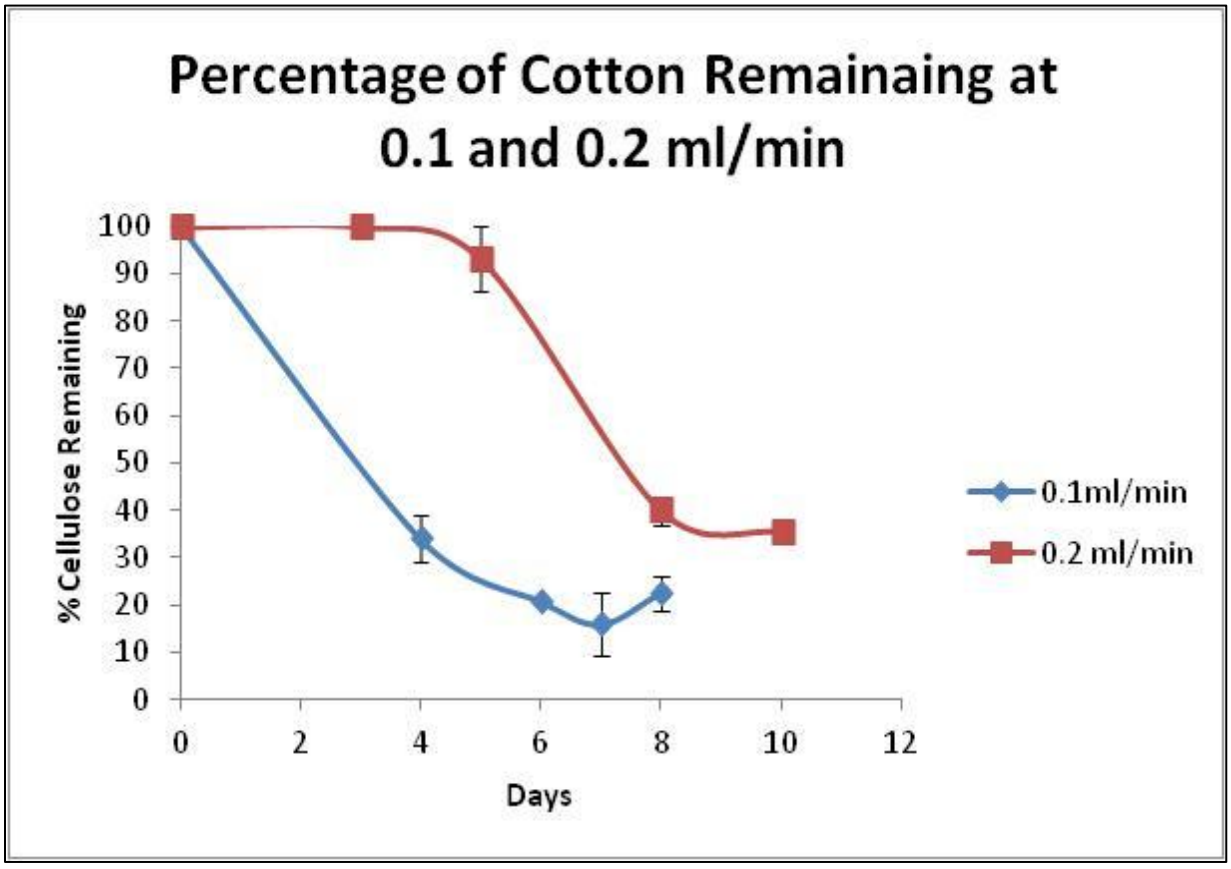
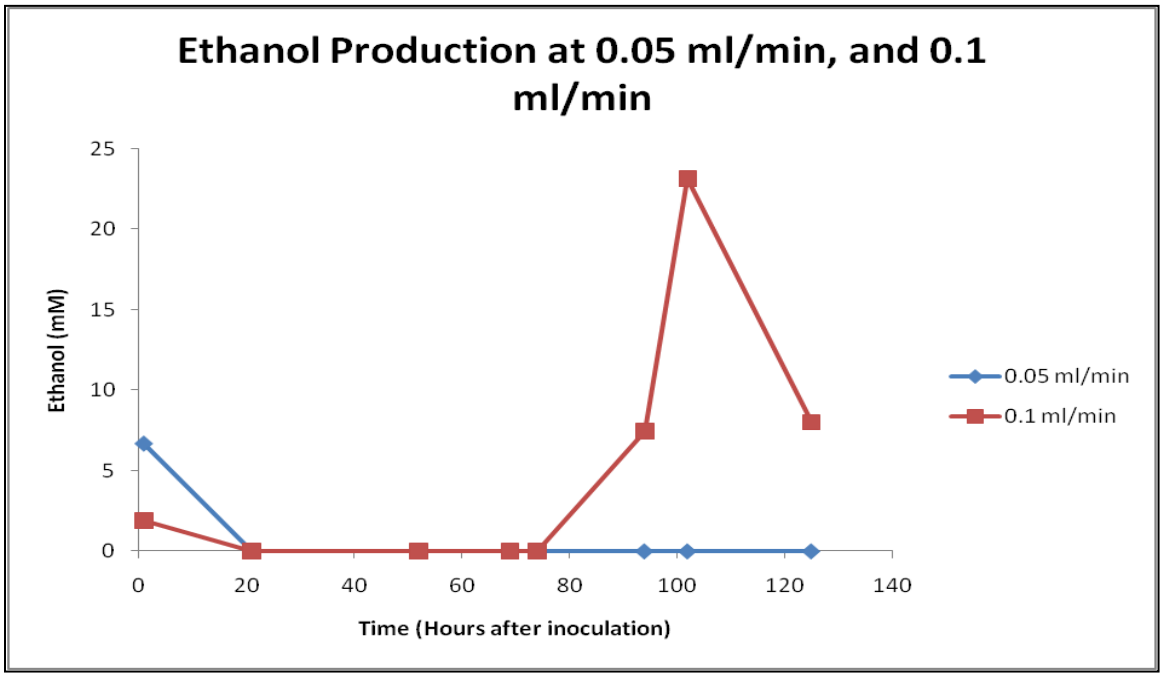


Figure 3.5: Total cellulose degradation at a flow rate of 0.1 and 0.2 ml/min

End product production at a flow rate of 0.1 ml/min and 0.05 ml/min (*D* of 0.75/hr and 0.37/hr)

The ability of the enriched community to produce value added end products, such as ethanol and acetate, was assessed at flow rates of 0.1ml/min and 0.05 ml/min ((*D)* of 0.75/hr, and 0.37/hr, respectively)). As 0.1 ml/min was found to have a faster hydrolysis rate then 0.2 ml/min, it was hypothesized that a continued reduction in flow may result in high ethanol concentrations. Effluent samples were analyzed using HPLC, and the concentrations of ethanol and acetate were reported in mM (Fig 3.6 A, B). Ethanol produced at a flow rate of 0.05 ml/min was below detection limit of the HPLC method (> 0.01 mM), while a peak of 23.1 mM was obtained at 0.1 ml/min (Fig. 3.6 A). Similarly, acetate levels were much lower at 0.05 ml/min, when comparing to the peak of 2.7 mM at 0.1 ml/min (Fig. 6 B). Furthermore, the overall ratio of ethanol to acetate at their peaks, at a flow rate of 0.1 ml/min was 8.5 :1.

A)



B)

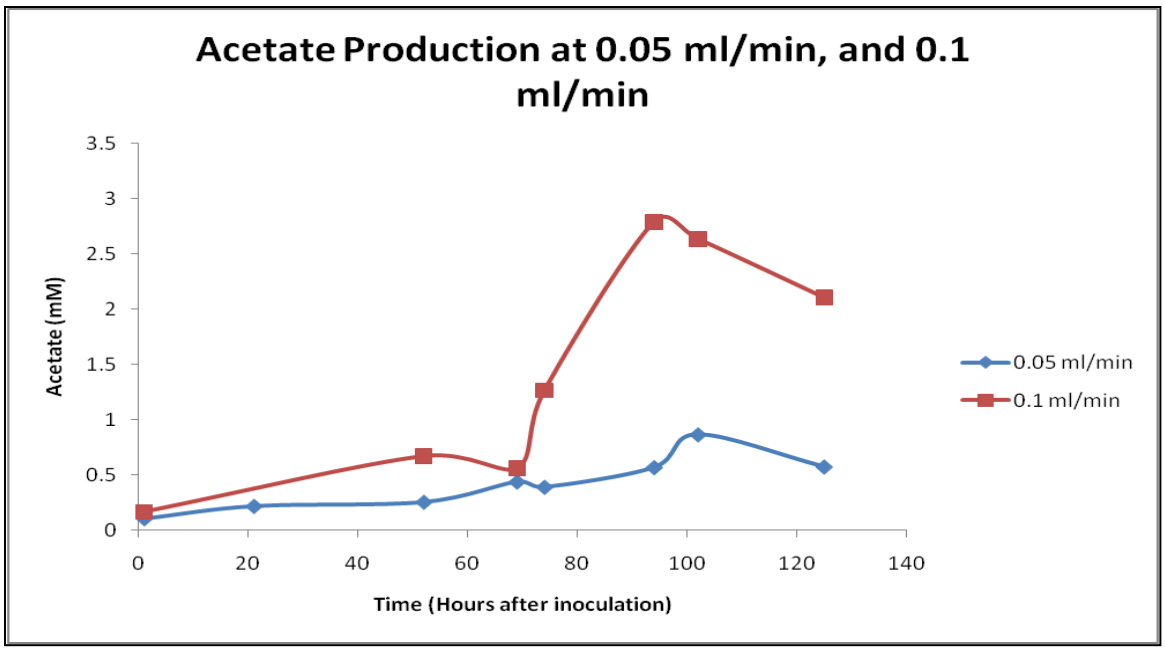


Figure 3.6: Ethanol and acetate production measured at a flow rate of 0.1 ml/min and 0.05 ml/min A) Ethanol production (mM) B) Acetate production (mM)

3.5. Discussion

Inoculation of the mixed community derived from active compost material into our sampling flow system visually demonstrated that members within this community poses the ability to consume oxygen and create anaerobic conditions, indicated by the colour change of resazurin (added in the medium) from pink/purple to colourless within the flow system, and most importantly the mixed community could also degrade cellulose (Fig. 3.2 A). The creation and maintenance of anaerobic conditions using only microbial metabolism is a considerable advantage for this mixed community in comparison to other strict anaerobic cellulose degrading pure culture system such as *C. thermocellum.* In general, pure anaerobic culture systems require the aid of reducing agents and compressed gas (Weimer & Ziekus, 1976), to create the anaerobic environment needed for effective cellulose degradation.

As the flow system turns anaerobic, not only did the medium changed from pink/purple to colourless, but also a yellow substance was produced on the cellulosic substrate and was quickly degraded soon after the colour changes (Fig. 3.2 B, C). The development of the yellow colour before the onset of the cellulose degradation could potentially be related to the yellow affinity substance that Ljungdahl et al. (1983) described in a pure culture *Clostridium* cellulose fermentation system.

The three distinct colour zones observed within the sampling flow system (aerobic aqueous phase (i.e. pink color region), anaerobic aqueous phase (clear to yellow region), and active cotton (the yellow to brownish-yellow cotton) (Table 3.1)) demonstrated that this continuous flow system allows for certain separation of the active community, which would otherwise be seen as a combined community in batch systems. All three zones were then assessed individually for their ability to consume oxygen and to break down cellulose. When

inoculated into fresh medium in batch, the results revealed that the solid phase active cotton as a source of inoculum produced the best degradation ability, compared to both the aerobic and anaerobic zone liquid inocula (Table 3.1). These results suggested that all the community members required for active cellulose hydrolysis and oxygen consumption were mainly found within the active cotton zone. Schwarz (2001) found that members from the *Clostridium* family are associated with both attached phase cellulose hydrolysis and the yellow colouring observed, which suggest that members from the *Clostridium* family may be responsible for the observed cellulose hydrolysis. Perhaps, using the solid material as a source of inoculation may facilitate the attachment process and decrease the initial attachment time needed. As an example of the advantage of attached phase hydrolysis, Lu et al. (2006) demonstrated that the cellulose-enzyme-microbe complex formed by direct contact to the substrate is more efficient at cellulose hydrolysis than aqueous phase cell free enzymes. For these reasons, using only active cotton as the source of inoculum for future enrichments would potentially select for a consortium capable of attached phase anaerobic hydrolysis. The sampling ports equipped in our continuous flow system would provide the ability for this form of sample collection without the need to stop the experiment.

With a system capable of enriching and separating the active cellulolytic consortia, a series of enrichments were performed to determine the stability of the community. A total of 8 enrichments were carried out using the sampling flowcell, transferring only active cotton out of sampling port D of one flowcell, and into sampling port E of another. Through observations, the cellulolytic capability of the community increased from roughly 17 days to be degraded to sampling port D, to roughly 7 days (data not shown). The DGGE community profile suggests that there was a change in major community members from the E_0 inoculum (enrichment 1) to

the first continuous flow enrichment (enrichment 2). Furthermore, throughout the continuous flow enrichment the community profile remained relatively unchanged (Fig. 3.3).

With a consistently stable cellulolytic community achieved, its ability to hydrolyze cotton was determined under medium and high flow rates, i.e. 0.1 ml/min and 0.2 ml/min ((D = 0.75/hr, and 1.50/hr)). Our result revealed that the hydrolysis under the medium flow rate was much faster than that under the higher flow rate. The higher flow rate suffered a considerable lag time, with hydrolysis only beginning to occur after 5 days. This may be attributed to increased dilution, which may have made initial attachment difficult. In comparison, at the same time point under the medium flow rate, approximately 75% of the initial cellulose were degraded (Fig. 3.5). By day 8 it is clear that the medium flow rate results in significantly faster hydrolysis. The medium flow rate may provide several advantages, in that it may be easier for the community to attach to the substrate, and any extracellular enzymes may be retained for a longer period. This may suggest that the medium flow rate allows for sufficient attachment to the surface with less cell washout, potentially resulting in an increased hydrolysis rate (Kroukamp et al., 2010). The ability of the community to produce anaerobic conditions, however, was not affected by the varying flow rates. With regards to a potential use in industrial setting, a lower flow rate would be advantageous as it would require less medium to achieve the same end results.

With a flow rate for efficient hydrolysis determined, the ability of the consortium to produce ethanol and acetate was assessed. As the medium flow rate resulted in efficient hydrolysis, it was hypothesized that a lower flow rate (0.05 ml/min, D = 0.375/hr) would be more advantageous. It was found that ethanol production at the medium flow rate peaked at 23.1 mM, while there were no significant peaks at the low flow rate. Though there was ethanol initially observed at the low flow rate, it is thought to be associated with the active cotton

inoculum which may contain residual ethanol (Fig. 3.6 A). Aside from the initial peak, the lack of ethanol at the low flow rate may be due to several factors such as: lack of nutrients arriving at the cellulolytic community attached to the substrate, potential end product inhibition, or excess residual planktonic cells consuming the ethanol. As there appears to be roughly 7 community members present, indicated by the DGGE community profile (Fig 3.4) – capable of traveling upstream and consuming the oxygen, and hydrolyzing the cellulose – it may be possible that at a reduced flow rate, insufficient nutrients make it past the initial oxygen consuming community and arrive at the cellulolytic community. Furthermore, as a yellow substance was produced by the community during cellulose degradation, suggesting *Clostridium* fermentation (Schwarz 2001) throughout the experiment, perhaps a increased number of planktonic cells may have consumed the residual ethanol. Also, a reduced flow rate may have failed to remove the ethanol at a sufficient rate, which could have potentially led to cell death.

Acetate production was also assessed along with ethanol, and the results follow a similar trend. A medium flow rate resulted in higher values than those of the low flow rate. This may indicate that the medium flow rate is more favourable for acetate production than the low flow rate, although acetate concentrations were much lower than those of ethanol at both flow rates. Comparing peak levels of ethanol to acetate, a ratio of 8.5:1 is observed. This value is much higher than that of pure culture *C. thermocellum*, under batch and continuous flow conditions (*D* of 0.05/hr and 0.37/hr), with reported ratios of 1:1.8, and ~1:1, respectively (Lynd et al., 1989). In addition, similar results using pure culture *C. thermocellum* were found by Carere et al. (2008), with an ethanol to acetate ratio of ~1.3:1 under continuous flow conditions, and at an identical displacement rate at used by Lynd et al. (1989). The displacement rate used by Carere et al. (2008) and Lynd et al. (1989) was significantly lower (0.05/hr, and 0.167/hr) than the

displacement rate in the current study (0.75/hr and 0.37/hr), with no reported change in the ethanol to acetate ratio at the different speeds in either study. Though the results of the present study may indicate that a significantly faster displacement rate to what has previously been reported results in a higher ethanol to acetate ratio, it was carried out in a much smaller reactor volume (8 ml compared to 1.7 L). However, if the reported displacement rate results in the same ethanol to acetate ratio after a scale up, it may be worthwhile to further investigate the reported rates.

As ethanol is a potential biofuel, this consortium may be particularly useful in an industrial setting coupled with Consolidate Bioprocessing. The enriched consortium is capable of both hydrolysis and fermentation of cellulose, is able to create and maintain a reduced environment under initially aerobic conditions, and results in a significantly higher ethanol to acetate ratios than previously determined in the literature (Lynd et al., 2005). In addition, end product inhibition and ethanol tolerance (which has been a frequently investigated topic in the literature)(Herrero et al., 1979; Herrero & Gomez, 1980; Jujjuri et al., 2007) appears to be bypassed all together by simply using a continuous flow system with an appropriate flow rate. The flow rate however must be slow enough to not result in cell washout, but be high enough to remove end products, which could inhibit certain activity at high concentrations.

3.6. Conclusions

In summary, a stable consortium capable of cellulose hydrolysis and fermentation under aerobic conditions and able to producing ethanol and acetate has been enriched under continuous flow conditions. The community retains its function and member after multi-enrichments and cryopreservation. A displacement rate of 0.75/hr yields an ethanol to acetate ratio of 8.5:1. Furthermore a *D* of 0.75/hr results in significantly faster hydrolysis than a *D* of 1.50/hr. This community may be useful in an industrial setting as it uses microbial metabolism to create desired (anaerobic) growth conditions without the added costs of reducing agents and compressed gas.

CHAPTER 4: METHOD DEVELOPMENT

4.1. Development of the sampling continuous flow systems

The first version of the sampling flowcell was roughly 1/3rd the size of the final version seen in figure 3.1. It also didn't contain any inoculation ports, but rather used tygon tubing for sampling (Fig 4.1, A). Unlike conventional flowcells (Fig 2.4), this system allows for sampling of the reaction chamber without stopping the experiment. After inoculation with a mixed community derived from active compost, the depletion of oxygen was observed, using resazurin as a oxygen indicator, and the change from aerobic conditions to anaerobic conditions occurred within approximately a hour. Therefore, in order to extend the change from aerobic conditions to anaerobic conditions, a larger reaction chamber would need to be made.

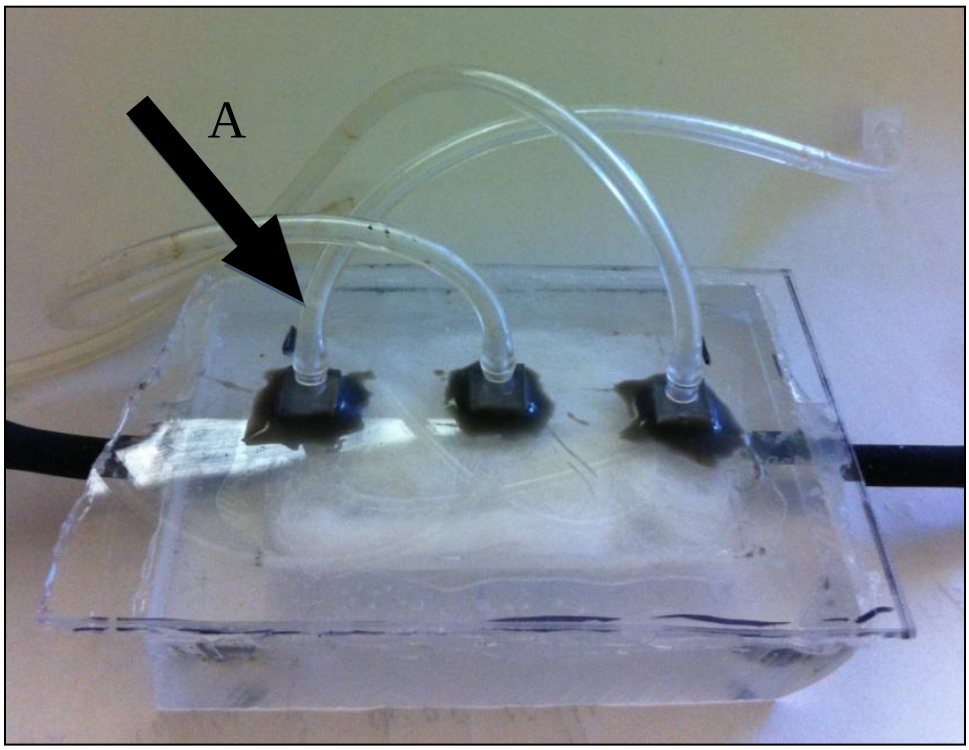


Figure 4.1: First version of sampling flowcell A) Tygon tubing sampling port

The second version was three times the size of the flowcell depicted in figure 4.1. This allowed for a large reaction chamber, and took longer for the transition from aerobic to anaerobic conditinos to occur, and made it possible to visualize the change. In addition, a inoculation device (Fig 4.2, B) was added for reliable inoculation of the flowcell.

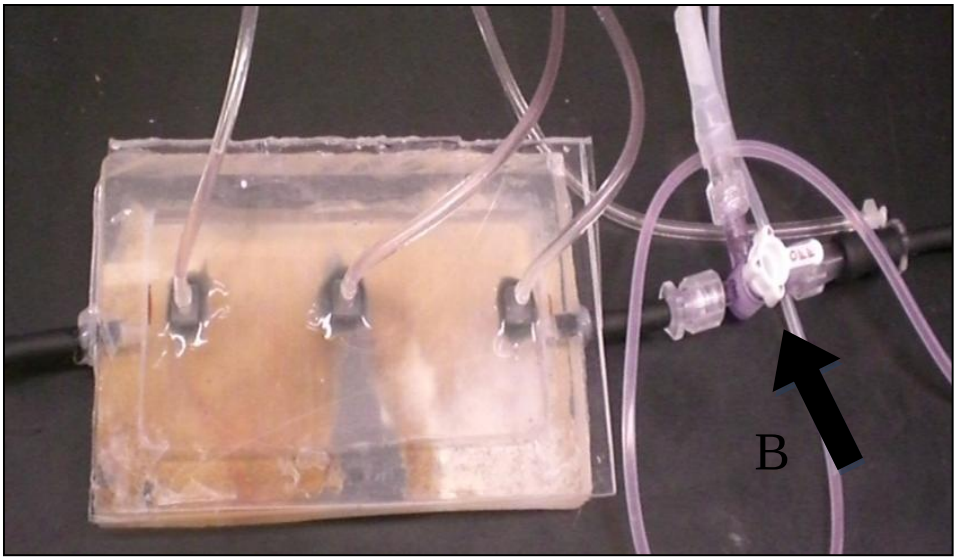


Figure 4.2: Second version of sampling flowcell B) Inoculation device

The third version contained butyl rubber inoculation ports (Fig 4.3, C), at both ends of the flowcell. This allowed for reliable inoculation into either side, without leaks. However, it was found that the attached phase actively degrading cotton was of interest, and couldn't be sampled using the tygon sampling tubes. Therefore new sampling ports that allowed for both solid and aqueous phase needed to be installed.

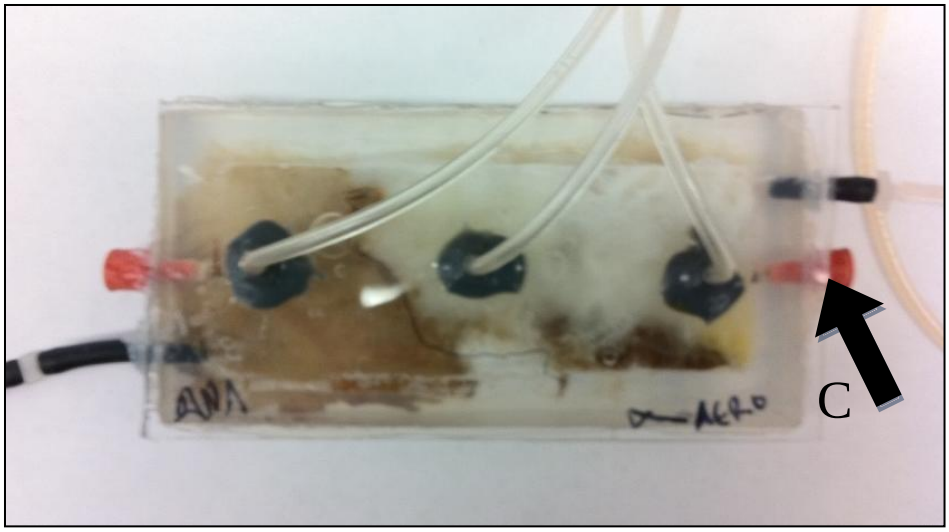


Figure 4.3: Third version of sampling flowcell C) Inoculation ports

The fourth version featured a new lid which contained large sampling ports that facilitated sampling of both solid and aqueous phase samples. These ports were designed to allow for sampling of active cotton, and were sealed with butyl rubber stoppers wrapped in parafilm (Fig 4.4, D). However sampling ports did not provide an air tight seal, and resulted in leaks of both oxygen and medium.

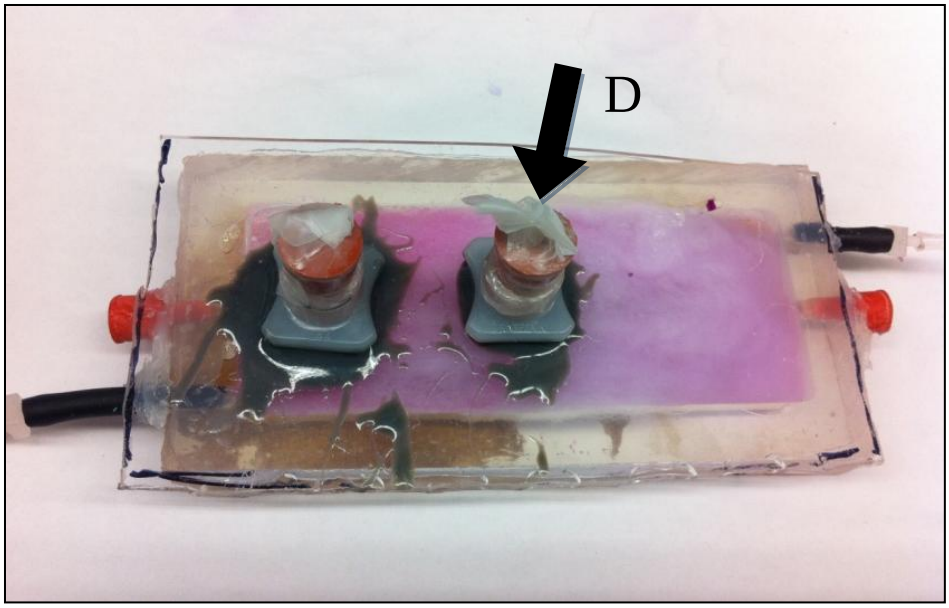


Figure 4.4: Fourth version of sampling flowcell D: Large inoculation ports

The final version replaced the unreliable butyl rubber stoppers with cryo vial screw tops (Fig 4.5, E). This allowed for both aqueous and solid sampling throughout the flowcell. Furthermore, the position on the sampling ports allowed for sampling of yellow cotton being actively degraded (Fig 4.6) , as well as aqueous phase liquid without stopping the experiment. All three zones (aerobic aqueous (Fig 4.7, F), active cotton (Fig 4.7, G), and anaerobic aqueous (Fig 4.7, H)) are visible, and using the sampling ports it was possible to sample each.

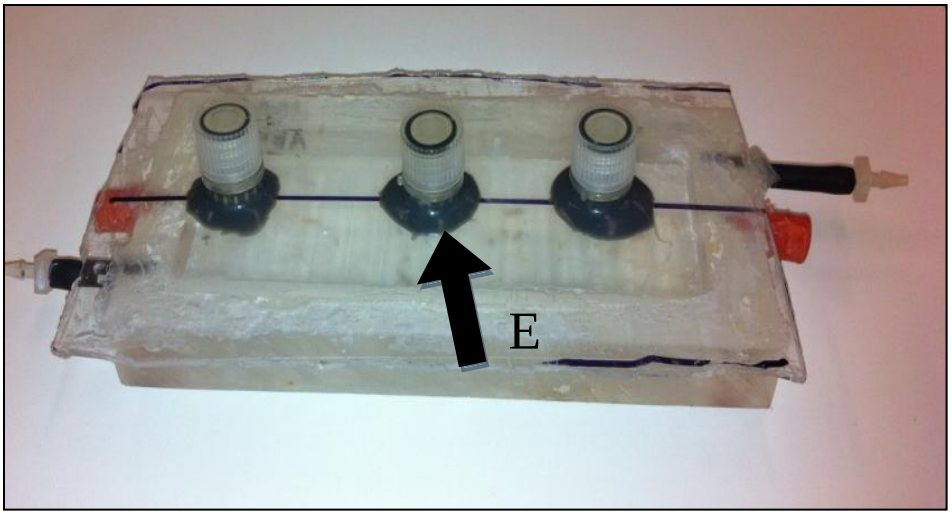


Figure 4.5: Final version of sampling flowcell E) Cryo vial screw top

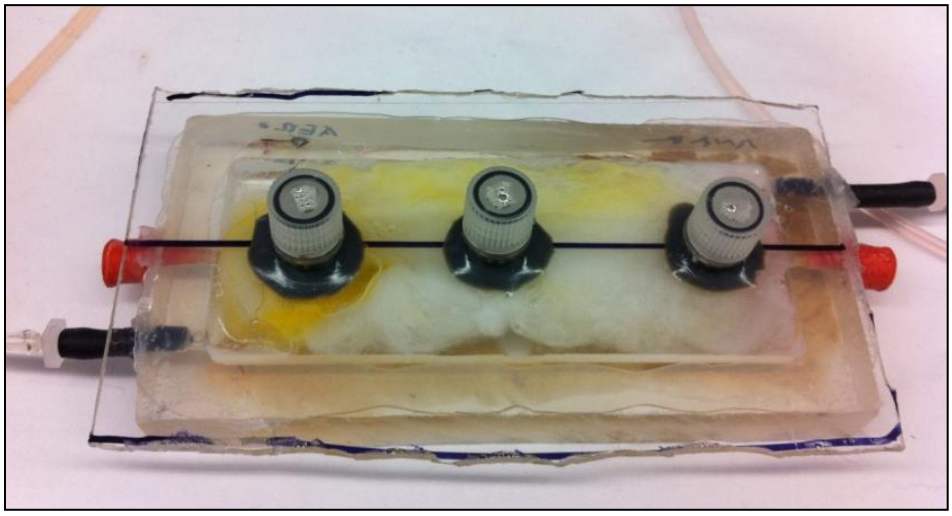


Figure 4.6: Sampling flowcell with actively degrading cotton.

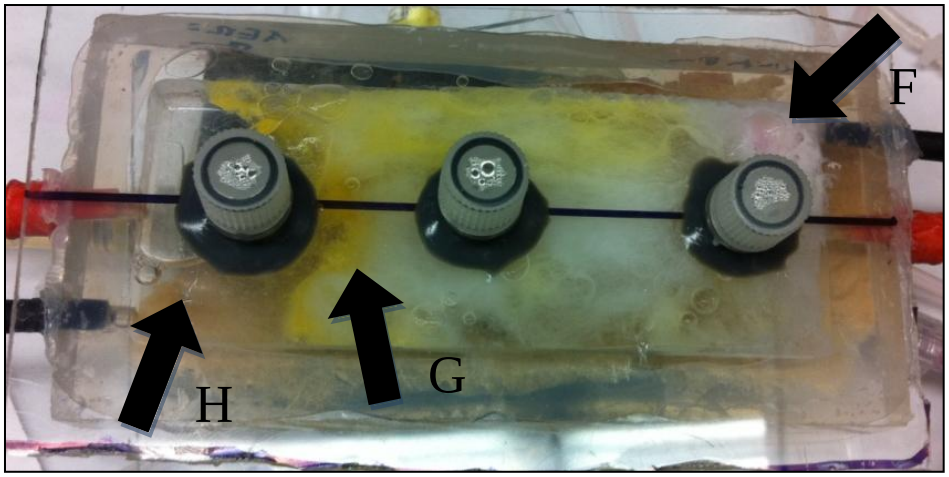


Figure 4.7: Sampling flowcell with F) aerobic aqueous zone
G) cotton being actively degraded and H) anaerobic aqueous zone

4.2. Development of the Gradient Flowcell

A gradient flowcell was developed in an attempt to enrich a cellulolytic community which could tolerate a high percentage of ethanol. The first version (Fig. 4.8) was constructed completely out of plexi-glass. However, as ethanol (which is detrimental to plexi-glass at high concentrations) was run through the test solute reservoir, it was found that the system would need to be constructed out of another material.

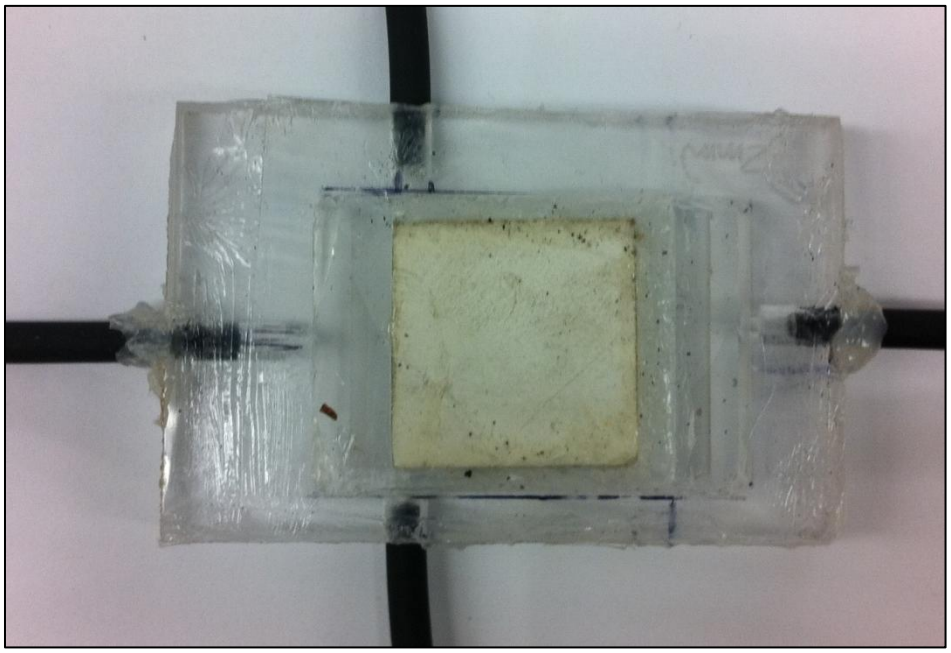


Figure 4.8: Plexi-glass gradient flowcell

The second version of the gradient flowcell was constructed out of stainless steel (Fig 4.9). This allowed for ethanol to be run at high concentrations without the risk of cracking or leaks. Furthermore, *swageloc* connectors were used in place of tubing and silicon sealant. This further helped with the reliability of the flowcell. However after visualizing the placement of the gradient using 50% bleach in the test reservoir, and a fluorescent pure culture *Pseudomonas*

(CT07) in the test chamber, the gradient appeared to be uneven and skewed to one side. This prompted the construction of the triangular flowcell.

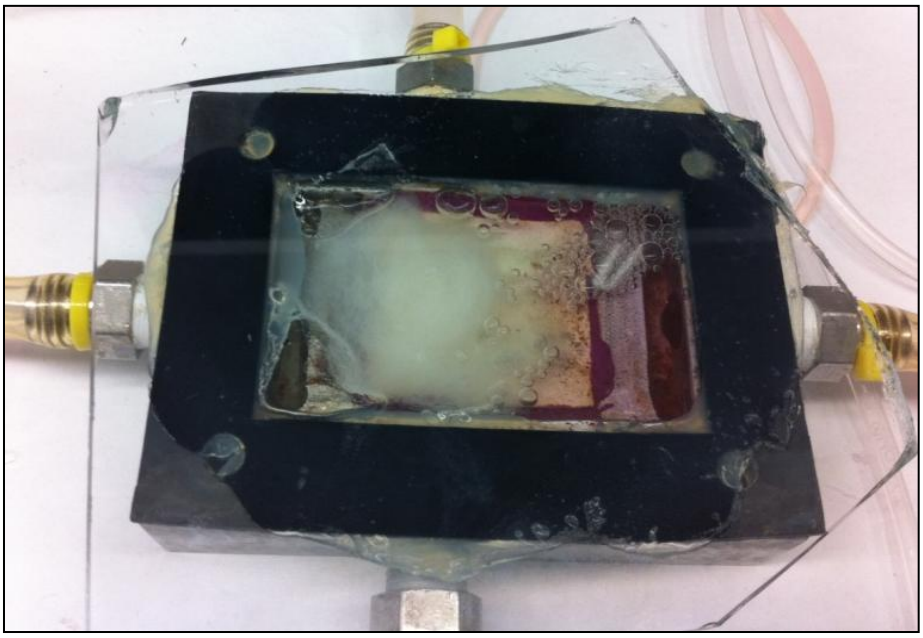


Figure 4.9: Stainless steel gradient flowcell

Rather than using a test solute reservoir (similar to the Gradient flowcell), two inflow tubes, one of which carried a test solute, and one of which carried medium, were used. As a proof of concept, bleach was run through the test solute tube (Fig 4.10, I) and a medium was carried through tube (Fig 4.10, J). To allow for inoculation, the flow system was first filled with medium, using only tube (Fig 4.10, J), inoculated with a pure culture of GFP *Pseudomonas* (CT07), and allowed to sit for 1 hour with no flow prior to the introduction of bleach. As seen in figure 4.10, which has been subjected to bleach for one hour, there is no fluorescence close to tube (Fig 4.9, I) as the pure culture (*pseudomonas* CT07) was killed, while the fluorescence intensity increased moving away from tube (Fig 4.9, I). As can be seen in figure 4.11, which has

been subjected to bleach for 3 hours, both the amount of fluorescent cells and intensity of the fluorescence has been dramatically reduced.

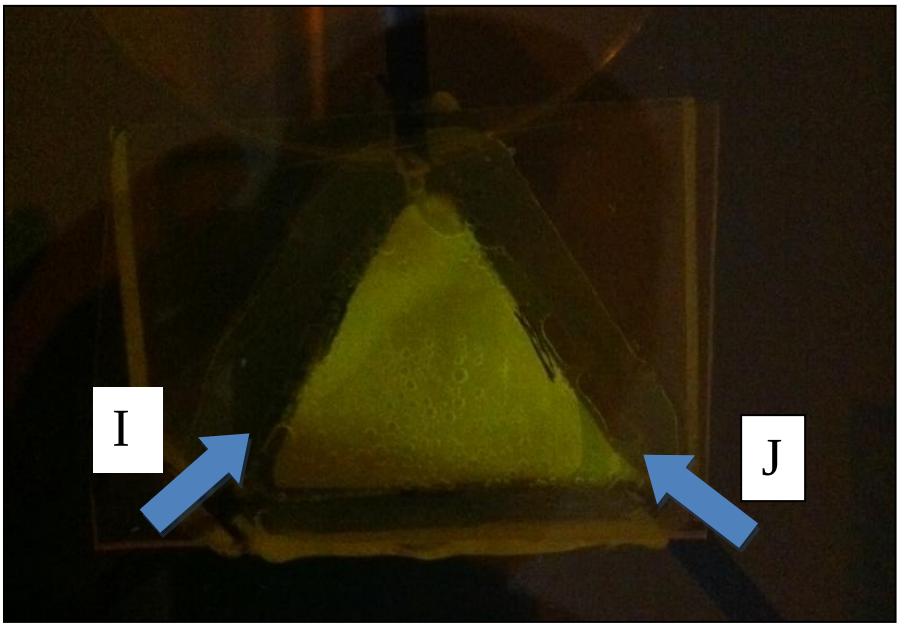


Figure 4.10: Plexi-glass triangular flowcell after 1 hour

Figure 4.11: Plexi-glass triangular flowcell growth inhibition after 3 hours

The final version of the triangular flowcell is much larger and features two sampling ports (Fig 4.12). The two ports were place in zones that would experience the most mixing: one in the middle of the reaction chamber, and one just prior to the effluent outlet. By sampling from only the zones of well mixed ethanol and medium, it would theoretically be possible to enrich for microbes tolerant to high solute concentrations.

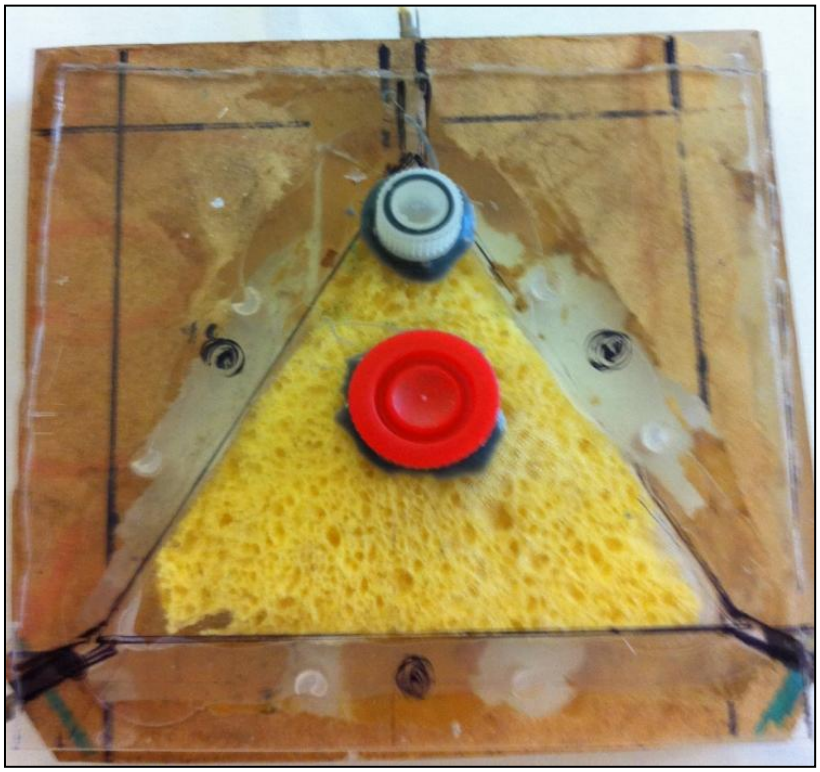


Figure 4.12: Large triangular flowcell filled with a cellulose sponge

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

All three of the desired objectives were completed in the current study. A system was developed which allows for enrichment and sampling of a cellulolytic bacterial community under continuous flow conditions without the need to sacrifice a flowcell or stop the experiment. Using the sampling flowcell, an attached phase cellulolytic microbial community was enriched using compost as an initial inoculum. Throughout the enrichment the community retained its ability to deplete oxygen, creating anaerobic conditions within the flowcell, and hydrolyze cellulose, resulting in the production of ethanol and acetate. This may be useful in reducing the cost associated with consolidate bioprocessing, as anaerobic conditions are achieved and maintained using microbial metabolism, rather than reducing agents and compressed gas. Lastly, it was found that ethanol and acetate production were the highest at a flow rate of 0.1 ml/min.

The continuation of this project would benefit by several experiments designed to help determine if the community would be suitable for industrial CBP. First, both higher and lower flow rates would be beneficial in determining the optimal flow rate for both cellulose hydrolysis and ethanol production, as well as observing if the ethanol to acetate ratio shifts as the flow rate increases and decreases. Second, a pre-treated substrate similar to what would be available on an industrial scale is necessary to determine if the community can function under real world conditions. For the purpose of this study raw cotton was used to easily asses for cellulolytic capabilities. Third, to further aid in overall cost reduction it would be interesting to determine if medium could be replaced with a waste product such as activated sludge or bunk wine, and if the culture would still be capable of cellulose hydrolysis and ethanol production. This may help offset the cost of an increase flow rate. Lastly, it would be interesting to test the community's ability to produce ethanol on a slightly larger scale using a flow-through system. To date all end

product experiments have been done using small amounts of cellulose (0.13 g), and it would be interesting to see if sustained ethanol production would be possible if larger amounts of substrate were to be used.

CHAPTER 6: REFERENCES

Alvira, P., Tomas-Pejo, E., Ballesteros, M., Negro, M.J. (2009). Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource Technology, 101*(2010), 4851-4861.

Bester, E., Wolfaardt, G., Joubert, L., Garny, K., & Saftic, S. (2005). Planktonic-cell yield of a pseudomonad biofilm. *Applied and Environmental Microbiology, 71*(12), 7792-7798.

Blenkinsopp, S. A., & Costerton, J. W. (1991). Understanding bacterial biofilms. *Trends in Biotechnology, 9*(4), 138-143.

Carere, C. R., Sparling, R., Cicek, N., & Levin, D. B. (2008). Third generation biofuels via direct cellulose fermentation. *International Journal of Molecular Sciences, 9*(7), 1342-1360.

Costerton, J. W., Stewart, P. S., & Greenberg, E. P. (1999). Bacterial biofilms: A common cause of persistent infections. *Science, 284*(5418), 1318-1322.

Costerton, J. W. (1995). Overview of microbial biofilms. *Journal of Industrial Microbiology, 15*(3), 137-140.

Datar, R., Huang, J., Maness, P. -., Mohagheghi, A., Czernik, S., & Chornet, E. (2007). Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process.*International Journal of Hydrogen Energy, 32*(8), 932-939.

Dumitrache, A., Wolfaardt, G. M., and Lynd, L. R. (2010). Surface microbiology of cellulolytic bacteria. In Manual of Industrial Microbiology and Biotechnology. Edited by Baltz, R. H., Demain, A. L., and Davies, J. E. ASMPress, Washington DC. 634-643.

Eveleigh, D. E., Stokes, H. W., & Dally, E. L. (1983). Recombinant DNA approaches for enhancing the ethanol productivity of *Zymomonas mobilis. Org Chem from Biomass,* , 69-91.

Haruta, S., Cui, Z., Huang, Z., Li, M., Ishii, M., & Igarashi, Y. (2002). Construction of a stable microbial community with high cellulose-degradation ability. *Applied Microbiology and Biotechnology,59*(4-5), 529-534.

Herrero, A. A., & Gomez, R. F. (1980). Development of ethanol tolerance in *Clostridium thermocellum*: Effect of growth temperature. *Applied and Environmental Microbiology*, *40*(3), 571-577.

Herrero, A. A., Gomez, R. F., & Wang, D. I. C. (1979). Development and characterization of ethanol tolerance in *Clostridium thermocellum. Electric Power Research Institute (Report) EPRI EA,* , 88-92.

Hill, J. (2007). Environmental costs and benefits of transportation biofuel production from food- and lignocellulose-based energy crops. A review. *Agronomy for Sustainable Development*, *27*(1), 1-12.

Jujjuri, S., Knutson, B. L., Thakur, A., Lynn, B., Dittert, E., Nokes, S., et al.,. (2007). Metabolic profile of wild-type and ethanol-adapted C*lostridium thermocellum* in continuous culture. *ACS National Meeting Book of Abstracts.*

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., & Igarashi, Y. (2004). Effective cellulose degradation by a mixed-culture system composed of a cellulolytic clostridium and aerobic non-cellulolytic bacteria. *FEMS Microbiology Ecology*, *51*(1), 133-142.

Kato, S., Haruta, S., Cui, Z. J., Ishii, M., & Igarashi, Y. (2005). Stable coexistence of five bacterial strains as a cellulose-degrading community. *Applied and Environmental Microbiology, 71*(11), 7099-7106.

Kroukamp, O., Dumitrache, R. G., Wolfaardt, G. M. (2010). Pronounced effect of the inoculum on biofilm development in flow systems. *Applied and Environmental Microbiology*, *76*(18), 6025-6031.

Leschine, S. B. (1995). Cellulose degradation in anaerobic environments. *Annual Review of Microbiology*, *49*, 399-426.

Lovitt, R. W., Longin, R., & Zeikus, J. G. (1984). Ethanol production by thermophilic bacteria. physiological comparison of solvent effects on parent and alcohol-tolerant strains of *Clostridium thermohydrosulfuricum*. *Applied and Environmental Microbiology, 48*(1), 171-177.

LSU – Louisiana State University. (2006). Structure and function of macro molecules. Department of biological science.

Lu, Y., Zhang, Y. -. P., & Lynd, L. R. (2006). Enzyme-microbe synergy during cellulose hydrolysis by *Clostridium thermocellum*. *Proceedings of the National Academy of Sciences of the United States of America, 103*(44), 16165-16169.

Lynd, L. E. E., Addy, M., & Kaffka, S. (2009). Squaring biofuels with food. *Issues in Science and Technology, 25*(4), 8-13.

Lynd, L. R. (2008). Energy biotechnology. *Current Opinion in Biotechnology, 19*(3), 199-201.

Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R. (2008). How biotech can transform biofuels. *Nature Biotechnology, 26*(2), 169-172.

Lynd, L. R. (2007). Thermophilic bacterium for biomass ethanol. *Industrial Bioprocessing, 29*(6), 4.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., & Pretorius, I. S. (2002). Erratum: Microbial cellulose utilization: Fundamentals and biotechnology (microbiology and molecular biology reviews (2002) 66:3 (506-577)). *Microbiology and Molecular Biology Reviews, 66*(4), 739.

Lynd, L. R. (1996). Overview and evaluation of fuel ethanol from cellulosic biomass: Technology, economics, the environment, and policy. *Annual Review of Energy and the Environment, 21*(1), 403-465.

Lynd, L. R., Grethlein, H. E., Wolkin, R. H. (1989). Fermentation of cellulosic substrates in batch and continuous culture by *Clostridium thermocellum*. *Applied and environmental microbiology*, 55(12), 3131-3139

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y. Y., Holtzapple, M., et al.,. (2005). Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology, 96*(6), 673-686.

Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of plolymerase chain reaction-amplified gene coding for 16S rRNA. *Appl. Environmental Microbiology*. 59: 695-700

Ng, T. K., Ben-Bassat, A., & Zeikus, J. G. (1981). Ethanol production by thermophilic bacteria: Fermentation of cellulosic substrates by cocultures of *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. *Applied and Environmental Microbiology, 41*(6), 1337-1343.

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J., & Zacchi, G. (2007). A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry, 42*(5), 834-839.

Peng, L., & Chen, Y. (2011). Conversion of paper sludge to ethanol by separate hydrolysis and fermentation (SHF) using *Saccharomyces cerevisiae*. *Biomass and Bioenergy, 35*(4), 1600-1606.

Schmer, M. R., Vogel, K. P., Mitchell, R. B., & Perrin, R. K. (2008). Net energy of cellulosic ethanol from switchgrass. *Proceedings of the National Academy of Sciences of the United States of America, 105*(2), 464-469.

Schwarz, W. H. (2001). The cellulosome and cellulose degradation by anaerobic bacteria. *Applied Microbiology and Biotechnology, 56*(5-6), 634-649.

Shaw, A. J., Podkaminer, K. K., Desai, S. G., Bardsley, J. S., Rogers, S. R., Thorne, P. G., et al.,. (2008). Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. *Proceedings of the National Academy of Sciences of the United States of America, 105*(37), 13769-13774.

Strobel, H. J., Caldwell, F. C., & Dawson, K. A. (1995). Carbohydrate transport by the anaerobic thermophile *Clostridium thermocellum* LQRI. *Applied and Environmental Microbiology, 61*(11), 4012-4015.

Waites. M. J., Morgan. N. L., Rockey. J. S., Higton. G. (2001). Industrial microbiology. Osney Mead, Oxford.

Wang, Z. & Chen, S. (2009). Potential of biofilm-based biofuel production. *Applied Microbiology and Biotechnology,* , 1-18.

Watanabe, T., Kanno, M., Tagawa, M., Tamaki, H., Kamagata, Y. (2011). Primary simple assay of cellulose-degrading fungi, *Mycoscience,* 1340-3540, 1-4.

Wood, B. E., & Ingram, L. O. (1992). Ethanol production from cellobiose, amorphous cellulose, and crystalline cellulose by recombinant klebsiella oxytoca containing chromosomally integrated zymomonas mobilis genes for ethanol production and plasmids expressing thermostable cellulase genes from clostridium thermocellum. *Applied and Environmental Microbiology, 58*(7), 2103-2110.

Yeung, C. W., M. Woo, K. Lee, and C. W. Greer. 2011. Characterization of the bacterial community structure of Sydney Tar Ponds sediments. *Canadian Journal of Microbiology*. 57: 493-503.