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Anaerobic Cellulolytic Microbial Communities From Various Sources Of Inocula Under Various Culture Conditions

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by

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ANAEROBIC CELLULOLYTIC MICROBIAL COMMUNITIES FROM VARIOUS SOURCES OF INOCULA UNDER VARIOUS CULTURE CONDITIONS

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ABSTRACT

To date studies focusing on microbial cellulose hydrolysis have focused on pure cultures such as *Clostridium themocellum*, or isolated environmental cellulolytic strains. Microbial communities fed with crystalline cellulose were cultivated in continuous culture, and optimal growth conditions and culture approaches have been investigated.

Inocula from different environments were tested to determine microbial cellulose hydrolysis and growth, as well as the effect of temperature and media composition. Differences in microbial hydrolysis existed not only between sources of inocula, but within inocula themselves. It was found that microbial consortia cultured from direct environmental inocula had more robust microbial activity than enriched inocula.

Cultures grown at 60℃ showed higher biomass-speci fic cellulose hydrolysis, resulting in more efficient cellulose hydrolysis. This study provides evidence of differences in sources of inocula for the culture of cellulolytic consortia, and suggests culture approaches for the further study of possible applications of microbial consortia in bioprocessing technology.

To my parents

For lifting me every step of the way

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1. INTRODUCTION

While debate over the world's remaining oil supply continues, one thing is clear: fossil fuels are a finite source. This fact cannot be ignored. There is a growing demand for local sustainable energy sources. One of the most promising natural resources North America has an abundance of is plant biomass. With extensive landmass, rich in forests and fertile agricultural soils, an undeniable source of energy is growing around us, in the form of lignocellulosic materials. The constituent sugars of lignocellulose represent a significant source of energy for microbial growth. Subsequent fermentative processes provide appealing alternatives to fossil fuels such as ethanol while offering other valuable commodities as well. Commercialization of cellulosic biofuels has been hindered by process costs, the root of which lies in the hydrolysis of cellulose to sugars. Current popular methods require the production of large amounts of cellulase to mediate hydrolysis, followed by anaerobic fermentation. A more promising approach is to degrade cellulose through microbial hydrolysis, with simultaneous fermentation. Not only does this effectively eliminate the cost of producing enzymes, but increases process efficacy due to interactions between microbe and enzyme.

To date cellulosic bioprocessing research has focused on pure cultures, although under environmental conditions a diverse microbial population exists. Are there multiple players in the degradation of cellulose? Does this combination of species synergistically degrade cellulose? What role does the source of the microbial community or the reactor conditions under which it is cultivated play in its ultimate cellulolytic performance?

Little literature has focused on cellulose degradation by a microbial community and even less on the interactions within these communities, and these studies look more at environmental aspects rather than biotechnological application. An in depth examination of how these communities function could potentially provide insight on means to increase the efficacy of cellulose hydrolysis, thereby reducing processing costs and making cellulosic biofuels more economically viable. The overall goal of this thesis therefore is to describe research that aimed to quantitatively compare cellulose hydrolyis within anaerobic microbial communities cultures from anaerobic digester sludge and garden soil.

2. HYPOTHESIS, OBJECTIVES, AND RATIONALE

This research tested the hypothesis that if a wide diversity of micro-organism exists in all environments, and the environmental pressures select the most robust cellulose degradation, then a similar community of organisms will arise from different communities, and microbial activity will also be similar. In contrast, differences in microbial activity would indicate differences in diversity amongst environments.

The overall goal of the research was to establish fundamental understanding of cellulolytic microbial communities and their ecology, possibly leading to bioprocessing applications.

Specific objectives were to determine if microbial community cellulose hydrolysis and growth are affected by:

1) different sources of inocula;

2) nutrient composition; and

3) two generally applied temperatures for the culture of thermophilic cellulose degrading anaerobes .

While the highest documented rates of anaerobic cellulose hydrolysis are associated with thermophiles operating optimally at 60℃, recent findings suggest wild cellulolytic communities degrade cellulose most extensively at 50℃ [22]. The effect of inocula on microbial activity was assessed at 50℃ and 60℃ in both an oligotrophic and a nutrient-rich medium, under both batch and continuous conditions.

3. LITERATURE REVIEW

3.1. Fossil fuels and alternatives

Fossil fuels are a precious commodity to humanity, which have enabled us to leap forward into the modern age. Fossil fuels power our cars, they are used to make our roads, our clothing, and our shopping bags. It is an absolute building block of our society, and our reliance on it runs deep. The world's energy consumption is expected to grow 50% by the 2030, and liquid fuels are the most consumed form of energy at 37% [60]. The world uses 83.6 million barrels of oil per day, and increases in consumption and cost are projected. The processes that produce crude petroleum and other fossil fuels work on a geological time scale, which cannot match our rates of consumption [60].

Almost all transportation systems in the developed world are powered by combustion engines operating on volatile liquid fuels. These fuels are integral to the infrastructure that makes our economy, and cannot easily be replaced by another form of energy [21]. The US department of energy recognizes that plant biomass is one of the most abundant and underutilized biological resources on the planet, and is seen as a promising feedstock for the production of fuels and raw materials [59]. The majority of plant biomass is contained in the cell walls, of which 75% is comprised of polysaccharides [21]. These polysaccharides are an ideal substitution for petroleum as a feedstock capable of producing fuels, chemicals and materials such as plastics [36]

Up to 170 x 10^9 tonnes of biomass are produced annually via photosynthesis. Of this annual production, less than 5 % is harvested, and of this 5 %, less than 5 % is used for

non-food related purposes [27]. This means we tap into less than 1 % of self-renewing source of energy.

3.2. Lignocellulose

3.2.1. The cell wall and cellulose

Familiarity with the constituents of plant biomass is essential to understanding lignocellulosic bioprocessing. Aside from chloroplasts, the photosynthetic organelles, the cell wall is the defining characteristic of a plant cell. This cell wall is a highly organized composite of different polysaccharides, proteins, and aromatic substances. The architecture of this wall differs from species to species, tissue to tissue, and even between regions of the same wall. There are eleven different sugars in the cell wall, four different linkage positions, with branching, which allows for a myriad of possible combinations of polysaccharides possessing different characteristics [9].

Cellulose is the largest constituent of the plant wall, making up 15-30% dry weight of plant biomass, consisting of (1→4)β-D-glucan chains composed of several thousand glucose monomers a few micrometers in length. These chains adhere into bundles spanning 36 chains across, with over-lapping chains extending hundreds of micrometers (Figure 3-1), forming cellulose fibres. Due to the β-linkage, and all of the chains in parallel, hydrogen bonding between chains along their length results in paracrystalline structure, which excludes water. The recalcitrance of cellulose against enzymatic hydrolysis is afforded by this crystallinity [9, 35].

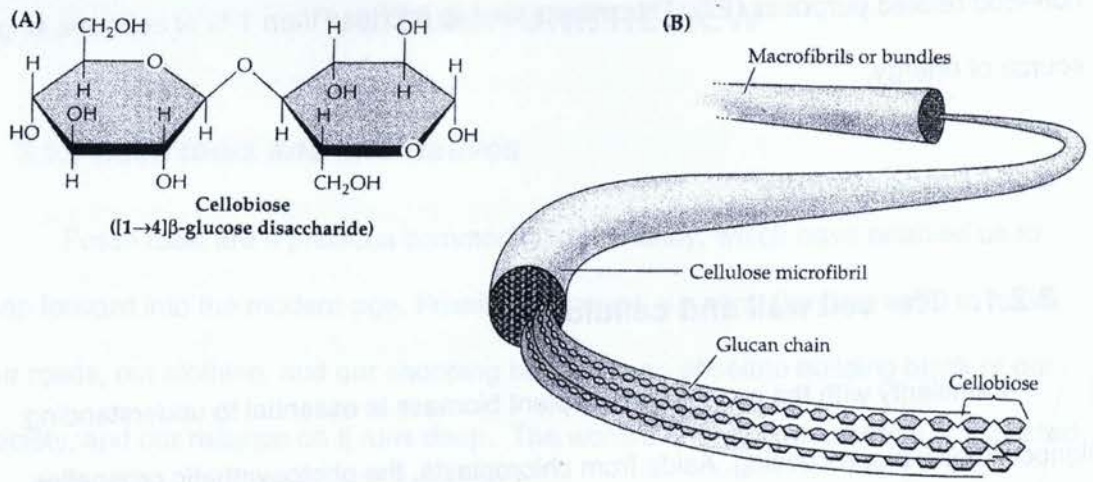

Figure 3-1 Cellobiose (A), Cellulose fibre (B) [9].

3.2.2. Cross-linking glycans

Although cellulose is the structural backbone of the cell wall in plants, several more components contribute to overall architecture. One crucial component is cross-linking glycans, "sticky" polysaccharides that coat microfibrils and interlink them into a network. These cross-linking glycans are often referred to as hemicellulose, a widely used but archaic term for all materials extracted from the cell wall with alkali solutions, regardless of structure.

Two major classes of cross-linking glycans exist, namely Xyloglucans (XyGs) and Glucuronoarabinoxylans (GAXs). XyGs link the walls of all dicot (woody) plants and half the monocots (herbaceous plants), while GAXs cross-link "commelinoid" monocots such as palms, bromeliads, and grasses. XyGs consist of glucose monomers linked by the same (1→4)β glucosidic bond, with the pentose sugar xylose linked at regular intervals (Figure 3-2). These side-linked xylans block crystalline formation, and instead act "sticky". GAXs on the other hand entirely consist of pentoses, with a xylan backbone

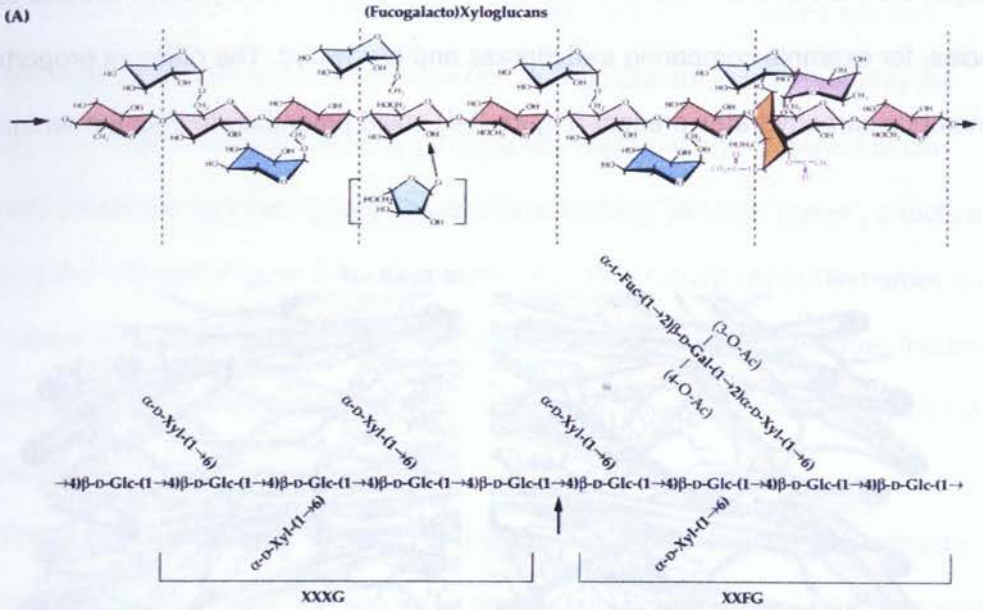

Figure 3-2 Xyloglucans, consisting of glucose (Glc) backbone chains, with xylose (Xyl) side-chains [9].

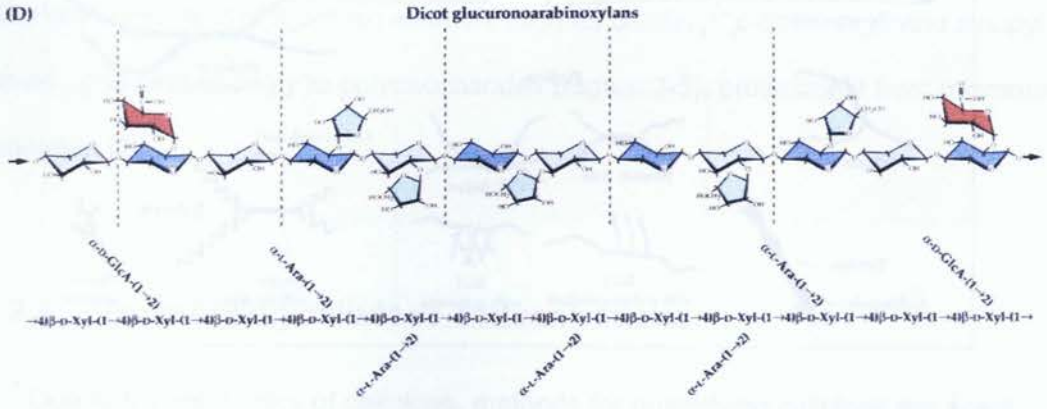

Figure 3-3 Glucuronoarabinoxylans, consisting of xylose (Xyl) backbone chains with arabinose (Ara) and glucuronic acid (GlcA) side chain.

with side-linking arabinose (Figure 2-3) [9]. The implications of this variety of structural linkages then is that the composition of plant biomass can change from species to species, for example comparing switchgrass and hardwood. The different proportions of monomeric carbohydrates present (Figure 3-4) could potentially impact fermentation.

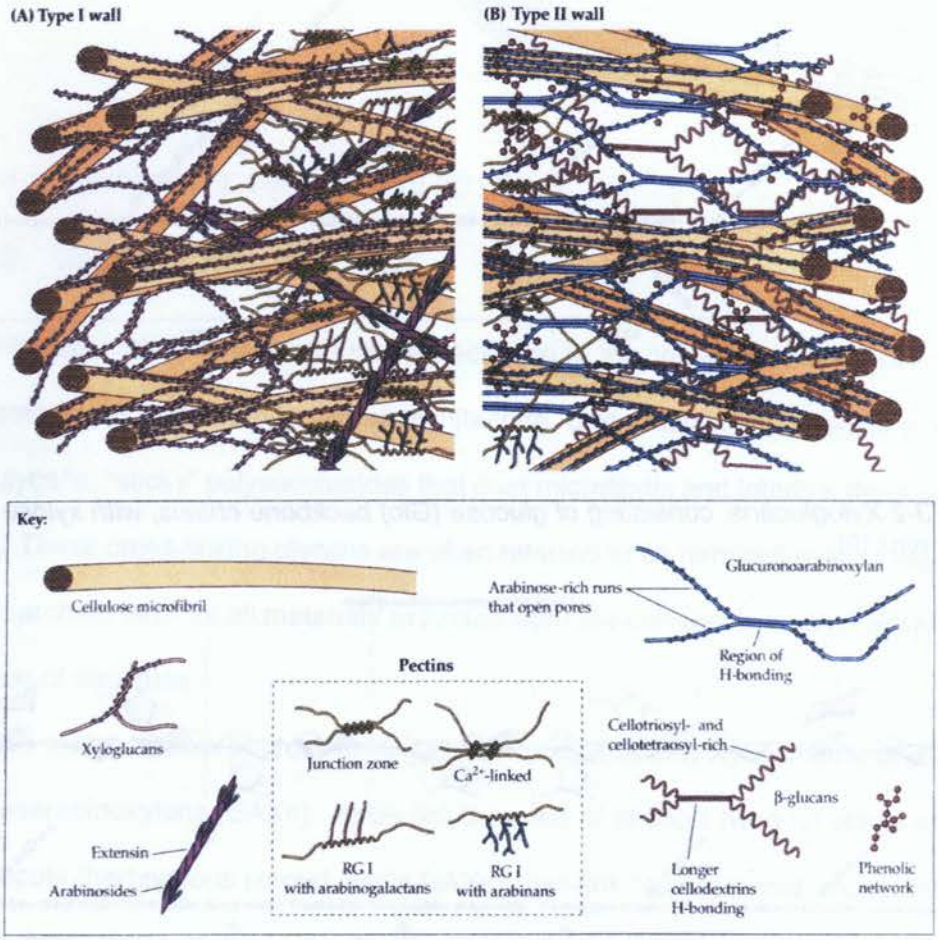

Figure 3-4 Cell wall architecture, demonstrating two variations common in cross-linking structure [9].

3.2.3. Pectins and lignins

Another important component of the cell wall are pectins. Pectins are well hydrated highly branched polysaccharides rich in D-galacturonic acid that play an important role in cell wall regulation, controlling cell wall porosity. Different pectin chains will condense by binding with calcium ions forming "junction zones", effectively hardening the cell wall (Figure 3-4). This condensation is brought about by removal of methyl esters from pectin via pectin methylesterase. It is possible that proper treatment of pectins may significantly soften plant biomass, especially in hardwoods, which have high amounts of pectin, thus increasing ezymatic degradative ability.

The final component contributing to the structure of the cell wall and ultimate plant biomass is lignin. From the latin wood *lignum* for wood, lignin is what gives wood its characteristic durability. Lignin composes 20-30% of all plant tissue, and after cellulose is the most abundant organic natural product. Degradation of lignin is limited to only a few aerobic fungi, and much ultimately ends up as humic acid in soil [11]. Lignins are comprised of aromatic alcohols such as coniferyl, *p*-courmaryl, and sinapyl alcohols , and bind strongly to polysaccharides (Figure 3-5), protecting it from microbial degradation [9].

3.2.4. Quantifying cellulose concentrations

Due to the insolubility of cellulose, methods for quantifying cellulose are scant and laborious. One approach is that of quantitative saccharification, developed by Jayme and Knolle (1960). This method entails hydrolysis of fermentation contents with

strong sulfuric acid, which hydrolyzes cellulose into its constituent glucose monomers. Soluble sugars can be quantified via HPLC [34], Miller's dinitrosalicylic acid colorimetric assay (DNS) [39], or a standard enzymatic glucose assay kit. DNS can only be used on crystalline cellulose, rather than lignocellulosic materials due to chemical interference [50] and acid hydrolysis conflicts with the alkaline reagents necessary in DNS to form color complexes [39]. Another widely used method for determining soluble carbohydrates is the colorimetric phenol-sulfuric acid reaction [16].

Another approach is the acid detergent fibre method of Goering and Van Soest (1970) that entails repeated washing of fermentation samples with alkali and organic solvents to remove non-cellulosic components, followed by drying and weighing [20].

Some studies choose to collect chemical oxygen demand (COD) values from fermentation, as COD can indirectly indicate hydrolysis of cellulose and subsequent fermentation [13, 53].

3.3. Consolidated bioprocessing

Initial investigations into cellulose hydrolysis focused on enzymatic studies, predominantly the aerobic fungal cellulases, in particular that of *Trichoderma reesei*. The study of only enzymatic action against hydrolysis is limited in scope, however. These cellulases are intrinsically bound to the organisms producing them, and the presence of these organisms have been shown to improve enzymatic activity under anaerobic conditions [33]. Understanding how these organisms interact with their substrate, and with other organisms, is an important step if we are to harness these microbial workhorses to perform biotechnological applications [3].

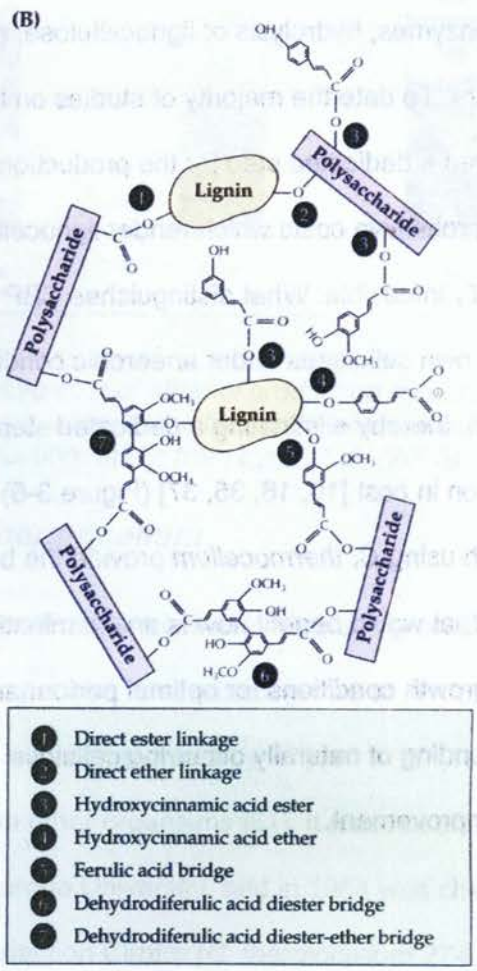

Figure 3-5 Lignin and associated bonds to polysaccharides, taken from Buchanan et al. *(2000)* [9].

One approach to microbial methods of utiliizing plant biomass that presents appeal is consolidated bioprocessing (CBP). CBP involves conversion of solid cellulose-based substrates into desired end-products (i.e. ethanol) in a single step fermentation using cellulolytic fermentative microbes. This single fermentation would include production of cellulolytic enzymes, hydrolysis of lignocellulose, and fermentation of both hexose and pentose sugars. To date the majority of studies on lignocellulose-based bioprocessing have involved a dedicated step for the production of cellulolytic enzymes. This process amounts to prohibitive costs which render lignocellulose-based bioprocessing economically infeasible. What distinguishes CBP is the capability of the microbes to produce their own cellulases under anaerobic conditions, coupled with simultaneous fermentation, thereby eliminating a dedicated step for enzyme production with a subsequent reduction in cost [10, 16, 35, 37] (Figure 3-6). Thorough method development and research using *C. thermocellum* provide the basis of our understanding of CBP. What would benefit now is an examination of cellulolytic consortia behaviour and growth conditions for optimal performance of CBP, in hopes that fundamental understanding of naturally occurring cellulose utilization could lead to further applications and improvement.

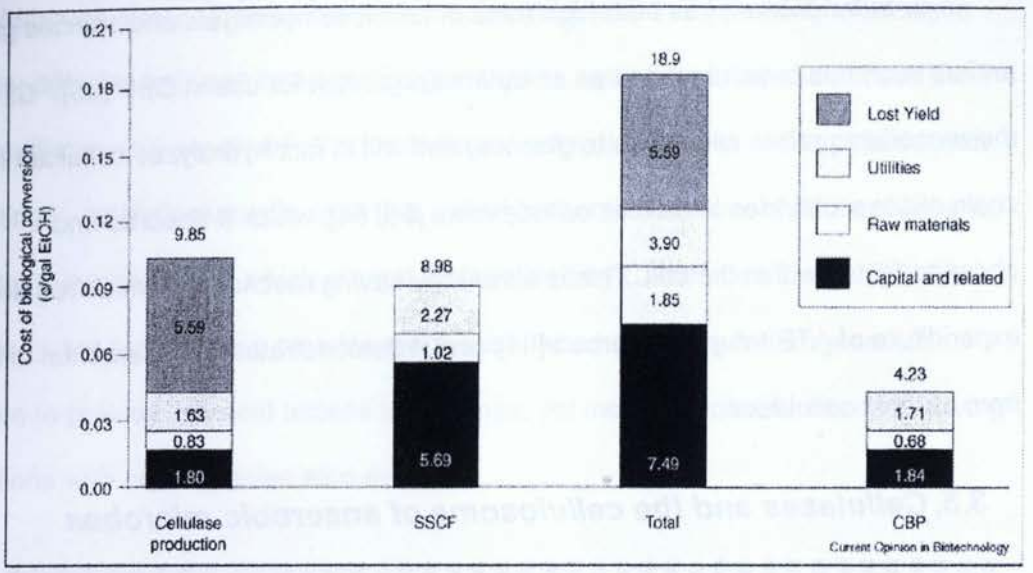

Figure 3-6 The comparative cost of ethanol production by consolidated bioprocessing (CBP) and by simultaneous saccharification and co-fermentation (SSCF) featuring dedicated cellulase production, taken from Lynd et al. *(2005).*

3.4. Clostridium thermocellum

Clostridium thermocellum is a cellulose-degrading cellulolytic thermophilic spore-forming rod-shaped gram positive anaerobe. It grows at an optimum temperature of 60℃ [24, 38]. This strain was first cultured by Vi ljoen *et al.* (1926) from horse manure, but in a mixed culture with other organisms [61]. It was not until 1948 that it was isolated by Dr. P. A. Tetrault of Purdue University, and in 1954 was characterized and submitted to the American Type Collection Centre (*C. thermocellum 27405)* by Mcbee [38]. Among other things yellow pigmentation was described, although this observation is not consistent with all cultures. *C. thermocellum* mainly produces ethanol, acetic acid, lactic acid [19, 34], with sometimes small amounts of butyric, formic, and succinic acid, as well as H2 [19, 38].

C. thermocellum has both high rates of cellulose hydrolysis and specific growth, and as such has been regarded as an optimal organism for use in CBP [35]. *C. thermocellum* prefers cellobiose to glucose, and will in fact hydrolyzes cellulose in small chain oligosaccharides known as cellodextrins [55, 64], which it absorbs and phosphorlyates within the cell. This is an energy saving mechanism which requires less expenditure of ATP to uptake carbon [64], and it demonstrates a fundamental difference from aerobic cellulases.

3.5. Cellulases and the cellulosome of anaerobic microbes

Aerobic fungi and bacteria secrete non-complex, free floating cellulases which have an affinity for cellulose, hydrolyzing the substrate into its constituent sugars, which are then absorbed by the nearby microbe. *C. thermocellum* and other anaerobic micro-organisms have a more "hands-on" approach to cellulose hydrolysis, firmly attaching themselves to their substrate via a complexed enzyme system known as a cellulosome [2, 3, 52].

Cellulosomes consist of a non-catalytic protein backbone or scaffoldin, firmly attached to the cell wall of the microbe. Catalytic subunits are attached to this scaffoldin, as well as carbohydrate binding modules (CBMs), which keep both the microbe and the catalytic subunits close to the substrate surface (Figure 3-7) [2, 3, 52] . It has been proposed that this proximity afforded by attachment provides advantage to the microbe by allowing access to soluble hydrolysis products in relatively high concentrations, before these products can equilibrate with the bulk solution [35]. This was quantitatively confirmed by *Lu et al.* (2006), who demonstrated that this attachment results in increase rates of cellulose hydrolysis, supporting the concept of cell-enzyme synergy [33].

Although similar in structure, cellulosomes express a large variety of catalytic subunits, differing between species. Exoglucanases and endoglucanases attack the cellulose fibres, and are clearly the fundamental catalytic subunits of the cellulosome. Interestingly, catalytic subunits exist that hydrolyze hemicelluloses, chitin, and lichens, the hydrolysis products of which are not metabolized by *C. themocellum* [3, 52]. It is likely these are present as a clearing mechanism when digesting heterogeneous substrate to provide physical access to cellulose, yet more complex functions involving interactions with other species also exist.

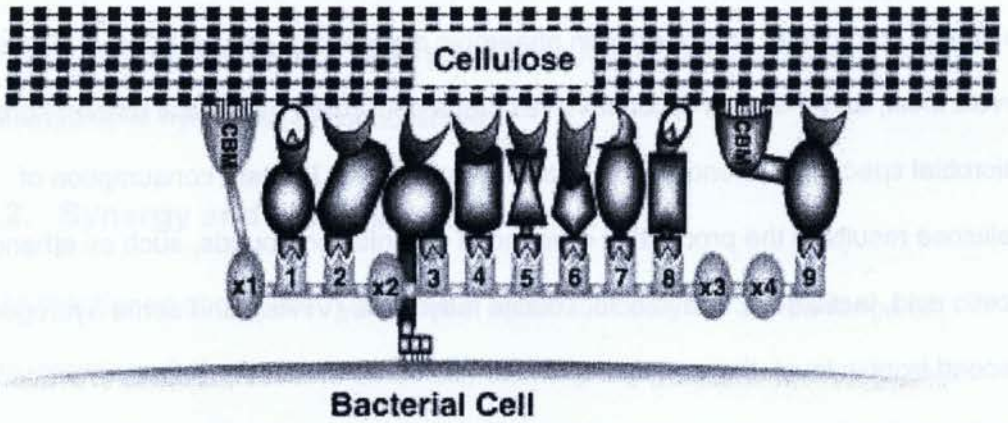

Figure 3-7 The structure of a hypothetical cellulosome, depicting a structural subunit which links the microbe to various enzymatic subunits and a carbohydrate-binding module (CBM) [52].

3.6. Interspecific interactions in cellulolytic ecology

Leftover products from cellulosome-mediated hydrolysis would constitute a significant source of energy in the natural setting. For instance, pentose sugars from hemicellulose released via the hydrolytic action of *C. thermocellum* cellulosomes would be a clear benefit to pentose-utilizing bacteria. The volatile fatty acids produced by the

fermentation of cellodextrins by cellulolytic microbes are also an energy source and a known substrate for other organisms [17, 25, 31]. It presents itself then that the tools and behaviour of cellulolytic anaerobes unlock a wealth of resources for an entire community, which will be discussed below.

3.6.1. Syntrophy and methanogenesis

Anaerobic cellulolytic organisms are the first link of a complex nutritional chain involving the conversion of plant biomass into fully-reduced end-products such as methane via alternative electron acceptors, such as sulfates or CO2. Within the microbial community participating in anaerobic digestion of cellulose, several trophic levels exist, and this conversion involves steps performed by several different groups of microbial species, a phenomenon known as syntrophy. Primary consumption of cellulose results in the production of reduced organic compounds, such as ethanol, acetic acid, lactic acid, formic acid, volatile fatty acids (VFAs), and some hydrogen. A second trophic level, the acetogens, consumes some of these products to produce acetic acid and more hydrogen. Finally, methanogens or sulphate reducing bacteria consume acetic acid, carbon dioxide/sulfates and hydrogen to produce methane or hydrogen sulphide, respectively. Methanogenic consumption of hydrogen produced by acetogenic bacteria is referred to as interspecies hydrogen transfer. The standard state free energy change of producing hydrogen and acetic acid from organic acids is non-spontaneous, with growth of acetogenic bacteria only possible because methanogens keep the hydrogen partial pressure exceedingly low [4, 25, 31]. Vice versa, methanogens are reliant on fermentative organisms to supply the necessary substrates, who in turn rely on cellulolytic microbes to provide an energy source. Thus, a complex

network of mutualistic interactions allows the existence of the anaerobic cellulolytic community. This is supported by documentation that coupled growth of methanogens and non-methanogens resultsin increased substrate utilization, and increased growth of both organisms [8, 17, 63].

In the wild cellulolytic arena then, both cellulose hydrolysis and methanogenesis are crucial for the continued flow of carbon, and these steps are moderately well understood. What deserves closer attention are the intermediary players and what role they play in enabling or even enhancing cellulose degradation. When such strong evidence of intrinsic mutualistic behaviour has been demonstrated within these communities, it is reasonable to postulate that these intermediary organisms would stimulate microbial hydrolysis of lignocellulose.

3.6.2. Synergy and biofilms

As mentioned above, the hydrolysis of lignocellulose yield a wide range of products, mainly carbohydrates discarded by cellulolytic organisms, as well as their fermentation products. These products are only partially reduced, and represent a sizable energy source that other microbes consume. These microbes would be attracted to such a food source, and it is highly likely that a consortium of micro-organisms would adhere to sites of cellulose degradation, forming a biofilm [14]. Non-cellulolytic organisms have been documented in close association with cellulolytic microbes, with more complete digestion of cellulose when compared to that of only the cellulolytic microbe [30].

This biofilm could confer multiple advantages to the cellulolytic organisms underneath such as

1) *Anaerobic Microenvironments.* Biofilms have been shown to generate strong physicochemical gradients in a very short distance, with large differences in pH and redox potential [15]. These gradients would play an important role in maintaining an environment sufficiently anaerobic and neutral for cellulolytic activity, allowing anaerobic cellulose hydrolysis in a wider diversity of environments. A degree of physical protection would be offered to the base-layer of cellulolytic microbes as well, possibly protecting them from biocidal compounds or predation by protozoa [14].

2) *Cross-feeding and inhibition.* Closely associated microbes consume fermentation products [17, 54] of the cellulolytic microbes, thereby alleviating inhibition [15]. Interestingly, it has been shown that some pentose-utilizing microbe growth rates are stimulated by increased acetate concentrations. A negative consequence however is that desired products may also be consumed.

3) *Nutritional benefits.* Co-cultures of *C. thermocellum* with non-cellulolytic organisms have been shown to mutually exchange vitamins that confer in vitro viability in media lacking nutrients [12, 41]. In terms of large scale biofuel production, this could result in simpler media, offsetting the cost of nutritional components.

4) *Acetogenesis and end-product consolidation.* Intermediary microbes metabolize the wide variety of volatile fatty acids, consolidating these into larger pools of substrate such as acetate, CO2 and H2 [17, 51, 54],which are main substrates for methanogens. Thus, intermediary microbes facilitate the flow of carbon and electrons from cellulose to methane.

Each one of these possible synergistic mechanisms would not directly increase the rate of cellulose hydrolysis, but rather facilitate this cellulolytic ecology. In an oligotrophic environment similar to that in certain environments, exchange of vitamins would be essential for growth. A biofilm would protect cellulolytic organisms from environmental stress and allow them to remain active in otherwise inhospitable environments. Chyi and Dague (1994) found that a mixed cellulolytic community taken from an anaerobic digester could degrade cellulose optimally at a pH of 5.5 [13], where *C. Thermocellum* and most cellulolytic isolates operate optimally at near neutral pH. This tolerance of pH merits more attention.

3.6.3. Co-cultures

It is difficult to discern a trend from studies comparing cellulolytic activity of a co-culture of a cellulolytic microbe with a non-cellulolytic species. Different species, substrates and culture methods are used. Weimer and Zeikus (1977) found that co-culture of methanogenic *Methanobacterium thermoautotrophicum* and *C. thermocellum* resulted in decreased ethanol yield, increased methane production, and decreased

latent period of growth, when compared to that of only *C. thermocellum* [62], supporting the concept of syntrophy. Ng *et al. (1981)* demonstrated that a co-culture of *C. thermocellum* and non-cellulolytic *Clostridium thermohydrosulfuricum* yield three times as much ethanol as the pure culture of *C. thermocellum* [44], supporting the concept that hydrolysis products not assimilted by *C. thermocellum* are captured by nearby non-cellulolytic microbes and fermented. Other cellulolytic-methanogenic co-cultures showed no increase in cellulose degradation, yet this was done in continuous culture, rather than batch [47] yet pH is maintained at neutral levels in a chemostat due to pH control, negating the benefit of acid-consuming methanogens. Some co-cultures show enhanced cellulose degradation while cultured on lignocellulosic substrates, rather than on pure cellulose [30], suggesting these organisms benefit from hemicellulosic substances devoid from purified cellulose.

All these studies demonstrate that interspecific interactions affect cellulose hydrolysis and growth of the organisms involved. Methanogens control pH, organisms benefiting from hydrolysis leftovers such as pentoses and cellodextrins stimulate cellulose degradation, while others consume fermentation products, relieving inhibition, while all microbes could very well form a protective biofilm, creating optimal growth conditions for the "bread-winning" cellulolytic providers. The complete reduction of substrate under anaerobic conditions is the result of intricate inherently-bound mutualistic interactions between a diverse network of microbial species, and this network has been well dissected and classified.

What is merited now is investigation of microbial communities as an entire system, rather than simply the sum of its parts. Many studies have focused on

synergistic cellulolytic communities in nature.

3.7. Cellulolytic communities

3.7.1. The Rumen

Ruminant animals harbour a large diversity of cellulolytic organisms, which play an essential role in the herbivore's nutrition. Playing host to bacteria, protozoa and fungi, the rumen provides a warm neutral environment for organisms to degrade plant biomass. It is largely agreed that bacteria play the largest role in determining the rate at which plant biomass is hydrolyzed and metabolized. The predominant cellulolytic bacteria present in the rumen are *Fibrobacter succinogenes, Ruminococcus flavefaciens*, and *Ruminococcus albus* [4, 40]. The attachment of these organisms to lignocellulose has been thoroughly documented, and both *Ruminoccocus* species are known to possess cellulosomes [40]. Protozoa comprise up to 50% of total biomass in the rumen [18], and several with cellulolytic abilities have been described, such as *Diplodinium* and *Eudiplodinium* [4] yet the majority predate on other microbes instead [18].

Rumen cellulolytic fungi constitute the order *Neocallimastigales*, or anaerobic chytridiomycetes [4] and were first isolated from a pellet of strained rumen fluid from sheep [45, 48]. To date sixteen species of rumen chytrids have been described, comprised of the genera *Neocallimastix, Piromyces, Caecomyces, Orpinomyces* and *Anaeromyces*, yet these fungi comprise only 1-4% of the total microbial rumen population [18]. These low populations are the result of bacterial suppression. When cultured in the absence of bacteria, chytrid cellulose hydrolysis rates are equal to that of

the whole rumen population [1]. In the rumen, anaerobic fungi are heavily outnumbered by bacteria but have a high specific activity for cellulose and therefore make a significant contribution to its degradation [1, 32].

Chytrid fungi also possess cellulosome-like structures similar to but different from those of bacteria [1, 32, 48]. These complexes merit closer inspection, due to chytrids high specific activity for cellulose.

While hydrolysis of lignocellulose in the host animal is probably the most important role these microorganisms play, their behaviour and contributions have yet to be full characterized [48]. The life-cycle of these fungi consists of two principal stages in which the motile zoospore alternates with vegetative thalli attached by a rhizoidal system to plant particles [1, 45, 48, 49]. Fungal rhizoids are effective at penetrating and weakening recalcitrant plant tissues and these microbes appear to have a special role in weakening plant cell walls. Their morphology as well extremely potent anaerobic fungal cellulases allow them to penetrate plant tissues with rhizoids, allowing access to cellulose and xylans, not only to fungi, but to rumen bacteria as well [11, 49].

A higher proportion of chytrids present in the rumen of animals feeding predominantly on dry biomass such as straw indicates the important role these fungi play in digesting more recalcitrant substrates [18]. Fungi have a more efficient degradative ability on some tissues in comparison to bacteria, in particular schlerenchyma [1], a tough ubiquitous plant tissue rich in lignin. Their relative success on heterogenous substrates such as plant biomass compared to that of pure cellulose also demonstrates the mechanical ability of fungal hyphae to penetrate and access recalcitrant substrates [18]. In a heterogeneous substrate then, the presence of fungi

would result in more thorough hydrolysis, suggesting possible application in enhanced hydrolysis of lignocellulosic biomass.

Recent advances in molecular biological detection indicate that these anaerobic fungi are not restricted to the rumen and in fact inhabit various environments, such as landfill sites and freshwater sediment [32].

Chytridiomycetes are a specialized group of fungi, one of the few if only groups of cellulolytic fungi that operate anaerobically. While proportionally less than their cellulolytic bacteria counterparts, this small group can approach the hydrolytic ability of an overwhleming majority. This, combined with the powerful pentrating ability of their hyphae, highlight chytridiomycetes are potential organisms for application in lignocellulosic bioprocessing.

3.7.2. Insects and cellulolytic endosymbionts

3.7.2.1. Termites

Approximately 60% of the terrestrial world is inhabited by termites, with high populations in the tropics, so high in fact they can outnumber grazing mammalian herbivores in terms of biomass density. A termite's ability to feed on wood is afforded by a mutualistic microbial community of both bacteria [56] and protozoa [7] harbored in the anaerobic intestinal hindgut. Of particular note are the anaerobic flagellated protozoa of the termite hindgut. These protozoa represent unique genera and species found virtually nowhere else in nature [7] stemming from one the deepest branches in the lineage of the eukaryotes, during the carboniferous era [42] These specialized flagellates can account for up to one third total weigh of a termite, and engulf wood particles where they are hydrolyzed and fermented to acetate and H2. The

physiological activity of flagellates within the termite hindgut is still unclear [42] however. It is unclear whether protozoa synthesize their own cellulase, or if instead endosymbiotic cellulolytic bacteria perform this task. In some case it has been show that the protozoa do in fact produce cellulase, but this is far from conclusive [4].

Protozoa play in important role not only by facilitating digestion of wood particles but by increasing the retention time of cellulose particles in the gut via phagocytosis, increasing exposure time to cellulases [7]. If termite cellulases are adsorbed onto cellulose particles engulfed by protozoa, they might continue to act, perhaps even synergistically, with protozoan enzymes. The intestinal microorganisms establish symbiotic associations not only with the termites but also with other intestinal microorganisms. For instance spirochaete bacteria, which are found in highest densities in termite intestines, adhere to the surface of protozoa, assisting in motility [7]. These spirochaetes have been shown to have important roles in anaerobic digester function stability [17], and seem to play roles in cellulose hydrolysis in different environments.

3.7.2.2. Microbial communities

In a recent study of hindgut microbial communities in termites by Tanaka *et al.* (2006) [56], the termite *Coptotermes formosanus* was fed wood dust, cellulose, cellobiose or glucose exclusively. Significant differences in both bacterial and protozoan components of communities existed between low molecular weight carbon diets (i.e. glucose, cellobiose) and high weight diets (i.e. cellulose, wood dust). DGGE results indicated a strong similarity in bacterial components (80%-60%) of gut biota between termites fed high weight diets and termites grown on solid wood, while gut biota of termites fed low weight diet populations were significantly different (40-45%). It is

interesting to note that when diets contained hemicellulose and lignins similarity to natural communities was highest (80%), compared to a diet of strictly cellulose (60%).

3.7.2.3. Protozoa

Three different species of protozoa, *Pseudotrichonympha grassi, Holomastigotoides hartmanni*, and *Spirotrichonympha leidyi*, were found in the hindgut of the termite *C. formosanus* that fed on diets containing carbon sources with high molecular weight. The three species were not present in the gut of termites on low weight carbon diets, with virtually no other protozoa present. These results indicate that protozoa play an active role in the flow of carbon in the termite hindgut. What this role is, or any direct cellulolytic activity by these protozoa has yet to be demonstrated however.

3.7.2.4. Coprophagy

Interestingly, worker termites that were fed on glucose for 30 days lost all protozoa, with DGGE results indicating drastic changes to bacterial population structure. More importantly, these termites lost viability on a cellulose diet, strongly demonstrating fundamental changes in microbial community influenced directly by substrate availability. When these termites were incubated with termites fed on cellulose diets, they quickly regained protozoa, and the ability to metabolize cellulose [56]. This is likely due to coprophagy. Dictyopteran insects such as termites and cockroaches are well known coprophages. Coprophagy provides access to concentrated microbial consortia, including protozoan cysts, growing on cellulose based substrates. Repeated ingestion of feces is no doubt required, however, because a successional colonization of the

various gut niches by microbes is typical in animals, including termites. Obligate anaerobes, for example, have to be preceded by facultative anaerobes, and a complex bacterial community has to precede protozoan populations [42].

3.7.2.5. Potential applications of termites and their endosymbionts

Tanaka *et al.*(2006) have posed potential applications for the mutualistic coprophagous behaviour, suggesting that undegradable materials (for example, aromatic compounds such as lignin, dioxin, etc.) can be mixed in artificial diets for termites, thus presenting opportunity for enriching for degradative micro-organisms. Another advantage of this system is that the dominant microbial community involved in the biodegradation will be enriched, and thus, a consortium of those microorganisms can be isolated, allowing for study of "unculturable" microbial communities. Also, xylanases related to those of expressed by rumen micro-organisms have been detected in arthropod digestive tracts, yet significant phylogenetic differences in protein sequence exist. With possible differences in hydrolytic activity and other properties, the appeal of novel microbial traits waiting to be uncovered for application poses a tantalizing opportunity for bioprospecting [6].

3.7.3. Cultured communities

Ueno *et al. (2001)* enriched a mixed cellulolytic community from compost consisting of straw and horse manure, and subsequent molecular analysis using denaturing gradient gel electrophoresis indicated the presence of 68 different organisms, which were classified into 9 groups depending on genetic similarity. The

majority were closely related to the subphylum *Clostridium/Bacillus*, with key players indentified as *Clostridium themocellum*-like organisms such as *Clostridium cellulosi* and new described *C. stramnisolvens [48]*, with a predominance of *Thermoanaerobacterium thermosaccharolyticum*, a pentose utilizing anaerobe [58]. These experiments were able to maintain a community batch culture in series in unsealed flasks with reproducible sub culturing [22].

The components of this community were isolated, reintroduced and demonstrated to have the same cellulolytic capability as the wild community [28]. This community was found to degrade cellulose optimally at 50℃ [28], rather than the conventional thermophilic temperature of 60℃, whic h is the optimal temperature of *C. thermocellum* [38]. Interestingly, inoculation of this community with *C. thermocellum resulted in the* replacement of the wild cellulolytic microbes when cultured at 60℃ [43], suggesting one can "tailor" a community by replacing component species. Kato *et al.* (2004) demonstrated that both the wild and constructed community degraded cellulose far more efficiently than a pure culture of the cellulose-degrading bacterium present [28].

In terms of biotechnological application, however, this matters little, as both constructed and wild communities were enriched aerobically, and contained aerobic organisms, along side the anaerobic organisms. These aerobic microbes neutralized pH and maintain zones of low redox potential away from the atmosphere-media interface, which allowed allowing anaerobes to grow. These conditions befitting anaerobic cellulose-degraders were afforded by their respiration of fermentation products to CO2 however[54], which is an unacceptable loss in terms of bioprocessing goals. To date

little work has been done to study the communities that arise from enrichment under anaerobic conditions.

3.8. Community profiling

In comparison with pure culture studies, a fundamentally different facet to mixed cultures is the understanding of community dynamics, or the changes in microbial species composition and proportions over time. Molecular techniques such as denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism permit "snapshots" of a community profile, which can be used to monitor community dynamics. Is a community in constant flux, with specie composition and proportion constantly in change, or does it stabilize, remaining constant? In terms of biotechnological application, focus lies on constant process performance. Can a community in flux produce constant process values? Kato *et al.* (2004) found that the above mentioned cellulolytic enrichment demonstrated a stable population throughout long-term sub-culture [28]. These experiments were done in open-system batch however, and it is possible that over longer periods under continuous systems, opportunities for community flux might be more likely to arise, as continuous cultures theoretically maintain continuous growth, and inhibition is relieved via pH control and media turnover.

In an indeterminate system such a continuous bioreactor, process equilibrium, or steady state, of a mixed community may not indicate fixed populations, rather constant behaviour despite the flux of individual populations under stable conditions. With increasing variation in populations, an "averaging effect" could result in functional stability, with multiple species competing to perform the same task, resulting in a

functional redundancy. This could partly explain the positive correlation between biodiversity and ecosystem stability [8].

Fernandez *et al.* (1999, 2000) ran two continuous bench-top reactors fed synthetic wastewater, and found that the less stable community resulted in more stable chemical oxygen demand (COD) removal, especially under perturbation [17]. Tolerance to pertubation would be a desirable quality in large-scale bioprocessing attempts, as there would be inevitable disturbances in reactor process, due to technical difficulties and maintenance.

Other large scale reactors have shown stable populations over long time periods [8]. It has been suggested that more diverse community will contain organisms with different capabilities, and this functional diversity allows for a shift in relative proportions of species present to adapt, thus maintaining process stability. Even in a non-disturbed system, if multiple species are present that perform the same task (i.e. fermentative bacteria), process behaviour would not change regardless of the population flux, due to this functional redundancy.

Some species are keystone however, a good example of which can be found in methanogenic reactors. Highly specialized methanogens are a low-diversity keystone group of organisms that drive the system and can not be removed without arresting reactor function. Fermentative bacteria on the other hand are a high-diversity group that can easily interchange with each other, still producing acetate and H2 which will be consumed by methanogens [8]. By possessing multiple pathways of fermentation via different species, interruption of methanogenesis could be avoided by both functional redundancy and diversity in a community. It seems altogether possible that a constantly

changing dynamic microbial community could maintain more stable behaviour patterns.

3.9. Summary and conclusion

With the rapid dwindling of fossil fuels, attention is being shifted to the production of liquid fuels derived fermented from lignocellulosic biomass. This biomass is complex and recalcitrant to hydrolysis, and under anaerobic conditions, this requires micro-organisms to with highly specialized enzyme complexes be effectively accessed, and these microbes can be found in a variety of forms and environments . These organisms play an important role in providing energy to a trophic cascade of organisms, whose separate roles in the anaerobic flow of carbon have been determined. It is essential however to view these functional groups of microbes as more than the sum of their parts, if we are to understand how they work on a fundamental level, as they are co-dependent and intrinsically bound to one another.

4. MATERIALS AND METHODS

4.1. Anaerobic continuous culture on insoluble cellulose

Microbial consortia were cultured in two Sartorius A+ BIOSTAT 2L benchtop autoclavable reactors (Sartorius AG, Goettingen, Germany). A pH of 7 was maintained via addition of 1M KOH. Reactors were stirred at 250 RPM, and temperature was maintained with an electric heating jacket. The reactors were autoclaved with the initial working volume of medium, to both sterilize and degas said medium. Oxygen was excluded by sealing every possible entry except for the inlet and outlet, as well as using oxygen impermeable Cole-Parmer norprene tubing (Cole-Parmer, Vernon Hills, Illinois, USA) in all lines leading from or to the reactor, feed carboy, or effluent carboy. Addition of medium and gas production resulted in the siphoning of reactor contents in excess of the one L up the effluent tube and into a waste carboy underneath. Medium to be added to the reactor was kept sterile under hypoxic conditions in a KIMBLE 20L bottom spigot glass carboy (Kimble, Vineland NJ, USA). Medium was added to reactor via an influent port, using a Watson-Marlow peristaltic pump (Wilmington, MA, USA) with the pump clamps enlarged to fit norprene tubing. Negative pressure in the reservoir headspace due to media removal was alleviated via connection of a gas-impermeable bladder filled with N_2. The medium carboy was continually mixed using VWR hi volume magnetic stir-plate (VWR, Missisauga, Canada) and the feed line was kept on a downhill gradient from carboy to pump to reactor, so as to prevent in-line sedimentation of avicel and

inaccurate feed concentrations (Figure 4-1). Feed concentrations from pumping and reactor input-output balance were quantified to confirm accuracy (Tables A-1, A-2). Conditions were maintained as constantly as possible to minimize disturbances in the microbial community, to determine if both functional and community stability arose.

4.2. Anaerobic batch culture in sealed serum vials

Serum vials containing 35 mL of medium were sealed with butyl rubber septa and aluminum crimps. Multiple sealed vials were then punctured with 22 gauge syringe tips and repeatedly flushed with N_2 and evacuated by vacuum using a three-way valve connected to a manifold. Vials were then autoclaved for one hour at 121 °C. Once cooled, sterile trace elements were added via 1-mL syringe. Vials were inoculated with 10% (v/v) inoculum and incubated at either 50 °C, or 60 °C, with shaking at 250 RPM.

4.3. Media composition

Avicel, consisting of purified 50 um cystalline celllulose particles (Sigma-aldrich, St. Louis, MO, USA), was added to all media. Two types of media were used. Nutrient rich modified RM medium [46, 57] contained 2 g/L urea, 2 g/L KH_2PO_4, 3 g/L K_2HPO_4, 0.2 g/L $MgCl_2 \cdot 6H_2O$, and 50 mg/L I $CaCl_2 \cdot 2H_2O$. Oligotrophic MEC medium contained 2 g/L KH_2PO_4, 3 g/L K_2HPO_4, 0.1 g/L NH4Cl, 50 mg/L $MgCl_2 * 6H_2O$, 10 mg/L $CaCl_2 * 2H_2O$, 1 mg/L rezazurin. Trace metals were added to all media after autoclaving as 1000x stock solutions resulting in the following working concentrations: 50 mg/l EDTA, 3.6 mg/L $FeCl_2 * 4H_2O$, 1.5 mg/L $CoCl_2 * 6H_2O$, 0.9 mg/L $MnCl_2 * 4H_2O$, 0.9 mg/L

$ZnCl_2$, 0.17 mg/L H_3BO_2, 0.01 mg/L $CuCl_2$, 0.09 mg/L $Na_2MoO_4*2H_2O$, 0.002 mg/L $NiCl_2*6H2O$. No reducing agents were added, as the microrganisms demonstrated the ability to generate fully-reduced condtions in sealed hypoxic media, as indicated by the disappearance of color in rezazurin. In batch vials, 0.5 g yeast extract was added. In reactors yeast extract was either added at varied concentrations or excluded altogether, and is specified accordingly for each experiment.

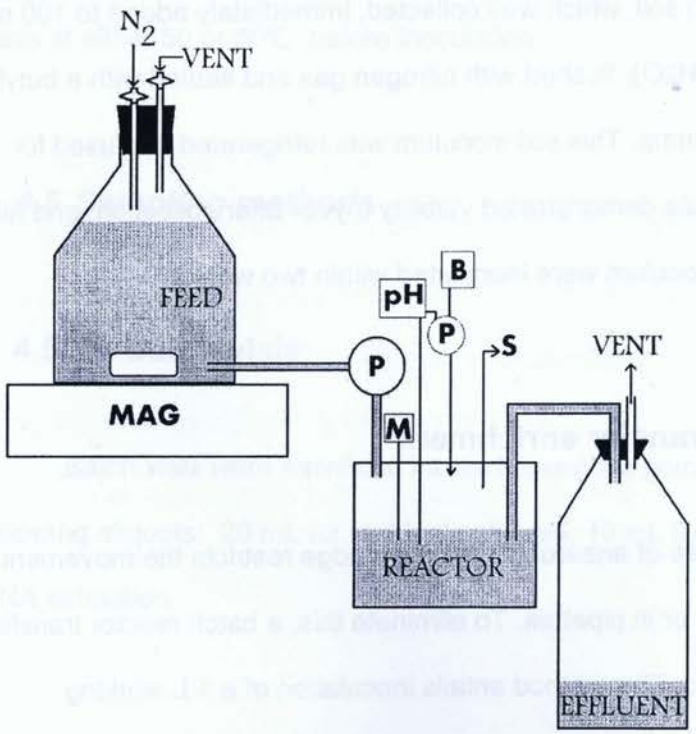

Figure 4-1 Schematic arrangement of the experimental apparatus for continuous culture of cellulolytic organisms. (P=pump, M=stirring motor, B= base control solution, S=sample line, MAG=magnetic stirplate).

4.4. Inocula

4.4.1. Sources of inocula

Inocula were taken from two sources. A 500 mL sample of anaerobic digester sludge was collected from Ashbridges Bay municipal wastewater treatment plant, Toronto, Ontario. The sample was used as inoculum for an enrichment batch reactor immediately, with the remainder refrigerated in a tightly sealed vessel. The second source was one gram of garden soil, which was collected, immediately added to 100 mL autoclaved deionized water (DiH2O), flushed with nitrogen gas and sealed with a butyl rubber stopper and aluminum crimp. This soil inoculum was refrigerated and used for multiple inoculations. This inocula demonstrated viability a year after collection, and all batch experiments using this inoculum were inoculated within two weeks.

4.4.2. Batch reactor transfer enrichment

The heterogeneous nature of anaerobic digester sludge restricts the movement of fluid, whether in reactor lines or in pipettes. To eliminate this, a batch reactor transfer enrichment method was devised. The method entails inoculation of a 1 L working volume anaerobic batch reactor with 100 mL of sludge, which was cultured at 60℃ under pH control at 7.0. After five days, compressed nitrogen gas was used to transfer 100 mL of reactor contents into a secondary reactor via O_2 -impermeable norprene tubing. This reactor was cultured under identical conditions to that of the primary reactor. After 5 days the contents were collected in sterile serum vials and refrigerated for further initial inoculations.

4.4.3. Inocula generation

To generate sufficient amounts of uniform active inocula for the multiple inoculations necessary for batch experiment replication, 100 mL of media was inoculated 5% (v/v) with either enriched sludge or refrigerated soil, and incubated for 5 days at either 50 or 60℃ before inoculation.

4.5. Sampling methods

4.5.1. Batch vials

Batch vials were sacrificed for each sampling point. Each batch vial yielded the following aliquots: 20 mL for residual cellulose, 10 mL for total protein, and 5 mL for DNA extraction.

4.5.2. Reactor sampling

Reactor sampling entailed collection of 65 mL of reactor contents, which was pipetted while being mixed to maintain sample homogeneity into two 20 mL aliquots for residual cellulose, two 10 mL aliquots for total protein, and 5 mL for DNA extraction.

4.6. Fermentation Analysis

4.6.1. Quantification of residual cellulose.

Residual cellulose concentrations from fermentations were determined using the quantitative saccharification (QS) method of Jayme and Knole (1960) [26]. 20 mL Fermentation samples were centrifuged in 50mL centrifuge tubes at 3600 RPM, with supernatant aspired and discarded. Pellets were then washed with 20 mL deionized water (diH2O) and centrifuged under the same centrifuge conditions, followed by aspiration. The resulting pellets were dried at 60°C. Desiccation was necessary for accurate subsequent acid hydrolysis, as well as preventing further microbial activity. Sample processing entailed pulverizing desiccated cellulose pellets with a glass stirring rod to increase surface area and promote thorough hydrolyis. Samples were then hydrolyzed with 72% sulfuric acid at 30℃ for one h our, followed by autoclaving for one hour at 121℃, resulting in a clear solution with s olubized cellulosic glucose. 800 uL hydrolysate aliquots were then were then quantified by HPLC with an Aminex 87x-H column (Bio-rad Laboratories Inc, Hercules, CA, USA) operated at 55℃ with a mobile phase of 25 mM sulfuric acid , at a flow rate of 0.6 mL/min. Test QS runs were performed with known amounts of hydrolyzed crystalline cellulose as described above to confirm accuracy (Table A-3).

4.6.2. Protein quantification

Protein samples consisted of duplicate 10 mL aliquots of fermentation

contents (batch or vial), centrifuged at 3600 RPM for 20 min, followed by aspiration of supernatant. Samples were then washed with 5 mL of DiH_2O, and centrifuged under the same conditions for another 20 min, followed by aspiration of supernatant, addition and resuspension of 2.5 ml DiH_2O. 2 ml aliquots of the sample were then transferred to a microcentrifuge tube, and centrifuged at 13 000 RPM for 10 minutes, followed by aspiration of supernatant, and stored at – 20 °C. Samples were processed by incubation in a water bath for one hour at 70 °C with 0.6 mL 0.1M NaOH to detach and lyse cells from cellulose. Samples were then neutralized using 0.6 mL 0.1M HCl and centrifuged at 13 000 RPM to separate solubilized protein from celllulose. Solubilized protein was then quantified using the Bradford Assay [5] with Biorad reagent (Biorad, Hercules, CA), and compared to known standards of bovine serum albumin (BSA). Sample aliquots ranged from 50 uL to 200 uL depending on sample protein concentration, to achieve a protein concentration within the range of the bradford assay.

4.7. Community Analysis

4.7.1. DNA extraction

1.5 ml reactor samples were centrifuged at 13 000 RPM for two minutes, and the resulting pellet was used for DNA extraction using Sigma GenElute DNA extraction kit (Sigma-Aldrich, St.-Louis, MO, USA). This method entails incubation of the sample pellet for 30 min at 37°C with lyzozyme, followed by incubation for 10 min at 55 °C with proteinase K, folowed by precipitation with 100% ethanol, and extraction via

centrifugation resulting in the elution of DNA. Extractions were performed in duplicate, and then mixed into samples which were frozen at 20 °C until subsequent sample processing.

4.7.2. Polymerase chain reaction (PCR)

PCR was used to amplify bacterial 16S rDNA fragments , using primers 357F-GC (5′-CGCCCGCCGCGCGCGGCGGGCGGGG

CGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3′)

and 518R (5′-GTATTACCGCGGCTGCTGG-3′) as the PCR primers. Each amplification reaction mixture (60 µl) consisted of 2.5 U Taq DNA polymerase (EMD Chemicals Inc., San Diego) , 4.8 µl of DNA sample, 6 µl of 10× PCR buffer, 1.2 µM of each primer, and a mixture containing each 125 mM deoxynucleoside triphosphate in a tube. After initial denaturation at 94℃ for 4 min, 35 cycles of 94℃ f or 30s, 54℃ for one minute, and 72℃ for 1 min were performed, and then the reactio n mixture was kept at 72℃ for 10 min. PCR products were stored at -20℃. PCR amplifi cation was confirmed (Figure A-1)

4.7.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE was used to approximate a profile of microorganisms present in a consortium at a given time, which can indicate changes in community structure over time. 50 uL of PCR product was used. The DGGE was performed using a D-Code system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Acrylamide (8%) gels were prepared and electrophoresed with 1×Tris–acetate–EDTA (TAE) buffer comprised of

0.2M Tris base, 0.02 M acetic acid, and 0.1 M EDTA (pH 8). The DGGE gel contained 20–70% gradient of urea and formamide in the direction of electrophoresis, created by casting a acyrlamide gel with two solutions, one with a high percentage of denaturants, the other with a low percentage, added in such a way that there is a higher proportion of denaturants in the acrylamide added first, with less added towards the end.

One hundred percentage denaturant consisted 40%(v/v) formamide and 7 M urea. DGGE was conducted at a constant voltage of 70 V at 60℃ for 16 h. The gel was stained with bromophenol blue.

4.8. Experimental design

4.8.1. Grass trimmings (Figure 5-1, 5-2)

To determine under what conditions cellulose degradation occurs in the environmental setting, environmental cellulose degradation was investigated using lawn trimmings. Freshly mown lawn trimmings were collected, wetted, and placed in a heap. Internal temperature of the heap was recorded. Once the temperature stabilized, samples from the heap were collected, weighed into 5 g samples. These samples were added to 100 mL sterile deionized water in 500 mL Erlenmeyer flasks which were then sealed, flushed with nitrogen gas, and incubated at 60℃ . Replicates were sacrificed in triplicate daily for five days, and assessed for pH change and lignocellulose degradation, with biomass strained and dried for weighing, and these dry weights were compared to initial control sample .

4.8.2. Batch #1: Enriched sludge cultured on two media (MEC, RM) incubated at two temperatures (Figures 5-3, 5-4)

To determine growth conditions for enriched digester sludge, batch vials containing either RM or MEC medium, both with 5 g/L Avicel and 1 g/L yeast extract, were inoculated with active sludge inocula incubated at 60℃ in the respective medium, and incubated at either 50 or 60℃ under shaking at 250 RPM. Vials were sacrificed in triplicate on day 2, 5, and 7, with triplicate controls at zero, resulting in 454 vials in total.

4.8.3. Batch #2: Enriched sludge and soil incubated at two temperatures (Figure 5-7, 5-8)

To compare growth rates of two inocula, batch vials containing MEC medium with 5 g/L Avicel and 1 g/L yeast extract were inoculated with either refrigerated soil or pre-enriched sludge enriched at 50℃. Inocu lated vials were incubated at 50℃ under shaking at 250 RPM. Vials were sacrificed in triplicate on day 2, 4, 5, and 7, with triplicate controls at time zero, resulting in 54 vials in total.

4.8.4. Batch #3 Unenriched soil cultured on two media at two temperatures (Figures 5-5, 5-6)

To determine growth conditions for soil, batch vials containing either RM or MEC medium, both with 5 g/L Avicel and 1 g/L yeast extract, were inoculated with unenriched soil and incubated at either 50 or 60℃ under shaking at 250 RPM. Vials

were sacrificed in triplicate on day 4.

4.8.5. Continuous #1: Enriched sludge cultured at 60 ℃ (Figure 5-9)

A one litre working volume reactor containing MEC media with 5 g/L Avicel and 0.1 g/L yeast extract was inoculated with 100 mL enriched anaerobic digester sludge to determine robustness and attempt to achieve steady-state, or constant values for cellulose hydrolysis rates and total protein concentration. The reactor was run for 35 days with a hydraulic retention time (HRT) of 24 h. pH was maintained at 7, with temperature maintained at 60℃.

4.8.6. Continuous #2: Enriched sludge cultured at 50℃ (Figure 5-11)

A one litre working volume reactor containing MEC medium with 5 g/L Avicel and 0.1 g/L yeast extract was inoculated with 100 mL enriched anaerobic digester sludge to test growth ability of treated inocula and attempt to achieve steady-state, or constant values for cellulose hydrolysis rates and total protein concentration. The reactor was run for 105 days with HRT of 24 h. The pH was maintained at 7, with temperature maintained at 50℃. This experiment was a replication of continuous attempt #1, with only the temperature changed.

4.8.7. Continuous #3: Unenriched soil cultured at 60℃ (Figure 5-

12A, 5-12B)

A one litre working volume reactor containing RM media with 5 g/L Avicel and either 0.1 g/L yeast extract (day 0-40), or 0.5 g/L yeast extract (day 40-100) was inoculated with one gram garden soil to test growth ability and attempt to achieve steady-state, or constant values for cellulose hydrolysis rates and total protein concentration outputs. The reactor was run for 100 days with HRT of 24 h. pH was maintained at 7, with temperature maintained at 50°C.

5. RESULTS AND DISCUSSION

5.1. Observation of environmental temperatures for moderate thermophilic cellulose degrading communities

To observe the behaviour of cellulolytic communities in the natural state, lawn trimmings were collected, wetted, and place into a heap. Temperature was measured in short intervals over three days, with a steady increase in temperature to 50℃ due to microbial activity (Figure 5-1). Once it was apparent that temperature was stable, samples were taken, and placed in sterile water, sealed and deoxygenated to observe cellulose degradation and pH decrease (Figure 5-2). The results in Figure 5-2 confirm the occurrence of anaerobic digestion of lignocellulosic biomass. The combined results of Figures 5-1 and 5-2 suggest that cellulose-degrading communities within the heap operated at 50℃, likely selecting for optimal cell ulose hydrolysis at this temperature. These results support the findings of Haruta *et al.* (2002), who found that compost heaps reached 50-60℃, and had the highest amounts of cellulose hydrolysis at 50℃ [22]. *C. thermocellum,* with robust cellulolytic activity, grows optimally at 60℃, however [38]. Which of these two temperatures would give the most desirable cellulose hydrolysis rates in continuous culture studies focused on bioprocessing applications?

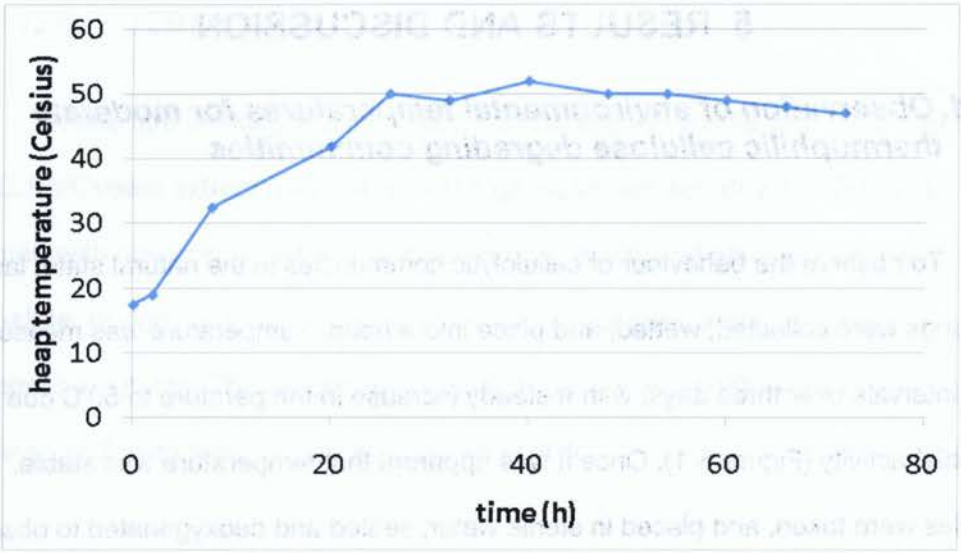

Figure 5-1 Temperature profile over time in a heap of lawn trimmings.

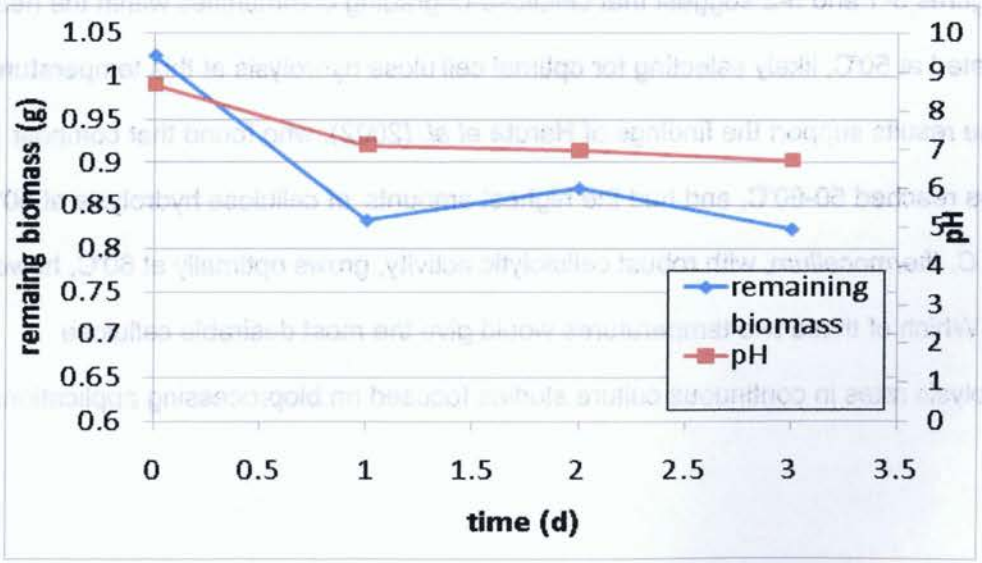

Figure 5-2. Lawn mowing heap sample biomass degradation and pH in batch at 60℃ vs. time.

5.2. Batch cultivation: Determination of optimal temperature and medium for cellulose degrading communities.

5.2.1. Investigation of optimal temperature for growth and cellulose hydrolysis for the enriched sludge community in two different media.

To determine suitable nutritional and environmental conditions for the culture of the enriched sludge community, batch experiments were prepared with both an oligotrophic medium (MEC), and a nutrient-rich medium (RM). Vials of each medium were inoculated with inocula enriched at 60℃ and i ncubated at either 50℃ or 60℃, with samples sacrificed for the quantification of cellulose and protein, as well as DNA extractions, over the span of a week. Figure 5-3 indicated that the enriched sludge community had both the highest levels of cellulose degradation and total protein when cultured in oligotrophic MEC medium at 50℃ , despi te the fact the inoculum was initially enriched at 60℃. However, results plotting biomass -specific cellulose hydrolysis indicate that cellulose was hydrolyzed more efficiently at 60℃ compared to 50℃ when grown on MEC medium (Figure 5-4). These results indicate that enriched sludge communities display higher cellulose hydrolysis at 50℃, yet more efficient cellulose hydrolysis at 60℃

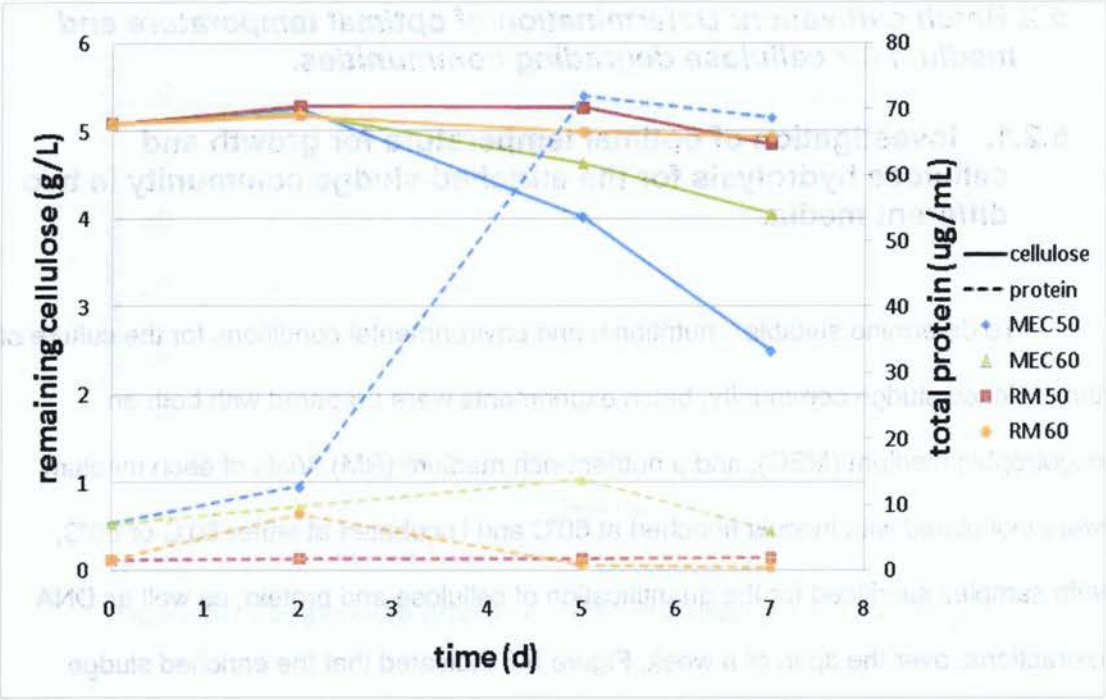

Figure 5-3 Total protein and remaining cellulose concentrations from 60℃ - enriched sludge grown in batch under two temperatures (50°, 60℃), in two media (MEC, RM) containing 5 g/L Avicel and 1 g/L yeast extract.

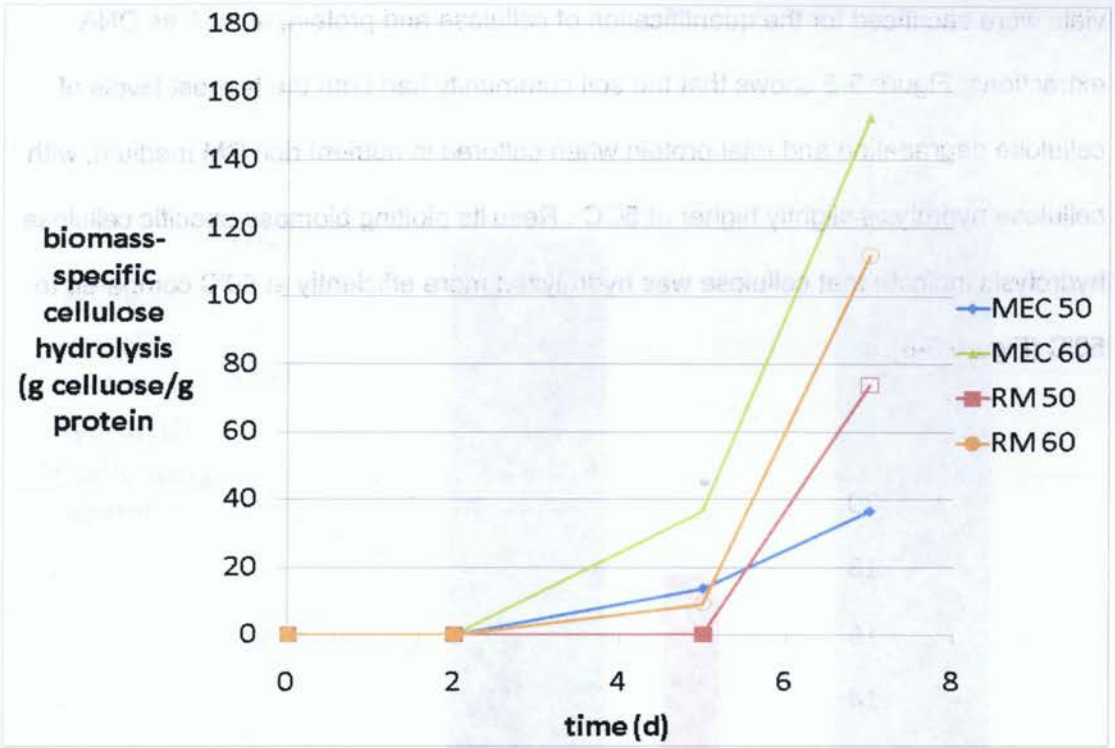

Figure 5-4 Biomass specific cellulose hydrolysis of 60℃-enriched sludge grown in batch at two temperatures (50°, 60℃) in two media (MEC, RM) , containing 5 g/L Avicel and 1 g/L yeast extract.

5.2.2. Investigation of optimal temperature for growth and cellulose hydrolysis for the soil community in two different media.

To determine optimal nutritional and environmental conditions for the culture of the soil community, batch experiments were prepared with both an oligotrophic medium (MEC), and a nutrient-rich medium (RM). Vials of each medium were inoculated with soil and incubated at either 50 or 60℃ . To scale down the number of vials needed for replication, vials were sampled for only one interval, at day four, as protein curves from previous experiments (Figures 5-3) indicate active growth at day 4-5. After four days,

vials were sacrificed for the quantification of cellulose and protein, as well as DNA extractions. Figure 5-5 shows that the soil community had both the highest levels of cellulose degradation and total protein when cultured in nutrient rich RM medium, with cellulose hydrolysis slightly higher at 50℃ . Resu lts plotting biomass-specific cellulose hydrolysis indicate that cellulose was hydrolyzed more efficiently at 60℃ compared to 50℃ (Figure 5-6).

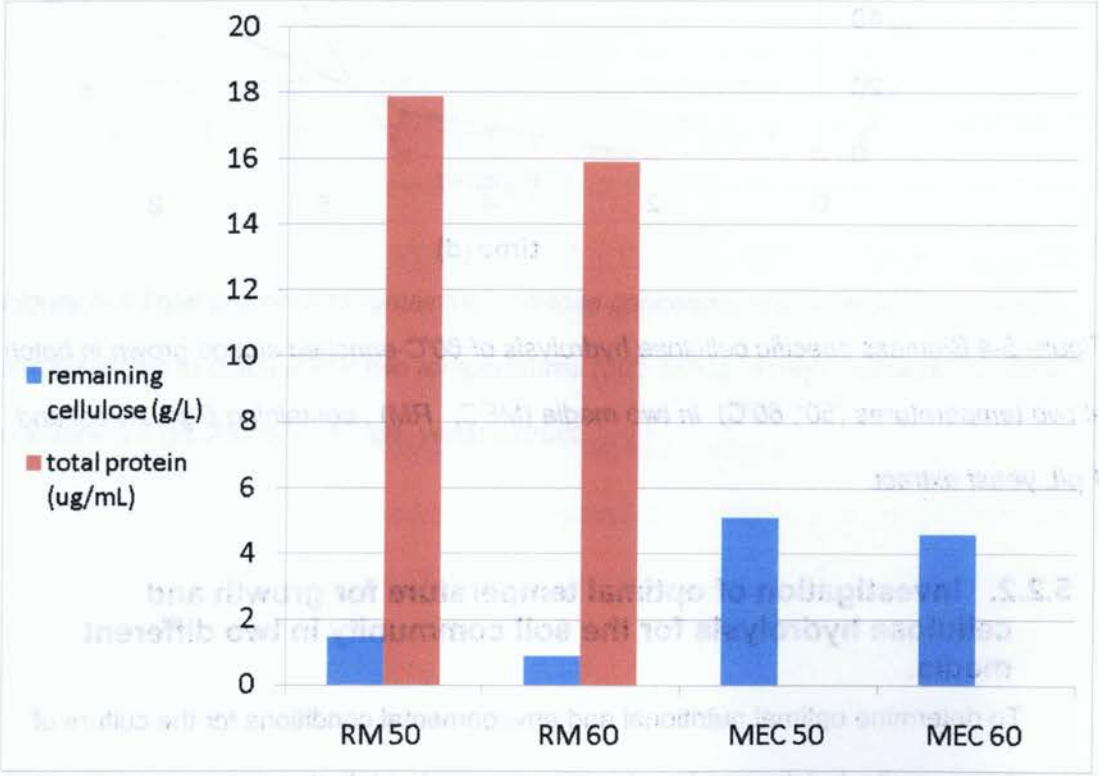

Figure 5-5 Total protein and remaining cellulose concentrations from soil grown in batch under two temperatures (50°, 60℃) on two media (ME C, RM) after 4 days, containing 5 g/L Avicel supplemented with 1 g/L yeast extract.

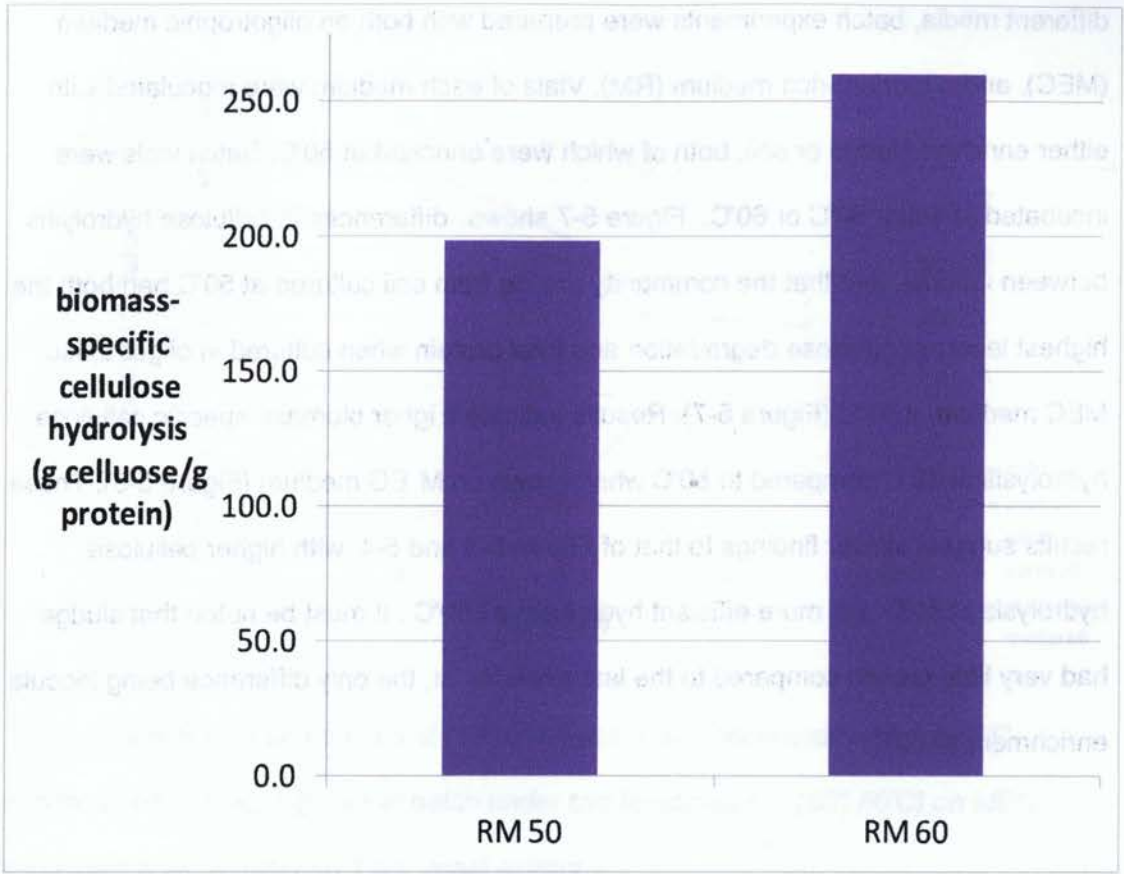

Figure 5-6 Biomass specific cellulose hydrolysis of soil grown in batch at two temperatures (50°, 60℃) and on two growth media(M EC, RM), containing 5 g/L Avicel supplemented with 1 g/L yeast extract.

5.2.3. The effect of different inocula on growth and cellulose hydrolysis using temperature and different growth media as criteria

To evaluate differences in microbial activity between inocula under two temperatures in

different media, batch experiments were prepared with both an oligotrophic medium (MEC), and a nutrient-rich medium (RM). Vials of each medium were inoculated with either enriched sludge or soil, both of which were enriched at 50℃. Batch vials were incubated at either 50℃ or 60℃. Figure 5-7 shows differences in cellulose hydrolysis between inocula, and that the community arising from soil cultured at 50℃ had both the highest levels of cellulose degradation and total protein when cultured in oligotrophic MEC medium at 50℃ (Figure 5-7). Results indicate higher biomass-specific cellulose hydrolysis at 60℃ compared to 50℃ when grown on MEC medium (Figure 5-8). These results suggest similar findings to that of Figure 5-3 and 5-4, with higher cellulose hydrolysis at 50℃, yet more efficient hydrolysis at 60℃. It must be noted that sludge had very little growth compared to the last experiment, the only difference being inocula enrichment at 60℃.

5.2.4. Discussion

Each experiment using enriched cultures for inocula, whether sludge or soil, had higher total protein and cellulose hydrolysis when cultured at 50℃ (Figure 5-3, 5-7), which is supported by Haruta *et al.*(2002) and Kato *et al.*(2004), who found that highest cellulose degradation in a cellulolytic community was achieved at 50℃ [22, 28]. Viljoen *et al.* (1926) found that the fermentation rate of a consortium grown on cellulose was highest at 65℃, These findings may not be contradictory, however. Haruta *et al.* (2002) and Kato *et al.* (2004) discussed the extent of total cellulose degradation, while Viljoen *et al.* (1926) discussed rates of fermentation product formation with respect to time.

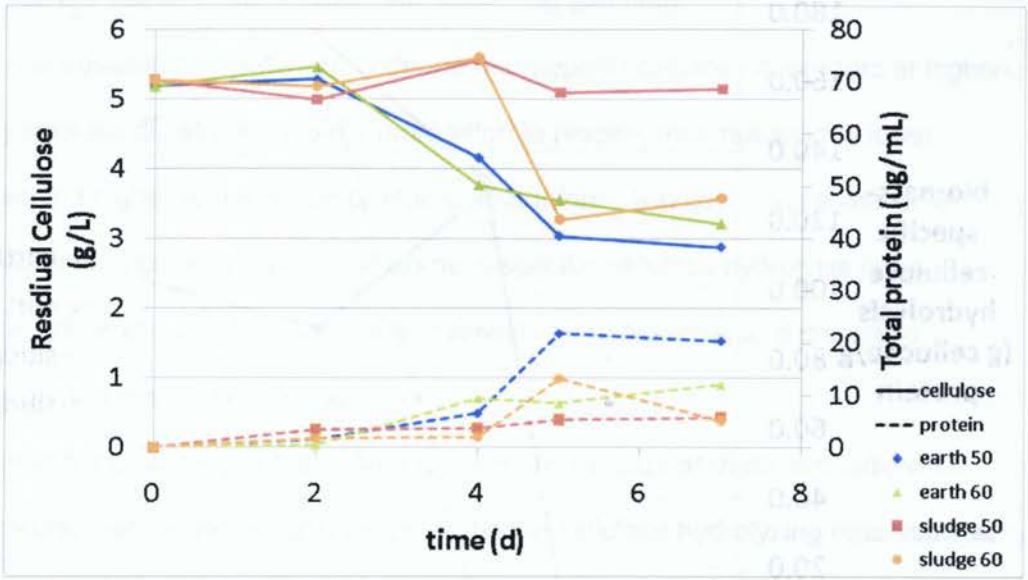

Figure 5-7 Total protein and remaining cellulose concentrations from 60℃ - enriched soil or sludge grown in batch under two temperatures (50°, 60℃) on MEC containing 5 g/L Avicel and 1 g/L yeast extract 1.

While total protein and cellulose hydrolysis differs amongst experiments, there appears to be a trend in biomass-specific cellulose hydrolysis (the amount of cellulose hydrolyzed per gram of total protein). In each case it seems that cultures at 60℃ have higher biomass-specific cellulose hydrolysis than their 50℃ counterparts, suggesting cultures have more efficient hydrolytic rates at the higher temperature (Figures 5-4, 5-6, 5-8).

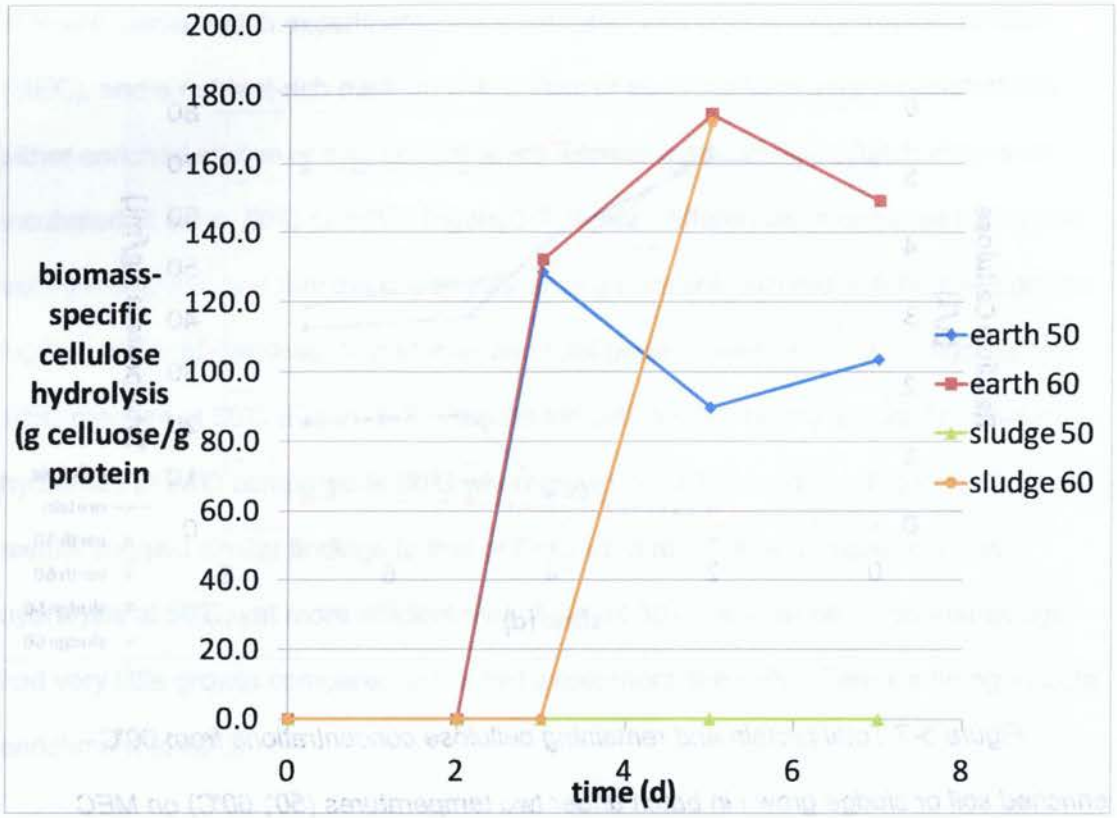

Figure 5-8 Biomass specific cellulose hydrolysis of 60℃-enriched soil or 60℃-enriched sludge grown in batch at two temperatures (50°, 60℃) MEC containing 5 g/L Avicel supplemented with 1 g/L yeast extract.

Lawn trimming piles reached temperatures of 50℃ (F igure 5-1), with evidence of anaerobic cellulose hydrolysis (Figure 5-2). Would an organism optimize its performance for its environment? Why do the most robust cellulolytic organisms such as *C. thermocellum* have higher optimal temperatures? Consideration must be given that the optimal growth conditions of a microbe might not produce the most effective results in terms of microbial cellulose hydrolysis for bioprocessing applications. Perhaps

converting less cellulose into more protein indicates a less harsh environment, where resources are easier to metabolize, with increased fecundity.

It is possible that culturing moderate thermophilic cellulose degraders at higher-than-optimal temperatures requires more effort to respire, thus resulting in lower biomass and higher fermentation products. This information could prove useful to bioprocessing application, as higher biomass-specific cellulose hydrolysis rates increase efficiency, with less substrate assimilated into biomass, and more into respiration and subsequent fermentation.

Another possibility is that different communities arise at these two different temperatures, with a decrease in the proportion of cellulose hydrolyzing organisms at 50℃, resulting in higher protein levels and lower cellulose hydrolysis. Community profiling with DGGE will aid in answering this question. If temperature-dependent community structure affects cellulose hydrolysis, these findings indicate that any mixed culture bioprocessing attempts need will temperature optimization.

Direct soil communities had the highest extent of cellulose degradation on nutrient-rich RM media, with little to no growth on oligotrophic MEC (Figure 5-5). Enriched community batch experiments results were highest when cultured on MEC (Figures 5-3, 5-7). This seems reasonable, as enrichments were done on MEC. Media used for existing work on cellulolytic communities bear close resemblance to the nutrient concentrations of RM media [23, 61] with higher levels of nitrogen available, compared to that of oligotrophic media, which are often used in pure culture studies [19, 29, 53]. The results of this thesis confirm that such a high-nitrogen nutrient-rich media are suitable for the study of cellulolytic consortia cultured from direct inocula.

The media selected for enrichment has an effect on successful sub-culturing, yet the consequences of enrichment temperature are less than clear. Sludge communities enriched at 60℃ had highest protein levels and cel lulose degradation when grown at 50℃ (Figure 5-3), while sludge communities enriche d at 50℃ showed the reverse, with better results at 60℃ (Figure 5-7). It is possibl e that enrichment reduces cellulolytic activity by loss of diversity. Community profiling would shed light on this question. It would interesting to observe activity in sludge without enrichment, yet this poses technical problems, due to the heterogeneity of sludge samples. Perhaps the answer is more complex than a loss of diversity, however. Primary enrichment of sludge in a reactor was done at 60℃. The first batch experimen ts were also at 60℃, followed by highest growth at 50℃(Figure 5-3, 5-4). When the s econdary enrichment temperature was reduced to 50℃ in subsequent experiments, sign ificantly less growth occurred, and this only at 60℃ (Figures 5-7, 5-8). It seems like ly that a change of temperature during serial enrichments reduces culture viability. The consortium cultured from soil was not serially enriched at different temperatures, which might explain its robust hydrolysis rates.

Whether enriched at 50℃ or 60℃, both experiments exhibited the highest amount of cellulose hydrolysis at 50℃. Yet the hig hest cellulose hydrolysis performances in the two enrichment batch experiments arose from different inocula, . What would have happened if soil cultures were included in the first batch experiment (Figures 5-3, 5-4)? Is soil superior to sludge as a source of inocula for robust cellulolytic organisms? Batch experiment #2 (Figure 5-5, 5-6) would suggest such in these isolated incidences, but much repetition would be necessary before any general statements

could be made.

It must be noted that throughout all batch experiments, biomass-specific cellulose hydrolysis was highest consistently at 60℃ (Figures 5-4, 5-6, 5-8), and that cellulose hydrolysis was highest overall when inocula was not enriched (Figure 5-5), suggesting enrichment is responsible for a loss of diversity.

The cumulative results of these experiments suggest that the source of the inoculum has an effect on the microbial hydrolysis of cellulose, and that better results occur without enrichment, on nutrient-rich media such as RM, with increased temperature resulting in increased efficiency in cellulose hydrolysis. Qualitative observations of batch vials (i.e. color change) indicated variability between batch vials, suggesting heterogeneity even within inocula and thus a certain degree of unpredictability. While the conclusions drawn from these findings are valid within these experiments, independent repetitions of these experiments using different inocula from the same source would need to be performed several times if any final conclusions on the general behaviour of communities and different inocula were to be made.

5.3. Continuous culture of cellulolytic communities

5.3.1. Continuous culture of enriched sludge communities at 60℃, HRT 24 h.

A sterile 1-L working volume reactor containing MEC medium with 5 g/L Avicel and 0.1 g/L yeast extract was inoculated with 100 mL enriched sludge prepared via transfer

enrichment in batch reactor. The reactor was stirred at 100 rpm and temperature was maintained at 60℃ . Once pH control was activated by decreasing pH levels (12 days post inoculation), the reactor was switched from batch to continuous mode with a HRT of 24 h. s shown in Figure 5-9, remaining cellulose in the reactor rose quickly to 5 g/L, indicating little to no cellulose hydrolysis was occurring, while protein levels dropped as well . These results were simultaneous with the development of grey coloration and odor change, the grey coloration possibly due to formation of FeS, suggesting the presence of sulfate-reducing bacteria. Community analysis using PCR-DGGE showed a change in community structure over time (Figure 5-10). A batch culture using the same inocula, inoculated on the same day as reactor inoculation and incubated at 60℃ demonstrates similarity to original reactor community profile, in that dominant banding patterns are similar. This batch culture demonstrated cellulolytic activity (confirmed visually) and after a week, no sign of the changes in community profile of the reactor were evident in this culture. This suggests that the common elements of batch and initial reactor community profiles are closely related to microbial cellulose hydrolysis.

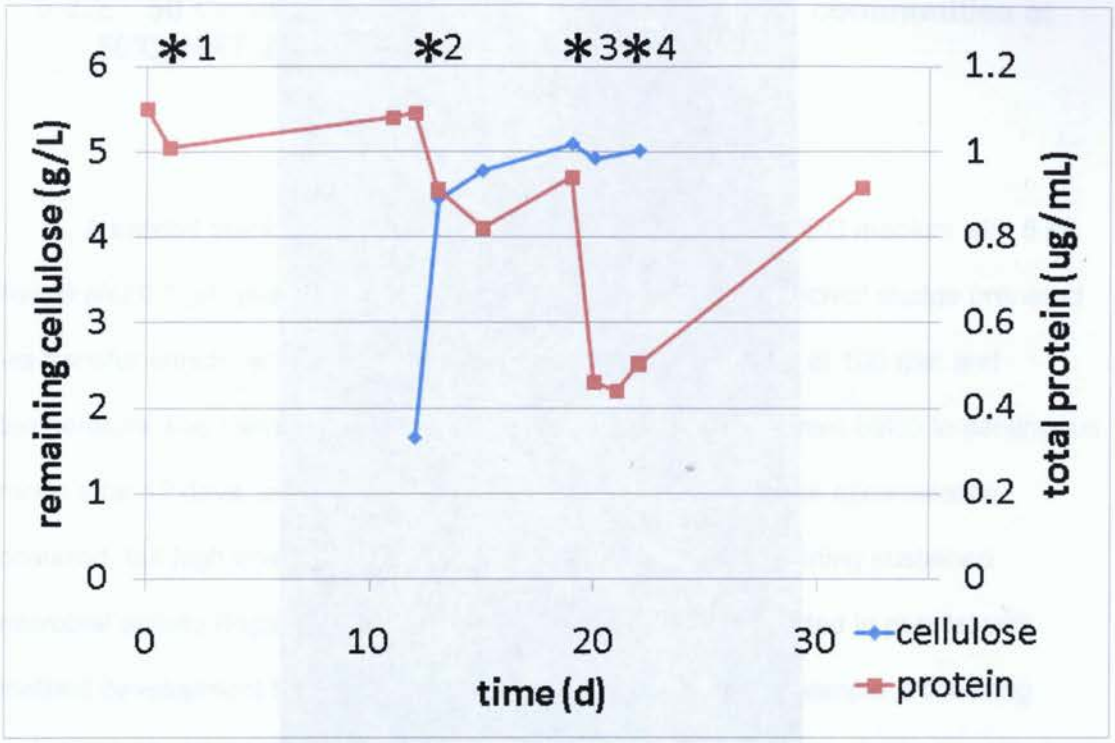

Figure 5-9. Total protein and remaining cellulose concentrations from 60℃ -enriched sludge reactor grown at 60℃ on MEC containing 5 g/ L Avicel supplemented with 0.1 g/L yeast extract, with the system switched from batch to continuous at day 12, HRT 24 hours.

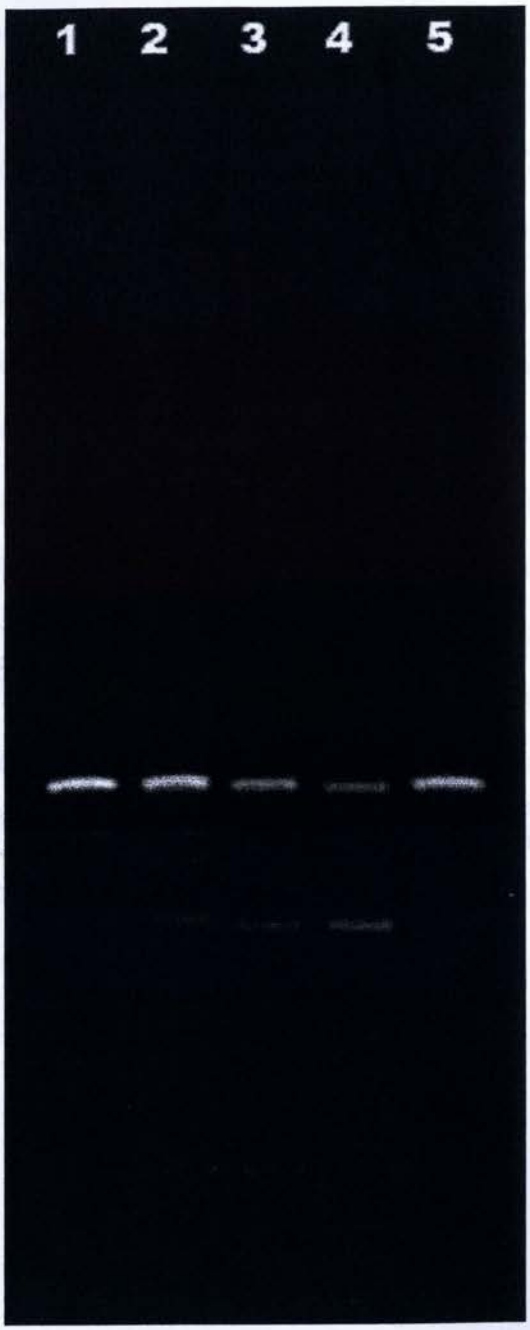

Figure 5-10 DGGE analysis of enriched sludge reactor, continuous at day 12, 60℃ , HRT 24 hours, MEC, 5 g/L Avicel. 1) Profile at inoculation 2) Profile at system transition from batch to continuous, day 12 3) Profile at day 21 4) Profile at day 22 5) Profile for week old batch vial inoculated with same inoculum as the reactor, cultured at 60℃.

5.3.2. 50 Continuous culture of enriched sludge communities at 50℃, HRT 24 h.

A second sterile 1-L working volume reactor containing MEC medium with 5 g/L Avicel and 0.1 g/L yeast extract was inoculated with 100 mL enriched sludge prepared via transfer enrichment in batch reactor. The reactor was stirred at 100 rpm and temperature was maintained at 50℃ . The reactor wa s shifted from batch to continuous mode after 12 days, and run for 100 days. High levels of cellulose accumulation occurred, but high levels of protein were also documented, indicating sustained microbial activity (Figure 5-11). These experiments were conducted in parallel with method development for HPLC analysis. Subsequent delays in sample processing limited real-time knowledge of reactor activity, and measures thus not taken to alleviate cellulose accumulation in a timely fashion.

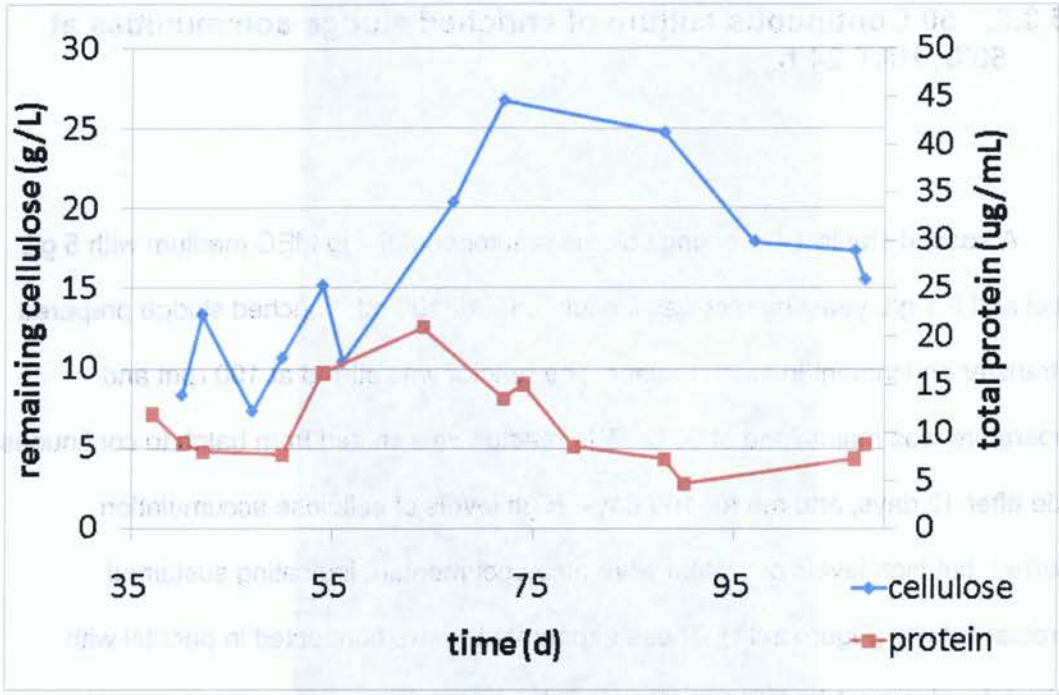

Figure 5-11 Total protein and remaining cellulose concentrations from 60℃ enriched sludge communities grown on MEC containing 5 g/L Avicel supplemented with 0.1 g/L yeast extract at 50℃, HRT 24 h.

5.3.3. Continuous culture of soil communities at 60℃, HRT 24 h.

Due to a lack of robust microbial cellulolytic activity in previous continuous attempts, as indicated by a lack of gas production, pH drop, and color change, fresh soil was used as inoculum for a third sterile one litre working volume reactor containing RM medium with 5 g/L Avicel and 0.1 g yeast extract. The reactor was stirred at 100 rpm and temperature was maintained at 60℃ . These par ameters were decided as a result of batch experiments involving soil cultures (see Figures 5-5, 5-6) The reactor was shifted from batch to continuous mode after 5 days, and run for 100 days (see Figure 5-

12A). As before, high levels of cellulose accumulation occurred during the initial forty days. Therefore, on day 40 two parameters of the reactor were changed. First, stirring was increased from 100 to 250 rpm to improve homogeneity and diminish sedimentation within the reactor. Secondly, to stimulate growth, yeast extract concentration was increased from 0.1 to 0.5 g/L. This resulted in a rapid decrease of remaining cellulose concentration, and a strong increase in yellow coloration, indicating the presence of *C. themocellum*-like microbes. From day 40 onwards, remaining cellulose concentrations decreased (Figure 5-12A)

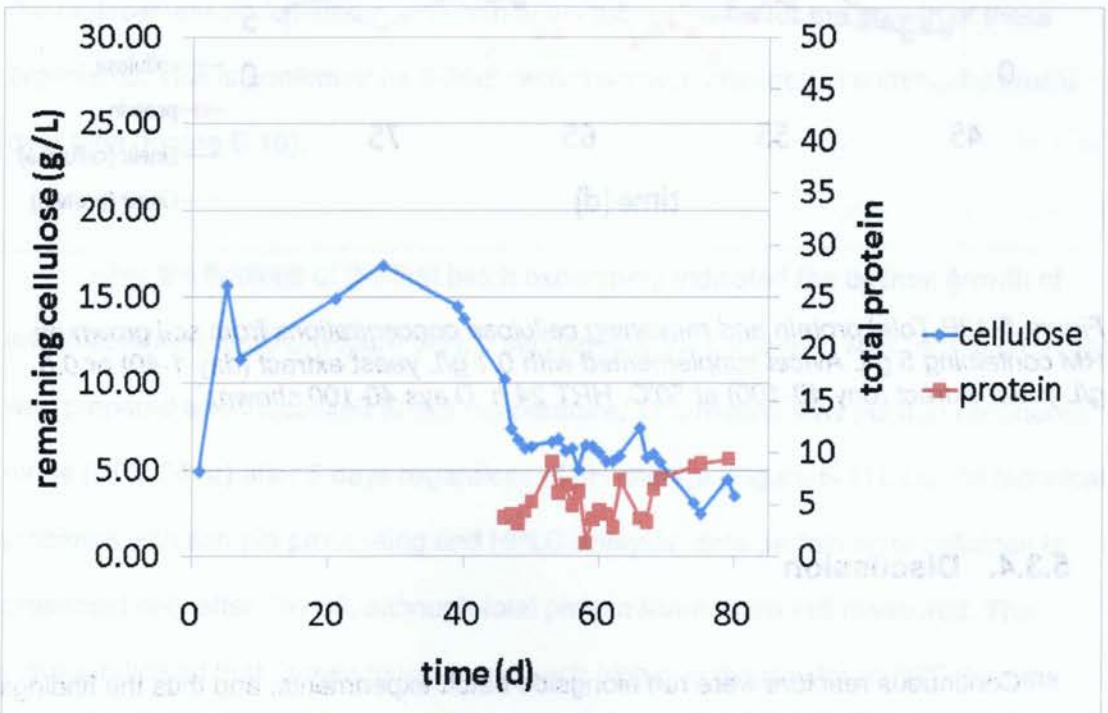

Figure 5-12A Total protein and remaining cellulose concentrations from soil grown on RM containing 5 g/L Avicel supplemented with 0.1 g/L yeast extract (day 1-40) or 0.5 g/L yeast extract (day 40-100) at 50℃, HRT 24 h.

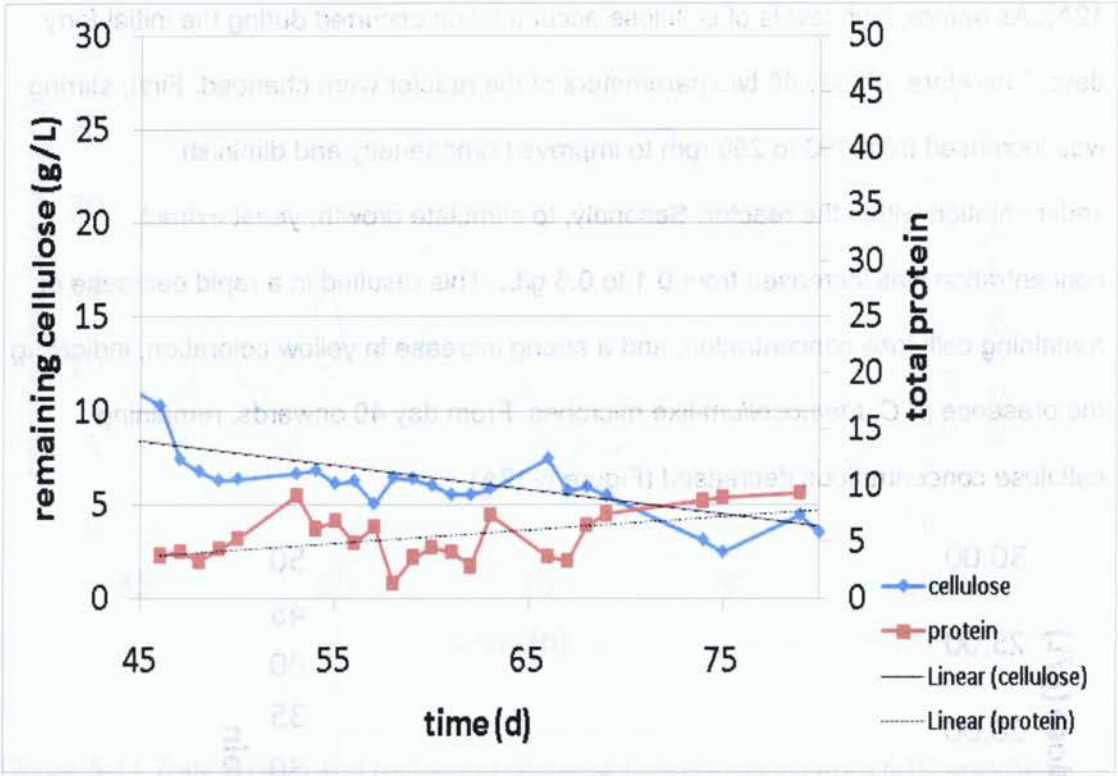

Figure 5-12B Total protein and remaining cellulose concentrations from soil grown on RM containing 5 g/L Avicel supplemented with 0.1 g/L yeast extract (day 1-40) or 0.5 g/L yeast extract (day 40-100) at 50℃, HRT 24 h. D ays 40-100 shown.

5.3.4. Discussion

Continuous reactors were run alongside batch experiments, and thus the findings of the batch experiments influenced the operational parameters of the reactors in an attempt to optimize growth conditions for environmental consortia. The first reactor was run with MEC and 5 g/L Avicel at 60℃ (Figure 5-9). This reactor was inoculated with enriched sludge and incubated until a pH drop indicated microbial activity. Once the

transition from batch to continuous (HRT 24hr) was made, cellulose rapidly rose to levels near 5 g/L, the reactor influent concentration indicating that little to no cellulose hydrolysis was taking place. Subsequent batch experiments indicated that the microorganisms reached their exponential phase of growth approximately near day 4-5 (Figure 5-3, 5-7). The strong concentrations of phosphate buffers in the media masked the drop in pH, and when a pH change was finally detected near day 12, the cellulolytic organisms would have stopped growing. Other pH tolerant organisms could have been allowed to develop in the delay, and when the switch to continuous culture was made, the replacement of depleted media with new media allowed for the growth of these organisms. This is confirmed by DGGE, which showed changes in community profile over time (Figure 5-10).

After the findings of the first batch experiment indicated the optimal growth of enriched sludge at 50℃ (Figure 5-3), a second reac tor containing MEC and 5 g/L Avicel was prepared and inoculated at this temperature. This reactor was put into continuous mode (HRT 24 hr) after 5 days regardless of pH change (Figure 5-11). Due to technical problems with sample processing and HPLC analysis, data pertaining to cellulose is presented only after day 35, although total protein levels were still measured. The results indicated that protein levels were much higher in the reactor at 50℃ than the previous reactor at 60℃, supporting the findings o f the first batch experiments. Reactor outputs were not stable however, with noticeable oscillations in both total protein and remaining cellulose concentrations. Near day 55 the glass drip tube for the feed line into the reactor was replaced for logistic reasons with a prototype drip tube fashioned from a

60 mL plastic syringe, as glass drip tubes were custom-made and not readily available. This allowed for routine replacement of drip tubes, thus reducing chances of feed carboy contamination, which had been problematic. Protein levels dropped and cellulose levels rose in the reactor. This could be explained by possible oxygen permeability of the plastic in the syringe, which restricted the growth of the anaerobic microbes. Again, these results were not acquired until long after the reactor was shut down as the samples were not processed until HPLC methods were reliable.

The operational parameters of the third reactor were set according to the third batch experiment, with fresh soil inoculated into RM media and incubated at 60℃, with the switch from batch to continuous (HRT 24hr) made after 5 days (Figure 5-12A). Initial cellulose concentrations showed a rapid increase in cellulose concentrations. Due to accumulation of cellulose in the reactor, at day 40, stirring speed was increased from 100 RPM to 250 RPM. Cellulose concentrations noticeably decreased and accumulation stopped. This confirmed that 100 RPM was insufficient stirring speed for a homogeneous cellulose suspension, which resulted in accumulation of cellulose. Until this point, all reactors had been run at 100 RPM, which shows that cellulose hydrolysis in all reactors could have been occurring, yet would have been masked by accumulation due to insufficient stirring. A stirring speed of 100 RPM was chosen assuming that environmental cultures are more sensitive to disturbance than microbes accustomed to culture, and minimizing disturbance would benefit their growth. It is worthwhile to note that through-out every experiment, reducing agents were omitted, showing that these anaerobic communities can tolerate low concentrations of oxygen, and in fact generate

6. CONCLUSIONS

The results of these experiments shed light on important factors regarding the culture of cellulolytic organisms. They suggest that both the source of inoculum and enrichment methods have a significant effect on activity of a cellulolytic community, suggesting differences in diversity exist between different environments. Specifically, differences in diversity indicate there is no cellulolytic community ubiquitous throughout all environments; different communities arise from different environments. There can be profound differences between environments (i.e. substrate availability, pH, ambient temperature), and the above work indicates that enrichment conditions effect the growth of consortia. Thus it is logical that each community would be tailored to its environment.

It was also demonstrated that environmental consortia can withstand the vigours of culture, such as rapid stirring rates, as well as tolerating oxygen and maintaining sufficient anaerobic conditions without the aid of added reducing agents. The results also suggest that cellulolytic communities benefit from the addition of supplements such as yeast extract on pure cellulosic substrates. It is especially interesting to note that the efficiency of cellulose hydrolysis is consistently higher at 60℃ compared to 50℃ , due to a shift from production of biomass to production of valuable fermentation products, suggesting improved bioprocessing production at higher temperatures. Due to the variable behaviour of microbial communities, it is recommended that these findings be replicated in the hopes of determining general trends in the behaviour of cellulolytic communities.

Molecular profiling using PCR-DGGE will provide insight into these experiments. For instance, comparison of a community grown at 50℃ and 60℃ would determine

APPENDIX Calibrations and confirmation of accuracy

6.1. Feed Pump output calibration

Samples taken in 20 mL from a Watson-Marlow peristaltic pump modified to hold L/S 14 Masterflex norprene tubing fed from a 20 L continually stirred bottom-spigot feed carboy containing 5 g/L avicel were collected, vacuum filtered on a glass fibre filter and dried (Table A-1). The theoretical amount of Avicel in 100 mL of a 5 g/l concentration is 100 mg. The average output was 102 +/- 3

Table A-1 Pump calibration outputs

Repetition	Avicel (g)
1	0.096
2	0.103
3	0.099
4	0.106
5	0.103
6	0.104
7	0.105
8	0.104
9	0.101
mean	0.102
std. dev.	0.003

6.2. Continuous reactor output calibrations

A sterile continuous reactor fed 5 g/L Avicel was run for two weeks with a 24 hour hydraulic retention time, and 250 RPM stirring. Samples were taken in 20 mL aliquots (theoretical yield 100mg), filtered and dried overnight at 60 °C (Table A-2).

Table A-2 Continuous reactor calibration outputs

Repetition	Avicel (g)
day 1	0.101
day 2	0.106
day 4	0.103
day 6	0.104
day 7	0.098
day 9	0.099
day 13	0.100
day 14	0.100
mean	0.101
std. dev.	0.003

6.3. Quantitative saccharification accuracy tests

100 mg of Avicel was subjected to quantitative saccharification as described above, with subsequent HPLC analysis to confirm accuracy of the procedure (Table A-3).

Table A-3 Confirmation of quantitiative sacharification accuracy

rep #	Quantified cellulose concentration (g)
1	0.106
2	0.105
3	0.098
mean	0.103
std. dev.	0.004

6.4. PCR test

DNA extraction samples taken from different experiments were subject to PCR and ran through and agarose electrophoresis gel to confirm successful amplification. Amplification was successful for DNA extractions, with no amplification in the negative control, and successful amplification in the positive control which consisted of Pseudomonas CT-07 genomic DNA (Figure A-1).

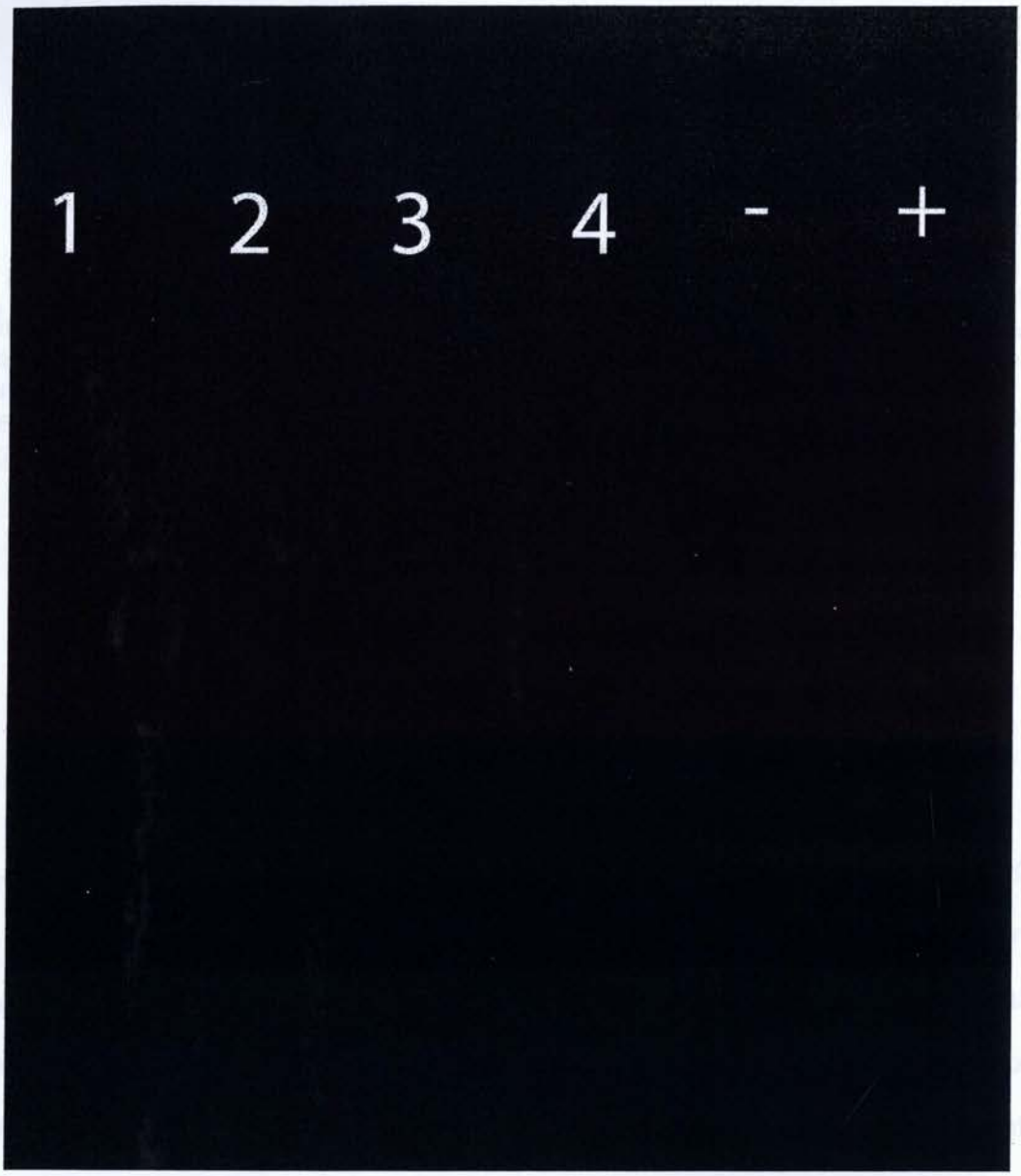

Figure 8-1 PCR test. Lanes 1-4 DNA extraction samples. Lane 5 (-) negative control, Lane 6 (+) positive control, consisting of Pseudomonas CT-07 genomic DNA.

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