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Development Of A Gradient System For The Establishment Of Cellulolytic Microbial Communities

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DEVELOPMENT OF A GRADIENT SYSTEM FOR THE ESTABLISHMENT OF CELLULOLYTIC MICROBIAL COMMUNITIES

by

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To My Parents (Sandra and Dominic), Andrew and Trevor

For supporting me and always keeping me strong

Development of a Gradient System for the Establishment of Cellulolytic Microbial Communities

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ABSTRACT

Cellulosic ethanol has shown promise as a feasible alternative fuel, especially if the hydrolysis of lignocellulosic biomass is done through a single step process known as consolidated bioprocessing (CBP). A major challenge for CBP, especially for large-scale industrial applications is the inhibition of cellulolytic microorganisms by ethanol. While recombinant DNA technology and microbial acclimatization by exposure have resulted in some increase in ethanol tolerance, the search remains for robust bacteria that can proliferate in industrially-relevant conditions. This study applied an anaerobic gradient system to provide a continuous spatial pathway for the selection of cellulolytic consortia with increased tolerance to ethanol. DGGE analysis showed that increasing concentrations of ethanol impacts the community profile. Biofilm formation of cellulose degrading communities has been found to be influenced by species diversity. Environmental gradients have shown promise for selective enrichment of cellulolytic consortia at desired conditions required for industrial application.

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CHAPTER 1: INTRODUCTION

1.1: BACKGROUND

With the increasing demand for energy, cellulosic ethanol has shown promise in the production of biofuels. The use of lignocellulosic biomass in the production of ethanol may provide a feasible solution (36, 37).

Optimal breakdown of lignocellulosic biomass can potentially be accomplished through such processes as consolidated bioprocessing (CBP) (36, 37, 39). Consolidated bioprocessing (CBP), is a single process that involves the use of cellulolytic microorganisms in the hydrolysis of cellulose into ethanol. The formation of a cellulose-enzyme-microbe (CEM) complex in CBP allows for efficient substrate utilization and greater concentration of cellulases. It is hypothesized that cellulose degradation by CBP involves the breakdown of lignocellulose into cellulose and hemicellulose, followed by enzymatic hydrolysis of carbohydrates into hexoses and pentoses, fermentation of sugars and finally the distillation into cellulosic ethanol. Among several challenges to the conversion of biomass by CBP is the intolerance of cellulose-degrading microorganisms to increased concentrations of ethanol. Tolerances of at least 5% (v/v) are required for cost-effective industrial production of ethanol (37).

Factors influencing ethanol tolerance of cellulolytic microorganisms include fluidity of the membrane and an imbalance in redox reactions (20, 21). The focus on recombinant technology for industrial application has shown tolerances of up to 8% (v/v) (13). However, ethanol tolerances as a result of microbial acclimatization of environmental isolates such as *Clostridium thermocellum* ranges from 4-8 % (v/v) (13, 49, 50).

Mixed community cellulolytic biofilms have the potential to enhance cellulose utilization through the complete degradation of both hexose and pentose sugars, resulting in increased production of ethanol (13). Due to the exposure of microbial consortia to naturally established gradients (pH, concentration), the application of gradient systems to environmental consortia may result in increased tolerance over time.

Gradient plates are used to evaluate tolerance of microbial consortia to various environmental stressors such as pH, concentration and temperature (57). Gradient plates can potentially be used in the evaluation of ethanol tolerance of cellulolytic microorganisms through prolonged studies involving the use of a temporal and spatial pathway. This study involved the application of an environmental gradient to the selection of microbial communities with increased tolerance to ethanol.

1.2: HYPOTHESIS AND OBJECTIVES

Conventional methods for enriching cellulolytic bacteria have resulted in the isolation of pure cultures and do not allow for the establishment of diversified microbial consortia. For an industrial application, there is a need to enrich mixed species consortia with an ethanol tolerance of at least 5% (v/v). It was hypothesized that with the widespread abundance of cellulose and cellulolytic microorganisms present in the environment, the provision of a temporal and spatial gradient will allow for the selection of interactive, cooperative microbial consortia at desired conditions. These conditions include those required for industry such as increased ethanol tolerance and decreased production of methane through the elimination of methanogens (pH tolerance).

The main objective involved the design of an anaerobic continuous flow system that provides a temporal and spatial continuum along which a microbial community may develop. This involved the assumption that an anaerobic community would behave similar to aerobic systems with regards to biofilm formation. The working hypothesis was that when first inoculated, the cellulolytic microorganisms would grow at a lower concentration of ethanol using cellulose as a carbon source. As competition within the biofilm increases, some of the cellulolytic microorganisms within the community may gradually adapt to higher ethanol concentrations (greater than 5% v/v) within the gradient through genetic exchange (conjugation) as well as natural adaptation (mutation, gene expression levels, altered gene structure).

Two specific objectives were formulated to evaluate the newly developed system:

1. To test its applicability to enrich for cellulolytic communities along an ethanol gradient, and

2. Investigate the dynamics of the resulting cellulolytic communities using molecular fingerprinting techniques (DGGE) and microscopy (CLSM).

1.3: LITERATURE REVIEW

1.3.1: Biofuels

1.3.1.1: Ethanol fermentation

Fermentation processes from any material that contains sugar could result in ethanol production (29). Sugars are directly converted into ethanol, whereas starches and cellulose require preliminary hydrolysis into sugars, thereby increasing the cost of the fermentation process.

1.3.1.2: Sources of biomass

Biomass used in the production of ethanol is widespread within the environment. These sources can be divided into four main categories, which include wood residues, municipal solid waste, agriculture residues and food crops (29). Wood residues are one of the largest current sources of biomass involved in the production of ethanol and can be obtained from pulp and paper mills, sawmills and furniture manufacturing (29). The use of wood residues has been applied to many processes such as consolidated bioprocessing (CBP) (36). Drawbacks to the use of wood residues include the use of aqueous acid to degrade the lignin, which destroys many of the other sugars in the process (29). Municipal solid waste (paper, cardboard, kitchen waste, yard trash) is the next largest form, used in the production of ethanol through acid hydrolysis (28, 30). Following acid hydrolysis as the pretreatment step, simultaneous saccharification and co-fermentation (SSCF) occurs, which involves the production of saccharolytic enzymes such as cellulases and hemicellulases. The second step results in the hydrolysis of carbohydrates present in the biomass to sugars. This is followed by the fermentation of various hexose sugars such as glucose, mannose and galactose and finally the fermentation of pentose

sugars such as xylose and arabinose (36, 43). Organic kitchen wastes have also been used in the production of hydrogen. Benefits include both the production of energy and removal of organic waste. The fermentation process involves substrate hydrolysis from a suspended state to a soluble state, readily utilized by hydrogen-producing bacteria (28).

Agriculture residues such as herbaceous crops, tall grasses, and wood crops, can be harvested from a single planting, reducing the costs for managing and maintaining crops (29). Lastly, food crops such as corn have been utilized in the breakdown of cellulose through the addition of extracellular enzymes followed by simultaneous saccharification and co-fermentation (SSCF) (36, 37). The most widely used sugar for ethanol fermentation is molasses, but higher concentrations of the sugar produces too much ethanol and require a prolonged fermentation resulting in incomplete sugar conversion (29). Starches such as corn, rice, potato and wheat may also be used for ethanol fermentation. Fermentation involving starches can be complex because starch is initially broken down into sugars before further fermentation into ethanol. Primary breakdown of starch involves the addition of enzymes such as α–amylase to prevent gelatinization, followed by a cooking process (140-180°C), which increases the cost of the fermentation process due to the high-energy requirements during both the cooking and enzymatic processes (29). Cellulosic materials such as lignocellulose compose 90% of the plant biomass, and provide feasible alternative to starches (29). Lignocellulosic materials are not always of practical use due to seasonal availability and high costs of transportation, as well as increased costs associated with delignification (29).

The United States (US) and Brazil have primarily focused on the use of food crops such as corn and sugarcane as the main substrates in the production of biofuels

(29). However, even if the current entire corn crop (grains) in the US were harvested for ethanol fermentation, only 15% of the total fuel requirement would be fulfilled (30). Therefore there is a need for a more abundant cellulosic source that is able to meet the current energy demand. In the U.S. there is a notable effort in looking at cellulosic biomass (43). The use of high-energy crops such as lignocellulosic biomass has shown promise (43). Therefore the focus on food crops has since shifted to the use of cellulosic materials such as switchgrass that are widely distributed within the environment. In 2005, lignocellulosic biomass (agricultural waste, forest residues, mill waste) had grown from 180 to 200 million dry tons per year, which has the potential to produce 16 billion gallons of ethanol per year (1). The U.S. department of energy has since stated that the use of cellulosic materials in the process of consolidated bioprocessing offers new possibilities for improvements in yield and cost (38).

1.3.1.2.1: Cellulosic biomass

Due to the natural abundance of cellulose, cellulosic biomass is one of the most feasible renewable sources for ethanol production (37). Cellulose is a major component of industrial and municipal waste (55). Cellulosic microorganisms are also an important component to many processes regarding carbon flux in the biosphere such as composting and anaerobic digestion (37). Anaerobic composting through the use of the sequential-batch anaerobic composting process, involves the breakdown of municipal solid waste without the use of aeration or mixing, into methane reducing energy requirements (7). Anaerobic digestion, involving the breakdown of biodegradable material by microbial consortia, is used in the treatment of wastewater and the production of renewable sources such as methane. Examples include the use of cellulosic materials from a wastewater

treatment plant in the production of biofuels through biogas desulphurization (46).

Cellulose has a crystalline structure and is composed of linear glucosyl residues held together by β-1,4 linkages. Cellulose molecules are packaged into protofibrils, which are in turn assembled into larger microfibrils and lastly cellulose fibers (37) (Fig. 1). Due to this structure, cellulose is highly resistant to microbial degradation and thus poses a significant challenge to processes such as industrial bioconversion.

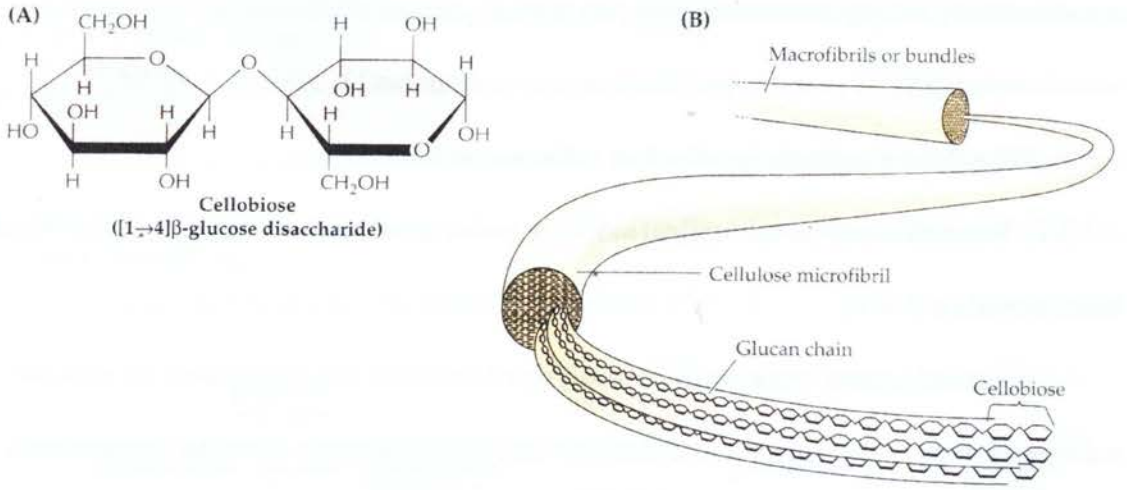

FIG. 1. Structure of cellobiose (a) and a cellulosic fiber (b) (3).

1.3.1.2.2: Lignocellulosic biomass

Plant biomass is primarily composed of cellulose (35-50%) and also includes biopolymers such as hemicellulose and lignin (20-35% and 5% respectively) encased within the recalcitrant matrix (37). This form of biomass is referred to as lignocellulosic biomass, and is where cellulose and hemicellulose are tightly bound to lignin through covalent and hydrogen bonds. This form of cellulose contributes to 90% of the total biomass found in the environment (29). Pretreatment of the lignin and disruption of the crystalline structure makes cellulose more accessible for hydrolysis. Use of lignocellulosic biomass for biofuel production has several advantages over food crops,

which include low cost, large-scale availability, reduced greenhouse gas emissions and environmentally benign production (29, 37, 51). Potential drawbacks include cost of transportation, storage and pre-treatment as well as limited seasonal availability (29, 43). However, with further research and development, the potential for lignocellulose is promising and has been used in a plant in Ottawa, Canada, since 2004 (29). Iogen Corporation, located in Ottawa, operates a 10-acre enzyme and cellulosic ethanol manufacturing facility producing over one million gallons of ethanol per year utilizing wheat, switchgrass, oat and barley straw as raw materials (22, 29).

1.3.3: Microbial processes involved in cellulose utilization

1.3.3.1: Simultaneous saccharification and co-fermentation (SSCF) & consolidated bioprocessing (CBP)

Currently, most work on the hydrolysis of cellulose has been based on enzyme-substrate interactions. Enzymatic hydrolysis that occurs separate from the fermentation process is referred to as separate hydrogen fermentation (SHF), whereas hydrolysis that occurs in the presence of microorganisms is termed simultaneous saccharification and fermentation (SSF). SSF requires enzyme and culture conditions to be compatible with regards to temperature and pH, for example, cellulases from *Trichoderma reesei*, have optimal cellulose degrading capabilities at pH 4.4 and 55°C (29).

Alternatively, simultaneous saccharification and co-fermentation (SSCF) refers to saccharification of cellulose and hemicellulose and further co-fermentation of sugars. The four-step process of simultaneous saccharification and co-fermentation (SSCF) involves the hydrolysis of cellulose using an enzyme-substrate complex (36, 37, 43). The first step involves production of saccharolytic enzymes such as cellulases and hemicellulases. The

second step results in the hydrolysis of carbohydrates present in the biomass to sugars. This is followed by the fermentation of various hexose sugars such as glucose, mannose and galactose and finally the fermentation of pentose sugars such as xylose and arabinose (36, 43). These four transformation processes can potentially be achieved in a single step using consolidated bioprocessing (CBP), which omits the specific step dedicated to producing large amounts of expensive cellulases (FIG. 2). CBP involves the pretreatment of lignocellulose into cellulose and hemicellulose using microbial enzymes, followed by the enzymatic hydrolysis of carbohydrates into hexoses and pentoses by thermophilic microorganisms, fermentation of sugars and distillation into cellulosic ethanol. Therefore CBP is advantageous in that there is lower cost and higher conversion efficiency associated with the breakdown of cellulosic biomass.

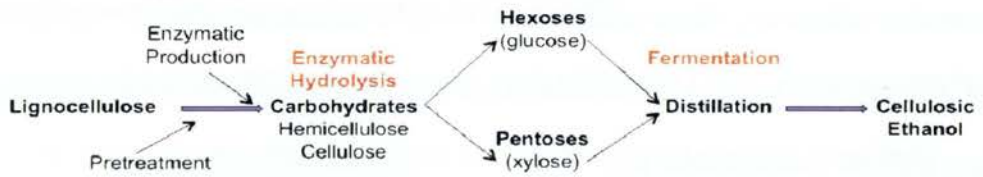

FIG. 2. Comparison of the single step process involved CBP to the four step process involved SSCF. CBP in comparison to SSCF lacks the step in dedicated cellulase production.

1.3.3.1.1 Cellulose-enzyme-microbe complexes (CEM) vs. cellulose-enzyme complexes (CE)

CBP can involve cellulose-enzyme-microbe (CEM) complexes to achieve higher hydrolysis rates. It has been hypothesized that the feasibility of CBP using CEM is that

the effectiveness of the cellulase (due to better concentrations of cellulases within the complex) is enhanced in comparison to CE complexes (36). CBP has a fourfold reduction in the cost of biological processing and a twofold reduction in the cost of processing overall (38). CBP involves the use of microorganisms with good substrate utilization capabilities and rapid degradation of other components from the pretreated biomass combined with high ethanol production (38). The closely associated CEM complex formed by cellulolytic bacteria such as *C.thermocellum* forms the cellulosome, which aids in attachment to cellulosic fibers and may result in enhanced cellulose degradation as opposed to the partial breakdown of cellulosic materials. A diversified cellulolytic community composed of both aerobes and anaerobes is able to breakdown both hexoses and pentoses, which may increase ethanol yields. This eliminates addition of expensive cellulases, which significantly reduces the cost associated with CBP. The use of thermophilic microorganisms involved in CBP also reduces costs associated with cooling after the pretreatment step.

The cellulase enzyme systems have proven to be quite costly due to the amount of enzyme required for the process. It has been reported that costs of cellulase is in the range of 10 to 20 cents per gallon of ethanol (36). Cellulase enzymes coupled with SSCF of hexose and pentose sugars has a biological processing cost of 18.9 cents per gallon and a projected selling price of 77 cents per gallon. This is equal to 1.08 dollars per gallon for the gasoline equivalent (Fig. 1.) (36). CBP on the other hand has a much lower processing cost of 4.2 cents per gallon and a selling price of 63 cents per gallon (Fig. 1.) (36). CBP has a gasoline equivalent of 88 cents per gallon in comparison to 1.32 dollars

per gallon spent in 2005 (36). Costs were determined by the United States Department of Energy (36, 54).

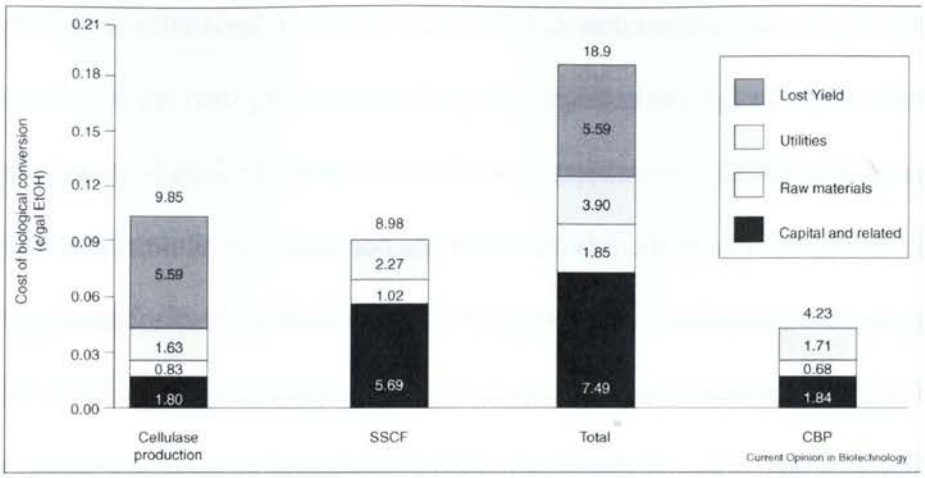

FIG. 3. Comparison of cost effectiveness of CBP and SSCF with a dedicated step in cellulase production. Adopted from Lynd et al. 2005 (36).

1.3.3.1.2: Thermophilic microorganisms used in CBP

The use of thermophilic organisms for both CEM and CE is advantageous with regards to the pre-treatment step in processing. Increased temperatures have been associated with higher rates of hydrolysis, a requirement for CBP (38). Herrero and Gomez (20) found that *Clostridium thermocellum* could be potentially used in the single-step process of CBP. The direct conversion of cellulose by *C. thermocellum* into ethanol, acetic acid, lactic acid, carbon dioxide and hydrogen gas, eliminates the requirement for the pre-treatment of the biomass (20).

For cellulolytic anaerobic microorganisms, the CEM complex plays a major role in the hydrolysis of cellulose (34). Also, the presence of cellulolytic microorganisms has been thought to increase hydrolysis rates of cellulose through the decrease in inhibitory products. Lynd et al. (36) found that cellulose hydrolysis rates involving cellulase activity for *C. thermocellum* using CEM were much higher than CE. This has been confirmed by

Reese and Mandels (36) who found that hydrolysis rates of various cellulolytic microorganisms were much higher when grown in culture as compared to enzymatic preparations. Furthermore, Jensen et al. (23) found that hydrolysis rates of cellulose were dependent on sessile bacteria in both leachate and rumen rather then species diversity of the community. The CEM complex has several advantages, which include easier access to hydrolysis products, due to the minimal distance between the cellulose degrading microorganism and the substrate, thereby allowing efficient uptake of oligosaccharides by the host cell (37). Other advantages include the concentration of cellulases due to close proximity of the cells to the substrate. Cellulose degrading microorganisms may compete with other non-adherent contaminants, which can ultimately increase the stability of industrial processes involving microbial cellulose utilization (36). In comparison, CE complexes have reaction rates that are generally at least two orders lower than those for most enzymes, and therefore require large amounts of expensive cellulase enzymes (58).

To date, there are no known naturally occurring microorganisms that exhibit rapid cellulose utilization and production of ethanol at a high yield (38). Various strategies have been suggested for the optimization of cellulolytic organisms involved in CBP, such as the native cellulolytic strategy and the recombinant cellulolytic strategy. The native cellulolytic strategy involves the genetic engineering of naturally occurring cellulolytic organisms to improve yield, titer, substrate utilization and efficiency through metabolic engineering and gene transfer (36). The recombinant cellulolytic strategy involves the engineering of non-cellulolytic microorganisms such as yeast with high product yields to express the heterologous cellulase system for efficient cellulose degradation (36). It has

been found that the use of non-cellulolytic bacteria, such as genetically modified yeast and bacteria were capable of fermenting both hexoses (glucose) and pentoses (xylose) resulting in greater efficiency of ethanol production (43). Although yeasts such as *Saccharomyces cerevisiae* produce ethanol at high yields through the utilization of substrate sugars, the expression of saccharolytic enzymes and their role in anaerobic cellulose degradation still remains to be determined (36).

C. thermocellum has been found to exhibit one of the highest rates of cellulose utilization in comparison to other thermophilic microorganisms. As a result of this finding, *C. thermocellum* is favourably used in the development of a one-step processing strategy used for the conversion of cellulose into ethanol (34). Due to the high substrate utilization capabilities of species of clostridia, further engineering to produce ethanol at high yields can be achieved as outlined by the native cellulolytic strategy.

1.3.4: Microbial degradation of cellulose

1.3.4.1: Commonly studied thermophiles

The use of thermophiles for cellulosic ethanol production involved in CBP is advantageous because of their ability to ferment a wide variety of monomeric and polymeric carbohydrates (13). Species of clostridia play a major role in the breakdown of plant material (16). In an industrial context, growth at high temperatures (50-70°C) reduces contamination of processes involving pure cultures and has been associated with increased hydrolysis rates (38). Since less money is spent on cooling and product recovery, the use of thermophilic microorganisms increases cost effectiveness (13). Other advantages include the ability of thermoanaerobes such as *Zymomonas* to ferment biomass polymers (cellulose, hemicellulose) directly into ethanol with high metabolic

rates. Thermophilic fermentations may also reduce the amount of energy required if a continuous ethanol recovery process at 60°C can be developed (33). Obstacles in the use of thermophiles include low substrate and ethanol tolerance, which may be overcome through genetic engineering, natural selection processes and further investigation of metabolism.

1.3.4.1.1: Clostridia

Species of clostridia; specifically the industrially relevant *C. thermocellum* and *C. cellulolyticum* in particular are gram positive, fermentative, anaerobic, cellulose-degrading microorganisms (31). *C. thermocellum* in particular ferments cellulose and cellodextrins to produce H_2, CO_2, ethanol and acetic acid (55). The metabolism of these microorganisms in the breakdown of cellulose is hindered by various regulatory factors. Weimer and Zeikus (55) found that the rate-limiting step involved in the production of ethanol by *C. thermocellum* was the solubilization of cellulose. Gueden et al. (16) found that nutrient deprived concentrations enhanced regulation and breakdown of cellobiose into ethanol as opposed to the breakdown into the main product acetate, as seen with complex media in *C. cellulolyticum*. This is a result of low nutrient conditions that are a reflection of the environment. Over time species such as *C. cellulolyticum* have evolved to break down cellobiose under these nutrient deprived conditions. Due to the crystalline structure of cellulose, the hydrolysis by microorganisms into cellobiose is accomplished through a network of several enzymes. This complex network of enzymes is known as the cellulosome. Cellulosomes exist on the cell wall of cellulolytic bacteria. These enzyme complexes are firmly bound to the cell wall but are flexible enough to bind tightly to cellulose (37).

1.3.4.1.1.2: Formation of the cellulosome

The cellulosome is important in anchoring species of clostridia to cellulose. The cellulosome of *C. thermocellum* is one of the largest (100 MDa) that have been described and the enzymes involved have been extensively studied (16). The multi-enzyme subunit exhibits intramolecular synergism with the help of binding molecules (22 amino acid residues) that orients the enzymes in the specific ratio and orientation to breakdown cellulose (FIG. 4) (51). These non-catalytic residues are conserved in all enzymes found within the cellulosome. These residues (also known as the dockerin domain) bind to cohesion modules resulting in the scaffoldin (51). Close contact between microorganisms and substrate is maintained by the cellulosome, which minimizes losses of hydrolytic products. With the use of genetic engineering, sequences of the cellulosome may be purified to study the mechanisms of attachment and formation on cellulosic fibers (51). This may be applied to other non-adherent microorganisms with high ethanol yields such as yeast, to promote good cellulose substrate utilization capabilities involved in the recombinant cellulolytic strategy. This could involve the genetic engineering non-adherent microorganisms to produce the cellulosome, which aids in cellulose degradation.

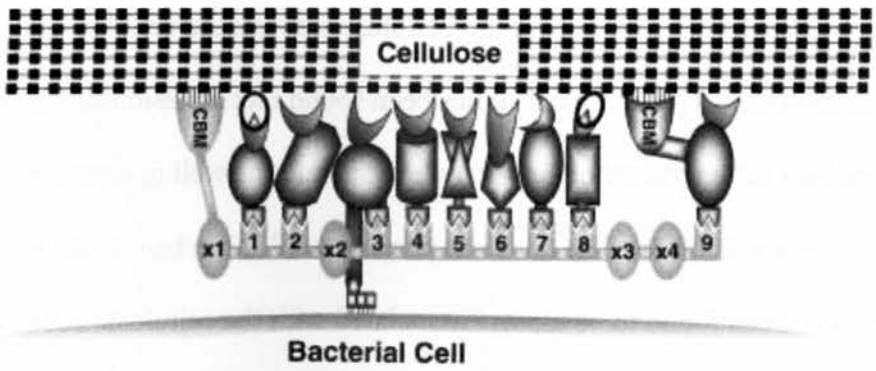

FIG. 4. The hypothesized structure of the cellulosome (52).

1.3.4.2: Biofilm formation by cellulolytic microorganisms

Substrate colonization by cellulolytic consortia is diverse in nature. Sources of cellulolytic inocula include anaerobic sludge, soil, compost, landfill leachate and manure (23). Microbial communities are diverse in many aspects including species profile, biofilm architecture and nutritional environment (23). It has been suggested that the biofilm composition of cellulolytic biofilms that colonize a biotic substratum differs from traditional biofilms with regards to substrate colonization and complexity of the biofilm architecture (39). Aerobic and anaerobic biofilms typically consist of a complex matrix of cells surrounded by extracellular polymeric substances (EPS) (Fig. 5) (39, 53). The active cells capture nutrients from the bulk aqueous phase. The surface-associated cells are resistant to many environmental stressors, which could be due to the diversity of the species present and their ability to modify their environment to survive.

In contrast, it has been suggested that the structure of pure culture cellulolytic biofilms is more ordered (39). *C. thermocellum* forms a structured monolayer of cells on the carbon (cellulose) substrate to obtain nutrients (Fig. 5.) (39). The hypothesized CEM complex involves the close association between microbial cells and the substrate, which allows for enhanced degradation and concentration of cellulases. Hydrolysis of cellulose primarily requires binding of enzymes to cellulose through the cellulosome, followed by the formation of the CEM complex. It has been found that adhesion-defective mutants of *C.thermocellum* have reduced cellulose hydrolysis rates as well as increased stability, resulting in reversion back to the adherent phenotype (37). In batch culture, species of clostridia have been found to be closely associated with the cellulosic substrate using cellulose-binding modules within the cellulosome (37). Lynd et al. (37) have speculated

that the CEM is a major component of cellulolysis. Since cellulose hydrolysis is a surface phenonmenon *C.thermocellum* attaches to the cellulose fibers resulting in degradation and utilization of soluble sugars for growth (21). Glycolipids present on the surface of *C.thermocellum* may serve as binding sites to substrates and may assist in the breakdown of the cellulose fiber related to full enzymatic attack (21).

Cellulose hydrolysis has also been described for ruminal bacteria (37). It is theorized that adhesion mediated by a glycocalyx offers several advantages including the concentration of enzymes at the cellulose surface, which allows first access of oligomeric products from cellulose hydrolysis to the adherent bacteria. This process not only protects ruminal bacteria from undesirable predatory attacks from protozoa and bacteriophages, but also preserves hydrolytic enzymes that could be cleaved by ruminal proteases (37).

The role of unattached cells in the degradation and hydrolysis of cellulose through extracellular enzymes is yet to be determined (39). The formation of EPS in anaerobic biofilms still needs to be further investigated.

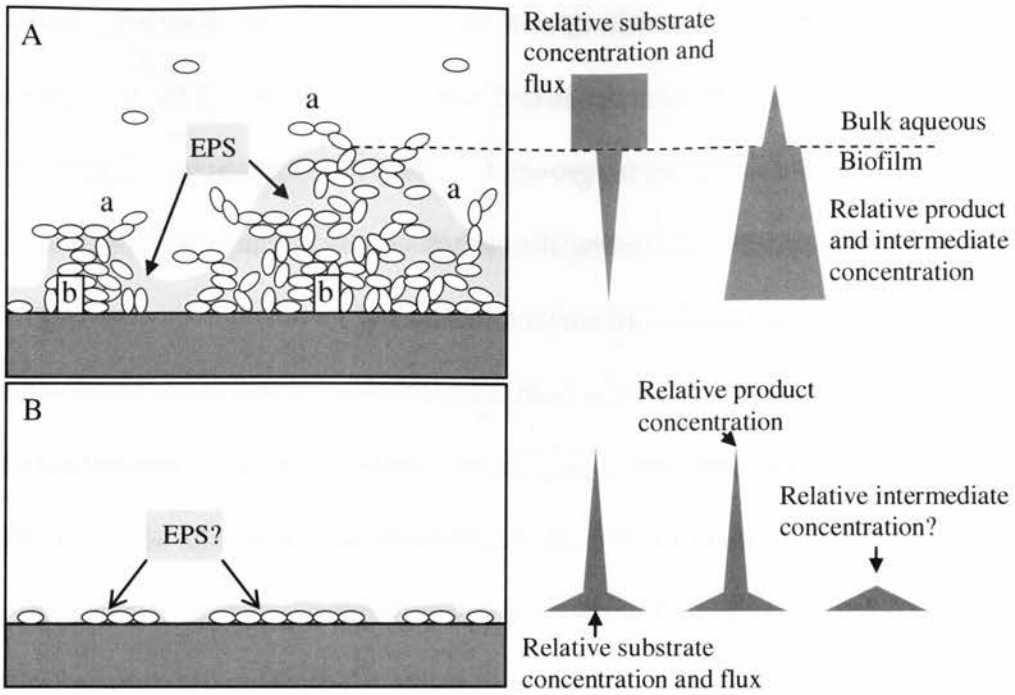

FIG. 5. The influence of abiotic and biotic substratum on biofilm formation. Biofilms that form on an abiotic substratum typically form a complex matrix surrounded by EPS where nutrients are obtained from the bulk liquid phase (a). It is possible that in pure culture cellulose degrading biofilms, a monolayer of cells is formed to obtain nutrients from the substratum (b) (39).

1.3.4.3: Genetic engineering of thermophiles

Gene transfer for the thermophilic anaerobe *C. thermocellum* has been described using biotechnological gene transfer involving electrotransformation (ET) (36). It has been reported that genetic engineering of *C. cellulolyticum* genes involving the expression of pyruvate decarboxylase and alcohol dehydrogenase resulted in both increased growth and production of ethanol as well as decreased production of lactate (16). Due to the inability of *C. thermocellum* to hydrolyze a wide variety of sugars such as xylose, research into the use of communities for complete hydrolysis of cellulose has been further conducted. Engineering of other non-thermophilic microorganisms such as

Escherichia coli has demonstrated promise. Using microarray technology it has been shown that the ethanolgenic *E. coli* mutant strain KO11 over expresses xylose metabolism genes thereby converting cellulose directly into ethanol with minimal undesired end-product formation (43).

1.3.4.3.1: Naturally occurring gene transfer

In the literature it has been found that cellulase genes are either randomly distributed (*C. thermocellum*), or clustered on the genome (*C. cellulolyticum*) (37). The clustered genomes of *C. cellulolyticum*, *C. acetobutylicum* and *C. cellulovorans* have been found to contain nine cellulosomal genes with a transposase gene in the 3' flanking region (37). This evidence denotes a common bacterial ancestor between the various species of clostridia or the occurrence of transposon-mediated horizontal gene transfer events. It has been suggested that the homologous cellulase genes between related organisms in various cellulose systems are a result of chromosomal rearrangement and horizontal gene transfer. Such examples include CBH1-like gene clusters in *Phanerochaete chrysosporium* and CelK and CbhA exoglucanases in *C. thermocellum* (37).

1.3.4.4: Ethanol titers of cellulolytic microorganisms

Ethanol titers for various strains of *C. thermocellum* have been found to be in the range of less than 26 g/L to 60g/L, and these discrepancies between titers are thought to be due to intolerance to ethanol (36). The reduced tolerance to higher concentrations of ethanol is a limiting factor in the potential use for industry (49, 50). Other reasons for the decreased yield are the inhibitory effects caused by the presence of organic acids and salts on thermophilic microorganisms (49, 50).

One possible method to decrease the inhibitory effects of organic acids is to target the genes involved in the synthesis of lactic acid (L-lactate dehydrogenase) and acetic acid (acetate kinase, phosphate acetyltransferase) (52). Shaw et al. (52) have shown that genetic engineering of *Thermoanaerobacterium saccharolyticum* resulted in a mutant strain able to produce ethanol as the only detectable end product in high yields (37g/L). Therefore metabolic engineering could be used to potentially maximize ethanol yields (36).

1.3.5: Environmental factors and tolerance

1.3.5.1: Ethanol tolerance

Ethanol tolerance is a key limitation in the production of cellulosic ethanol. Tolerances of up to 8% have been found for species of clostridia (20, 21, 32, 33). Studies have shown that inhibition is a result of an increase in membrane fluidity as well as changes in membrane composition. Ethanol affects membrane physiology by partitioning between lipid bilayers and interfering with lipid-lipid and lipid-protein interaction (21). The physical effect of ethanol to "tighten" the membrane results in a more fluid membrane. Herrero and Gomez (20) have shown that increased fluidity can be attributed to a blockage in glycolysis resulting in the inhibition of glycolytic enzymes involved in the breakdown of hexose into glyceraldehyde-3-phosphate as demonstrated by *C. thermocellum*. Lovitt et al. (33) found that decreased tolerance affected the metabolism of microorganisms such as *C. thermohydrosulfuricum*. Inhibition was a result of a redox imbalance, which was contradictory to results presented by Herrero and Gomez (20) that attributed intolerance to membrane composition. It was found that there was twice the concentration of NADH in comparison to NAD^+ in glycolysis (33). *C.*

thermohydrosulfuricum has two alcohol dehydrogenase enzymes, a primary alcohol dehydrogenase that produces ethanol and a secondary alcohol dehydrogenase that consumes ethanol and produces NADH (32). *C. thermohydrosulfuricum* can ferment a wide variety of substrates such as glucose and xylose with a higher tolerance to ethanol than *C. thermocellum*. Certain species of Clostridia have a higher tolerance to ethanol as a result of differences in membrane composition. This has been attributed to differences in regulation of carbon and electron flow pathways resulting in enzymatic activity alterations (33).

1.3.5.1.1: Genetic engineering of cellulolytic microorganisms

Low ethanol tolerance can be overcome by adaptation of the microorganisms to high ethanol concentrations or by development of high ethanol-tolerant mutants (13). Increased tolerance has been found mainly for genetically engineered thermophiles (13, 33). Tolerances of greater than 4% (v/v) and 2-3% (v/v) ethanol were found for mutant and wild type strains, respectively (33). Similarly *T. ethanolicus* wild type and mutant (39E-H8) strains had tolerances of up to 6% (v/v) and 8% (v/v) (4, 13).

1.3.5.1.2: Naturally occurring gene transfer

For species of Clostridia, research has shown that maturity of the culture effects environmental tolerance. Rani and Seenayya have shown that over an extended period of time strains of *C.thermocellum* (SS21 and SS22) were able to tolerate concentrations twice that of results found in literature reaching concentrations of up to 8% (v/v) (49).

1.3.5.1.3: Influence of temperature

Temperature influences ethanol tolerance of thermophiles. Growth and ethanol tolerance has been found to increase for mutant strains such as *C. thermohydrosulfuricum*

at lower temperatures (33). Although Lovitt et al. (33) found that lower temperatures (45-50° C) have been found to increase tolerance of microbes, the optimal temperature for increased yield (56 g/L) and tolerance of cellulolytic microorganisms such as *Thermoanaerobacter* A10 has been found to be 60°C (13, 33). Narisawa et al. (45) has found that temperature is a key factor controlling competition in a bioreactor between exogenously added bacteria such as *C. thermocellum* and an indigenous cellulolytic mixed community.

Mathematical models such as The Levenspiel Model can be used to study microbial growth and the effect of toxicity on microorganisms (13). This model has been used to study the effect of toxic metabolites such as ethanol on yeasts and *T. ethanolicus* (13). Georgieva et al. (13) concluded that continuous addition of ethanol progressively inhibited microbial growth. The optimum temperature for growth of *Thermoanaerobacter* A10, isolated from an Icelandic hot spring is 70°C. At 70°C the maximum ethanol tolerance was observed at 4.7% (v/v) whereas growth was completely inhibited at 5.6% (v/v). The highest tolerance (greater than 5.1% v/v) of the thermophile was observed at 60°C (13). Studies using The Levenspiel Model have shown increased tolerance and optimal yield of *Thermoanaerobacter* at lower temperatures in the range of 55-60°C. Using this relatively simple model, Georgieva et al. (13) found that ethanol exhibits non-competitive inhibition on microbial growth.

1.3.5.2: pH tolerance

In anaerobic digestion by mixed communities, pH can be limiting. Anaerobic digestion is accomplished by hydrolysis, acidogenesis and methanogenesis. The hydrolysis of the biodegradable particles is a rate-limiting step in acidogenesis involving

sludge (6). Methanogens are involved in the conversion of organic acids or CO_2 and H_2 into methane gas (6). Studies involving enzymatic hydrolysis have shown that pH and temperature are the most important factors affecting hydrolysis of cellulose and biomass growth rate involved in CBP (6). Chyi and Dague (6) demonstrated that the production of methane gas by cellulolytic microorganisms decreased as the pH decreased. By converting the amount of methane gas into chemical oxygen demand (COD) an overall increase in soluble COD at pH 5.6 was found, as a result of increased hydrolysis and reduced methane production (6). The optimum pH of hydrolysis and acidogenesis was determined to be approximately 5.6 (6). An important factor to note is that cellulose degradation by some species of clostridia such as *C. straminisolvens* has been found to be limited under a pH of 6.0. It has been found that aerobic microorganisms within a community neutralize the pH through acetic acid consumption, which could potentially allow for the unwanted optimal growth of methanogens (25). Future studies involving gradient systems may result in the adaptation of species of clostridia to lower pH values, preventing the growth of methanogens thereby eliminating the dependence on aerobic microorganisms to neutralize the solution.

1.3.6: Beneficial applications of communities

1.3.6.1: Synergistic effects of communities

To date, pure cultures have been used in studies to evaluate the potential application of CBP. There is a potential use for mixed communities involved in CBP in industrial settings. This is due to the difficulty in the utilization of pure cultures without pre-treatment or sterilization (18). Diverse microbial consortia may potentially eliminate the need for pre-treatment or sterilization. In nature, microbes exist as communities carrying

out various natural processes such as the breakdown of cellulose. Typical approaches used to enrich for a community have led to isolation of pure cultures. Scientists have attempted to reconstruct a stable community through the isolation of the various microorganisms that are present (18). It is difficult to mimic an environmental community through the isolation of various constituents. Although it provides an advantage of clarification of the role of each bacterium in the community, the individual function may differ when present in a community. Therefore these techniques were not designed to enrich for consortia consisting of interactive and cooperative members.

Within the environment, cellulosic materials such as lignocellulose are most probably degraded with the cooperation of many microorganisms (18). The breakdown of cellulose by cellulolytic bacteria can be enhanced by the presence of non-cellulolytic bacteria (18). The presence of both cellulolytic and non-cellulolytic bacteria in a complex community has been found to increase the degradation efficiency of cellulose (18).

As demonstrated by Haruta et al. (18), the profiling of a complex community can be conducted through denaturing gradient gel electrophoresis (DGGE). Disadvantages of this method include the inability to determine all of the components within the community as well as the biases in steps of DNA extraction and PCR amplification (18, 25). Using DGGE analysis, the 16S rRNA fragments from the DNA extracted are amplified using the Polymerase Chain Reaction (PCR) (10, 18, 24, 44). The amplified product is resolved on a DGGE gel. The resulting bands, which contain 16S rRNA gene sequences are then excised from the DGGE gel, eluted in water, and sequenced. Sequence results are compared to a database such as GenBank using sequence similarity search programs like BLAST (10, 19).

Haruta et al. (18) found that through the cooperation of aerobic and anaerobic bacteria such as *Pseudoxanthomonas taiwanensis* and *Clostridium thermosuccinogenes* a microbial community capable of degrading rice straw with high efficiency was isolated. This was due to the relationship among microorganisms through their metabolism. Examples of synergistic interactions found in literature include the presence of aerobes that metabolize inhibitory products such as lactate and acetate, which enhance cellulose utilization by cellulolytic microorganisms and increase the production of ethanol. Cellobiose is rapidly degraded by aerobic metabolism, reducing its inhibitory effect on species of clostridia and leading to enhanced substrate utilization (24).

The ability for the community members to coexist is a result of a highly oxygenated upper phase at the liquid-air interface and an anaerobic lower phase at the liquid-substrate interface creating a reductive condition (25). There are several factors that contribute to the success of the cellulose degrading community. Aerobic isolates such as *Brevibacillus agri* in a community consume the oxygen through the utilization of substrates such as peptides and amino acids found in yeast extract and peptone (25). Several species of clostridia have been found to be inhibited by metabolites derived from cellulose such as cellobiose. Aerobic species within the community degrade the various metabolites, which enhance the cellulose degrading capability of the anaerobic bacteria (25). Excretion of low-molecular-weight compounds may also increase the cellulose degrading capability of cellulolytic organisms. Addition of boiled supernatant from *Pseudoxanthomonas sp.* was found to enhance the cellulolytic capability of *Clostridium straminisolvens* (25). Through these findings, the function of a thriving cellulolytic

community can be attributed to the interactions that occur among the various species present.

1.3.7: Environmental gradients (temporal and spatial gradients)

In most experimental settings, spatial and temporal pathways required for communities to establish are not provided, which could effect the time required for naturally occurring gene transfer such as conjugation to occur. Instead of providing pathways, which allow for the community to develop naturally, scientists engineer communities in order to obtain the desired product(s). For example, in a traditional chemostat, the pH of the system can be altered in order to obtain a thriving pure culture at a specific value. By allowing a temporal and spatial pathway as seen in a gradient plate the microorganisms within the system may be able to naturally adapt to pH. Therefore there is a need to develop a continuous temporal and spatial pathway to study microbial communities. The existing methods do not allow for continuous pathways. Also, there is a need to develop fundamental techniques to manage cellulolytic microbial consortia in an industrial setting. These include the use of gradient systems to study microbial tolerance to inhibitory factors such as ethanol and pH.

The growth of microorganisms is greatly influenced by various gradients such as pH, temperature, moisture, nutrients, pressure and antimicrobial agents (56, 57). The adaptation of microbial consortia to such gradients have allowed for the inhabitation of various environmental niches. Gradient plates offer the advantage of providing both a continuous and spatial pathway for microbial consortia to develop (57).

The gradient that developed in a ceramic-based system has been previously defined using pH microelectrodes (57). The concentration gradient measured between the highest

concentration and the point of stabilization (lowest concentration) was approximately 9 mm (Fig. 6.) (57). These values may be applied to other ceramic-based gradient systems with similar construction. Other applications include the use of a ceramic-based gradient system to study the tolerances of cellulose degrading communities to various environmental stressors (ethanol, pH).

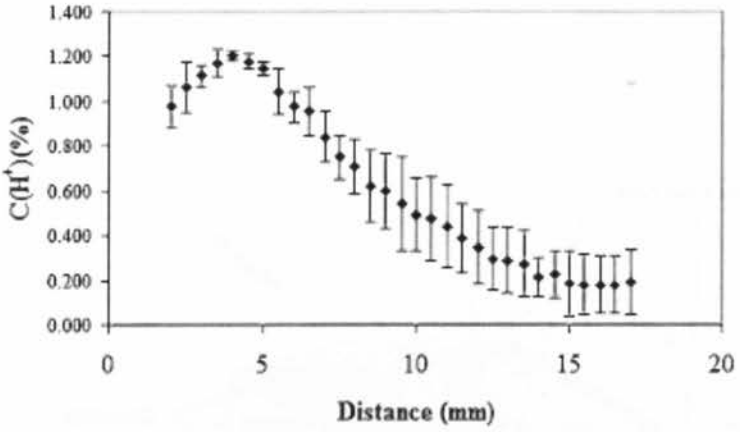

FIG. 6. Verification of concentration profiles using pH microelectrodes (57).

1.3.7.1: Materials used in the construction of a stable gradient

Gradient systems used to study various influences such as the effects of nutrient flux, pH and oxygen tolerance on microbial growth commonly use gels to establish concentrations. Due to various consequences of gel deterioration as a result of long-term incubations needed to study complex microbial communities, ceramic materials have been used as an alternative (Fig. 7.) (57). Gradient plates provide several conditions to support microbial adaptation, which can be a result of gene transfer, competition, synergism and cooperation. Since bacterial adhesion has been correlated to improved genetic exchange, the study of biofilms in gradient plates offers potential for

improvement of bioconversion efficiency (57). The limitation to the current design is that the concentration range occurs over a short distance of approximately 10 mm, which makes it difficult to determine the concentration, or pH at any point (57).

As outlined by Wolfaardt et al. (57), a pH gradient was successfully established in a 4mm thick ceramic tile by a constant supply of 0.5 M HCl at flow rate of 120ml/h. Microelectrodes were used to measure the pH gradient after a steady-state was established after 15 hours (57). It was found that measured concentration profiles were correlated with simulated concentration profiles (57).

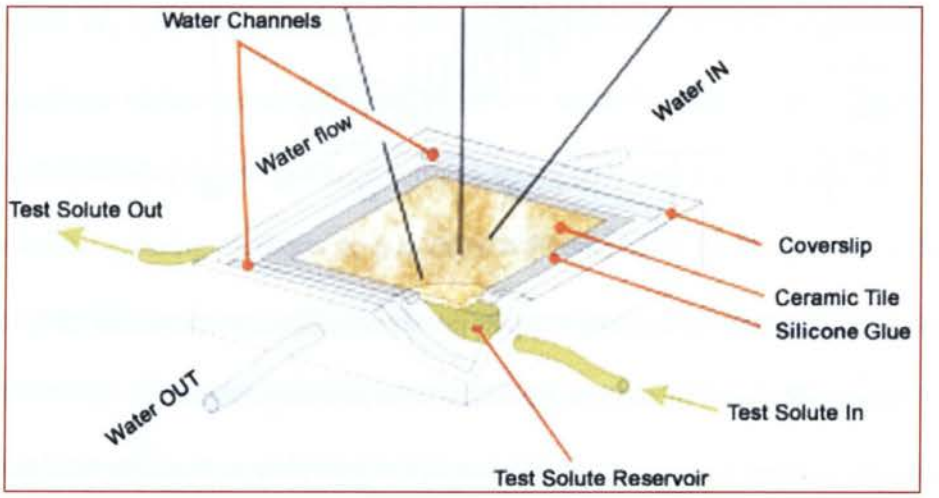

FIG. 7. Schematic diagram of a gradient plate flow cell (57). Flow cells are milled from Plexiglas, containing a ceramic tile (28 mm x 28 mm x 4 mm) and microscope coverslip (45 mm x 50 mm).

The application of gradient systems to cellulose degrading communities may provide an alternative to the study mechanisms of inhibition of cellulolytic microorganisms, such as ethanol tolerance, encountered within industry. Ethanol

tolerance has been found to be an increasingly important inhibitory factor in industrial applications. The use of cellulose degrading mixed communities has shown that cooperation between microbes may enhance the hydrolysis of cellulose. Furthermore, environmental isolates involved in CBP may result in an increase in environmental tolerance through competition, synergism and genetic exchange. This study aimed to apply a ceramic-based system to study the tolerance of cellulolytic communities to ethanol.

CHAPTER 2: EXPERIMENTAL

2.1: ABSTRACT

Cellulosic ethanol production has shown promise when achieved through consolidated bioprocessing (CBP). A major challenge for CBP is the inhibition of the cellulolytic microorganisms by ethanol. While recombinant technology and microbial acclimatization by exposure have resulted in some increase in ethanol tolerance, the search remains for robust bacteria that can proliferate in industrially-relevant conditions. A system was developed to expose microbial communities to an ethanol gradient for the enrichment of cellulolytic consortia with increased tolerance to ethanol. This anaerobic continuous flow system, allowed for cultivation of cellulolytic communities with an increased degree of ethanol tolerance. While minimal changes in community profile could be detected along ethanol gradients using DGGE analysis, biofilm formation of the resulting cellulose degrading communities has been found to be dependent upon species diversity as a result of environmental fluctuations. The use of gradient plates in the selection of cellulolytic consortia at desired conditions has shown promise for the use in industrial applications.

2.2: INTRODUCTION

With the increasing demand for energy and the depletion of fossil fuels, renewable energy sources are desirable. In the next few decades, the reliance on fossil fuels will increasingly become unsustainable (27). Ethanol is a practical alternative as a reliable source due to renewable capabilities and cleaner burn (1). The use of lignocellulosic biomass in the production of biofuels has proved to be a feasible solution (36, 37). Cellulosic biofuel production can reduce greenhouse gas emissions by 80% and produce significantly less smog (13, 43).

Optimal breakdown of lignocellulosic biomass can potentially be accomplished through such processes as consolidated bioprocessing (CBP) (36, 37, 39). This process utilizes cellulolytic microorganisms for the breakdown of cellulose, and is optimized for the direct conversion of cellulose into ethanol. Previous emphasis has been on the addition of extracellular enzymes combined with simultaneous saccharification and co-fermentation (SSCF), which has proved to be cost ineffective (36). CBP however, utilizes the formation of a cellulose-enzyme-microbe (CEM) complex that allows for efficient substrate utilization and greater concentration of cellulases. A major challenge to CBP is the intolerance of many cellulolytic microorganisms to higher concentrations of ethanol. Tolerances of at least 5% (v/v) are required for industrial production of ethanol (37).

Ethanol intolerance has been attributed to various factors, which include metabolism, fluidity of the membrane as well as an imbalance in redox reactions. An increase in membrane fluidity is related to a blockage in glycolysis (21, 35). Glycolysis is also affected by the increase in glycolytic intermediates hypothesized to be as a result

of protein-membrane interactions (32, 33). An increase in the NADH/NAD^+ ratio results in a redox imbalance and is a result of two alcohol dehydrogenases (ADH). One ADH produces ethanol and the other consumes it and produces NADH. Ethanol tolerant mutants such as *T. pseudoethanolicus* 39E (4) lack the ADH enzyme that consumes ethanol, resulting in a decrease in the build-up of NADH and therefore an increase in the production of ethanol.

Studies to date have focused on the engineering of microorganisms with either high ethanol production or good substrate utilization capabilities to accomplish both processes simultaneously. The focus on the engineering of microorganisms for industrial use has proven successful with tolerances of up to 8% (v/v) (4, 11, 13) but there is still a need for microorganisms that have higher tolerances of ethanol coupled with efficient substrate utilization.

Microbial communities enhance the utilization of cellulose through the complete breakdown of recalcitrant materials such as lignin. A mixed community biofilm has the potential to degrade both hexose sugars (glucose) and pentose sugars (xylose) for more efficient product recovery and therefore enhanced production of ethanol (13). Actively degrading cellulose communities found in soil environments are diverse in nature with consistent breakdown of cellulosic materials. Studies have shown that tolerances of environmental isolates such as *Clostridium thermocellum* range from 4-8% (v/v) (13, 26, 49). Exposure to naturally established gradients within the environment allow for increased tolerance of microbes to various stressors (pH, concentration) as a result of gene flow (9, 57). Selection of a community that can naturally degrade cellulose with an ethanol tolerance of greater than 5% (wt/vol) will be cost effective (37).

To date, the use of gradient plate flow cells to examine the tolerance of cellulose degrading communities to ethanol, pH and other factors inhibiting growth has not been evaluated. Gradient plates offer the advantage of prolonged studies over time, which allows for natural adaptation of microorganisms through genetic exchange as well as competition for resources. Gradient plates also allow for a continuous and spatial pathway for microorganisms to develop (57). This study aimed to use environmental gradients to select for communities that can adapt to higher concentrations of ethanol over time.

2.3: MATERIALS AND METHODS

2.3.1: Growth medium

The growth medium was prepared anaerobically. Serum vials (10 ml) contained RM medium (47) composed of 2 g/L Urea, 2 g/L KH_2PO_4, 3 g/L K_2HPO_4, 1 g/L yeast extract, 0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.05 g/L $CaCl \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$ and 5 g/L Avicel. Vials were sealed with a butyl stopper (Cole Parmer, Montreal, Canada), aluminum top and flushed with a gas (N_2)/vacuum cycle every 5 seconds for a total of 5 minutes before being autoclaved at 121°C for 20 minutes.

2.3.2: Sources of Inocula

Inocula were obtained from various sources, including soil, compost and an anaerobic digester; anaerobic sludge was collected from the Ashbridges Bay municipal wastewater treatment plant in Toronto, Ontario, soil samples were gathered from Ryerson University as well as Apsley, Ontario, while composted samples from grass clippings were obtained from Aurora, Ontario. All soil and compost samples were collected into anaerobic vials that contained 100 uL of sterile distilled water, which was degassed with N_2 and sealed with a butyl stopper (Cole Parmer, Montreal, Canada). Pure culture strains of *C. thermocellum* (27405) were obtained from the American Type Culture Collection (ATCC, VA, USA).

2.3.2.1: Enrichment of cellulolytic communities

Cellulolytic enrichment of samples was obtained by successive culturing in RM medium (47). Prior to inoculation, vials were sterilized with ethanol. Cultures (1 ml) were transferred using a sterile 1 ml syringe into vials that contained 8 ml of RM medium and were incubated at 60°C. After three days, the inoculum was transferred into new medium

at the same temperature conditions. This was repeated up to four times to achieve successful enrichment.

2.3.3: Cultivation of a cellulolytic biofilm communities under conditions of continuous flow

2.3.3.1: Development of a gradient system for the cultivation of ethanol-tolerance cellulolytic microbial consortia

Gradient flow cells were constructed out of Plexiglas as described by Wolfaardt et al. (57). Flow cells contained a ceramic tile (28 mm x 28 mm x 4 mm) to allow for diffusion of the test solute and establishment of an ethanol gradient. Modification of the design previously described included the use of Norprene tubing to prevent the evaporation of ethanol as well as addition of a cellulosic substrate. Three cotton (cellulose) strands were placed parallel with the direction of medium flow, but perpendicular to that of an ethanol gradient. Cellulose strands were positioned at least 2 mm below the coverslip in order to provide steady state concentration profiles (FIG. 8.) (57). Gradient plates were boiled in de-ionized water for 10 minutes to remove air bubbles from the porous ceramic plate, sterilized for 30 minutes with a 10% bleach solution and flushed with de-ionized water overnight.

2.3.3.1.1: Materials used in the construction of gradient systems

The current gradient plate model was designed for aerobic systems using silicone tubing, which is permeable to gas exchange. After several trials, growth was observed only at very low concentrations of ethanol (0, 1%). The tubing was then switched to Norprene (Cole Parmer) to prevent gas exchange and allow for a defined gradient (FIG. 8).

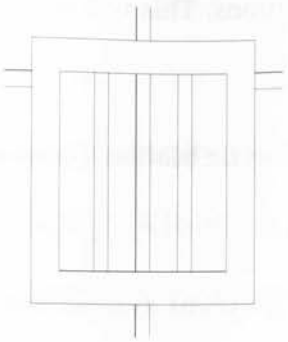

FIG. 8. Schematic of a gradient plate flow cell that was modified to include the cellulosic substrate (cotton) as well as Norprene tubing to prevent the evaporation of ethanol.

Silicone adhesive was replaced with liquid viton, which prevented leaks that occurred between connections. Liquid viton®, a fluoroelastomer, provided exceptional resistance to degradation by ethanol, in comparison to silicone adhesive, resulting in a stronger and tighter seal. Liquid viton®, was able to effectively bind to the Plexiglas and was further used for subsequent trials.

2.3.3.2: Preparation of medium

Medium was prepared aseptically (Fig. 9). A 2L flask that contained RM medium (47) composed of 2 g/L Urea, 2 g/L KH_2PO_4, 3 g/L K_2HPO_4 and 1 g/L yeast extract, was autoclaved at 121°C for 20 minutes. After autoclaving, medium was degassed for 1.5 hours with N_2, before further reduction by the addition of 30 g/L cysteine and addition of trace elements (0.2 g/L $MgCl_2 \cdot 6H_2O$, 0.05 g/L $CaCl \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$). Trace elements were autoclaved and filter sterilized using a 0.45 um filter prior to preparation of medium.

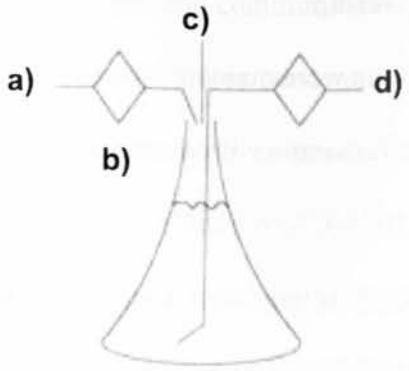

FIG. 9. Schematic diagram of 2L flasks used to deliver media and test solute(s). Flasks were sealed with a butyl rubber stopper that contained Norprene tubing as well as a port for ventilation (a), a sterile filter to prevent contamination (b), an injection port for the addition of cysteine and metals (c), as well as an outlet to allow flow of solutes (d).

Ethanol reservoirs of 15% (v/v) and 30% (v/v) were prepared using denatured ethyl alcohol. De-ionized water was autoclaved in a 2 L Erlenmeyer flask. After sterilization, flasks were degassed with N_2 for 90 minutes. Ethanol was added immediately through a sterile injection port into the flask.

Prior to the use of ethanol reservoirs for the continuous supply of ethanol to the gradient system, a syringe filled with a 30% ethanol solution was utilized to establish the gradient. The solution was injected into the system twice daily, and the gradient plate was clamped off to prevent loss of ethanol and ensure diffusion from the reservoir into the ceramic plate. Growth was only observed at ethanol concentrations of 0% which extended the entire length of the flow cell, therefore it was assumed that ethanol was evaporating through the silicon tubing preventing the formation of a gradient, which was attributed to the lack of continuous flow and that a continuous supply of ethanol was required to establish a gradient.

2.3.3.3: Continuous flow set-up

Flow cell experiments were maintained under strict anaerobic conditions within an anaerobic cabinet (Coy Laboratory Products Inc., MI, USA). An eight-channel Watson Marlow peristaltic pump 205S/CA (6.6 ml/hr) was used to deliver media and test solutes (such as ethanol to investigate tolerance of microbes to an ethanol gradient) to a gradient plate incubated at 60°C.

A continuous flow system was used to examine environmental tolerance of cellulose degrading bacteria. The system was continuous due to the constant replenishment of media and test solutes. Erlenmeyer flasks sealed with rubber stoppers and Norprene tubing (to prevent the evaporation of ethanol) was used to deliver solutes through a peristaltic pump into a gradient flow cell contained in an incubator (60°C). In order to maintain anaerobic conditions the effluent was collected into sampling vials within the anaerobic cabinet (FIG. 10).

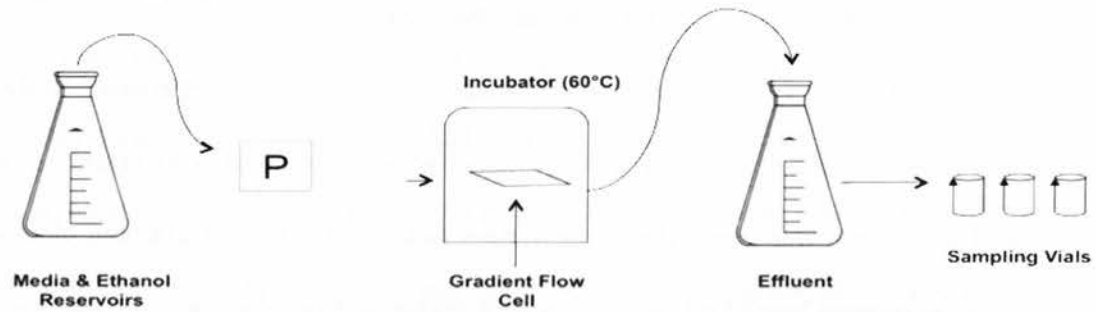

FIG. 10. Schematic diagram of an experimental continuous flow set-up. Media and test solutes were delivered through a peristaltic pump (P), into a gradient flow cell contained in an incubator. Effluent was used to inoculate sampling vials.

Continuous inoculations of soil, compost and sludge samples (to provide a wide variety of cellulolytic microorganisms from various source environments) every few days

were used to provide diverse cellulose degrading communities along the ethanol gradient. Effluent samples were collected into vials at various concentrations of ethanol.

2.3.4: Qualitative analysis of ethanol tolerance over time on cellulolytic communities in batch culture

Effluent (1 ml) from the gradient plate was sampled into 10 ml serum vials that contained 8 ml of RM medium without cysteine, and concentrations of ethanol ranging from 0-6% (v/v). Samples were incubated at 60°C for 14-35 days. One set of trials involved the collection of samples into vials that contained either Avicel (5 g/L) or cotton. The reduction of the medium and a colour change of resazurin from pink to clear were observed as a positive confirmation of growth. Effluent was sampled every 1-2 days into 10 ml serum vials under strict anaerobic conditions at the various ethanol concentrations over a period of 14-35 days. Batch experiments of effluent collection into vials, were repeated several times to determine prolonged exposure of a community to ethanol over time.

Samples that grew at higher concentrations of ethanol (4-6%) were used as sources of inocula. Samples (1 ml) were transferred to 10 ml serum vials with increased ethanol concentrations of 4-8% (v/v) and incubated at 60°C for 30 days to observe adaptation of strains to higher concentrations of ethanol over time.

2.3.5: Amplification of DNA using PCR

2.3.5.1: DNA extraction

DNA extractions were conducted on all samples using the ZR Soil Microbe Kit™ (Zymo® Research, Orange, CA, USA). The presence of DNA was confirmed using 2% agarose electrophoresis.

2.3.5.2: PCR amplification of the 16S rDNA fragment

Several PCR protocols were evaluated to optimize reaction conditions. Protocols were adapted from Muyzer et al., Mahmood et al. and Pearce et al. (40, 44, 48). The forward and reverse primers used were 357F-GC (5' CGCCCGCCGCGCGCGGCGGGCGGGGCACGGGGGGCCTACGGAGGCAGCAG-3') and 517R (5'-ATTACCGCGGCTGCTGG-3')/518R (5'-GTATTACCGCGGCTGCTGG-3') respectively and were described by Muyzer et al. (44). These primers were designed to target the variable V3 region of the 16S rRNA fragment. The resulting PCR product was approximately 190bp, as confirmed by alignment with the 16S rRNA gene of *E. coli.*

PCR was conducted using EconoTaq™ DNA Polymerase (Lucigen®, Middleton, WI, USA). This allowed for the concentrations of both the polymerase and $MgCl_2$ (a catalyst) to be altered, to discourage non-specific binding. The reaction mixture (50 uL) contained 20 ng of template DNA, 5 μM of each primer, 10x PCR buffer, 25 mM of $MgCl_2$, 10 mM deoxynucleoside triphosphate mixture and EconoTaq™ DNA Polymerase (5 units/uL) (Lucigen®). The outlined modified PCR protocol was adapted from Muyzer et al. (44). This consisted of a touchdown procedure, which included 20 cycles of a denaturation step of 1 minute at 94°C, 1 minute of annealing at 65°C (decreasing 1°C every second cycle until 356°C) and 72°C for 1 minute. The last 10 cycles consisted of a denaturation step of 1 minute at 94°C, 1 minute of annealing at 55°C and 72°C for 1 minute. The protocol included an initial denaturation step of 94°C for 5 minutes and a final primer extension step of 72°C for 5 minutes. PCR was performed using the

Eppendorf Mastercycler Pro (VWR, Toronto, Canada). PCR products were confirmed using electrophoresis with a 2% agarose gel.

2.3.6: Determination of community structure using DGGE

Amplified 16S *rRNA* gene products were separated using a D-Code™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Separation was carried out on a 8% polyacrylamide gel by electrophoresis with a 30-55% (top to bottom) gradient of increasing concentrations of urea and formamide, where 100% denaturant contained 40% (v/v) acrylamide and 7 M Urea (8). Twenty microliters of PCR products were loaded into each well, and DNA was concentrated in the well by running at 30 V for 15 minutes. The gel was run at 130 V for 4.5 hours at 60°C in Tris-Acetate-EDTA (TAE) buffer (40mM Tris base, 20 mM Acetate, 2mM EDTA (pH 8) (8). The resulting gel was stained for 10 minutes in TAE buffer containing ethidium bromide (10 ug/ml) and destained for 15 minutes in TAE buffer. Following washing, the gel was placed on the BioDoc-IT imaging system (UVP) for visualization and further transferred to the UV transilluminator to excise the bands. DNA was eluted from the gel into 30 uL of MilliQ water overnight at 4°C (59). Eluted DNA was amplified with PCR using the primers 357F-GC and 518R. The resulting PCR products were run on a DGGE gel, as outlined above.

2.3.7: Confocal Laser Scanning Microscopy (CLSM) to examine growth along environmental gradients

In situ colonization of cotton strands by environmental consortia was examined using confocal laser scanning microscopy (LSM 510, Zeiss, Toronto, Canada). Biofilms were stained for 1 hour with 1ml of the nucleic acid stain Syto 9 (5 mM) (Invitrogen, CA,

USA), flushed with media for 20 minutes and visualized with a 63x objective. Thirty-five three-dimensional Z-stack images of biofilm structure were generated for the evaluation of microbial colonization on cellulosic strands through the use of a Zeiss LSM Image Browser.

2.4: RESULTS & DISCUSSION

2.4.1: Ethanol tolerance of microbial consortia obtained along environmental gradients

Microbial communities within nature are diversified; therefore continuous inoculations from a wide variety of sources may result in the emergence of cellulolytic microorganisms with increased ethanol tolerance. The ability of community members contained within the effluent to tolerate higher concentrations of ethanol for application to industrial settings was the focus of the current study and further identification of microbial communities within vials was conducted using DGGE.

Vials that contained RM media were not reduced with cysteine (remained pink), in order to examine the reducing capabilities of microorganisms contained within the effluent. The change in colouration from pink to clear indicated growth within each of the vials. Effluent was collected into vials that contained either Avicel or cotton to determine whether or not there was preferential growth, since the carbon (cellulosic) source within the gradient plate was cotton. Qualitative examination of cellulolytic communities using an environmental ethanol gradient showed no specific preference for a cellulosic substrate (Table 1), with ethanol tolerances of up to 5% v/v (Table 3).

Effluent collected from gradient systems that grew at higher concentrations of ethanol (4-5% v/v) was re-inoculated into subsequent vials that contained fresh media (results not shown). Results, not shown, indicated that over an extended period of time (2-3 weeks), strains were able to adapt to higher concentrations (6% v/v) of ethanol. Serial enrichments of cellulolytic consortia obtained from gradient plates may result in increased tolerance of effluent samples collected over time. The inoculation of samples

that grew at higher concentrations of ethanol (6% v/v) into fresh medium may result in increased tolerance over time by exposure as indicated in results above.

Ljungdahl et al. (31) has shown that *C. thermocellum* produces a yellow affinity substance when fermenting cellulose. Qualitative results (Table 1) indicate a very low ethanol tolerance of *C. thermocellum* due to the absence of the yellow affinity substance. *C. thermocellum* has an ethanol tolerance of 4-8% (v/v) depending on the duration of ethanol exposure (49). Therefore growth of *C. thermocellum* was expected in the vials collected at the highest concentration of ethanol (5% v/v). Reasons for the lack of growth at higher concentrations of ethanol include competition between microbes within their microenvironment (45), lack of a defined ethanol gradient, deterioration of the cellulosic substrate, and amount of cells yielded to the effluent (as a result of a shear force created during inoculation). The growth of cellulolytic microorganisms and the lack of the yellow affinity substances produced by *C.thermocellum,* may be due to the presence of a closely related microorganism. Competition within the microenvironment may also cause fluctuations in growth, for example in vials with ethanol concentrations of 2% (v/v) and 4% (v/v) (Table 1), over time. Competition may be a result of the depletion of the cotton substrate, thereby resulting in the loss of certain microorganisms or the ability of other microbes to adapt to higher concentrations of ethanol in order to obtain nutrients.

Surface-associated microbes may have increased ethanol tolerance in comparison to planktonic cells found within the effluent. The inoculation of RM medium using cellulosic strands obtained from the gradient system may result in microorganisms with an ethanol tolerance of greater than 5% (v/v), due to acquired resistance to ethanol and

other inhibitory products such as lactate and acetate, between community members found within mature biofilms (39, 53).

Since growth was observed at an ethanol concentration of 5% (v/v), it has been verified that gradient plate systems can be used to establish industrially relevant microbial communities. Modifications to the current design can include the construction of flow cells out of steel to withstand higher concentrations of ethanol over time for better resistance to leaks/cracks, as well as the measurement of the established ethanol concentrations using probes that contain a sensor to measure volatile substances.

TABLE 1. Qualitative analysis of an actively degrading cellulosic community obtained from soil and compost grown on Avicel and cotton over a 14-day period.

Day	Ethanol Concentration (%)							
	0		2		3		4	
	A	C	A	C	A	C	A	C
4	++	+	-	ns	ns	ns	-	ns
5	++	+	-	-	ns	ns	-	-
6	+	+	-	+	ns	ns	+	-
8	+	+	-	+	ns	ns	+	-
9	+	+	-	+	ns	ns	-	-
10	+	+	+	+	ns	ns	-	+
13	+	+	+	+	+	+	-	-
14	+	+	-	+	+	+	-	-

*A: Avicel as the cellulosic substrate
C: Cotton an the cellulosic substrate
+: Clear colouration
++: Yellow colouration
-: No growth observed
ns : No sample collected

TABLE 2. Prolonged studies of an environmental gradient over a 35-day period inoculated with soil, sludge and compost samples and collected into vials that contained Avicel.

Day	Ethanol Concentration (%)					
	0	1	2	3	4	5
1	-	-	-	-	-	ns
20	+	+	+	-	-	ns
22	+	+	+	-	-	ns
24	+	-	+	-	-	ns
26	+	-	-	-	-	ns
27	+	+	+	+	-	ns
30	+	ns	ns	+	-	-
33	+	+	-	+	+	+
35	+	-	+	+	+	+

*+: Clear colouration
++: Yellow colouration
-: No growth observed
ns : No sample collected

2.4.2: Examination of increasing concentrations of ethanol on community profiles of anaerobes

DGGE analysis of environmental ethanol gradients showed that increasing concentrations of ethanol may affect the community profile (Fig. 11). For example, fluctuations within banding patterns observed in lanes 8 (0% v/v), 10 (2% v/v) and 11 (3% v/v) (Fig. 11) may be attributed to increasing concentrations of ethanol. Other minor fluctuations within the community profile are potentially a result of the continuous inoculations from various source environments. These include differences in the banding patterns of effluent collected into vials that contained either Avicel (lane 6) or cotton

(lane 7), which are contrary to results observed in Tables 1 and 2 (Fig. 11). Therefore growth of microbes on various cellulosic substrates (Avicel, cotton) may differ in community structure and ethanol tolerance. Prolonged exposure to ethanol over time may also affect the community profile of effluent collected on different days (lanes 1-5, Fig. 10).

Narisawa et al. (45) has shown that addition of exogenously added bacteria such as *C. thermocellum* and *C. straminisolvens* changes the community profile by increasing competition within the indigenous community. Competitive interactions may play an important role not only in the emergence of ethanol tolerant strains, but also the loss of key community members in prolonged studies. The positive control (*C. thermocellum*) is present within vials that contained either Avicel or cotton (Table 1), at very low concentrations of ethanol. Since the natural tolerance of *C. thermocellum* is 4% (49), the lower (0% v/v) tolerance observed in Table 1 may be attributed to competition of related microorganisms at higher concentrations as well as the loss of a defined gradient. DGGE confirms the presence of *C. thermocellum* in vials containing cotton that were not observed qualitatively (Fig. 11). The presence of a band in all samples that appears to be the positive control (*C. thermocellum*) may be a closely related species that does not produce a yellow affinity substance, which would explain results observed in Table 1. The absence of *C. thermocellum* in lanes 15 and 16 (Fig. 11.) may also be attributed to discrepancies in DNA extractions. DNA from each of four samples of the positive control was extracted and purified at different times. DNA from *C. thermocellum* in lanes 14 and 17 was extracted more recently than lanes 15 and 16. Results can be attributed to discrepancies in the DNA extraction procedure such as partial lysis of cells and elution

efficiency. Since DNA was eluted into 100 uL of elution buffer, DNA used for subsequent PCR reactions (after centrifugation) may have been less than required. A clean-up step using potassium acetate, and resuspension in distilled water, may increase the amount of purified DNA and remove inhibitory substances for downstream applications.

Studies by Kato et al. (24, 25) and Haruta et al. (18, 45) have shown that key members in an actively degrading cellulosic community include *C. thermocellum* and *C. straminisolvens* as confirmed by DGGE analysis, as well as aerobic species such as *Pseudoxanthomanas* and *Brevibacillus*. *C. thermocellum* and *C. straminisolvens* are sole cellulose degrading bacteria. The former provide metabolites to other members within the community while the latter have high carboxymethyl cellulase activity (25, 45). Aerobes such as *Pseudoxanthomanas* sp. and *Brevibacillus* sp. that exist in the upper oxygenated fraction may co-exist with anaerobes due to the close relationship regarding metabolism or metabolites, which may play an important role in the current results (18, 24). Aerobes secrete low molecular weight compounds that aid in the degradation of the cellulosic substrate. Kato et al. (24) found that boiled supernatant from *Pseudoxanthomanas* sp. enhanced cellulose degradation by a pure culture of *C. straminisolvens* CSK1. Aerobes that metabolize inhibitory products such as lactate and acetate may enhance cellulose utilization by cellulolytic microorganisms and increase the production of ethanol. Similarly, cellobiose, found to repress cellulose degradation by species of clostridia, is rapidly degraded by aerobic isolates resulting in increased substrate utilization (24). Aerobic bacteria also supply an anaerobic environment, which is essential for strict anaerobes such as *C. thermocellum* and *C. straminisolvens*. Aerobes consume the oxygen

through the utilization of substrates found in yeast extract and peptone such as peptides and amino acids (24). It has been reported by Kato et al. (24) that aerobic isolates neutralize the pH of the media, to provide an optimal pH (7.5) for cellulolytic microorganisms such as *C. straminisolvens* to grow. Mixed community cellulolytic biofilms involved in the active degradation of cellulose may be further studied through visualization with CLSM.

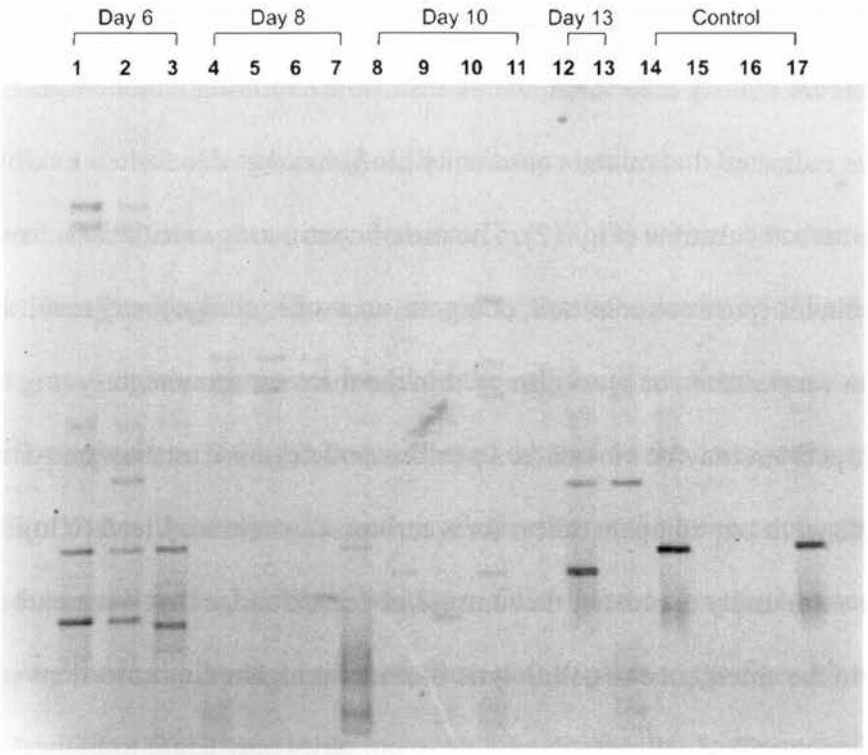

FIG. 11. Denaturing gradient gel electrophoresis banding patterns of cellulolytic bacterial 16S rRNA genes amplified by touchdown PCR using primers 357F-GC and 518R on Day(s) 6,8,10 and 13. Banding profiles correspond to growth (from samples over 14 days) on cotton at ethanol concentrations at 0% (lanes 3,4,9) and 2% (lanes 1,7,13) respectively. Growth was observed in vials with Avicel at concentrations of ethanol at 0% (lanes 2, 5, 8), 2% (6, 10), 3% (11) and 4% (13) respectively. *C. thermocellum* ATCC 27405 controls (lanes 14-17) were used as molecular markers.

2.4.3: Colonization of cellulosic material by anaerobic cellulolytic mixed communities

Images obtained with CLSM illustrated that mixed community cellulolytic biofilms (Fig. 12, 13, 14) have architecture more similar to biofilms that obtain nutrients from the aqueous phase (multiple cell layers; varied spatial orientation) rather than cellulolytic pure culture biofilms that obtain nutrients from the attachment substratum (postulated to be typically one or few cell layers) (39). In cellulolytic pure culture biofilms cells are closely associated, with the substrate forming a monolayer (39). Results have indicated that mixed community biofilms may also form a monolayer of cells on the carbon substrate (Fig. 12). The use of continuous inoculations from a wide variety of sources (pure cultures, soil, compost, anaerobic sludge) may result in increased competition, cooperation, or synergism within the microenvironment.

Competition may be viewed as a positive and negative interaction. The ability of microorganisms to out-compete others for a carbon substrate may lead to loss of cellulolytic community members. In contrast, competition for that same carbon substrate may result in the emergence of cellulolytic bacteria at higher concentrations of ethanol. Therefore, a diversified cellulose degrading community may adapt to higher concentrations of ethanol along the environmental gradient as a result of competition.

Cooperation of microorganisms from a wide variety of source environments may result through quorum sensing. Quorum sensing involves the regulation of gene expression through the production and release of chemical signal molecules called autoinducers that increase in concentration as a function of cell density (42). The detection of an autoinducer leads to an alteration in gene expression and subsequent

physiological activities, as demonstrated by Gram-positive and Gram-negative bacteria (42). Mixed community cellulolytic consortia may therefore use quorum sensing to alter gene expression to obtain an enhanced tolerance to ethanol.

Similarly, synergistic interactions involving mutually beneficial applications to mixed community members may also play a role in increased ethanol tolerance of microbes. For example, mutually beneficial interactions between aerobes and anaerobes in a cellulolytic microbial community, as described above, may not only play a role in enhanced cellulose degradation, but enhanced ethanol tolerance through the decrease of inhibitory effects such as the tightening of the cellular membrane.

In some cases, one species may dominate over others within the community (Fig. 12). Further visualization using Gram staining may show the presence of both gram-positive and gram-negative microbes found on the cellulosic substrate, opposed to one particular microorganism. The inhibitory effect of ethanol on the community may be attributed (Fig. 12a) with heavy colonization of microbes at the lowest concentration of ethanol and fewer cells at higher concentrations. Other mixed community anaerobic biofilms may be more similar to aerobic biofilms with regards to the formation of a complex matrix (Fig. 13). Although the presence of a glycocalyx has been well described for ruminal bacteria (37), further research is required to determine the roles of a glycocalyx for other anaerobes.

The presence of aerobes (18, 25) from various source environments may contribute to the complex structure of cellulolytic biofilms. Cells surrounding the complex matrix of cellulolytic biofilms appear to form chain-like structures in the aqueous phase around the cellulose fibers, which have been hypothesized to contribute

through the release of extracellular enzymes (39). Wolfaardt et al. (56) found that colonization of gel surfaces and cooperation by biofilm consortia and free-floating planktonic cells may result in enhanced degradative efficiency of microbes. This theory may be applied to an interactive cellulolytic biofilm system resulting in recruitment of new members and further attachment, with high cellulose degrading efficiency (56).

It can be concluded that biofilm formation of cellulose degrading communities has been found to be dependent upon species diversity, as a result of environmental fluctuations and source environments (23). The environment that the sources of inocula (soil, compost, sludge) were obtained will be diverse in nature. Environmental fluctuations in nutrient concentration, pH or temperature may alter community diversity. Environmental fluctuations that result in source environments may alter the species diversity within the community. Therefore, biofilm formation of cellulolytic communities in gradient systems may be attributed to the species diversity within the environment.

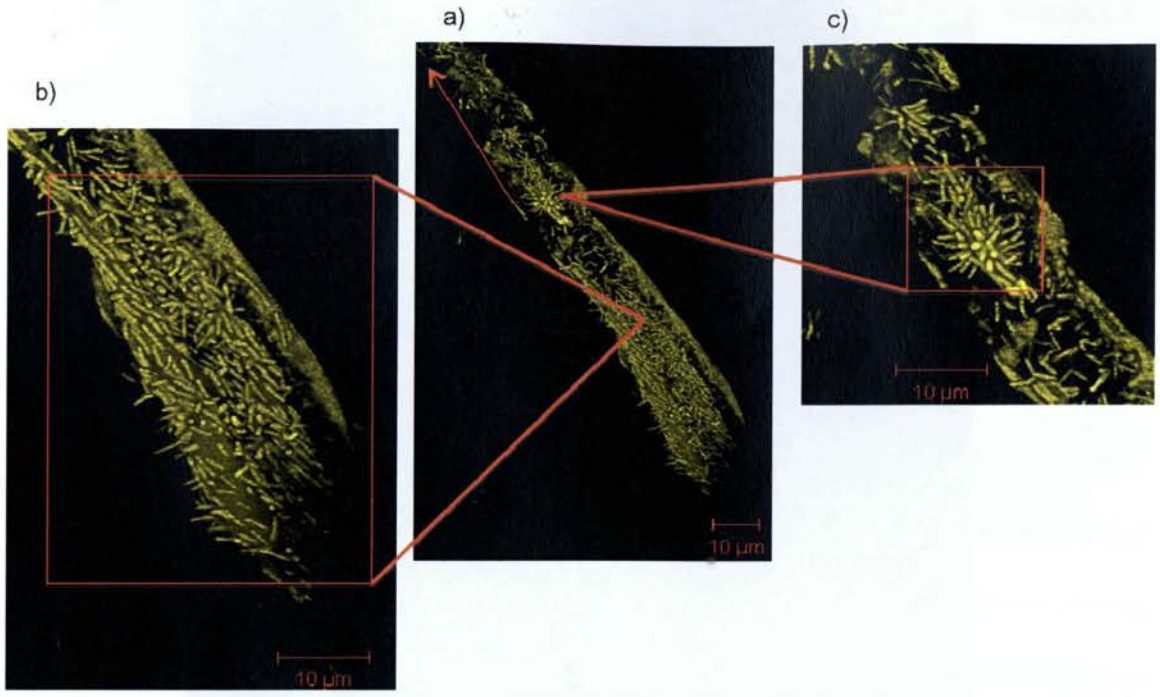

FIG. 12. CLSM images of a 14-day-old cellulolytic biofilm on a cotton fiber stained with Syto 9 (a). In subsections (b, c), cells were closely associated with the cotton substrate where nutrients were obtained. Increasing ethanol gradient depicted by an arrow (a).

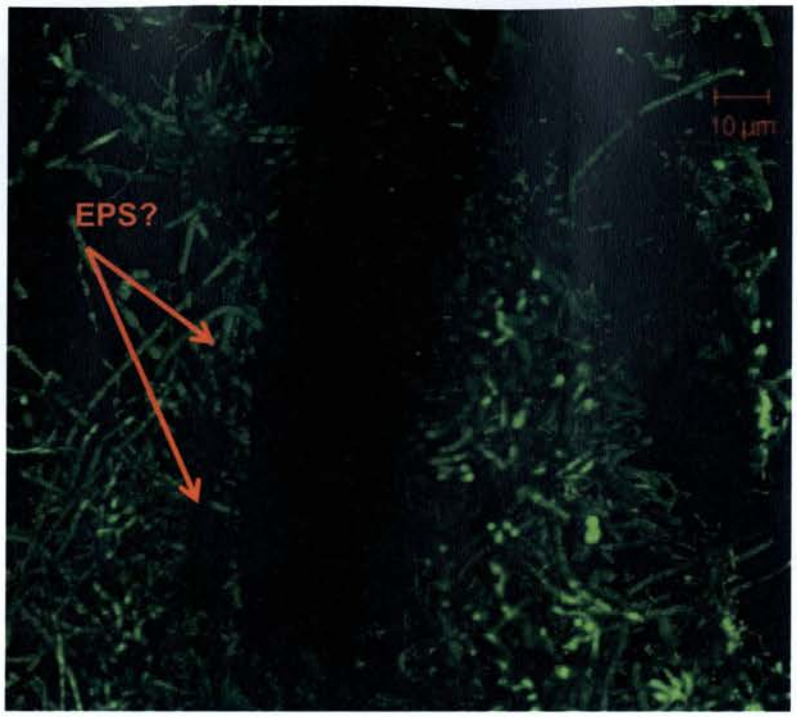

FIG. 13. Colonization of several cotton fibers by a mixed community, observed with CLSM after 35 days. Cells (stained with Syto 9) are maintained in a relatively stable orientation within the intricate matrix closely associated with the substrate suggest a potential role of EPS in anaerobic biofilms.

FIG. 14. Cells arranged in chain-like structures within the aqueous phase from a 35-day flow cell. Cells were stained with the nucleic acid stain Syto 9.

Many studies have focused on the genetic engineering of cellulolytic microbes for increased tolerance to ethanol (2, 13, 20). Fewer studies have focused on the genetic adaptation of wild-type microorganisms (11, 49). In this study, tolerances of up to 5% (v/v) were observed. This demonstrates the potential applicability of environmental gradients to the study of ethanol tolerance. DGGE analysis showed that the community profile was not significantly impacted, although fluctuations observed may be attributed to increased concentrations of ethanol, sources of cellulose and exposure time. Biofilm colonization of cellulose degrading communities may be a result of species diversity found within various environmental sources. Results showed that cellulolytic communities might behave as cellulolytic pure culture biofilms, which colonize biotic substrates, due to the monolayer of cells formed on the substrate (Fig. 12). Other results showed that mixed community biofilms may have a structure more similar to biofilms formed on an abiotic substrate, due to the complexity of the matrix observed (Fig. 13). The close proximity of microbial cells to the cellulosic substrate during colonization (Figs. 12, 13) suggests the potential formation of a CEM complex, a key component to CBP, resulting in minimization of the diffusion distance between substrate and microbe. Furthermore, the use of environmental gradients to study ethanol tolerance may have future applications to larger-scale industrial cellulosic ethanol production by CBP.

CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

3.1: Future Development and Modification of gradient systems for industrial application

Since increased tolerance (5% or greater) was observed over a period of 35 days, prolonged exposure of communities to increasing ethanol gradient over time may result in higher tolerances of microbes. Pure ethanol (absolute ethanol) should be used in further experiments to eliminate impurities, which may affect results. Due to cracking of flow cells constructed out of Plexiglas to higher concentrations of ethanol, highly resistant materials such as steel will aid long-term investigations. The ethanol gradient may be measured using capacitance probes, which measure the dielectric permittivity of a substance and can be further used to measure volatile substances.

Although Norprene tubing prevented the rapid evaporation of ethanol into the anaerobic environment that was observed with the Silicone adhesive, prolonged exposure to organic solvents results in softening, loss of strength and swelling of tubing. Viton® tubing, although more expensive, is recommended for construction since it is less permeable to oxygen and can resist higher concentrations of ethanol. Liquid viton® should be used for sealing the ceramic tile within the flow cell instead of Silicone adhesive to allow for the establishment of a defined gradient, prevent the diffusion of media into the ethanol reservoir and ensure adequate flow. The elasticity of liquid viton® makes it difficult to manipulate the adhesive, and so further construction of anaerobic gradient systems will involve the use of joints to connect the flow cell to the tubing to allow for a tighter seal and prevent leaks.

The gradient plate design should be modified to include a microscope coverslip on hinges that will allow for the visualization of biofilm colonization on cotton strands at various time intervals. Cotton strands will also be used as sources of inocula into vials with RM media to study ethanol tolerance of biofilms in comparison to those obtained in the effluent. This will aid in the further understanding of the tolerances of microorganisms observed within both the effluent and the cotton strands. DGGE work will aid in the identification of the community members found within the effluent or on the cotton strands, which may examine the resistance of cellulolytic microbes found within biofilms.

Qualitative results have indicated that the increased ethanol tolerance of cellulolytic communities is not attributed to the type of cellulosic substrate. However, the production of ethanol and subsequent hydrolysis of cellulose may differ. Therefore future studies are required. This includes quantitatively measuring cellulose breakdown by weighing cellulosic biomass before and after degradation. The production of ethanol and other inhibitory products such as lactate can be measured using HPLC. This is important to determine the cellulosic substrate that will result in both increased tolerance and production of ethanol. Studies involving the use of other cellulosic substrates such as wood chips (from pulp and paper mills), used in industrial applications, are required and may result in increased tolerance and production of ethanol.

Serial flow cells will also be used for prolonged studies. Since the cotton is degraded after a period of 35 days, the addition of a second gradient system in tandem will allow for long-term investigations. After 35 days the first flow cell may be compromised for analysis. This promotes the idea of biofilm-biofilm communication,

whereby the effluent of one system is used as a source of inoculum for the second system. The communities of all systems used in long-term investigations can be studied using DGGE and microscopy to study changes in species profile and biofilm colonization.

3.2: Identification of cellulolytic communities using molecular fingerprinting techniques

Although increasing concentrations of ethanol may be attributed to changes in community structure, the role of community members found within the effluent still remains to be determined. In order to better understand the growth of microbes on various cellulosic substrates, DGGE profiles of sources of inocula, cells from cotton strands and cells contained within the effluent should be conducted. Corresponding banding profiles will determine whether cells collected within the effluent were yielded from the biofilm or the initial sources of inocula. Comparison of banding profiles from all sources of inocula will also aid in the identification of community members found within the effluent and yield information on the respective source environment.

Due to problems associated with DGGE such as poor quality gels (fuzzy bands, poor separation, biases in DNA extraction and PCR), the use of other techniques such as terminal-restriction fragment length polymorphism (t-RFLP) and fluorescent in- situ hybridization (FISH) may be used in future studies.

Similar to DGGE, t-RFLP is also an rRNA-based gene targeting technique. This technique involves the labeling of primers with fluorescent probes to tag PCR products. Amplified PCR products are then digested with a restriction endonuclease and separated by capillary electrophoresis (14). An electrophoretic profile is generated, which is

characteristic of the microbial community. Advantages include rapid and semi-quantitative analysis (due to the use of fluorescent probes). Lee et al. (28) studied cellulose-degrading community dynamics and found that the molecular method was less time consuming then traditional isolation techniques such as DGGE.

FISH is a quantitative technique used in both the enumeration of microbial cells as well as determination of community structure. FISH combined with CLSM allows for direct visual resolution of microbial cells including both slow growing and non-culturables, which may be encountered with anaerobic cellulolytic microorganisms (14). The protocol involves the treatment of microbial cells with a fixing agent followed by the hybridization of microbial fluorescent probes. Cells are visualized after washing by CLSM. This technique may be applied to the study of cellulolytic microbial communities obtained from gradient plates. Chin et al. (5) used FISH to study methanogenic cellulose degrading communities using general probes for archaea and bacteria and specific probes for the genera *Methanobacterium, Methanosarcina* and *Methanosaeta.* It was found that *Methanosaetaceae* and *Methanosarcinaceae* dominated the methanogenic rice field.

3.3: Visualization of biofilm attachment

Results have indicated a potential role of extracellular polymeric substances in the establishment of cellulolytic biofilms (EPS) (FIG. 13). Further investigation into the presence and role of EPS is required. It has been hypothesized that EPS may trap hydrolysis products and concentrate cellulases within the matrix resulting in enhanced cellulose breakdown involved in the CEM complex. EPS may also concentrate extracellular enzymes that are excreted by microbial cells within the aqueous phase.

Visualization of EPS in future trials involves the use of lectin staining. Lectins are proteins that bind sugar molecules. Lectin stains such as concanavalin A (ConA) target carbohydrates predominately found within the EPS. Staining with both a nucleic acid stain and a carbohydrate stain, will allow for distinction between microbial cells and EPS. McSwain et al. (41) used ConA (lectin) and Syto 63 (nucleic acid stain) to visualize EPS distribution in aerobic flocs and granular sludge.

Gram staining can be used to differentiate between microorganisms colonizing the cellulosic substrate as well as those found in the aqueous phase. The Gram stain is a fundamental technique based on cell wall differences and is used in microbial classification. Gram-positive microorganisms differ from gram-negative organisms in that their wall mainly consists of peptidoglycan and lacks the complex outer membrane of lipopolysaccharides (12).

Forster et al. (12) applied a novel fluorescent dye hexidium iodide to the assessment of Gram status to study wastewater populations. Hexidium iodide (HI) is a nucleic acid binding dye that allows assessment of Gram status by differential absorption through bacterial cell walls (12). HI selectively stains gram-positive microorganisms. The use of novel fluorescent dyes may provide a robust and rapid alternative to traditional Gram staining (12). This technique may be applied to differentiate between gram-positive and gram-negative microbes involved in the degradation of cellulose.

Although the gradient system has shown tolerances of up to 5% (v/v), further modifications to the gradient plate design are required to optimize results. More in-depth analysis into identification of community members and the role of aerobes in the degradation of cellulosic substrates may be accomplished through DGGE and FISH.

Differentiation between community members within the cellulolytic biofilm may be conducted through the use of fluorescent dyes to determine Gram status as well as lectin staining to determine the presence of EPS.

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CHAPTER 5: APPENDIX

5.1: DNA Extraction Protocols

DNA was extracted using various commercial extraction kits, in order to test their effectiveness. These kits included the Ultraclean™ Soil DNA Isolation Kit (Mo Bio® Laboratories) and the ZR Soil Microbe Kit™ (Zymo® Research). Both kits have been found to be quite efficient with regards to the extraction of DNA from soil samples, giving fairly high yields. The ZR Soil Microbe Kit™ however is much faster, simpler and efficient.

Culture samples from flow cell 3 (0-4%) were processed using the Ultraclean™ Soil DNA Kit (Mo Bio® Laboratories). The remaining samples from flow cells 2 & 3 (0-4%) were analyzed using the ZR Soil Microbe Kit™ (Zymo® Research).

The presence of bacterial DNA was confirmed using PCR amplification. Several PCR protocols were necessary to optimize reaction conditions. Protocols were adapted from Muyzer et al. (44), Mahmood et al. (40) and Pearce et al. (48). The forward and reverse primers used were 357F-GC (5' CGCCCGCCGCGCGCGGCGGGCGGGGCACGGGGGGCCTACGGAGGCAGCAG-3') and 517R (5'-ATTACCGCGGCTGCTGG-3')/518R (5'-GTATTACCGCGGCTGCTGG-3') respectively and were obtained from Muyzer et al. (44). These primers were designed to target the variable V3 region of the 16S rRNA fragment. The resulting PCR product was approximately 190bp, as confirmed by alignment with the 16S rRNA gene of *E. coli*.

The protocol outlined by Mahmood et al. (40), using the primers 357F-GC and 518R, produced distinct bands corresponding to the desired product (Fig. 15). There was

however non-specific binding that occurred in other lanes, as seen by the presence of products larger than those expected. In an attempt to minimize the non-specific binding, primer specificity was examined using the 517R primer as described by Pearce et al. (48) in place of the 518R (Fig. 16). The resulting bands were more intense and the effect of non-specific binding was greatly reduced.

The Muyzer et al. (44) touchdown PCR protocol was also followed during the optimization process using the 517R primer (Fig. 17). The positive control (*Pseudomonas* sp. CTO7) had a single band visible on the gel, which was an improvement from the Pearce et al. (48) method. However, the DNA amplification was inefficient and some non-specific binding as a result of primer dimers were seen. At this point the PCR kit was changed from Titanium™ Taq® DNA Polymerase (ClonTech®) to EconoTaq™ DNA Polymerase (Lucigen®). This allowed for the concentrations of both the polymerase and $MgCl_2$ (a catalyst) to be altered, to discourage non-specific binding. Since $MgCl_2$ catalyzes the binding of DNA polymerase, modification of the amount added may result in more efficient binding and therefore an increase in PCR products.

All samples were analyzed utilizing the protocol outlined by Muyzer et al. (44), using the 518R primer with the EconoTaq™ kit. It was determined that the amount of non-specific binding decreased when this reaction was performed (Fig. 18 & 19). The method was slightly modified by increasing the number of cycles in the final stage to 10 from the original 5. It was found that there was greater specificity and more efficient DNA amplification (as seen in Fig. 20.), when compared to the same samples in Fig. 16. The modified Muyzer et al. (44) protocol produced the best results and was used in further PCR.

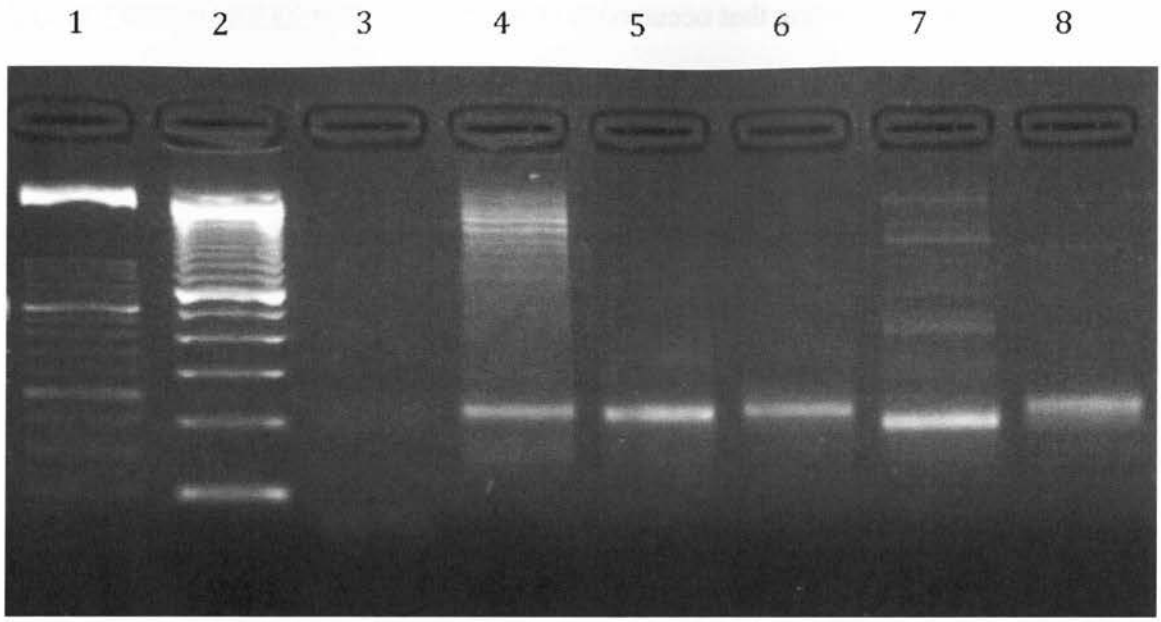

FIG. 15. 2.0% agarose gel showing PCR products from the Mahmood et al. (40) protocol (Titanium Taq PCR Kit). The 196 bp product was confirmed with 150bp and 100 bp molecular markers (lanes 1 & 2) respectively. *E. coli* was used as a positive control for the 196 bp fragment and H_2O was the negative control. Avicel was used as the cellulosic source and effluent from the flow cell was collected at concentrations of ethanol of 0% (lane 7) and 3% (lane 6) over a 14-day period. Similarly growth was observed with a cotton substrate at an ethanol concentration of 3% (lanes 5, 8).

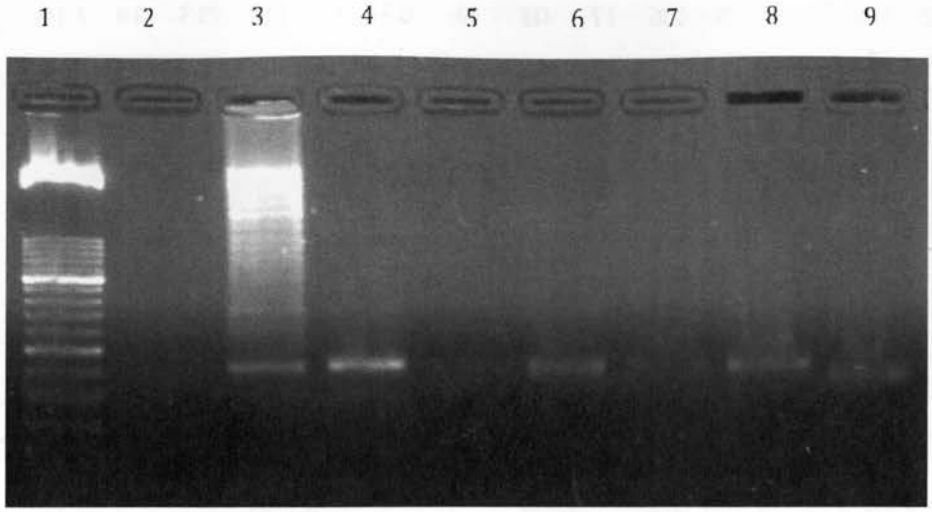

FIG. 16. 2.0% agarose gel showing PCR products from the Pearce et al. (48) protocol (Titanium Taq PCR Kit). The 196 bp product was confirmed with a 50bp molecular marker (lane 1). *Pseudomonas sp.* CTO7 was used as a positive control for the 196 bp fragment and H_2O was the negative control (lanes 3,2). Avicel was used as the cellulosic source and effluent from the flow cell was collected at concentrations of ethanol of 0% (lane 9), 2% (lane 4, 8) and 3% (lane 6, 7) over a 14-day period. Similarly growth was observed with a cotton substrate at an ethanol concentration of 3% (lane 5).

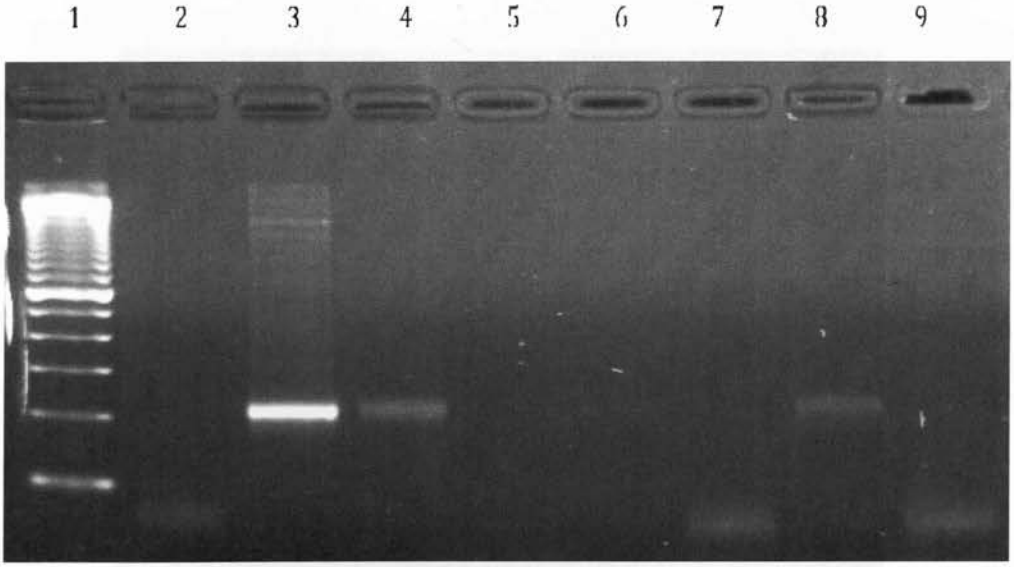

FIG. 17. 2.0% agarose gel showing PCR products from the Muyzer et al. (44) protocol (Titanium Taq PCR Kit). Lanes 1, 2 and 3 represent the molecular marker (100 bp), the negative control (H_2O) and the positive control (*Pseudomonas sp.* CT07). Avicel was used as the cellulosic source and effluent from the flow cell was collected at concentrations of ethanol of 0% (lane 9), 2% (lanes 4, 8) and 3% (lanes 6, 7) over a 14-day period. Similarly growth was observed with a cotton substrate at an ethanol concentration of 3% (lane 5).

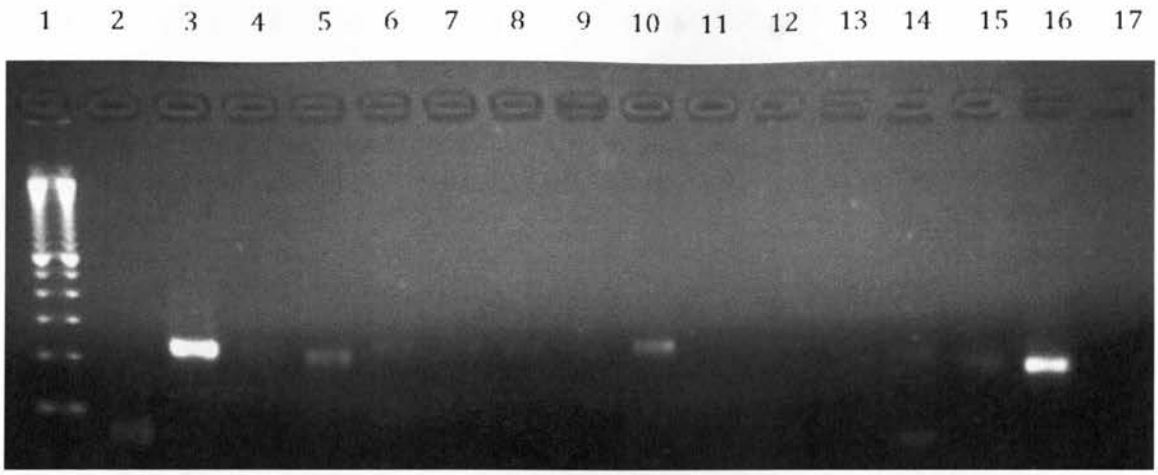

FIG. 18. 2.0% agarose gel showing PCR products from the Muyzer et al. (44) protocol (EconoTaq PCR Kit) Samples 1-15. Lanes 1, 2 and 3 represent the molecular marker (100 bp), the positive control (*Pseudomonas sp.* CT07) and the negative control (H_2O). Avicel was used as the cellulosic source and effluent from the flow cell was collected at concentrations of ethanol of 0% (lanes 4, 5, 8, 15, 16), 2% (lanes 6, 10, 14, 17) and 3% (lane 13) over a 14-day period. Similarly growth was observed with a cotton substrate at ethanol concentrations of 0% (lane 7), 2% (lane 9) and 3% (lane 11) respectively.

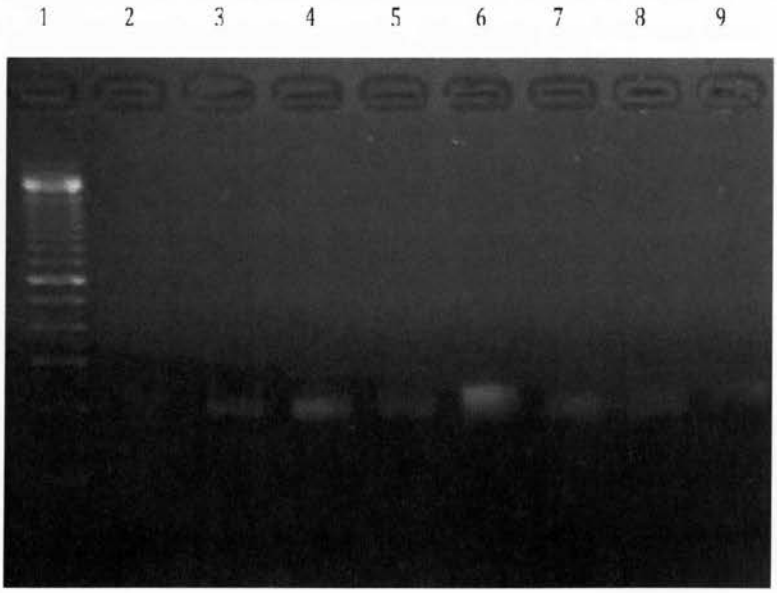

FIG. 19. 2.0% agarose gel showing PCR products from the Muyzer et al. (44) protocol (EconoTaq PCR Kit) Samples 16-22. The 196 bp product was confirmed with a 100bp marker (1ane 1). Avicel was used as the cellulosic source and effluent from the flow cell was collected at concentrations of ethanol of 3% (lane 6, 7, 8), and 4% (lane 2) over a 14-day period. Similarly growth was observed with a cotton substrate at ethanol concentrations of 0% (lane 3), 2% (lane 4), 3% (lane 9) and 4% (lane 5) respectively.

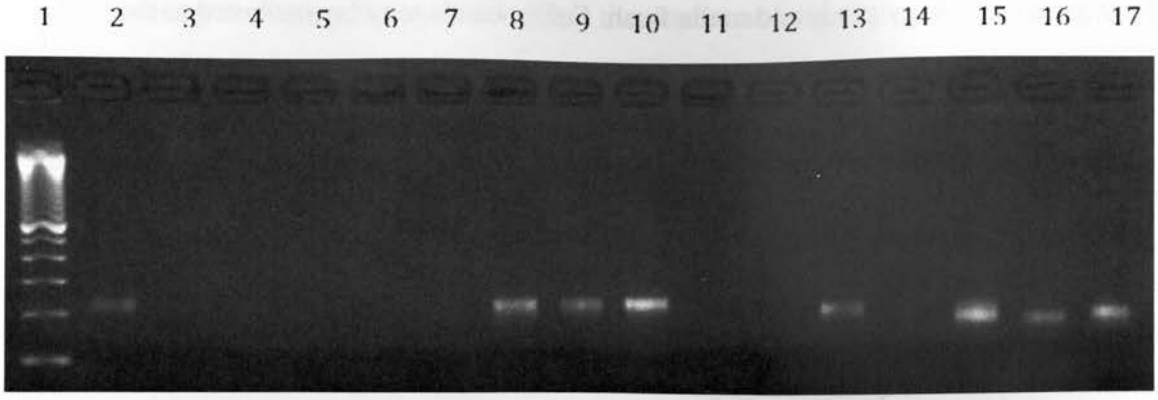

FIG. 20. 2.0% agarose gel showing PCR products from the modified Muyzer et al. (44) protocol (EconoTaq PCR Kit) Samples 1-15. Lanes 1, 2 and 3 represent the molecular marker (100 bp), the positive control (*Pseudomonas sp.* CT07) and the negative control (H_2O). Avicel was used as the cellulosic source and effluent from the flow cell was collected at concentrations of ethanol of 0% (lanes 4,5,8,15,16), 2% (lanes 6, 10, 14, 17) and 3% (lane 13) over a 14-day period. Similarly growth was observed with a cotton substrate at ethanol concentrations of 0% (lane 7), 2% (lane 9) and 3% (lane 11) respectively.

5.2: Troubleshooting DGGE protocols

Issues associated with DGGE analysis included the handling of gels. Glycerol (2% v/v) was added to each solution prior to formation of the gradient using an automated system to deliver gradient solutions in the appropriate ratio (Table 3). Glycerol was added to increase flexibility of gels and to reduce breakage during staining, removing plates and viewing on the transilluminator (15). In subsequent DGGE trials, glycerol did not seem to greatly enhance the flexibility of the gel and may have interfered with the gradient due to the viscosity of the substance.

Before the use of an automated system to deliver gradient solutions, a manual pump was used for delivery. The absence of bands on the DGGE gel may be attributed to the lack of a defined gradient. The automated delivery system, combined with a 0% stacking gel was used to enhance results. Several conditions were also modified to enhance results. Since poor quality gels have been attributed to the freshness of reagents, all reagents used were

of molecular biology grade and made fresh. Fuzzy bands may be attributed to the diffusion of samples into adjacent wells, therefore electrophoresis was started as soon as samples were loaded (15). Gels stored overnight, were wrapped in Kim wipes and saturated with TAE buffer to prevent evaporation. Samples were also loaded in the middle lanes of the gel in order to prevent gel smiling/frowning, which may interfere with results.

Although several gel percentages were used (6%, 8%, 10%), an 8% gel was found to have the greatest band separation (between 200-400 bp) and subsequently produced the best results. Larger separations have been used to prevent blurring patterns as those observed in Figure 21, since bands are dispersed over a larger area. The gradient percentages of 30% and 55% (Figs. 21, 22) were modified to 20% and 70% since many of the bands were concentrated on the lower half of the gel. This increase in gradient did not affect the results and no further DGGE bands were visualized, contrary to Green et al, (15) who found that a broader denaturant range decreased the appearance of poor quality gels. In some cases, banding patterns were not observed with samples that had been confirmed using 2% agarose gel electrophoresis. This may be attributed to air bubbles trapped within the plates, which hinders the migration of DNA through increasing concentrations of denaturant (15).

The protocol outlined by Cui et al. (8) was modified to 16 h at 100 V (17) or 3 h at 250 V. Although longer run times have been found to produce better quality gels (15), all protocols used produced similar results. Run times are important in order to prevent poor separation and fewer bands. Notably, the protocol from Cui et al., with a DNA concentration step, had the most reproducibility and accountability (8).

Staining/destaining time did not affect the results as long as the staining time ranged from 10-20 minutes and destaining ranged from 15-30 minutes. The UVP transilluminator malfunctioned, so images obtained for subsequent gels were taken with the Polaroid camera (Fig. 22) in comparison to that of the digital camera (Fig. 21) and were of poor quality.

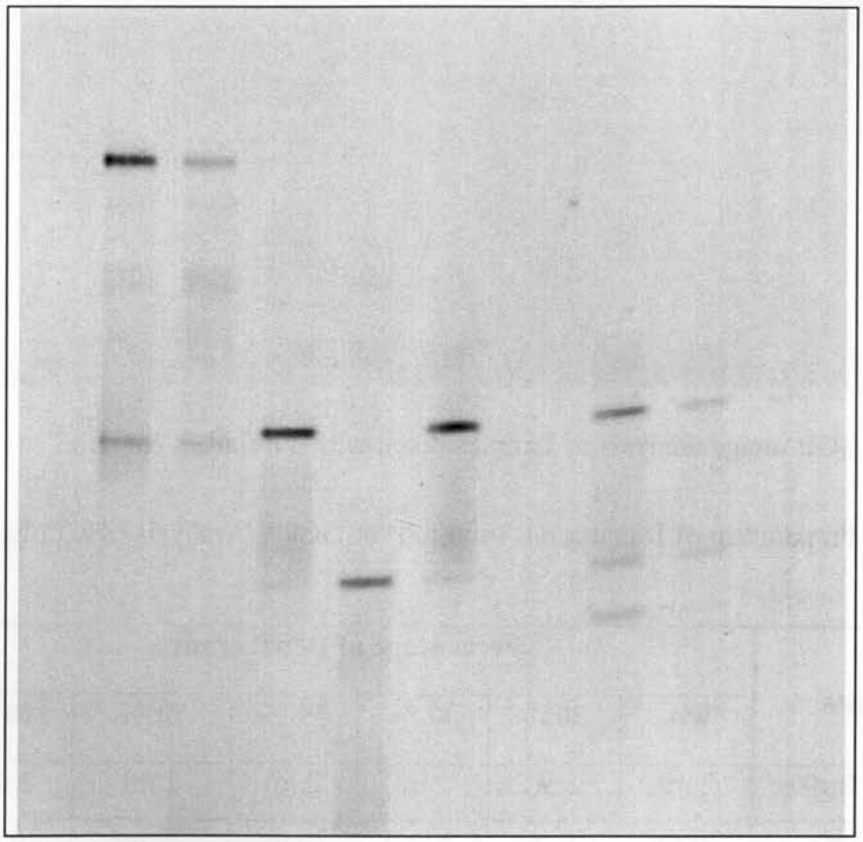

FIG. 21. DGGE image analysis of samples taken with a UVP transilluminator.

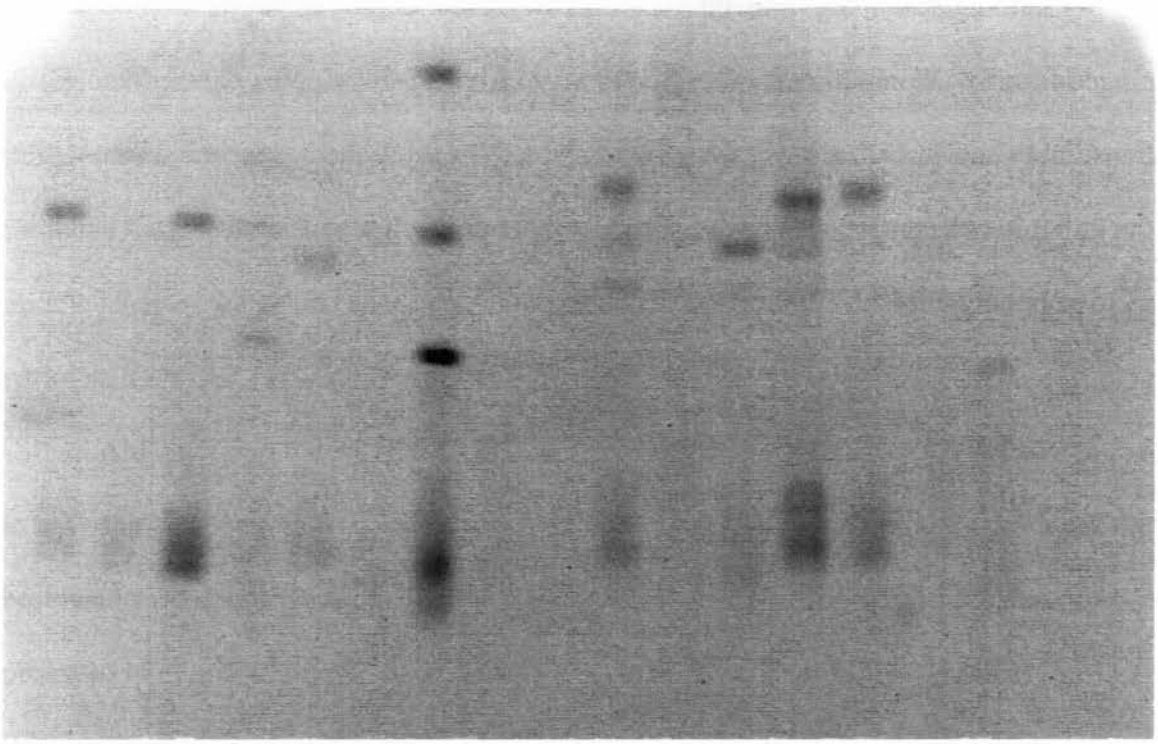

FIG. 22. DGGE image analysis of samples taken with a Polaroid camera.

TABLE 3. Preparation of Denaturants required for DGGE Analysis (8% Polyacrylamide Gel).

Reagents	**Percentage of Denaturant**					
	0%	**20%**	**30 %**	**55 %**	**70 %**	**100 %**
50X TAE Buffer	2 ml	2 ml	2 ml	2 ml	2 ml	2 ml
40% Acrylamide/Bis	20 ml	20 ml	20 ml	20 ml	20 ml	20 ml
Formamide	_	8 ml	12 ml	22 ml	28 ml	40 ml
Urea	_	8.4 g	12.6 g	23.1 g	29.4 g	42 g
H_2O	78 ml	To 100 ml	To 100 ml	To 100 ml	To 100 ml	To 100 ml

- All reagents are from G biosciences (Maryland Heights, MO, USA) and Bio-Rad (Hercules, CA, USA) unless otherwise indicated.