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AUTOMATED SOLID-SUBSTRATE CULTIVATION OF THE ANAEROBIC BACTERIUM CLOSTRIDIUM THERMOCELLUM

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AUTOMATED SOLID-SUBSTRATE CULTIVATION OF THE ANAEROBIC BACTERIUM CLOSTRIDIUM THERMOCELLUM

THESIS

A thesis submitted in partial fulfillment of the requirements
For the degree of Master of Science in Biosystems and Agricultural
Engineering in the College of Engineering at the University of Kentucky

BY

Mathew Ruwaya

Director: Dr. Sue E Nokes

Biosystems and Agricultural Engineering

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2016

ABSTRACT OF THESIS

AUTOMATED SOLID-SUBSTRATE CULTIVATION OF THE ANAEROBIC BACTERIUM

CLOSTRIDIUM THERMOCELLUM

The organism *Clostridium thermocellum* grows on cellulosic substrates and produces ethanol, acetate, lactate, formic acid, and CO_2. The organic acids produced alter the growth environment in which the bacteria grows and ultimately inhibit bacterial growth. One method which has been used successfully to maintain the system at acceptable growth conditions is to intermittently flush out the spent media and metabolic products and replace with new fermentation media. Our goal was to design and build an automated system that will automatically flush the spent media from the growing culture and resupply new media without manual intervention. An automated control system was designed and built to control growth parameters. Heated water was pumped through the jacket of each culture vessel and used to regulate the reactor temperature. Sensors for pH and temperature were connected to a central data acquisition system and NI LabVIEW software was used to control each of the components through the signals provided by the data acquisition system. Peristaltic and vacuum pumps were used to supply growth media and acquire reproducible samples for HPLC analysis with limited contamination. In a series of trials, targeted temperature and moisture conditions were achieved and new media was passed through each reactor using a time trigger. More product was produced in manual and automatically flushed cultures than in batch.

KEYWORDS: Automation, Solid State Fermentation (SSF), pH, *Clostridium thermocellum,* LabVIEW

Mathew J. Ruwaya

April 31st 2016

AUTOMATION OF MEDIA REPLACEMENT FOR THE SOLID-SUBSTRATE CULTIVATION OF THE ANAEROBIC THERMOPHILE CLOSTRIDIUM THERMOCELLUM

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Chapter One: Background Information and Subject Review

1.1 Introduction

Over the last 50 years researchers have discovered various microbes which are capable of producing cellulases. These microbes include mycobacterials, actinomycetales, eubacterials and fungi (Cen & Xia, 1999). Many fungi and bacteria produce a cocktail of 'free enzymes' with single or several catalytic units which degrade polysaccharides(Resch et al., 2013). As such, these microbes have been targeted for use in the production of low cost cellulolytic enzymes (endoglucanases, exoglucanases and glucosidases) to be used in production of sustainable fuels from agricultural residues. In an industrial process the fermented waste is protein enriched and as such can be used as bio-fertilizer or animal feed. The production of cellulases and the degradation of agricultural residues can be performed using solid-substrate cultivation (SSC)which is widely considered as a more practical alternative than liquid fermentation (SmF) (Pandey, 2003).

Clostridium thermocellum grows on cellulosic substrates in the absence of free water (Chinn & Mbaneme, 2015). Substrates include agricultural residues and byproducts of forestry or food processing, and energy crops. Intermediate products of SSC using *C.thermocellum* include cellobiose and cellodextrins (Demain, Newcomb, & Wu, 2005). These intermediates can be further converted to ethanol, acetic acid, lactic acid, hydrogen and carbon dioxide by *C. thermocellum.* The organic acids produced alter the growth environment on which the bacteria grow and ultimately inhibit growth. In a study (Dharmagadda, Nokes, Strobel, & Flythe, 2010) it was determined that intermittent flushing of SSC cultures of this bacterium with fresh media reverses

end product inhibition and extends cellulose conversion by removal of accumulated organic acids (Dharmagadda et al., 2010). The same set of experiments demonstrated that decreasing the time between flushes also improved bacterial growth as evidenced by the accumulation of organic nitrogen in SSC as a marker for cell protein. The motivation for the current study was derived from these experiments. We hypothesized that a method to automatically flush the system under pre-set conditions would allow for the determination of optimum growth conditions.

1.2 Solid State Cultivation Overview

SSC has been used for the production of enzymes, bio transformations involving organic solvents and saccharification of complex organic matter (B.V. Kilikian, 2014). Lab scale and industrial applications of SSC claim to achieve high productivities of stable compounds at reduced cost (Hölker, Höfer, & Lenz, 2004) . Implementation of SSC technology is impeded by limitations in heat and mass transfer, system control and bioreactor design as well as product purification techniques.

Advantages	Reference
High end product yield (e.g. *Monascus* pigment and fungal spores are produced in high yields in	(Velmurugan et al., 2011)**(; U. Hölker et al., 2004)**
Lower demands on sterility due to low moisture content (Aw) in SSC	**(Tim Robinson & Nigam, 2003)**
Procedure simple and lower energy requirements	**(Rao, 2010; T. Robinson, Singh, & Nigam, 2001)**
Mixed microbial cultivation	**(W. Bellon-Maurel et al., 2003)**

Table 1: Summary of advantages of solid substrate cultivation (SSC) over liquid (submerged) fermentation (SmF) described in the literature.

Furthermore, pH and moisture gradients developed in SSC reactors influence microbial growth kinetics and present engineering challenges because of the heterogeneity introduced into the reactor(Couto & Sanromán, 2006) .

The agro-industrial residues used in SSC are comprised of structural polysaccharides such as cellulose, hemicellulose, and lignin which are abundant in nature. Efficient hydrolysis of these cellulose poses the greatest challenge due to β-1, 4 glycosidic linkages between glucose molecules which are resistant to hydrolysis. Solid substrates also present physical challenges in SSC applications. Large substrate particles allow for better aeration but reduced surface area. Substrate particles that are too small may lead to reduced aeration. Fine and coarse substrate particles can be mixed to ensure an optimal bed packing density and ultimately achieve better product yields (Nigam & Pandey, 2009).

Physical parameters such as temperature, aeration, pH and mass flow rate are important factors in a SSC process (W. Bellon-Maurel, O. Orliac, & P. Christen, 2003). To maintain the system at optimum growth conditions, physical and chemical parameters must be regulated to promote optimum cell replication. Systems involved in controlling the chemical parameters of a growing microbial cultures measure redox potential, dissolved oxygen or carbon dioxide, carbohydrate (carbon) level or nitrogen levels. There are a limited number of sensors that measure chemical parameters compared to physical properties such as temperature, pressure and flow rate. The low moisture (*A*w) content in SSC aided with poor thermal conductivity of the substrate make heat transfer and temperature more difficult to control (Pandey, 2003).

Another way of following cellular activities is to monitor the level of key intermediates such as nicotinamide adenine dinucleotide (NAD^+) and adenosine triphosphate (ATP). The former is an indicator of catabolic activity and the latter of anabolic activity(Schneider & Gourse, 2004)

Flourimetric techniques have been developed to monitor $NADH_2$ in whole fermentation broths while combined enzyme and flourimetric analysis have been developed for ATP assay. ATP is a useful marker in measuring cell viability because it is present in metabolically active cells and experiences a rapid decline during cell death (necrosis)(Wiegand & Hipler, 2008).

1.3 End product inhibition in metabolic pathways

End product inhibition (or feedback inhibition) is common in many bacterial and fungal species. During end product inhibition the end product of a metabolic pathway limits the activity of an enzyme in a preceding step. Once the activity of an initial step is affected the formation of substrate for later steps is limited. (Yang et al., 2012)and others have confirmed the presence of this form of inhibition in the bacterium *C. thermocellum*. The primary products in *C. thermocellum* fermentation are acetate, ethanol, lactate and formate (Levin, Islam, Cicek, & Sparling, 2006). (Herrero, Gomez, & Roberts, 1985) et al and several other authors have confirmed inhibition of this bacterium by ethanol. In culture this bacterium produces 0.08 to 0.26g ethanol/g glucose equivalent (Rani, Swamy, Sunitha, Haritha, & Seenayya, 1996). *C. thermocellum* has an ethanol tolerance of $5gL^{-1}$ before it is inhibited(Chinn & Mbaneme, 2015). In one study (Williams, Combs, Lynn, & Strobel, 2007) genes for oxidative stress protection, electron transfer, sulfur and nitrogen acquisition and DNA repair mechanisms were identified in responses for other physical (heat) and chemical (furfural) stressors in *C.thermocellum* metabolism. This suggests a unique response for individual stressors/inhibitors which can be tracked to the individual genes for possible manipulation.

Gradual ethanol adaptation may be used to develop new ethanol tolerant strains (Scott, Trotin, & Daugulis, 1997). In a similar experiment *C.thermocellum* strains exhibiting growth in medium containing 0.5% (v/v) ethanol were selected and transferred to media containing 0.25% ethanol

increments successively. Two strains tolerant of 3.5 % and 5.0% (v/v) ethanol were obtained and labelled GA and TA respectively (Rani et al., 1996). Gradual adaptation is also very common with the ethanol producing fungus *S.cerevisiae*. This fungus has been used in several experiments targeted at adapting its growth to increased levels of ethanol(Y. Wang et al., 2015) (Dinh, Nagahisa, Hirasawa, Furusawa, & Shimizu, 2008)(Ismail & Ali, 1971). In one such experiment (Fiedurek, Skowronek, & Gromada, 2011) 8 different strains with high ethanol yields (73.11 to 81.78% of theoretical) in culture was selected from 24 existing strains . One of these strains was selected and used for adaptation in 5-15% ethanol cultures. After multiple subsequent two clones (*S.cerevisiae* ER-A and ER-M) were found to have tolerances for ethanol at 15%. Even though labor intensive and time consuming this technique is economical by allowing the microorganism to self-adjust.

Genetic shuffling has been suggested and used as one of several methods to improve ethanol tolerance in *C.thermocellum*. This mechanism allows for combinations of useful genetic traits into beneficial phenotypes to counter inhibition. Brown et al., 2011 demonstrated that alcohol dehydrogenase genes are involved in improved ethanol tolerance in *C.thermocellum* by resequencing the genome of an ethanol tolerant mutant. When mutant alleles of the alcohol dehydrogenase gene were cloned into a replicating plasmid and inserted into *C.thermocellum* bacteria the new strain showed significant growth in up to 40g/L ethanol. Williams et al., 2007 postulated that moderate concentrations of ethanol lead to loss of integrity of the fluid membrane in *C.thermocellum*. Timmons et al. (Timmons, Knutson, Nokes, Strobel, & Lynn, 2009) suggested a model to demonstrate that ethanol adaptation was a result of changes in the fatty acid rigidity. In this work the authors reported that ethanol adapted strains have more fatty acids with chain lengths > 16:0 and more 16:0 plasmogens in their membrane proteins compared to the parent strain.

Gas stripping can be used a method to reverse end product inhibition. In this method CO_2 or N_2 gas is forcefully applied through the substrate bed. This stream of gas will carry volatile substances and fusel oils into the condensate. This method has been used successfully in very high gravity fermentation (VHG) applications. In a fermentation study (L. Wang, Zhao, Xue, & Bai, 2013) gas stripping was employed in a continuous VHG fermentation using *Saccharomyces cerevisiae* to obtain an ethanol rich condensate (189g/L) while maintaining residual glucose levels under 0.1g/L. In an industrial application this would mean greater ethanol yield and the stripping gas (N_2) can be reused. If applied to SSC moisture changes and harmful effects of N_2 gas to the bacterium (Munsch-Alatossava & Alatossava, 2014) would have to be monitored and corrected.

1.4 Solid State Cultivation Bioreactors

Bioreactor design in SSC is still developing and difficulties in controlling mass transfer, heat addition and removal have not been overcome completely (Hongzhang, Fujian, Zhonghou, & Zuohu, 2002). Bioreactors are constructed to suit the microbiology and biochemistry of particular microorganisms. In each case factors such as the optimal cell concentration, oxygen concentration and heat evolution rate influence the final design. Widely used bioreactors are divided into four general categories based on aeration and agitation strategies. The four categories are tray, packed bed, rotating drum and mixed forcefully aerated bioreactors(Mitchell et al., 2006).

Tray bioreactors are simple in design and have been in use for centuries for the production of traditional foods such as *tempe* and in the production of spore inoculum for the *koji* fermentation (Lotong & Suwanarit, 1983). Tray bioreactors are characterized by a static bed mixed infrequently and air circulated around the bed (Mitchell et al., 2006). In the *koji* process, wooden trays were initially used for soy sauce production by *Aspergillus oryzae (Tim Robinson & Nigam,*

2003). Over time these have been replaced with modern materials such as plastic and aluminum and successfully used for numerous commercial and research applications (Raimbault, 1998)

Packed bed reactors have been used for the production of enzymes, organic acids and secondary metabolites. Packed bed reactors consist of a static bed that is mixed infrequently while O_2 or CO_2 is forcefully passed through the reactor in aerobic and anaerobic SSC respectively. The substrate is supported by a perforated base through which aeration occurs. In a packed bed reactor design the most important phenomena are the axial and radial temperature gradients. Heat transfer is mainly through conduction and at large scale, water jacketing of the bioreactor walls is not efficient since the outer jacket will only influence about 20cm in from the outer wall. Water evaporation from the bed may also occur. Efforts should be made to keep the substrate from drying out. A pressure drop within the bioreactor may also be observed consistent with the way the microorganism fills intermolecular spaces in the substrate.

Roussos (Roussos, Raimbault, Prebois, & Lonsane, 1993) designed and constructed a pilot scale packed-bed reactor with internal heat transfer plates called the 'Zymotis' bioreactor. The bioreactor (4-12kg dry matter) performance was determined in the production of cellulase using *Trichoderma harzianum*. Equal productivities were obtained for small scale experiment using 18g medium capacity column SSC fermenter and 'Zymotis' bioreactor. Uniformity of growth and absence of temperature gradients was also reported when the gaps between the plates was less than 5cm(Roussos et al., 1993).

Rotating drum bioreactors (RBD's) are designed to promote aeration and heat removal by the rotational movement of the horizontal drum. Challenges in scale up, injury to mycelia cells during growth during the early growth phase, aggregation of the media, and growth retardation due to particle attrition, microbial contamination and problems maintaining optimal

temperatures have all been reported with the use of rotating drum bioreactors. The rotation rate or stirring speed will influence the amount of heat generated and gaseous transfer within the reactor as well as of the amount of microorganism shear. Ziffer (D. Mitchell, Berovic, & Krieger, 2000) used RBD's for the commercial production of penicillin using wheat bran. The plant contained 40 RBD's measuring 1.22m in diameter and 11.28m in length. The bran was mixed with nutrient solution externally before being added to the vessel and water was sprayed on the substrate bed surface to aid in temperature control.

The fourth group of bioreactors consists of mixed, forcefully aerated bioreactors(Mitchell et al., 2006). The subgroups of this reactor design consist of continuously mixed bioreactors and intermittently mixed bioreactors. Agitation in a bioreactor limits the occurrence of temperature, moisture and pH gradients. The selection of which reactor type to use in this group is dependent on how well the microorganism resists shear force and strain within a bioreactor.

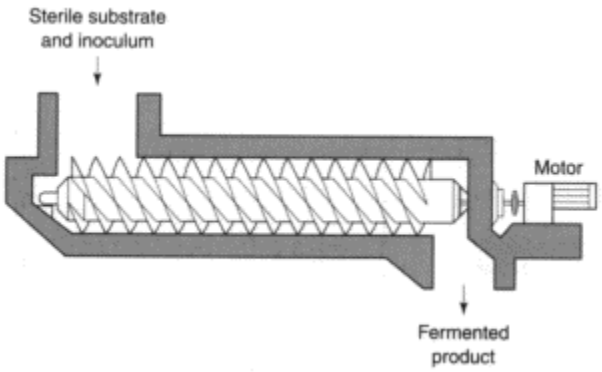

Figure 1: General screw fermentation reactor (van de Lagemaat & Pyle, 2004)

Other variations of bioreactors include tunnel fermenters and continuous screw bioreactors (Fig. 1). J.van de Lagemaat (van de Lagemaat & Pyle, 2004) conducted SSC experiments using *Penicillium grabum* cultivated on a tannin-rich model substrate in the production of tannase (tannin acyl hydrolase). Analysis was performed on samples of

polyurethane foam (PUF) cubes impregnated with liquid media used as substrate. The cubes were analyzed for amounts of initial inoculum, extracellular tannase, gallic and tannic acid. His findings showed the production of significant amounts of the desired product using a screw-type SSC reactor.

1.5 Objectives

The main objective of this experiment was to develop an automated measurement and control bioreactor system to intermittently replace media in order remove metabolic end-products and thereby regulate the growth of the thermophilic anaerobic bacterium *C.thermocellum*. The proposed system must regulate temperature and pH while limiting oxygen concentration for optimal cultivation of the anaerobic bacterium. Using the automated system, the specific goals of the subsequent experiments were:

1. To compare the effectiveness (in terms of the total mass of end products) of periodic anaerobic media replacement (flushing) in low and high solids *C. thermocellum cultures grown on corn stover versus batch cultures.*

2. To compare the effectiveness of an automated flushing mechanism versus manual flushing in regulating the end product formation of the bacterium grown high solids pretreated corn stover.

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Chapter Two: Bioreactor Design and Construction

2.1 Introduction

A well designed and constructed bioreactor system is necessary for controlled, reproducible and statistically relevant studies. Such a system can serve as a model for large scale industrial SSC operation. Our goal was to design and assemble reactors that would maintain the anaerobic and thermophilic conditions for the bacterium *C.thermocellum*. The design addresses limitations on the efficiency of the material transport phenomena in SSC while providing a method to continuously cultivate thermophilic bacteria and reduce inhibition with limited personnel intervention. In particular the following set-points are required.

- The bioreactor system should allow control of the internal environment properties such as oxygen concentration, pH, temperature and shear stress.
- The automated system should be capable of automatically flushing the growing culture and apply new media without manual intervention.
- The system should provide a mechanism to generate and collect useful samples for metabolite analysis using High Performance Liquid Chromatography (HPLC).
- The bioreactor should also allow for anaerobic operation during media supply, sampling and waste removal.
- Stringent safeguards against contamination
- Reproduce the desired environmental control conditions through continuous computer monitoring, analysis and feedback control of the fermentation control environment.

2.1.1 Overview of Operating Variables

C. thermocellum requires carbon, nitrogen, trace elements and micronutrients (e.g. vitamins) for optimal growth. An understanding of fermentation biochemistry is essential for developing a medium with a suitable formulation. The formulation of the fermentation medium greatly affects the yield, rate and product profile. Changes in the concentrations of certain nutrients may result in unexpected results. Some trace elements may have to be avoided – for example, minute amounts of iron reduce yields in citric acid production by *Aspergillus niger* (Vandenberghe, Soccol, Pandey, & Lebeault, 1999).

Bacterial cultivation is influenced by multiple factors that include temperature, pH, nature and composition of the medium, oxygen and carbon dioxide concentration. Cell growth is also affected by the fermentation type (e.g. batch, fed-batch, continuous), feeding with precursors, mixing and/or agitation and shearing within the fermenter. Variations in these factors will affect: the rate of fermentation; the product spectrum and yield; the organoleptic properties of the product (appearance, taste, smell and texture); the generation of toxins; nutritional quality; and other physical and chemical properties.

The largest obstacle in solid state applications is scale up to industrial levels results due to engineering complications in maintenance of pH, temperature and oxygen concentration during cultivation. Fermenters and bioreactors should provide optimal growth conditions for microorganisms to achieve conversion and/or production of biological products (Krahe, 2000) In addition to the biochemical requirements listed above, good practice in fermenter design embodies cardinal rules necessary for a fully function bioreactor system. Such rules include maintenance of sterile conditions in areas directly in contact with the growing culture, absence or removal of dead spaces, clean valves and use of reactors capable of operating under positive

pressure. As such periodic wiping of surfaces and equipment with 70% ethanol is useful as a safety measure to prevent contamination and remove unwanted matter.

2.2 Materials and Methods

2.21 Bioreactor and Equipment Set-Up

The objective of this work was not only to overcome the problems of end-product inhibition but also to develop a mechanism to continuously monitor and control the growth parameters of the bacterium *C thermocellum*. In this regard, the pilot scale bioreactor system incorporated a number of subsystems to interact efficiently and maximize bacterial growth. These subsystems included temperature control, pH regulation, O_2 removal, nutrient delivery and sampling.

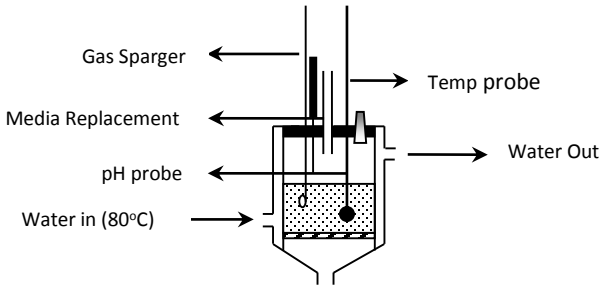


Figure 2: Schematic view of the SSC bioreactor. Reactor configuration and sensor positioning are indicated.

A static glass bioreactor (Kimble, NJ) with a normal capacity of 60g solid substrate was used. Each bioreactor measured 2.5cm ID and 5cm in length with a water jacket surrounding a microbial growth chamber. The reactor bottom consisted of a ceramic filter (20µm pore size) to hold the substrate but allow fluids to pass when a vacuum is applied. Three such bioreactors were connected in series and heated water (~80°C) was steadily pumped through the jacketed reactors by a peristaltic pump (model HV7553-70 Masterflex IL). The observable online variables

were temperature, pH and CO_2 flow rate. Probes were located in the substrate layer allowing a continuous measurement of key growth parameters. Sensor signals for these parameters were amplified and sent to a data acquisition device. The latter converted the analog values to digital forms and transmitted them to the computer.

A rubber stopper was fixed to each reactor to create an airtight seal. Each reactor is inoculated with inoculum (~0.15mg dry cells/g substrate) at the beginning of the experiment. To maintain optimum growth conditions for anaerobic bacteria carbon dioxide gas was passed through each reactor (approximately 40ml/min) to create an oxygen free headspace

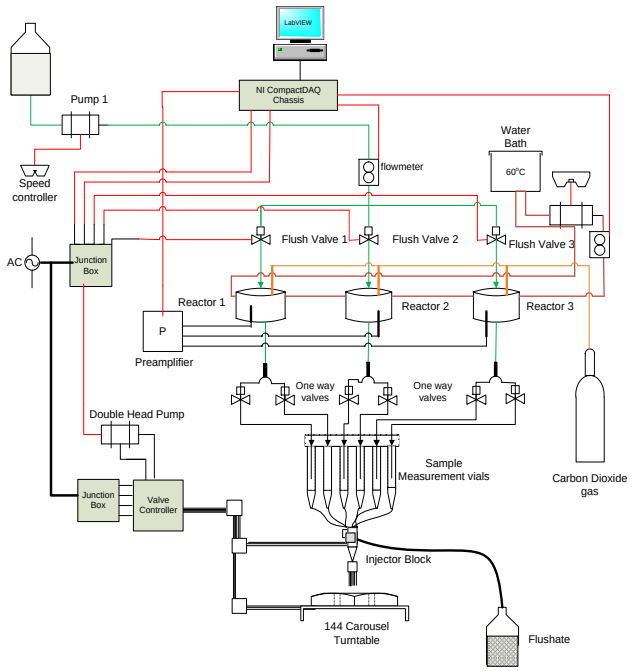


Figure 3: Equipment set-up and computer configuration with respect to the reactor's major functions.

A Tedlar gas sampling bag (GD0707-7000) was connected to the reactor and used as a carbon dioxide reservoir. After gassing the culture the bag would fill up with carbon dioxide

creating a headspace with positive pressure on the substrate mass. Heated water (~80^{o}) was pumped through a water jacket in each reactor. Media replacement for each reactor was controlled by the application software using a time value trigger.

2.2.1.1 Automation and Control

Successful automation requires that the constraints and limitations of the present SSC measurement and control mechanisms be matched with new technology (hardware and software). Some innovation is also required to satisfy the performance criteria for a fully functional bioreactor system. For the fermentation system described in this work, programmable devices (sensors, pumps, valves etc.) were required to provide a sufficient supply of nutrients together with the removal of toxic or inhibitory substances. To produce a high productivity system temperature, pH and oxygen concentration were also closely monitored and controlled. Figure 4 illustrates the mechanism used to intrinsically control media replacement in the solid matrix and monitor bacterial growth parameters in our bioreactor. Media replacement for each reactor was controlled by the application software using a time value trigger.

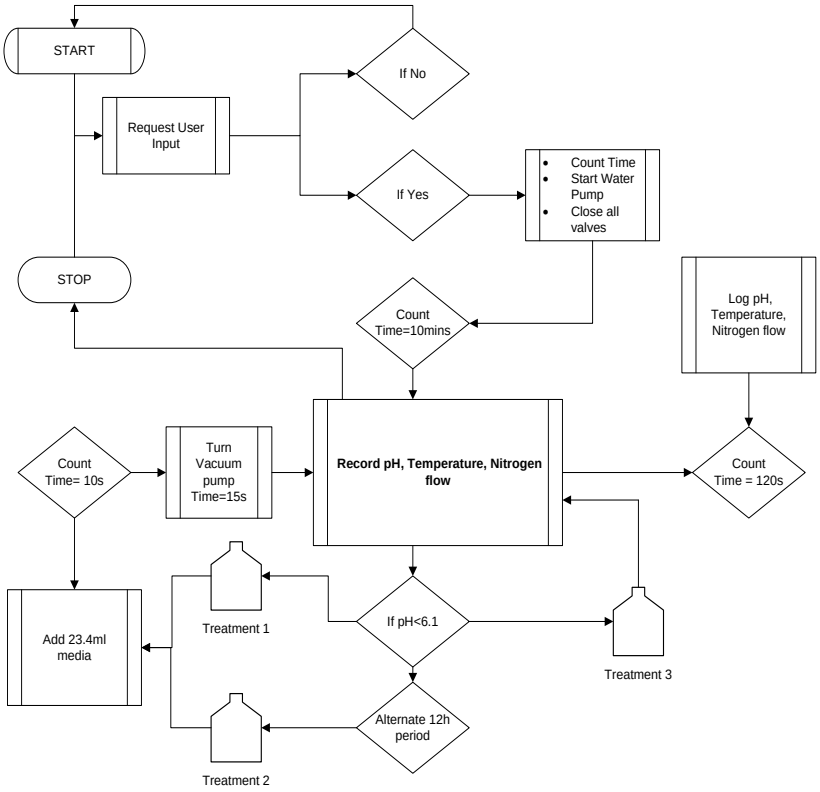


Figure 4: Process flow diagram for automated control and triggered flushing.

2.2.1.2 LabVIEW programming

National Instruments data acquisition hardware and LabVIEW software was used in this experiment. The NI CompactDaq data acquisition and control system was used in our SSC automation. A NI CompactDaq system consists of a chassis, NI C-Series modules and a windows host computer/device connected over USB, Ethernet or 802.11 Wi-Fi. This system provides a reliable and versatile method for wide range of analysis, control and display functions. The NI CompactDaq uses a NI-DAQmx driver which can be interfaced with LabVIEW, C/C++, Visual Basic and Visual Studio.NET programming languages.

A LabVIEW program is referred to as a virtual instrument (VI). The Virtual Instrument consists of 2 windows, the front panel and the block diagram. The front panel consists of the user interface made up of controls and indicators. The block diagram consists of the brain or code for the VI. The graphical nature of LabVIEW mimics data flow in flowcharts. Functions in the block diagram specify the functionality of controls and indicators in the front panel. Data is passed between functions on the block diagram by wires with different wire color representing different data types. The code used in the automation is shown in appendix G.

The NI 9178 cDAQ chassis carries different modules that perform digital and analog input and output functions. The chassis also contains in-built timers specific to each input module connected. Several C-series modules were required for our application Five NI9481 relay module are used to controlling the pump and valve switching mechanism. The NI 9211 module conveyed thermocouple input signals between the sensor and the application software. The NI9205 module conveyed raw voltage signals from the pH amplifier. This module also performed various signal conditioning processes such as amplification, transformation and linearization.

2.22 Temperature Control

C. thermocellum exhibited optimal growth at temperatures between 55 and 60^{o}C. This temperature was achieved by using a jacketed vessel supplied with a continuous stream of heated water. A 5.25 gallon water reservoir (Fisher Scientific, model Isotemp 120, MA) was used to supply the bioreactors with heated water. In this design the water jacket also upset the heat generated in the substrate matrix by microbial activity. Thermocouples are generally used as temperature sensors in SSC and SmF systems. A thermocouple consists of two dissimilar metals welded together to form a junction. Under ideal conditions the electromotive force (emf) produced by a

thermocouple circuit is a result of the *Seebeck effect*. This state that that a voltage potential is produced by temperature differences in a thermocouple circuit. The relationship between emf and junction temperatures in a thermocouple circuit is given by the *Seebeck* coefficient (α_{AB}).

$$\alpha_{AB} = \left[\frac{\partial(emf)}{\partial T}\right]_{open\ circuit}$$ (Webster, 1998). [Equation 1]

In this equation *A* and *B* are two dissimilar metals that make up the thermocouple. In addition to the Seeback effect, the Peltier and Thompson effects also contribute to the total emf of the circuit. These are calculated using the following equations.

Thompson effect

$$Q_\sigma = \sigma I(T_1 - T_2)$$ (Fraden, 2004)

Were: Q_σ *= Heat production per unit volume*

σ *= Thompson coefficient*

T_1*=Temperature at junction 1*

T_2*=Temperature at junction 2*

Peltier effect

$$Q_\pi = \pi_{AB} I$$ (Fraden, 2004)

Were: Q_π *= Peltier heat*

π_{AB} *= Peltier coefficient*

I *= Electrical current*

A type J rugged transition joint thermocouple probe with standard dimensions from Omega.com (model TJ36-ICSS-316U-2 ½, Omega, CT) was chosen for our application. The

selection of a thermocouple for use depends on the temperature range to be measured, uncertainty level required and the environment at which the measurement is made. Temperature ranges are also affected by sheath material and diameter. The thermocouple voltage output as a function of time for our chosen thermocouple is shown in the reference tables attached (Appendix E, *Omega Engineering Inc*).

Heat Transfer in Bioreactor

Energy can be exchanged through the wall of the bioreactor by conduction or convection. Energy can be transferred by convection through the walls of a bioreactor using a water jacket or heating element. Free convection occurs when energy is transported through bulk fluid movement induced by density differences. Heat transfer in solids is mainly by molecular motion and interaction. (D. A. Mitchell, von Meien, & Krieger, 2003) Fourier studied the mathematical theory of heat conduction. Conduction heat transfer through solids is due to molecular vibration. In a jacketed bioreactor the change in energy inside the bioreactor will manifest itself as a change in temperature (D. Mitchell et al., 2000). The constant of proportionality is called the material thermal conductivity (*k*) (Vandenberghe et al., 1999). The heat generated is removed by forced convection. The significance of this heat removal depends on the size of the bioreactor. The surface area to volume ratio is larger in lab scale bioreactors than large scale fermenters. Using a

water jacket is therefore more efficient in small bioreactors.

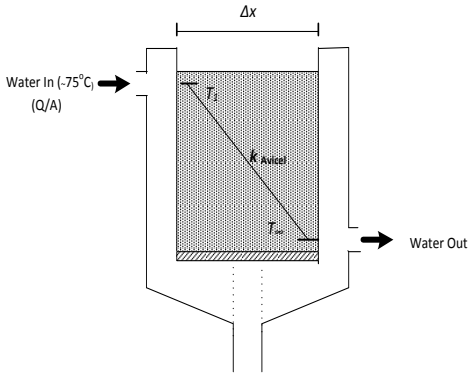


Figure 5: Conduction though jacketed bioreactor and biomass filled area at steady state.

For a reactor space loaded with cornstover:

$$Q/_A = -k\frac{dT}{dx} \quad \text{(Fourier's eqn) (Narasimhan, 1999)}$$

$$= k\frac{(T_1 - T_2)}{\Delta x}$$

$$Where\ k = material\ thermal\ conductivity$$

$$T_1 = Water\ temperature$$

$$T_2 = Substrate\ bed\ temperature$$

The heat transfer problem was solved by assuming the carbon substrate placed in each bioreactor forms a cylindrically shaped block of cornstover. Heat transfer occurs on the cornstover block by forcing heated water (free fluid stream) on the cornstover block. The thermodynamic properties in the table below are found in literature:

Cellulose	Water
Density (ρ) =1500kg/m^3	T_o=26.85°C
Thermal Conductivity (k) = 0.06W/mK	T_∞=75° C
Specific heat capacity (C_ρ) =1440J/kgK	k_{water} = 0.58W/mK
Thermal diffusivity (α) = 2.78x10 m^2/s	v_{water} = 3.78x10^{-7} m^2/s

Table 2: Thermodynamic properties for cellulose and water

The thermal conductivity depends on the material, for example, the various materials found in nature and used in industry have varying thermal conductivities (W/m K) listed in Table 4 (see appendix A1).

Mass Transfer in Bioreactor Design

The bioreactor can be treated as a unit by drawing a boundary around the outside of the bioreactor and considering the exchange of mass to its surroundings (David A. Mitchell, Krieger, & Berovic, 2006). For the bioreactor described in this work a fresh supply of nutrients was added to the bioreactor during a flush sequence and old media is removed from the bioreactor. The headspace carries mass (CO_2, O_2 and water vapor) and energy into and out of the reactor. Oxygen presence in the headspace or substrate matrix is undesirable but may occur. Carbon dioxide was introduced into the bioreactor via a gassing jet positioned in the substrate layer. A one way valve positioned at the top the reactor was used to discharge spent gases. The change in energy in bioreactor can therefore be shown as a change in the phase of water between the liquid and vapor states inside the bioreactor. The outlet gas is likely to have had more water, CO_2 and O_2 hence the gas flow needed to be intermittently applied to prevent complete drying of the substrate layer.

2.23 pH Control

A pH measurement provides a direct method of quantifying the activity of hydrogen ions in a given solution. For the design described in this work a direct measurement of pH was possible because a liquid film was maintained at the surface of the substrate to allow ionic equilibrium. In quantitative pH measurements the flow of hydrogen ions produces a voltage signal which was used to determine the pH value using the Nerst equation(Eggins, 2002). The sensitivity of a pH probe is about 59.2 mV/pH unit at 25°C and varies at 0.003 pH/°C/pH units away from pH 7 (Cheng & Zhu, 2005).

$$pH = -log[H^+]$$

$$E_{pH} = E_{constant} + (2.3 \times \frac{RT}{nF}) \times log[H^+] \qquad \text{Nerst Equation}$$

Where: $E_{pH} =$

$Voltage\ difference\ between\ sensing\ electrode\ and\ reference\ electrode$

$E_{constant} = Voltage\ difference\ in\ a\ solution\ with\ pH = 7V$

$R = Gas\ constant(8.314J/K * mole)$

$T = Temperature\ in\ Kelvin\ (K)$

$n = Number\ of\ Valence\ Electrons\ per\ Mole\ (1\ for\ H^+)$

$F = Faradays\ Constant\ (96500J/V * mole\ e^-)$

$$E_{pH} = E_{constant} - 1.98158 \times \frac{10^{-4} volts}{Kelvin} * T * pH$$

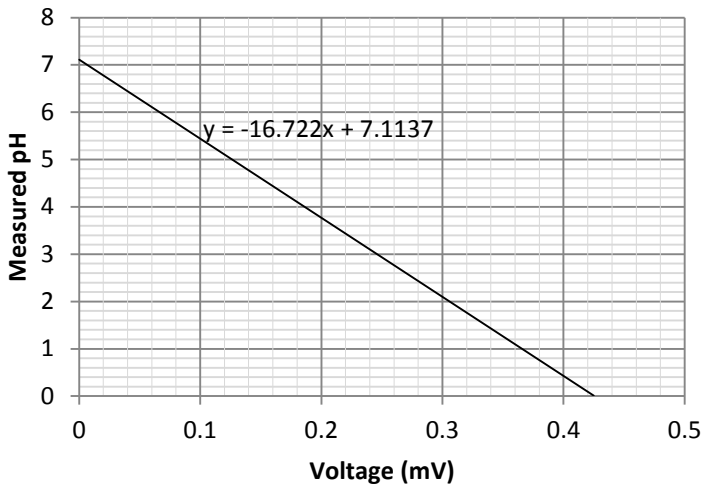


Figure 6: Default pH calibration function

A typical pH circuit is shown appendix F. Due to high impedance mismatch, the pH circuit required a high impedance preamplifier. The unity gain buffer (operational amplifier with a gain of 1) has high input impedance relative to the sensor, and low output impedance relative to the DAQ board. The pH amplifier also strengthened and stabilized the signal making it less susceptible to electrical noise.

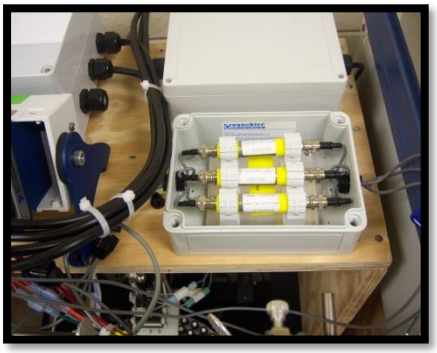


Figure 7: Unity gain amplifier set-up (model PHAMP-1)

2.24 Anaerobic Control

A fermentation bioreactor requires a pure supply of nitrogen gas for optimal microbial growth. To achieve this, an almost pure stream of carbon dioxide gas was passed through a gas filtering mechanism. Carbon dioxide was continuously bubbled through the basal media and at intermittent intervals in the culture vessel. Copper oxidizes slowly in the presence of oxygen, corroding to produce a brown or green tarn. At higher temperatures (above 300°C) the process is much faster and produces mainly black copper oxide. The oxide can be reduced by hydrogen gas, which is a moderately strong reducing agent, producing a shiny, clean copper surface (Hungate 1982). The oxidation and reduction of copper metal was used in our experiment to purify a gas stream needed for a fermentation reactor

Equations for the reactions are:

$$2Cu(s) + O_2(g) \rightarrow 2CuO(s) \qquad (i)$$

$$CuO(s) + H_2(g) \rightarrow Cu(s) + H_2O(g) \qquad (ii)$$

The gas scrubbing mechanism (Fig 9) consisted of a vertical column of coarse copper filings packed in a Pyrex glass column (25mm ID). A cylindrical glass shield was placed around the inner column to minimize heat loss and protect the user. The inner column was heated electrically to about 350°C by a coil of nichrome wire wrapped around the column. The inner column was narrowed at the top and bottom and attached to 7/16 inch rubber tubing.

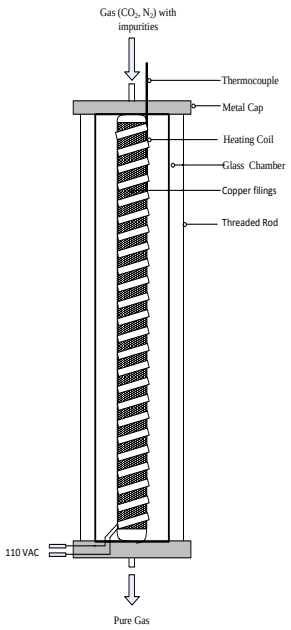


Figure 8: Gas scrubbing mechanism for CO_2 and N_2.

The conditions needed for the forward reaction to occur were achieved using a gas scrubbing mechanism described in the aforementioned section. The desired temperature was reached by adjusting the setting on the MiniTrol controller (model PL312 Glas-Col, IN). When set to a particular setting, the controller proportionally changed the power output between 5% to a 100% (full power output).

To ensure maximum contact of the gas and copper filings the gas stream (CO_2 or N_2) entered the gas column at the top and exited at the bottom. The column temperature was also closely monitored and controlled to promote the chemical reaction. In order to measure the temperature a Type J thermocouple (model TJ36-ICSS-316U-2 ½, Omega, CT) was inserted between the copper column and the insulated heating coil as shown in the diagram.

To obtain a more accurate temperature reading, the junction of the thermocouple was placed such that it touches the surface of the inner glass column between adjacent heating coil strips.

Lead wires were connected to the data acquisition module interfaced with the LabVIEW program.

Figure 9: Minitrol PL312 temperature controller

2.25 Sensor Accuracy

The precision of the sensors used in this work is not discussed in great detail. However to obtain an estimation of the error from each sensor and data acquisition an error analysis was conducted using equation 2.1. The uncertainty due to a series of electrical elements is a function of individual uncertainties of the electrical instruments (Fornasini, 2008). If two or more elements are to be added then the absolute error in the result is the square root of the sum of the absolute errors of the inputs:

$$\varepsilon_{Tot} = \sqrt{\sum \left(\frac{\partial f}{\partial x_i}\right)^2 (\Delta x_i)^2} \qquad (2.1)$$

Where:

Δx_i= propagated uncertainty in x

f= function describing the desired property

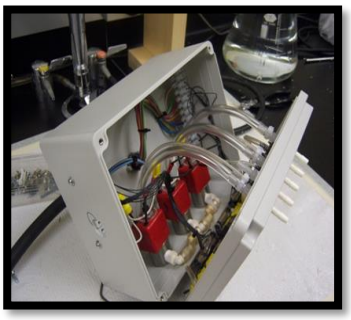
ε_{Tot}=total error

x_i=measured independent quantities of the function f

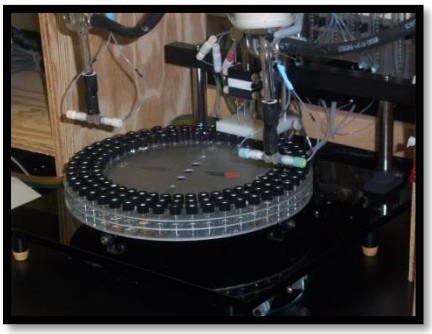
The total error of any measured value has two components, a systematic error which is easily corrected by a proper calibration and random uncertainty which can be enumerated but cannot be removed. For example drifts in reference voltage may cause the thermocouple to provide faulty readings. This usually happens in systems that have their reference voltage supplied form batteries. It is important to maintain a clean junction and the junction on our probe was brushed as such. The connection at the copper - copper and constantan - constantan junctions can accumulate oxides and other forms of contamination. It is also preferable to have thin thermocouple wire. As the diameter of the wire increases, it becomes increasingly difficult to position the thermocouple in the metal casing (shaft). The junction may end up contacting the walls of the thermocouple casing.

A pH electrode is characterized by its zero point and its slope. A two-point calibration is chosen for greater precision. The pH probes (S350CD - HT, Garden Grove, CA) were calibrated using standard pH solutions. Standard buffered solutions at pH 2.0, 4.0 and 7.0 were used to calibrate the standard probe. Voltage signals were obtained at interval 30s until a stabilized reading was observed.

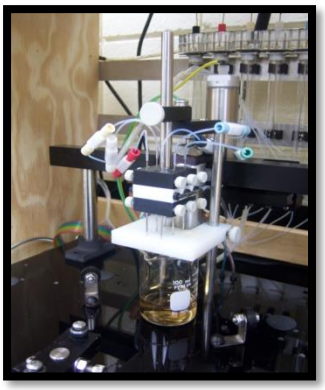
2.26 Sampling



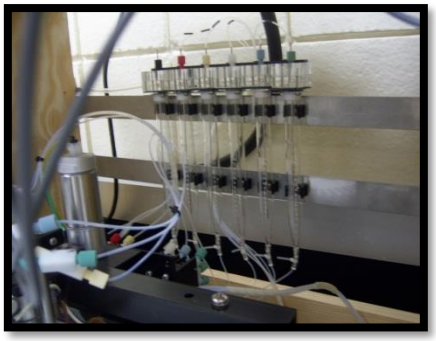
Pneumatic Control Valves



Turntable with 144 vial carousel



Six needle injector block.



Sample collection cylinders

Figure 10: Sampling system components

Two millimeter samples were collected during each flushing sequence. Flushing was initiated by a timed or pH dependent mechanism and the flushed media was collected and processed for HPLC analysis. Automatic sampling components from an autosampler (model 27-010-014, Hanson Research Dissoette, Chatsworth, CA) were integrated into the pH measurement and control system. Each component was connected to a miniature valve system (Fig 7) controlled by the application software. LabVIEW uses a 1-D array (see appendix C) stored in a text file to determine the flushing sequence.

2.3 Results and Discussion

A lab scale model was developed to establish control measures required to generate optimum conditions for *C. thermocellum* growth. The mechanism which consisted of mechanical and electrical components was designed and built to automatically flush a growing culture of *C. thermocellum*. Fresh media was applied to mitigate the inhibition effects of fermentation products (Dharmagadda et al., 2010)) using a time trigger. Electrical valves and flowmeters were set up to control the media supply and dry CO_2 flow to the growing cultures. All bioreactors were packed with dry sodium hydroxide pretreated (section 3.2.2) corn stover an optimal growth temperature of 63°C was maintained.

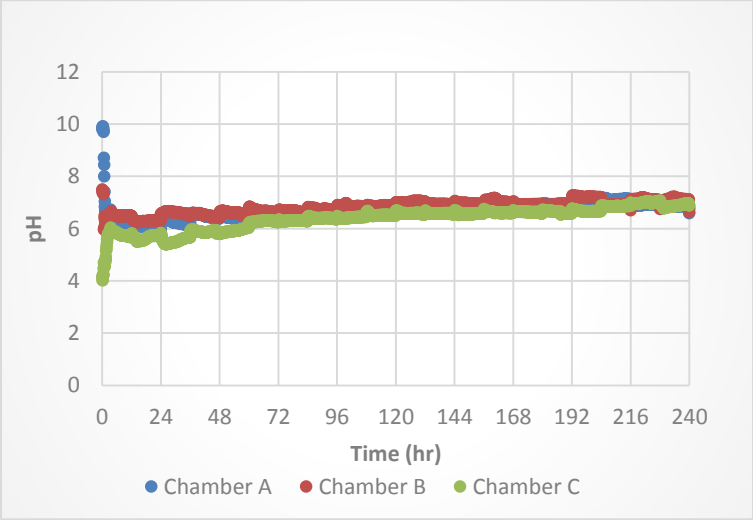


Figure 11: Automatically controlled pH measurement over 10 days.

Fig 11 shows pH measurements over time in automatically controlled pH cultures over 10 days. In SmF pH was maintained at 7.1 using pH probes inserted in the substrate and automatically applying a batch of new media into the substrate bed.

2.3.1 pH (H^+) based flushing

An attempt was made to initiate the flushing schedule in the automated reactor design based on the pH. A pH set point of 7.1 was used and controlled using pH probes inserted in the substrate. When the pH dropped below 4.5 a flushing sequence was triggered and a batch of fresh media was automatically applied into the substrate bed. Inoculation, sampling and flushing procedures for cultures grown using this technique were similar to those of SmF and SSC cultures above. Measurements of pH in SSC were not possible. pH measurements rely on H^+ ions to be evenly distributed within an aqueous medium. In SSC such an aqueous environment is not available due to the low moisture content. As such our pH measurements were inconsistent and changed sporadically after each measurement.

2.4 References

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Chapter Three: SSC and SmF cultivation in automated versus non-automated reactors

The effect of periodic media replacement on end-product formation in high and low solids *C. thermocellum* cultures grown on corn stover.

3.1 Introduction

Several studies postulate that a sufficient supply of nutrients, the removal of toxic or inhibitory substances together with a steady carbon substrate is crucial to control a constant microbial growth environment for long term culture. However, bacteria are capable of adjusting their metabolic functions according to their process inputs resulting in changes to the products or their product ratios. A kinetic model to determine effects of a change in the substrate or a variable associated with it can be described by a differential equation with the parameters of the equation taking into account the effect on growth of key state variables such as the temperature, pressure and water activity of the substrate bed (D.A. Mitchell; N.Krieger; M. Berovic., 2006). Using a model substrate for a carbon source reduces the complexity of hydrolyzing a potential feedstock material such as corn stover or miscanthus but favors higher yields of organic solvents and sugars. (Dharmagadda , Nokes, Strobel, & Flythe, 2010) used Avicel as a model substrate in an experiment to determine the effect of flushing *C.thermocellum* cultures to reverse product inhibition.

Microcrystalline cellulose is composed of glucose units connected by β-1-4 glycosidic linkages. Avicel PH101 is a microcrystalline cellulose powder (50 µm particle size) prepared by treating alpha cellulose (type I_β) with mineral acids (Thoorens, Krier, Leclercq, Carlin, & Evrard, 2014). In comparison lignocellulosic biomass consists of amounts of hemicellulose (20-40%), cellulose (30-50%) and lignin (15-25%). Our substrate of choice (cornstover) is a mixture of stalks, leaves, husks

and cobs of the corn plant remaining after harvesting the corn kernels. As a result corn stover is composed of variable amounts of cellulose, hemicellulose and lignin (Weiss, Farmer, & Schell, 2010). Hemicellulose connects lignin and cellulose fibers. It is composed of galactose, glucose mannose, xylose and arabinose(Freese, Schmidt, & Fischer, 2005). Lignin holds in place cellulose and hemicellulose giving the plant rigidity and impermeability. Lignin is made up of numerous phenolic groups which cannot be utilized in fermentation. Other compounds found in lignocellulosic feedstocks include salts, minerals, phenolics and fatty acids (Jönsson et al., 2013).

Compared to fermentation experiments using Avicel, reactions involving biomass feedstocks are subjected to a pretreatment step since lignin in lignocelluloses prevents access of cellulose hydrolyzing agents. An effective pretreatment method will deconstruct the 3-dimensional structure of lignin while breaking down cellulose and hemicellulose. Specifically, pretreatment of cellulosic material will reduce the amount of intact lignin, reduce cellulose crystallinity, and increase the surface area of exposed cellulose so that cellulases have more binding sites for the hydrolyzing enzymes (Hatakka, 1983)(Dias et al., 2010).

Acid and base pretreatments provide a low cost, shorter reaction time and less inhibition from the substrate (Akinosho, Yee, Close, & Ragauskas, 2014a) . However microbial cultivation using these methods present significant challenges such as the need for adequately designed reactors to meet acid or base solutions, elevated temperature or pressure requirements. Furthermore in SSF flushing and process variables can be difficult to control due to the non-uniform substrate resulting in the formation of gradients within the substrate matrix. It is therefore important to perform a separate study on the effect of flushing cultures with pretreated biomass feedstocks (solid state and liquid cultures) as the carbon substrate in a static vessel. The work below describes a method to develop and compare the effectiveness of an automated flushing mechanism versus

manual flushing in regulating the end product formation of the bacterium grown high solids pretreated corn stover.

3.2 Materials and Methods

3.2.1 Experimental Design

Six different treatments were established to determine the effects of flushing, moisture levels and automation in bacterial cultures.

I. Low Solids (6% solids) with flushing
II. Low Solids (6% solids) without flushing
III. High Solids (75% solids) with flushing
IV. High Solids (75% solids) without flushing
V. Automated Flushing – Low Solids (6%)
VI. Automated Flushing – High Solids (75%)

Apart from the variables (i.e. flushing, solids content and automation) under investigation all parameters were kept constant including temperature, flushing schedules, media composition and gas used to regenerate anaerobic conditions.

3.2.1.1 Treatments

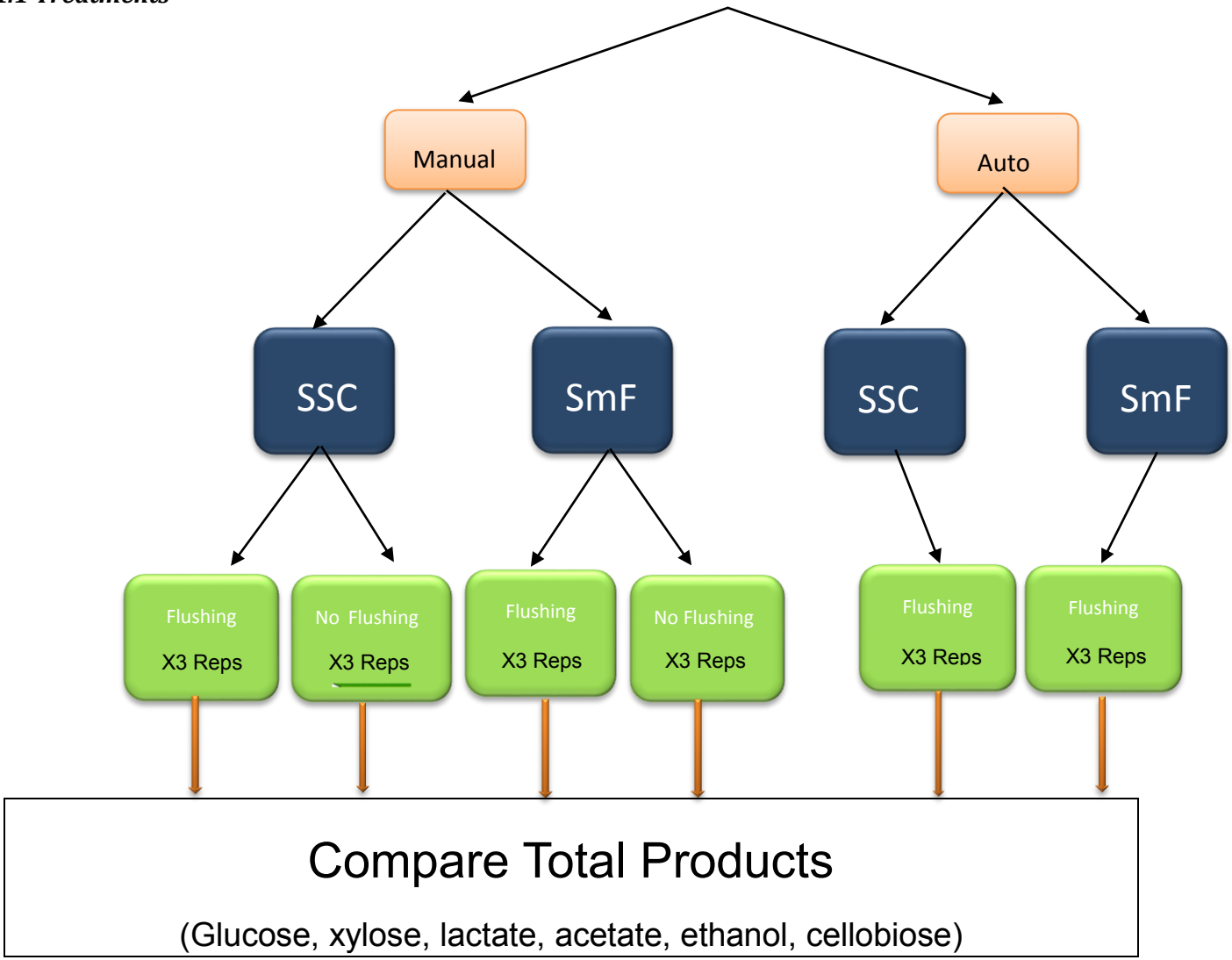

Figure 12: Experimental design schematic demonstrating the experiments conducted to compare metabolic end product concentrations produced in manual and automatic flushing in both solid substrate and liquid cultures.

3.2.2 Substrate Pretreatment

In general, alkali treatments have less severe operating conditions than dilute or concentrated acid pretreatments (Brodeur et al., 2011) which require high temperatures and longer retention times. Sodium hydroxide is widely used in the paper and pulp industry and was chosen for the pretreatment of our feedstock. The loading (0.1 gram alkali/ gram biomass) was achieved by adding 392grams of NaOH, 4.23kg corn stover (7.5% moisture content wet basis) and 75L of deionized water to a 25 gallon polypropylene container (Fig 14). Substrate digestibility was increased by continuous recirculation of solubilized NaOH for 48 hours. A liquid pump was used for recirculation at ambient conditions (waste heat from the pump raised the temperature from 25 ^{o}C to approximately 28^{o}C). To rinse the biomass, the NaOH solution was drained, the vessel was filled with tap water (~75L) and recirculated for 1 hour. The rinse process was repeated three times until the solids reached a neutral pH. Drying was achieved by placing the solids in a temperature controlled chamber set at 30^{o}C and low humidity (final moisture was ~6.8%). The solids were then sealed in plastic bags to store for use in our fermentation experiments.

Figure 13: Reactor for feedstock pretreatment

3.2.3 Media

A mixed solution of nutrients is critical for the formation of a thermophile medium (T-media) required for maintaining a constant and defined growth environment. The basal media (per liter) used in all of our SSC and SmF was prepared as follows:~ 1530mg Na_2HPO_4 ,1500mg KH_2PO_4, 500mg NH_4Cl_2,500mg $(NH_4)_2SO_4$, 90mg $MgCl_2 \cdot 6H_20$,30mg $CaCl_2$, 400mg yeast extract, 10ml vitamins, 5ml modified metals, 500mg cysteine hydrochloride, 1ml rezasurin and 4000mg sodium carbonate. The media was adjusted to pH 6.7 with NaOH and sterilized by autoclaving (20 min, 121^oC at 21psi). The media was maintained under a CO_2 atmosphere. The vitamin solution contained (per liter 100mg pyradoxamine 2HCl, 200mg riboflavin, 200mg thiamine HCl , 200mg nicotinamide, 200mg CaD pantotheinate, 100mg lipoic acid, 10mg p-aminobezoic acid, 5mg folic acid, 5mg biotin, 5mg cobalamin (Co B_{12}), 100mg pyridoxal HCl, and 100mg pyridoxine. The modified metal solution contained (per liter): 500mg Na_4EDTA, 200 mg $FeSO_4.7H_2O$, 10mg $ZnSO_4 \cdot 7H_20$, 200mg $MnCl_2.4H_2O$, 20mg H_3BO_3, 20mg $CoCl.6H_2O$, 1mg $CuCl_2.2H_20$, 2mg $NiCl_2.6H_2O$, 3mg $Na_2MoO_4.2H_2O$, 10mg $Na_2WO_4.2H_2O$, 1 mg Na_2SeO_3.The media solution was autoclaved at 120^oC and 20 psi for 20 minutes. One hundred millilitres of sodium bicarbonate (4g/50ml) buffer is added after cooling using CO_2 gas as a blanket to keep maintain anaerobic conditions in the culture. In the automated system the T-media reservoir (2L) was kept on top of a hot plate (50^oC) to avoid contamination and maintain sterility.

3.2.4 Organism

Clostridium thermocellum ATCC 27405 was a gift from the lab of Dr Michael Flythe (USDA, FAPRU). The original culture was obtained from the American Type Culture Collection (Rockville, MD) and grown in basal media before redistribution as glycerol stocks. A bacterial culture from the stock

stored at -80^{o}C was grown for 24hours in Balch tubes (pH 6.7) at 63^{o}C containing 10ml basal medium supplemented with 0.5g (1x 6cm) filter paper (Whatman #1). This initial culture was used to inoculate 54ml of filter paper containing media. After 48h of growth, this secondary culture is used as standard inoculum stock for use in solid substrate cultivation (SSC) and submerged fermentation experiments (SmF).

Inoculum viability was checked by measurement of dehydrogenase activity within the bacterium. This measurement appraises the quality of the inoculum (Turner, Sandine, Elliker, & Day, 1963) (Roussos et al., 1997). The agents used for this procedure are generally tetrazolium salts such as 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) or tetrazolium red (2, 3, 5-triphenyltetrazolium chloride). When reduced these salts give a colored formazan crystal. In this experiment 1% (w/v) of 10% 1, 3, 5 –triphenyltetrazolium chloride was added to T-media. When respiration occurs (viable cells respiring) a color change resulting from a redox reaction gives a qualitative indicator for bacterial growth. The media broth also consisted of a basal medium molten agar substitute (Gelrite) in Petri dishes which was inoculated with 0.1 ml aliquots of newly prepared standard inoculum in an anaerobic chamber. Gelrite gellan gum is a naturally derived polysaccharide used as agar substitute for bacterial cultures requiring high incubation temperatures (Shungu et al., 1983)(Wery, Cambon-Bonavita, Lesongeur, & Barbier, 2002). Compared to agar Gelrite has better optical quality and less than half the amount of gellan gum as agar is required to reach the equivalent gel strength. Furthermore it has been shown that Gelrite may yield higher viable cell recoveries than similar media solidified with agar (Lin & Casida, 1984; Shungu et al., 1983).In our experiment plates were brought out for analysis and photography under visible light at 12 hour intervals for 2 days

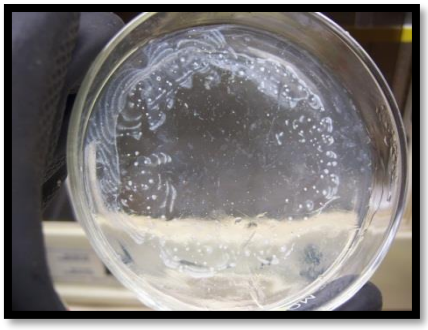

Figure 14: *C thermocellum 27405* culture grown on a solid Gelrite matrix

Figure 15: Discoloration of tetrazolium red using *C.thermocellum after 48hrs*

Figure 16 shows red discoloration of the tetrazolium dye after 48hrs of growth. However the widespread discoloration on the entire gel matrix suggests abiotic reduction from non-biological reductants. These reductants which may be present in growth media have been shown to reduce tetrazolium salts depending on pH, incubation time and reductant concentration(Bhupathiraju, Hernandez, Landfear, & Alvarez-Cohen, 1999). Among many other reagents cysteine–HCL present in our complex thermophile media is a likely culprit for abiotic reduction in this test.

3.2.5 Reactor

SSC was carried out in chromatography polypropylene reservoirs attached to a borosilicate glass barrel. (Kimble-Chase 420401-2510). Each column measured 10 cm long with an internal cross

sectional area of 2.5cm^3 and a total volume capacity of 49ml. The biomass in each column was supported by a High Density Polyethylene (HDPE) bed with 20 micron porosity. Each reactor was sterilized with bleach (20%) and rinsed with deionized water prior to loading with feedstock.

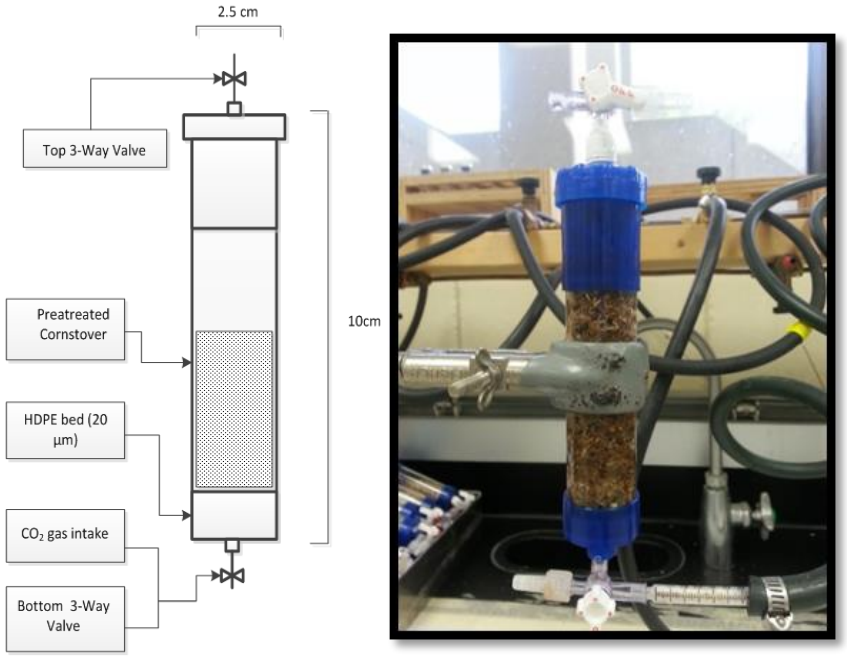

Figure 16: Polypropylene reactor for manual SSC and SmF cultivation

3.3 Experimental Set-Up

In SSC and SMF cultivation 3g or 6g of NaOH pretreated corn stover was added to each reactor respectively. In SSC and SmF 20.7 and 46.2 ml of inoculum were added to each reactor respectively. Carbon dioxide was passed through a cotton plug and flushed through the bottom of each reactor for 30 minutes to regenerate anaerobic conditions and distribute the inoculum around the biomass. Flushing was achieved by connecting a luer lock syringe fitted with neoprene tubing (gassing jet) from a gassing manifold to the bottom of the column After flushing with

carbon dioxide from the bottom of the reactor the gassing jacket was connected to the top valve and used to 'force' out all the liquid from the previously added inoculum. Fresh T media (20.7ml in SSC and 46.2ml in SmF) was added through the top to maintain the desired moisture for SmF (6% solids) and SSC cultivation (75% $_{wb}$ moisture content). A one milliliter sample was collected (day 0) without replacement and stored for HPLC analysis.

After adding fresh media, a stream of CO_2 gas was passed from the bottom of each reactor for 30 minutes to create an oxygen free headspace. Columns were then placed in an incubator set at 63^{o}Cfor 72 hours without shaking

Figure 17: Flushing columns loaded with cornstover

3.3.1 Flushing and sampling protocol

Flushing was achieved by forcing out spent media while draining a small liquid sample before adding fresh media. To add fresh T media (20.7ml in SSC and 46.2ml in SmF) was added through the top to maintain the desired moisture for SmF (6% solids) and SSC cultivation (75% $_{wb}$ moisture content).

To ensure anaerobic conditions were maintained columns were gassed with CO_2 for 30 minutes after each flushing procedure. Flushed SSC cultures were sampled manually through a release valve at the bottom of each column. Reactor flushing followed by sampling was conducted at 0hrs, 24hrs, 48 and 72hrs. Sampling was achieved by opening the outlet valve on each bottom of the reactor and collecting a 1.5ml sample from each treatment/control replicate. Samples were centrifuged (11,655g for 15minutes) and the supernatant collected and stored in microcentrifuge tubes at -20^{o}C.

3.3.2 Analysis

Identification of fermentation is generally accomplished through comparison of retention times and spectra to reference standards. Calibration standards were prepared from stock solutions of 20g/L cellobiose, 40g/L glucose, xylose 20g/L, 100mM lactic acid, 100mM acetic acid and 100mM ethanol. A 5 point calibration curve was developed from the desired concertation of each standard. Mixed standards were made by adding a volume of each stock solution corresponding to the concentration required in each working standard. Sterile apparatus and new HPLC vials (MicroSolv 9502S-3WAP) were used for standards and sample preparation. Stored samples were thawed at room temperature and centrifuged for 15 min at 11,655g. One milliliter of the particulate free supernatant was collected in HPLC vials and placed on the auto-sampler tray.

HPLC was performed with a Dionex Ultimate 3000 LC System comprising of a WPS-3000 auto-sampler and an ISO-3100SD Isocratic analytical Pump. Analysis was carried out using a Aminex® HPX-87H column (300mm x 7.8mm, 9µm particle size, 8% cross linkage, Hercules, CA) coupled to a micro guard cation H refill cartridge (3.0 cm x 4.6 mm, Bio-Rad, Hercules, CA) and a micro guard de-ashing refill cartridge (3.0 cm x 4.6mm, Hercules, CA). The mobile phase consisted of eluent A

with 5mM H_2SO_4 and eluent B with deionized water (Nanopure Analytical Ultrapure Water System, model D11901). The chromatographic conditions for the 35minute run were a flow rate of 0.4 ml per min, the temperature at 50 ^{O}C and the injection volume at 20µL. For quantification, the carbohydrate analytical column was attached to a refractive index detector (Shodex model number RI-101). The entire LC system was controlled and acquired data through Chromeleon 7.1 software (Thermo Scientific ™ Dionex ™ Sunnyvale, CA).

3.3.3 Statistics

The data collected were analyzed as a full factorial design (2x2x2 factorial). In all submerged fermentation and solid substrate cultivation experiments, three replicated (reactors) were used for each treatment. Cultivation method was regarded as a main effect in the ANOVA. PROC ANOVA of SAS (version 9.3) was used to determine if there were any statistically significant differences between treatments. If differences existed, Tukey's range test (Appendix B) was performed to determine aggregate differences among the treatments.

3.4 Results

Treatments were evaluated by comparing the concentration of fermentation products produced by each treatment at 24hr intervals. The data below show total concentration of metabolic products for batch cultivations the concentration of metabolic products produced since the last flushing procedure for flushed cultivations. Total products (g) were calculated by multiplying individual product concentrations by their molar mass and summing with other products. Cultivations were conducted for three days.

3.4.1 End Products from Liquid fermentation of Corn Stover by C. thermocellum; Manual Flushing vs Batch Fermentation

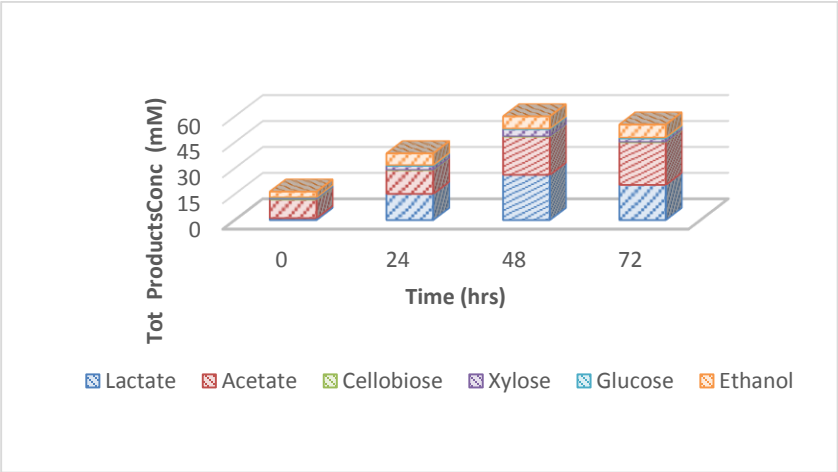

Figure 18: Total product concentration in batch SmF of *C. thermocellum* on corn stover over 72 hours

Figure 18 shows the concentration of fermentation products produced by *C. thermocellum* in reactors that were not flushed. In SmF 46.2 ml of standard inoculum was added to dry biomass for initial growth. The liquid portion of the inoculum was removed and 46.2ml of new T media was added to achieve 6% solids content. The product concentration measured at day 0 likely represents residual products of fermentation from the inoculum liquid. Each point in time represents three replications destructively sampled, so for example, cultures sampled at 24 hours were not the same cultures analyzed on day 0.

The product concentration measured on day 1 includes any product that was present on day 0 plus what was produced or consumed by the microorganism. The results shown in figure 19 indicate that the acetate, lactate and ethanol were present on day 0. Product concentrations increased with increasing cultivation times. Ethanol concentrations increased from ~3mM on day 0 to 8.85 mM after 48hrs. The largest concentration of organic acid in batch SmF cultures was

obtained as lactate with a peak concentration of 26 mM measured at 48 and 72 hours. A small decrease in ethanol concentration (1.32mM) was observed between 48 and 72hs of cultivation.

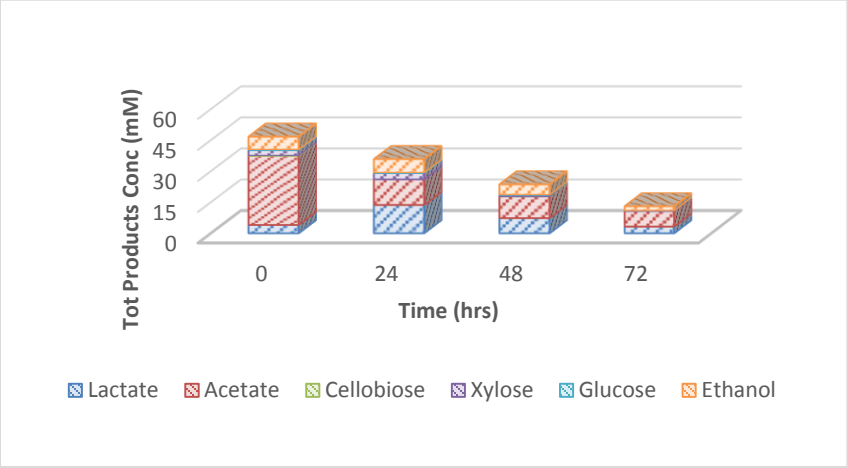

Figure 19: Product concentration measured in manually -flushed SmF of *C. thermocellum* on corn stover cultured over 72 hours.

Figure 19 shows the concentration of fermentation products produced by *C.thermocellum* grown on corn stover in manually - flushed SmF reactors. Because media is removed every 24 hours, data represent products formed during 24 hours following flushing. Therefore concentrations shown indicate the amount of product formed during the 24 hours after flushing. For example, the acetate and lactate present on day 0 were flushed out, and the lactate and acetate concentrations shown on day 1 were produced by the micro-organism during the previous 24 hours. Figure 19 shows decreasing product concentrations after every flush. After 24, 48hrs and 72 hrs 6.06 mM, 5.07mM and 2.16 mM ethanol was produced respectively

3.4.2 End Products from Solid-Substrate Cultivation of Corn Stover by *C. thermocellum*: Comparison of Manually-Flushed vs Batch

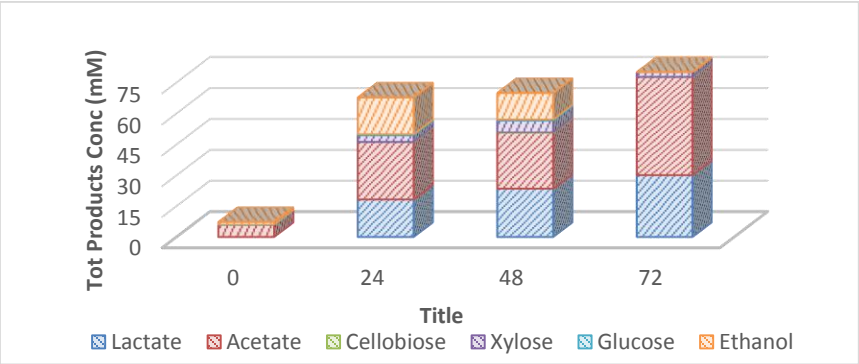

Figure 20: Total products formed in batch SSC reactors

Fermentation products in batch SSC cultures were compared to manually flushed SSC reactors. According to Figure 20 total product concentrations increased between 0hrs and 72hrs in batch SSC reactors. Ethanol concentrations decreased after 24hr cultivation period while lactate concentrations increased.

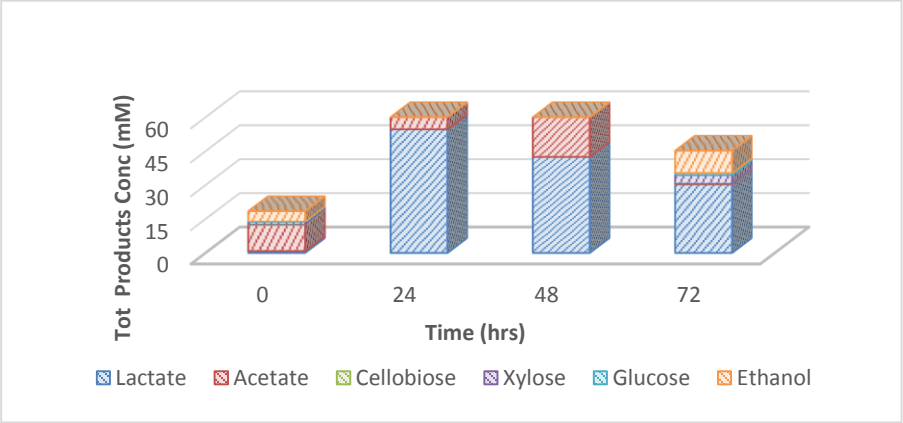

Figure 21: Total products formed in manually flushed SSC reactors

In flushed SSC cultures product concentrations were unevenly distributed. The highest amount of lactate (54.6mM) was obtained after 24hrs of growth. Lactate concentrations declined thereafter. The highest amount of ethanol (9.8mM) was obtained after 72hrs of cultivation

3.4. 3 End Products from Solid-Substrate Cultivation of Corn Stover by *C. thermocellum*: Manually-flushed vs Automatically-Flushed based on Time

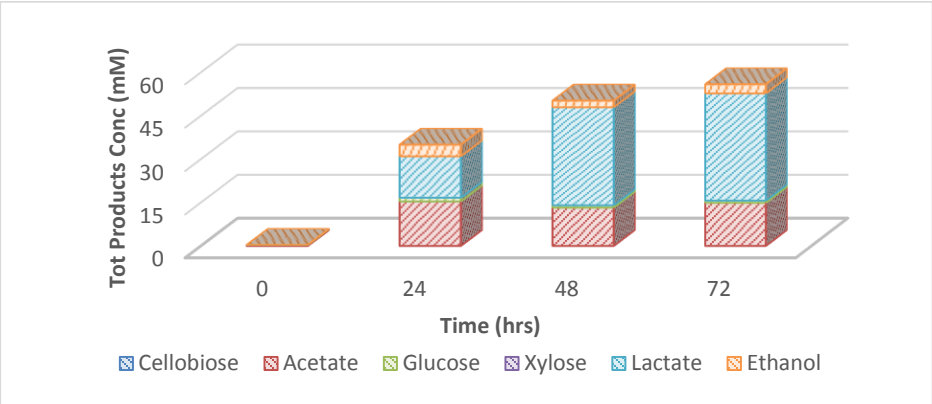

Figure 22: Total products formed in automatically flushed SSC reactors.

Fermentation products in manually flushed SSC cultures were compared to automatically flushed SSC reactors. According to figure 22 ethanol was detected in almost similar amounts (~1mM) after 24, 48 and 72 hrs of cultivation. In automatically flushed SSC cultures (Figure 22) more lactate was produced after 24, 48 and 72 hours of cultivation.

3. 4. 4 End Products from Cultivation of Corn Stover by *C. thermocellum*: Automatically-flushed SmF flushing versus Automatically-flushed SSC

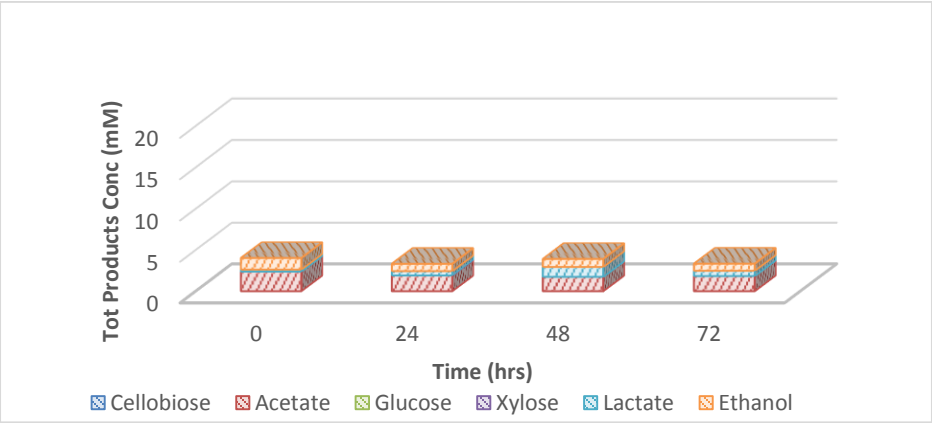

Figure 23: Total products formed automatically flushed SmF reactors

Fermentation products in automatically flushed SSC cultures were compared to automatically flushed SmF cultivations. In automated SmF flushing Figure 25 shows that the concentration of fermentation produced after 24, 48 and 72 hrs was almost constant. Results for the same experiment in SSC (Figure 24) show varying product concentrations in higher amounts at each time period

3.4.5 pH (H^+) based flushing

Fig 25 shows the products in automatically controlled pH cultures over 10 days. Increase in the concentration fermentation products were coupled with a drop in pH. The pH drop triggered a flush and new media was added into the reactor. Product concentration increased after flushing the reactor with new media.

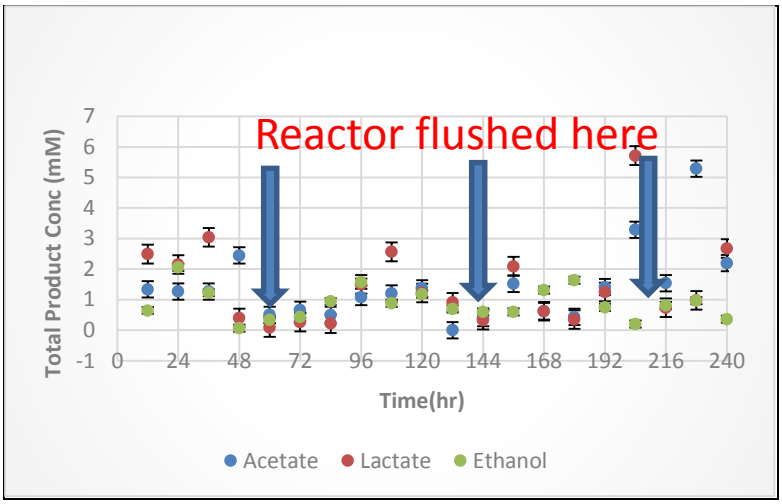

Figure 24: Product concentration over time using pH trigger

pH measurements in SSC were not possible because of the low moisture conditions. pH is the negative log of the hydrogen ion concentration. For an accurate measurement these ions have to be evenly distributed in an aqueous environment. Moreover, temperature affects the accuracy and speed of response of the pH electrode. Higher temperatures will alter the material being measured and significantly. When measuring pH using a pH electrode the measurement from the electrode is affected by temperature based on the Nernst Equation Temperature compensation can be achieved manually or automatically but the temperature sensing probe would have to be positioned in proximity to the pH electrode to achieve reliable readings.

3.4. 6 Total Product

For this experiment xylose, lactate, acetate, glucose and ethanol concentrations were recognized as fermentation products during analysis. Their concentration was summed and the amount of total product (g) in each treatment class was calculated as follows:

$$Total\ Product\ (per\ treatment) = \sum (\ X_{Ethanlol} \times \frac{46.0844}{1000} \frac{g}{mol} \times V \frac{ml}{1000}\ + X_{Acetate} \ldots .)$$

$$X_{Ethanol}\ = Total\ product\ concentration\ in\ mM\ after\ x\ hours\ of\ cultivation$$

$$Molar\ mass\ _{Ethanol} = 46.0844\ g/mol$$

$$V = volume\ removed\ or\ flushed$$

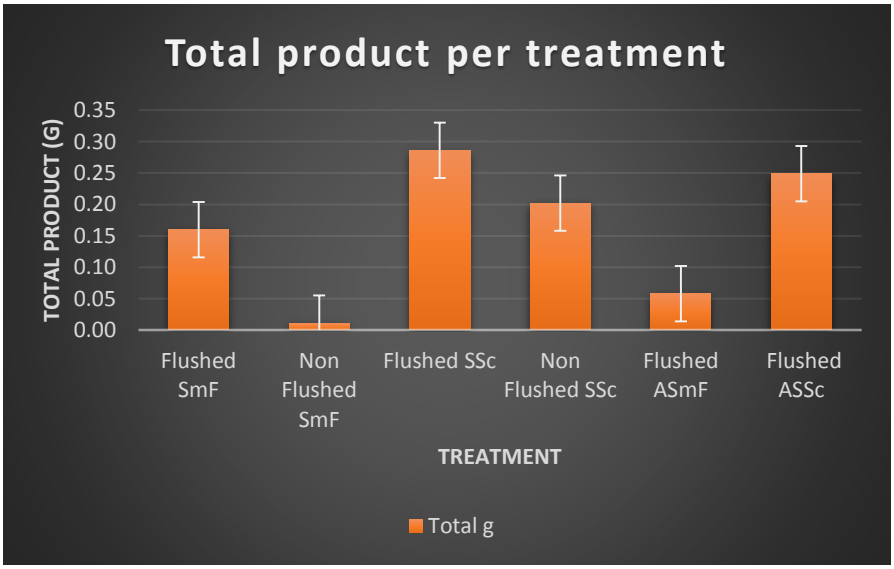

Figure 25: Total product in grams per treatment

Figure 25 shows the amount of product in terms of weight for each treatment class under comparison. Total product was higher in flushed SmF and SSC cultures when compared to non-flushing SmF and SSC cultures respectively. Automatically flushed cultures in both SSC and SmF produced lower product amounts than manually controlled cultures. Statistical analysis showed a difference between treatments ($p<0.05$).

Dependent Variable: Total Product

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.17389894	0.03477979	23.90	<.0001
Error	12	0.01746067	0.00145506		
Corrected Total	17	0.19135961			

Figure 26: Cultivation methods ANOVA

The post-ANOVA Tukey test revealed differences among treatment means grouped by main effect. There was a statistical difference between manually flushed SmF cultures and non- flushing SmF cultures. In contrast there was no statistical difference between manually flushed SSC and non – flushing SSC cultures over the 72hr period. There was a statistical difference between manually flushed SmF cultures and automatically flushed SmF cultures. However was no statistical difference in manually flushed SSC cultures and automatically flushed SSC cultures. This outcome suggests plausible similarity between the manual and bacterial cultures generated via the automated system.

3. 5 Discussion

Flushing bacterial cultures with new media (without carbon source) extended microbial growth time. Product yields at the beginning of the experiment (time zero) reflect the amount of measured metabolites from the inoculum. After this sample was collected a flushing sequence was initiated to remove these inhibitory components while leaving *C.thermocellum* bound to the substrate. Over each 24 hour period our ethanol yield was lower than reported average concentrations of approximately 3g/L (Akinosho, Yee, Close, & Ragauskas, 2014b) under similar

growth conditions. However variations in product concentrations were observed among treatments. In non-automated SmF (6% solids) ethanol was present in larger concentrations in cultivations that were flushed versus cultivations that were not flushed. In both non-automated SmF and non-automated SSC total product was greater in flushed cultures versus cultivations that were not flushed. Total cumulative product was greater in automated SSC than automated SmF.

Our product yields were lower than those reported in literature. Residual amounts of NaOH after cornstover pretreatment might have caused poor bacteria. A possible solution would be to further wash biomass before cultivation. Alternatively bacterial cultivation can be attempted without NaOH pretreatment. It is also plausible that intermittent flushing with CO_2 had an adverse effect on our growth cultures. There is evidence(L. Wang et al., 2013) that CO_2 sparged into the reactor or produced during fermentation will strip ethanol produced by the bacterium. This and other results from our experiment suggest the need for further experiments to improve the proposed design, data collection and analytical methods.

Although cultivation procedures were similar in both manual and automatically flushed cultivations there were key differences in reactor configuration and temperature regulation. This could explain the lower product concentrations in automatically flushed cultivations than in manually flushed cultures. In addition anaerobic conditions in automatically flushed cultures were not sparged with carbon dioxide after each flushing sequence like cultivations in manually operated reactors. This was because the operator was not physically present to initiate gassing at the time of flushing in accordance to the design for an automated system

3.6 Conclusion

A SSC system with manual flushing was developed using polypropylene chromatography columns loaded with cornstover. All conditions were identical to the automated system. Samples of flushed media were acquired and used for HPLC analyses. In our experiment the automatic media replacement improved bacterial growth substantially by removal of accumulated inhibitors and waste. Cultivations that were flushed continuously resulted in comparatively greater biomass conversion yields of ethanol, acetate and lactate than cultivations from static treatments.

3.6 References

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Chapter Four: Future Work

4.1 Biomass pretreatment

The initial step in lignocellulosic fermentation usually involves mechanical reduction in particle sizes followed by enzymatic hydrolysis, acid hydrolysis or dissolution in an ionic liquid. Some pretreatment procedures have been reported to generate by-products which include compounds which can be inhibitory to cellulase enzymes as well as bacterial growth (Palmqvist and Hahn-Hägerdal, 2000 and Palmqvist et al., 1996). Organo-solvent hydrolysis is fast and easy to perform but is hampered by non-selectivity and formation of significant degradation products (Fan et al., 1982). The dilute acid damages the sugar chain polymers of hemicellulose, cellulose and lignin without discrimination. The result is an aqueous mixture of sugar degradation products and more aromatic compounds which may be toxic to *C.thermocellum* during hydrolysis. The absence of these inhibitors in pretreatment methods such as high temperature steam and enzymatic hydrolysis make them more appeasing compared to solvent based and ionic liquid pretreatments Rana et al., 2014). Furthermore enzymes are preferred in catalyzing lignocellulose because other limited expense and high selectivity compared to chemical treatments (acids and bases).

Ionic liquids (ILs) have also been reported to be a more effective alternate to dilute acid or ammonia pretreatments (Nimonia 2015). Their use should be investigated in our pretreatment of cornstover to increase available sugars. ILs are organic salts which exist in liquid form at ambient temperatures. They are able to solubilize and precipitate cellulose for hydrolysis. Ionic liquids are diverse in their chemistry but have also been reported in literature to have some significant toxicity level (Li et al., 2013) therefore possessing a potential adverse reaction during hydrolysis

by *C.thermocellum*. Experimental evidence (Datta et al., 2010) reports a reduction in cellulase activity in the presence of a common ionic liquid, 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]). Currently various methods are employed to recover ILs including flushing the biomass with large amounts of water to recycle the reagent (Datta et al., 2010 and Ouellet et al., 2011). If ILs were used in our set-up the flushing schedule can be used as a twofold measure to remove inhibitors and recover the ILs.

4.2 Simultaneous Saccharification and Fermentation

Simultaneous saccharification and fermentation (SSF) refers to the combination of hydrolysis and fermentation of resulting sugars steps mainly because of the similarity in process conditions both processes (Kádár, Szengyel, & Réczey, 2004). The advantages of this process include reduced equipment costs and reduced contamination. For SSF processes to be successful standard operating conditions have to be identical. In the fermentation of glucose to ethanol thermotolerant yeasts capable of withstanding temperatures above 40^{o}C have been coupled with cellulolytic bacteria with temperature optima in the same range.(Ballesteros, Oliva, Negro, Manzanares, & Ballesteros, 2004). Wahono et al., 2015 demonstrated the feasibility of SSF by combing *Saccharomyces cerevisiae* and *Trichoderma Reesei* and optimizing ethanol production (11.810 gL^{-1} from 3.249 $g.L^{-1}$). A NaOH pretreatment was employed resulting in a reduction in lignin (6.94%) and improved cellulose availability.

Some cellobiose fermenting yeasts have been identified for potential use in SSF because of additional β-glucosidase activity that can speed up SSF reaction while promoting high initial ethanol yields. For example simultaneous saccharification and fermentation was used to improve xylose conversion by a recombinant strain of Saccharomyces cerevisiae (Olofsson, Rudolf, & Lidén,

2008) . The simultaneous (SSF) process involves: saccharification catalyzed by glucoamylase and fermentation of glucose to ethanol by yeast. (Murthy, 2006)

Similar microbe combination involving *C.thermocellum* should be investigated. Cellulases produced in the SSC of *C.thermocellum* can be to hydrolyze agricultural residues and utilized by fermentation microbes with similar growth conditions to yield ethanol. Such an approach could help significantly reduce operational costs in a full scale application of this fermentation procedure.

4.3 References

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Appendices

Appendix A: Glass reactor dimensions

Quantity		Symbol
Capacity	60	*mL*
Disc Diameter	40	*mm*
Porosity	Fine	
Approx. Stem .Length	75	*mm*
Approx. Stem O.D	10	*mm*
Approx. Overall Height	155	*mm*
Approx. Overall Body O.D	70	*mm*

Independent parameters of each bioreactor

Material	Thermal Conductivity ($Wm^{-1}K^{-1}$)
Copper	400
Aluminum	240
Cast Iron	80
Glass	1.05
Water	0.61

Thermal conductivities of common materials

Appendix B: Statistical Analysis using SAS®

Syntax:

```
data FAM;
input treatments $ TotalProduct@@;
datalines;
A 0.009 A 0.014 A 0.009
B 0.161 B 0.192 B 0.126
C 0.220 C 0.190 C 0.200
D 0.270 D 0.315 D 0.273
E 0.160 E 0.310 E 0.270
F 0.084 F 0.030 F 0.060
;
run;
proc anova data=FAM;
class treatments;
model TotalProduct=treatments;
means treatments/tukey;
run;
```

Output:

The SAS System

The ANOVA Procedure

Class Level Information

Class	Levels	Values
treatments	6	A B C D E F

Number of Observations Read	18
Number of Observations Used	18

The SAS System

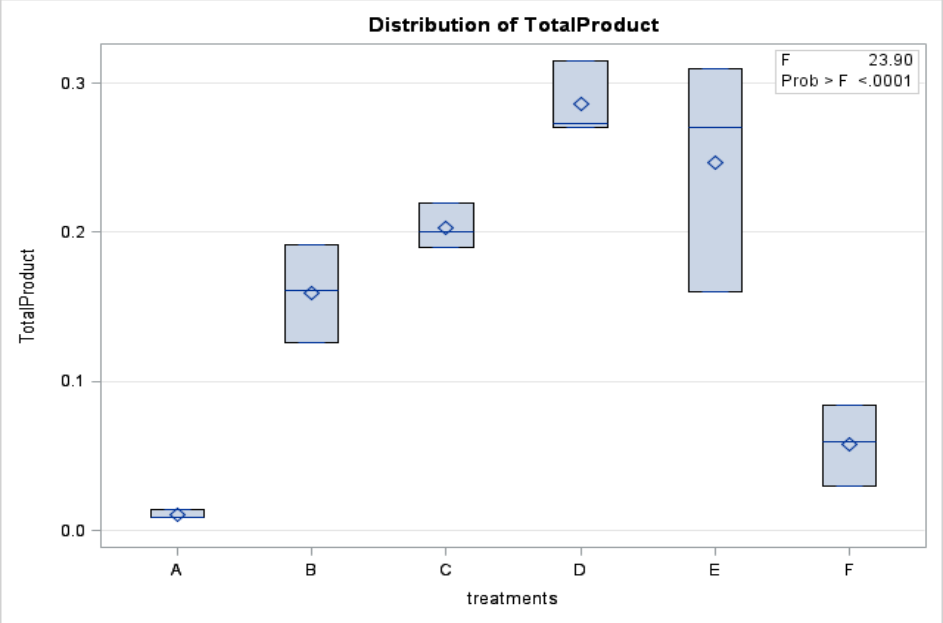
The ANOVA Procedure

Dependent Variable: TotalProduct

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	5	0.17389894	0.03477979	23.90	<.0001
Error	12	0.01746067	0.00145506		
Corrected Total	17	0.19135961			

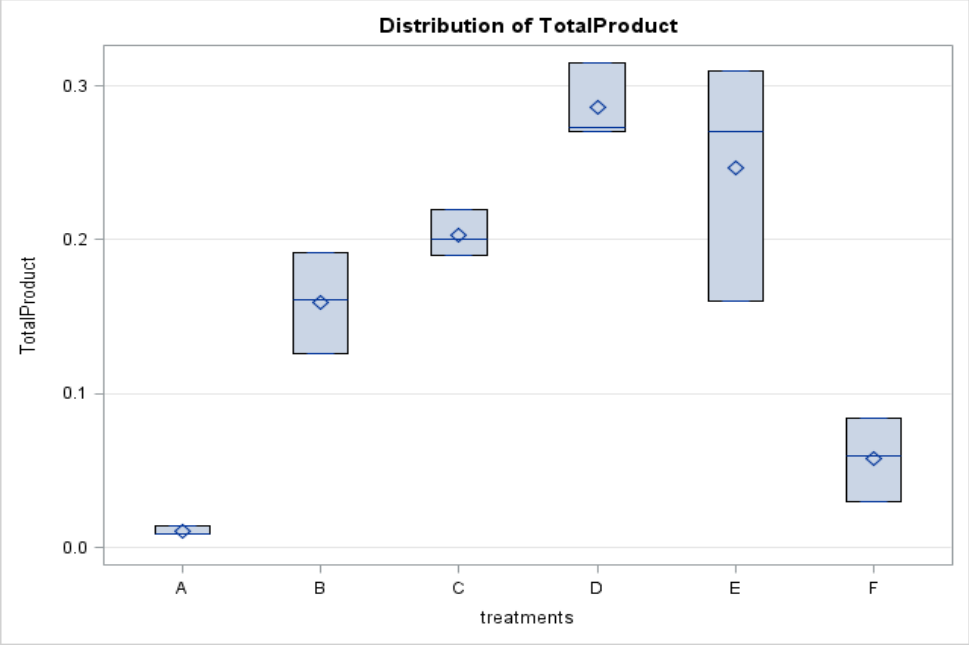
R-Square	Coeff Var	Root MSE	TotalProduct Mean
0.908755	23.73361	0.038145	0.160722

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatments	5	0.17389894	0.03477979	23.90	<.0001



The SAS System

The ANOVA Procedure



The SAS System

The ANOVA Procedure

Tukey's Studentized Range (HSD) Test for TotalProduct

Note: This test controls the Type I experimentwise error rate, but it generally has a higher Type II error rate than REGWQ.

Alpha	0.05
Error Degrees of Freedom	12
Error Mean Square	0.001455
Critical Value of Studentized Range	4.75020
Minimum Significant Difference	0.1046

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	treatments
A	0.28600	3	D
A			
B A	0.24667	3	E
B A			

Means with the same letter
are not significantly different.

Tukey Grouping		Mean	N	treatments
B	A	0.20333	3	C
B				
B	C	0.15967	3	B
	C			
D	C	0.05800	3	F
D				
D		0.01067	3	A

Appendix C: Thermocouple Calibration

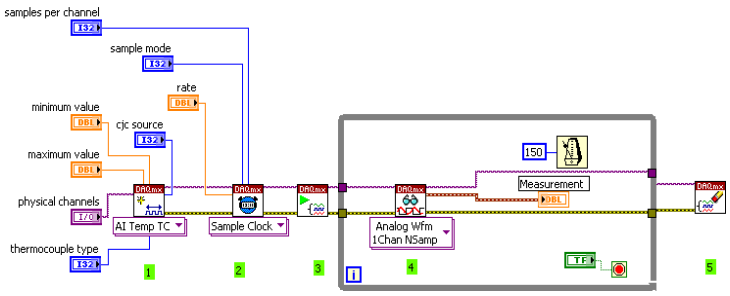
$$Reynolds\ number(Re) = \frac{\mu_\infty D}{v} = \frac{0.03157\ m/s \times\ 0.025m}{0.000378kg/ms} = 2.08$$

$$Prandt'l\ number\ (Pr) = \frac{v}{\alpha_{water}} = \frac{3.78 \times 10^{-7} m^2 s}{1.4 \times 10^{-7}\ m^2/s} = 2.7$$

$$Nusselt\ (Nu) = 1.1CRe_D^n Pr^{0.31} = 1.1 \times 0.891 \times 2.08^{0.330} \times 2.7^{0.31} = 1.698$$

$$Heat\ transfer\ coefficient(h) = \frac{Nu_D k}{D} = \frac{1.698 \times 0.58\ W/mk}{0.025m} = 39.393 W/m^2 K$$

$$Biot\ number\ (Bi_{R)} = \frac{hR}{k_{Avicel}} = \frac{39.393 W/(m^2\ K) \times 0.0125m}{0.06 W/mK} = 8.206$$



Block diagram for temperature measurement VI

1- Open a DMM session.

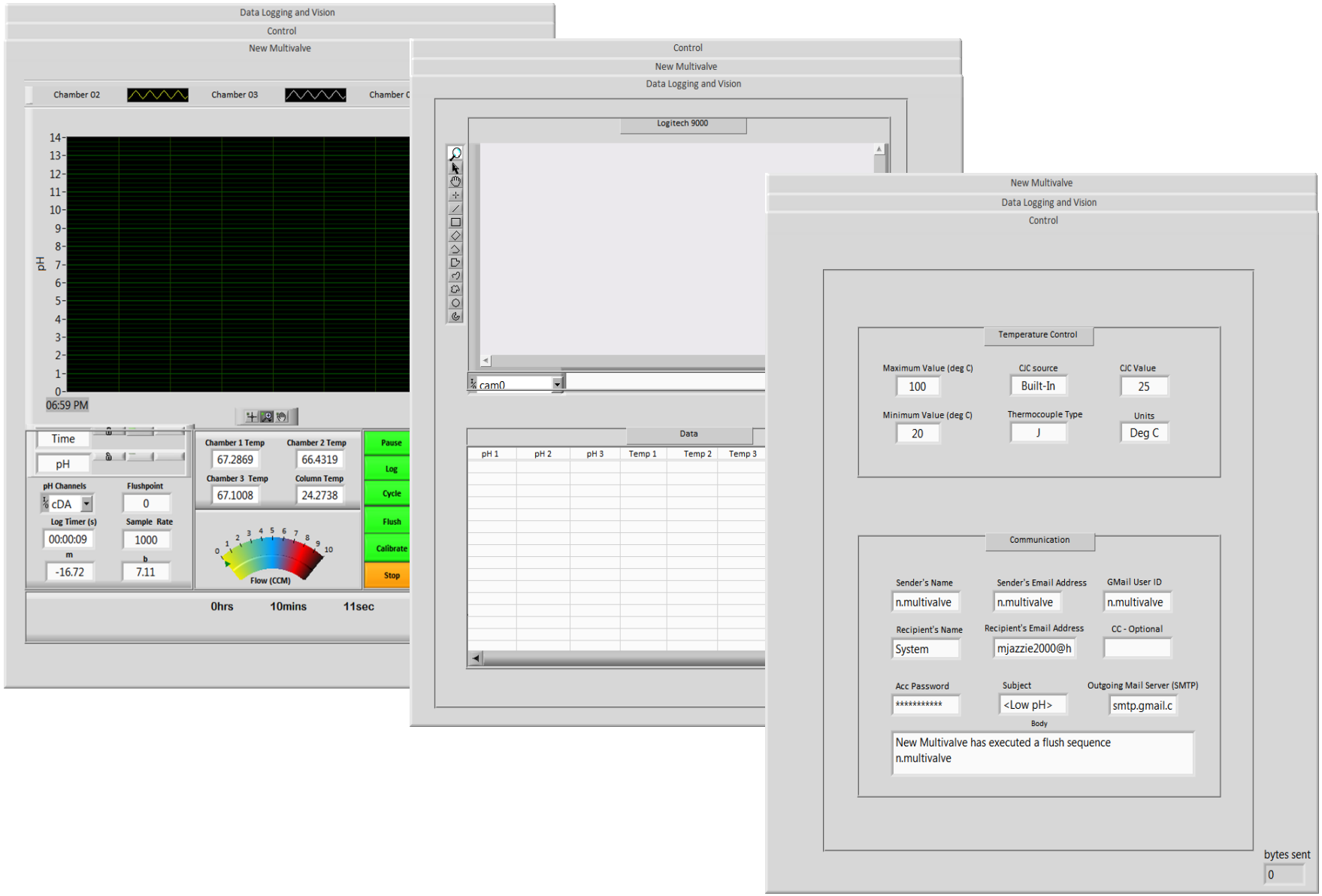
2- Configure the function and resolution.

3- Configure the transducer type.

4- Configure the thermocouple type and Fixed Ref Junction type.

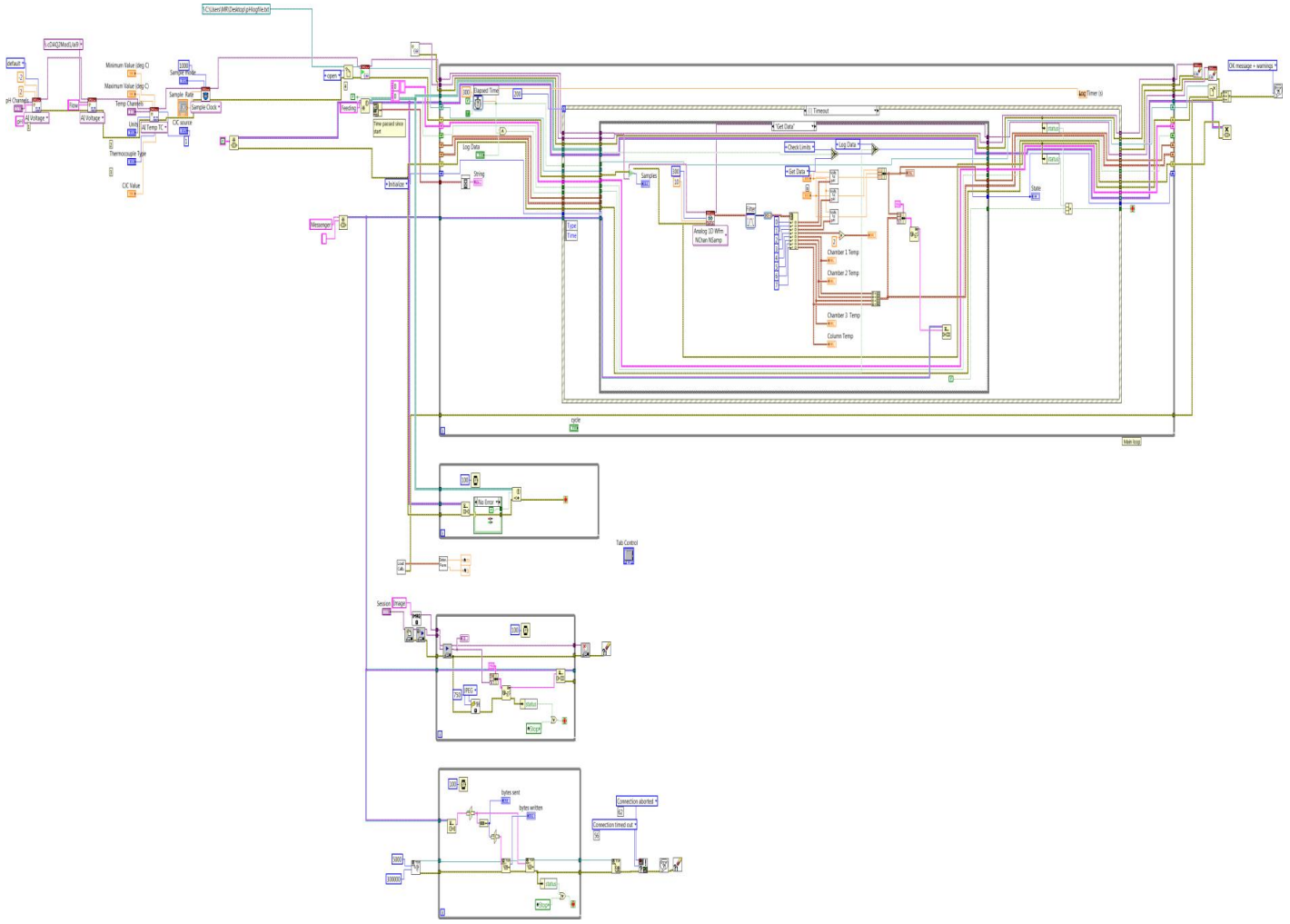
5- Clear Task.

Appendix D: LabVIEW Graphical User Interface



GUI for automated fermentation system

Appendix E: LabVIEW Code



Block diagram for pH, temperature and automated flushing control

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