COUNTERCURRENT ENZYMATIC SACCHARIFICATION OF LIGNOCELLULOSIC BIOMASS AND IMPROVEMENTS OVER BATCH OPERATION

A Thesis

by

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ABSTRACT

Transportation fuels are the major driver for fossil fuel production, a burden that many countries have tried to ease by blending fossil fuels with biofuel substitutes such as ethanol. Current U.S. ethanol production relies on fermentation of starchy biomass (e.g., corn), which competes with food. Using lignocellulose avoids competition with food; however, it is difficult to digest using traditional batch saccharification. This work investigates countercurrent saccharification as an alternative that reduces enzyme requirements. Compared to baseline yields for standard batch saccharification, countercurrent saccharification reduces enzyme requirements by 5 to 37 times.

Initial studies identified Solka-Floc as an acceptable substrate to represent treated biomass; it is readily available and reliably consistent from sample to sample. To measure yields, batch saccharifications were performed at various enzyme loadings and reaction times. Two relatively low enzyme concentrations were selected for further study: 2 and 5 mg protein/g biomass, or 2.6 and 6.4 mg protein/g glucan, respectively. Both of the selected loadings are below levels suggested for commercial use.

For each enzyme loading tested, a multi-stage, semi-continuous countercurrent train was constructed. The first experiments used trains consisting of eight 1-L bottles loaded with α-cellulose, using the same concentrations as the batch experiments. Liquid and solid phases were countercurrently contacted, with transfers of each phase occurring every second day for approximately 4–6 weeks, until steady-state operation was maintained for at least 10 days. At 2 and 5 mg/g enzyme loadings, total sugar yields

reached 55.9% and 85.3%, respectively. A follow up study using 16 bottles and 2 mg/g loading produced total sugar yields of 73.4%.

In the interest of future scale-up, alternatives to the cycloheximide and tetracycline antibiotic cocktail were investigated. Preliminary results suggest that chloroform or a volatile essential plant oil may be effective. Future work is needed to confirm the antimicrobial strength of these compounds at low concentrations, which is needed to limit enzyme inhibition.

DEDICATION

This thesis is dedicated to my family, who have always been there for me and allowed me the freedom to make my own choices. I will be forever grateful for their guidance and support.

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NOMENCLATURE

CVS	Control verification standard
DNS	Dinitrosalicylic acid
DC	Digestible content in % of dry solids
DI H_2O	Deionized distilled water
D_m	Standard deviation of slope
D_r	Standard error of slope
EB	Enzyme blank
Gal	Mass of galactose
[Gal]	Concentration of galactose
GalC	Galactan fraction of dry biomass
Glu	Mass of glucose
[Glu]	Concentration of glucose
GluC	Glucan fraction of dry biomass
HPLC	High performance liquid chromatograph
ID	Inner diameter
L_s	Liquid retained in waste solids
m	Slope
MC	Moisture content
m_d	Dry mass desired
$M_{l,i}$	Liquid mass in Bottle i

m_t	Target air-dry mass
OD	Outer diameter
OPEC	Organization of the Petroleum Exporting Countries
PFR	Plug flow reactor
rpm	Rotations per minute
SB	Substrate blank
S_{out}	Waste solids
SS	Steady-state
TS	Total solids
UV-vis-NIR	Ultraviolet-visible light-near infrared
V	Volume of sample
$V_{inositol}$	Volume of inositol standard
$V_{l,i}$	Volume of liquid in Bottle i
V_{reac}	Reaction volume
V_{sample}	Volume of liquid samples
Xyl	Mass of xylose
[Xyl]	Concentration of xylose
XylC	Xylan fraction of dry biomass

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CHAPTER I

INTRODUCTION

As human population continues to expand, energy resources are becoming increasingly scarce. To satisfy current and future demand for liquid transportation fuels, researchers have increasingly turned to renewable sources, such as biofuels, to produce alternative fuels. Existing hydrocarbon technology relies on non-renewable reserves of ever-diminishing crude oil and natural gas, reserves that are becoming more difficult and more expensive to extract. In 2009, 6.67 trillion kg of coal and 3.52 trillion kg of crude oil were extracted worldwide (International Energy Agency, 2012a; International Energy Agency, 2012b). These staggeringly large amounts of energy extraction come at large costs, both in terms of long-term environmental impact and human lives. Coal mine collapses (Upper Big Branch Mine, 2010) threaten workers. Oil spills in the Gulf of Mexico (Deepwater Horizon, 2010) and Alaska (Prince William Sound 1991) have long-term consequences for the affected environment and local populations, which are not soon forgotten by the public. Recently, the Arkansas pipeline spill (Mayflower, March 2013) caused the evacuation of many residents and raised concerns over future pipeline projects routed through delicate ecosystems.

Historically, the total imported crude oil has been high in the United States. In 2011, approximately 52% of U.S. net petroleum imports were sourced from countries belonging to OPEC. This reliance on countries with unstable political and social climates threatens supply and weakens U.S. leverage abroad. The large percentage of imports also

contributes heavily to the U.S trade deficit. In 2011, the U.S. net petroleum imports totaled 8.4 million barrels per day, representing 45% of domestic demand for that year (U.S. Energy Information Administration, 2012).

In the interest of decreasing foreign energy dependence and demand for non-renewable sources, first-generation biofuel technology focused on converting starch and sugar-rich feedstocks into fuel. Industrially, ethanol is produced from sugarcane (Brazil) or corn (United States). Corn grain is a common livestock feed because of its highly digestible content of starches, sugars, and soluble fiber, with total digestible nutrients generally over 85% (Chase and Hibberd, 1987). These properties also make it an attractive feedstock for biofuel production. Because of rapid commercialization of corn-to-ethanol processes, corn grain available for food and livestock feed has declined, which inflates the cost of food products. During the advent of these early biofuel technologies, corn devoted to energy production rose sharply. Notably, from 2005–2010 corn consumption for biofuels climbed from 14% to 37%. More recently, this dramatic increase has slowed, and is projected to slow substantially during the next 5 years (U.S. Department of Agriculture, 2012). As recently as 2006, and as far back as the early 1970s, corn prices were relatively stable at $2–$3/bushel. Since 2006, the price has more than doubled, averaging approximately $7/bushel in the first six months of 2013 (Capeheart, 2013). Because of this substantial increase, the costs of dependent agricultural commodities, such as meat and dairy, have been affected. To provide resources for the world's expanding population, and the associated demands for food and energy, a more sustainable energy alternative must be explored.

As an alternative to food crops, biofuels can be produced from lignocellulose. Rather than converting corn grain to biofuels, the corn stover, frequently discarded, can be converted. First, the lignocellulose is pretreated to enhance digestibility. Then, a sugar-rich solution is produced via enzymatic hydrolysis using commercial enzyme blends. This sugar solution can then be fermented to alcohol using yeast.

For decades, countercurrent technologies have been employed to increase yields, improve heat transfer, and improve upon batch technologies, when feasible. Application of countercurrent saccharification to produce sugars for ethanol production has the potential to greatly reduce both primary and secondary costs associated with ethanol production.

Table 1-1 summarizes literature on countercurrent saccharification related to this work. The independent and dependent system variables are summarized and compared to conditions used within this work.

The research presented in the following chapters has the following major objectives:

- Determine an optimal substrate for testing
- Develop testing methods that optimally balance speed, cost, and precision for evaluating sugar production
- Create baseline data using batch saccharification
- Design new countercurrent apparatus to increase yields
- Determine optimal feed location for enzyme loading
- Test options for volatile, recoverable antibiotics

Table 1-1 Summary of countercurrent saccharification studies

Source	Mode of Operation	Substrate	Number of Stages	Enzyme Addition Point	Enzyme Load	Solids Concentration (% solids by mass)	Conversion (%)	Product Concentration (g/L)	Improvement Factor (yield:batch yield) (same enzyme load)
Jeffries, Schartman	Batch	Paper fines	–	–	5.56 FPU/g carb.	4%	46.90	54**	–
			–	–	5.56 FPU/g carb.	8%	32.80		–
	Psuedo-countercurrent	Solka-Floc control	3	Moving, all stages	5.56 FPU/g carb.*	4%	65.20		1.39
					5.56 FPU/g carb.*	8%	47.80		1.46
Fox, et al.	Batch	Sugarcane bagasse	–	–	Not given	11%	44.30	48.7†	–
	Countercurrent PFR		3	Not given		11%	56.40	62†	1.27
This work	Batch	Solka-Floc	–	–	1.98 FPU/g glucan	10%	19.53	19.5	–
			–	–	4.94 FPU/g glucan	10%	41.69	41.6	–
			8	4	1.98 FPU/g glucan	10%	55.91	64.9	2.86
	Countercurrent	α-cellulose	8	5	4.94 FPU/g glucan	10%	85.34	102	2.05
			16	8	1.98 FPU/g glucan	10%	73.39	67.4	3.75

*With 11.11 FPU/g carb. β-glucosidase added

**Only reported value for batch with conditions: 8% solids concentration, 5.56 FPU/g carb., plus 11.11 FPU/g carb. β-glucosidase

†Calculated sugar product concentrations from available yield and loading data

1 FPU is defined as the amount of enzyme required to release 1 μmol/min of glucose into solution

1 IU is defined as the amount of enzyme required to digest 1 μmol/min of substrate (cellulose); therefore, 1 IU = 1.111 FPU via correction for water of hydrolysis

CHAPTER II

ANALYSIS METHODS

2.1 Introduction

For any scientific study, regardless of the subject matter or context, the methods by which data are collected, analyzed, and presented are a primary concern. Not only do the experimental methods need to be sound, but the tools and analytical methods must be selected appropriately for the results to be significant. In the context of biofuels, there is frequently the need to measure compositions using chromatography.

For multi-component analysis, there are several methods available, depending on the material to be tested. Gas chromatography is well suited for volatile organic mixtures that can be easily vaporized and separated. When components cannot tolerate high temperatures or do not easily vaporize, liquid chromatography is preferred. High performance liquid chromatography (HPLC) is the premier liquid chromatography method, frequently used as a validation tool in pharmaceutical testing to determine purity and compliance (U.S. Federal Drug Administration: CDER, 1994).

2.2 Materials and Methods

2.2.1 DNS Reagent

The dinitrosalicylic acid (DNS) assay uses chemicals that inactivate enzymes and, upon heating, react with reducing sugars to develop a color, the intensity of which is proportional to concentration. This blend of chemicals (DNS reagent) consists of deionized water (DI H_2O), 3,5 dinitrosalicylic acid, sodium hydroxide, sodium

potassium tartrate, sodium metabisulfite, and phenol. Stock solutions of this reagent were prepared in batches of varying sizes and stored in foil-covered flasks in a refrigerator because of the light- and temperature-sensitive nature of the components. Excess reagent was stored for a period not to exceed two weeks before being discarded as hazardous organic waste. For detailed preparation instructions and warnings see Appendix A.

2.3 DNS Testing

2.3.1 Sample Preparation

To produce viable results, preparation of samples for analysis by the DNS method requires several carefully timed steps. Termination of enzyme activity was accomplished by adding DNS reagent to samples immediately following incubation. To ensure samples reacted for exactly the correct duration, timing was critical. The color development step was the primary source of inconsistencies. The development period was to be exactly 5.0 minutes, leaving only 5–6 seconds to remove a sample from the boiling water and place it into an ice bath to quench the color development reaction. Because of the large number of samples required for batch reactions, precise timing was difficult to maintain, even with assistance.

2.3.2 Analytic Equipment

Color was measured using a Hitachi U-4100 UV-vis-NIR spectrophotometer, utilized a matching pair of quartz cuvettes to establish a background reading and sample absorbance at the specified wavelength (540 nm). There were some challenges associated with using this equipment. The spectrophotometer was located in a facility

shared with several departments, meaning scheduling time on the device was difficult, worsened by the fact that samples had to be tested immediately because of the light sensitivity of the color development solution.

Reliability played a larger part in delays. Occasionally, another researcher would mishandle the cuvettes, requiring professional repairs, a process that frequently meant a week-long delay. Additionally, there were several instances when the photomultiplier (PMT) detector was unable to communicate with the equipment and repairs again delayed analysis.

Even when the process proceeded smoothly and no outside forces delayed analysis, the analysis method – from sample preparation to dilution and measurement – was time-consuming and labor-intensive. One of the primary goals of developing a modified DNS assay was to reduce labor and turn-around time for results; thus, this procedure was not successful.

2.3.3 Testing Method

The DNS assay is widely used to operate under extremely specific conditions for determining cellulase activity using the filter paper assay (Ghose, 1987). Compared to other available analytical methods, it proved less repeatable and reliable at the conditions of the study. Concerns about repeatability stemmed from the necessity to dilute samples so that the sugar concentration fell between a fairly narrow range of standards. When the samples were being tested to determine sugar content, this proved difficult with no prior knowledge of expected performance under the conditions of the study, even when a fifth, higher concentration sugar standard was included in calibrations. The other potential

concern with dilution was injection into the cuvette. The procedure calls for 0.200 mL of sample be diluted with 2.5 mL of water. This was accomplished by using high-precision auto pipettes, with new tips for each sample, to ensure exact volumes in every sample run. Repeatability was further questioned because of the color development step, which was sensitive even to seconds of delay. Any minor mistakes in this step could lead to triplicate samples erroneously reporting very different results.

Outside of data integrity, some operational concerns arose, including waste handling and time commitment, as mentioned. Although the process itself took less time to produce a result after a batch assay had been completed, the fact that every step was so labor intensive meant that results were actually obtained slower than could be accomplished with other methods. Once analysis was complete, the waste had to be properly discarded. It could not simply be diluted into laboratory waste water, because it contained a high concentration of organic solvents and acids. Disposal consisted of collecting all post-analysis waste, including rinsing all test tubes prior to disposal, and any excess or unused DNS reagent in a common container. Twice a month, this waste container was neutralized to a pH of approximately 7, and brought to the satellite waste disposal facility within the department.

2.4 HPLC Testing

2.4.1 Sample Preparation

The sensitivity and precision of HPLC equipment necessitates more caution when preparing samples for analysis. The basic steps follow: (1) boil sample to deactivate enzymes, (2) homogenize sample, (3) centrifuge sample to separate bulk

solids, (4) filter centrifugate to remove suspended fine particles, and (5) analyze sample in HPLC equipment. A thorough procedure for HPLC sample preparation and testing conditions is found in Appendix C. Because an autosampler was used with the HPLC, it was possible to create sequences of up to 100 vials, including standards, which allowed for a week of data to be analyzed during the following weekend.

Because samples were terminated through thermal deactivation of enzymes, and no temperature- or light-sensitive components were introduced into the sample during preparation, it was possible to freeze the sugar solutions at any step during the process. If samples were frozen, they were always allowed to return to room temperature and vortexed for 10–15 seconds to re-homogenize the sugar solution, which tends to form a concentration gradient as it freezes. The availability of long-term storage eliminated many concerns related to equipment availability, because samples could simply be thawed and tested whenever there was enough time to complete a sequence.

2.4.2 HPLC Methods

To prepare each sample for HPLC testing required several consumable materials, including microtubes, 1.0-mL syringes, cellulose acetate filter cartridges, and glass autosampler vials. Although all of these combined pieces produced packaging waste, there was no chemical waste produced. Even the waste liquid from the HPLC was neutral enough to be diluted and flushed down laboratory drains. Thanks to the autosampler and injector system in the HPLC, sampling was both automated and extremely consistent. By removing the human element from final liquid sampling, a large source of error was eliminated. The autosampler and injector also allowed for

extremely precise calibration of standards, a benefit utilized by increasing from four to eight standard concentrations containing all expected product components. These more robust calibrations meant results obtained from HPLC had a very high level of confidence. Because the HPLC required extremely small samples (10 μL per injection), excess sample liquid could be retained and retested if results were questionable.

2.5 Results and Discussion

2.5.1 DNS Test Method

One of the goals of this first study was to determine a faster and cheaper method for testing large batches of sugar samples so that future studies involving large matrices of variables could be screened efficiently without taxing laboratory resources and workers. The DNS method was chosen because of its relatively simple testing procedure and speed.

Samples were prepared for analysis in several steps, beginning with preparation for batch (see Appendix F) followed by adding the DNS reagent to terminate enzymatic activity. This "dead" enzyme solution was then placed in boiling water for exactly 5 minutes to develop the color of each sample. After 5 minutes, the samples were quenched in ice water to halt further color development. Following development, samples were then diluted to allow for precise measurement using a Hitachi U-4100 UV-vis-NIR spectrophotometer with matching quartz crystal cuvettes. Care was taken when handling the DNS reagent because of its toxic and staining properties (see Appendix A). The DNS method is fully detailed in Appendix B.

A calibration of sugar standards provided the data required to calculate sugar concentrations from absorbance readings. A sample sugar calibration (Figure 2-1) shows the range bracketed by the standards.

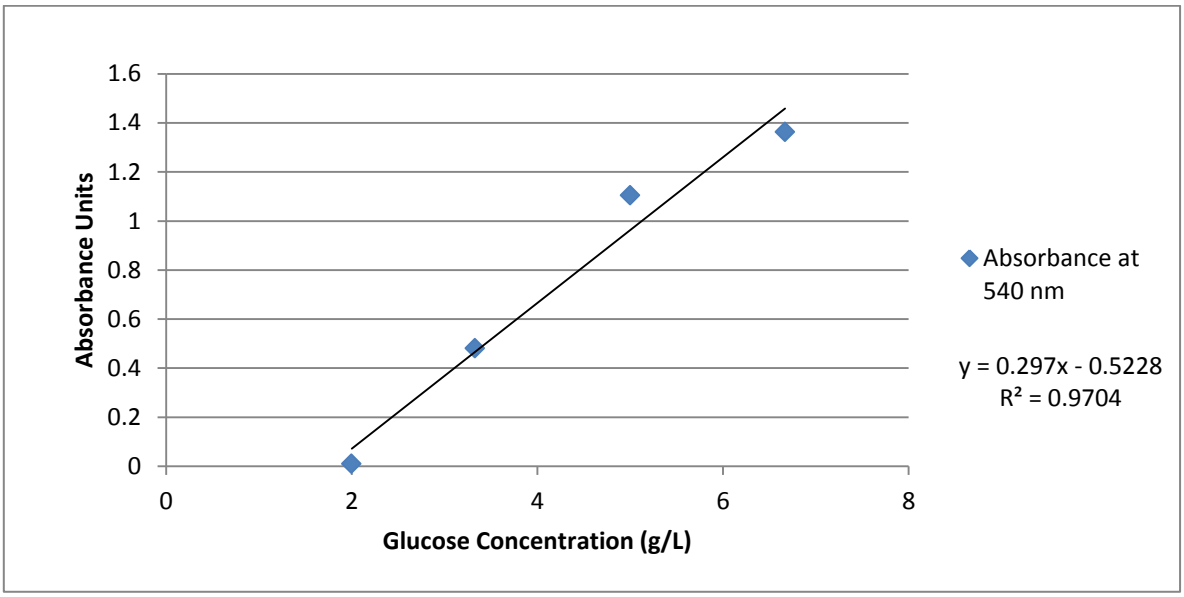

Figure 2-1 Sample DNS sugar calibration

A common issue using this method was diluting samples so that they reliably fell within the calibration range, and were therefore confident results. Even between replicates of the same conditions, results were very inconsistent. The data collected from a 1-day saccharification assay illustrate this point (Table 2-1). Although enzyme blanks were more well-behaved, substrate blanks showed a similar inconsistency (Table 2-2).

Table 2-1 Absorbance measurements for samples after 1-day saccharification assay

Enzyme Concentration (mg protein/g biomass)	Sample Identifier	Absorbance Units	Average Abs.	Std. Dev.
10	P1-10	1.392	1.360333333	0.082209
	P2-10	1.267		
	P3-10	1.422		
20	P1-20	1.842	1.652333333	0.292893
	P2-20	1.800		
	P3-20	1.315		
33	P1-33	1.790	2.119	0.285154
	P2-33	2.272		
	P3-33	2.295		
48.4	P1-48.4	2.113	2.1	0.163887
	P2-48.4	1.930		
	P3-48.4	2.257		

Table 2-2 Absorbance measurements for blanks after 1-day saccharification assay

Enzyme Concentration (mg protein/g biomass)	Sample Identifier	Absorbance Units	Average Abs.	Std. Dev.
10	EB1-10	0.106	0.0985	0.010607
	EB2-10	0.091		
20	EB1-20	0.209	0.2025	0.009192
	EB2-20	0.196		
33	EB1-33	0.41	0.4095	0.000707
	EB2-33	0.409		
48.4	EB1-48.4	0.62	0.6135	0.009192
	EB2-48.4	0.607		
Substrate Blanks	SB1-10	0.178	0.099388	0.064561
	SB2-10	0.0651		
	SB1-20	0.093		
	SB2-20	0.207		
	SB1-33	0.123		
	SB2-33	0.041		
	SB1-48.4	0.037		
	SB2-48.4	0.051		

In addition to frequent, unpredicted inaccuracies, the analysis method itself was messy and time consuming, requiring several hours of rapid, precise steps to obtain results. There were many opportunities for error, even for a practiced hand. Because of very tight time constraints between steps, there was no time to double check work. Finally, even when performed correctly and cleanly, the chemicals used must be disposed of as organic waste. After several frustrating rounds of experiments, HPLC analysis was reconsidered as a viable testing method.

2.5.2 HPLC Reconsidered

After mounting concerns over the precision and reliability of the DNS method became too great, HPLC testing was reconsidered as a viable analysis method. Originally avoided because of testing costs, it became clear that the costs were justifiable if the alternative was a result with no confidence. The primary problems with DNS were precision and reliability, areas where HPLC measurements excel. Fit lines to sugar standards used for calibration regularly reported $R^2 > 0.998$, with fits to glucose standards consistently having $R^2 = 1$. Although there was variability between replicate samples, as with any assay, the variability was significantly lower.

HPLC analysis comes with its own issues, the foremost being reliable operation and maintenance. Because it is so sensitive, HPLC equipment must be operated according to very strict procedures to ensure analytic columns are maintained, pumps are not damaged, and contaminants and clogging from algal growth are prevented. As anyone who has used an HPLC can attest, the equipment can be very difficult to fix once

problems arise. To prevent costly damage and lengthy downtimes, strict maintenance procedures were followed with every sequence to keep equipment in optimal condition.

Even under pristine conditions, HPLC operation is costly. The equipment itself is expensive, as are the materials consumed in sample preparation. To reduce these costs, every effort was made to find suppliers who provided bulk quantities of the necessary components to reduce unit costs. In addition to bulk purchasing, by buying materials that are not individually packaged or sterile, when sterility is not required, not only are costs reduced, but so is waste production and preparation time. By reducing waste, finding low-cost products that still meet specifications, and properly maintaining the equipment, HPLC analysis became a viable, and preferred, method of sample analysis.

CHAPTER III

BATCH ANALYSIS OF VARIOUS HYDROLYSIS SUBSTRATES

3.1 Introduction

Of the various reactor schemes available, batch reactions are widely used because of their simplicity and versatility. Within a single vessel, many different reaction conditions can be tested. Batch reactors can be ideal for processes that require extended reaction time; a well-designed and controlled batch reactor can require very little user input during the reaction. Because the vessel can be sterilized between cycles, batch reactions are the preferred operating mode for producing most liquid biofuels.

Despite the many benefits, there are associated drawbacks to batch reactors. Chief among these is that continuous production tends to be less expensive because there is no lost production during the dead time when a batch reactor is cycled. Another concern is maintenance and cleaning, a regular and time-consuming task when operating batch reactors.

To simulate lignocellulosic biomass used in commercial processing, a suitable model substrate must be chosen. To select the best candidate, various substrates were considered and their merits and performance were compared. Once this selection was complete, a baseline had to be established against which to compare future results.

The goals of this study follow:

- Determine an acceptable substrate analog to pretreated lignocellulose.

- Establish a baseline of yields for the selected substrate at various enzyme concentrations and reaction times.
- Select conditions to be used in countercurrent experiments.

3.2 Materials and Methods

3.2.1 Substrate

The candidate substrates included some materials used previously in MixAlco research, such as shredded office paper (Rapier, 1995). Also tested were shredded Whatman ashless filter paper circles, clean printer paper, and Solka-Floc, a refined pulp containing cellulose and hemicellulose. During later countercurrent experiments, α-cellulose from Sigma Aldrich was used to ensure uniform composition and quality, once it had been confirmed that the α-cellulose exhibited the same reactivity as Solka-Floc. Optimum solids concentration was determined by varying from 5 to 15% solids.

After initial experiments gauging substrate performance, 10% solids concentration using α-cellulose powder was selected. With this solids concentration, there was ample room for mixing and sufficient free liquid for analysis. α-cellulose powder provided the most repeatable results and the most consistent sampling.

3.2.2 Citrate Buffer

To maintain an optimum pH for enzyme activity, a buffer solution is required. Sodium citrate buffer solution was prepared using de-ionized water (DI H_2O), citric acid monohydrate powder, and sodium hydroxide (NaOH) pellets. Citric acid monohydrate is added to DI H_2O to create a 1-M solution and pH is adjusted using NaOH to a target of

4.5. This stock solution is diluted to 0.1 M and further adjusted using NaOH and hydrochloric acid (HCl) to a final pH of 4.8 (Appendix D).

3.2.3 Antibiotics

To prevent microbial growth, which consumes produced sugar, an antibiotic cocktail is used. The two components of this cocktail are a solution of tetracycline (10 g/L in a 70% ethanol/30% DI H_2O solvent) and cycloheximide (10 g/L in DI H_2O). The tetracycline solution is stored in airtight containers in a freezer maintained at –10°C (14°F). The cycloheximide solution is stored in airtight containers wrapped in foil to prevent light exposure in storage refrigerators (Appendix E). Relevant safety information is also provided in Appendix E.

3.2.4 Enzyme Solution

The enzyme was Novozyme CTec2, a proprietary blend of glucanases, β-glucosidases, and hemicellulase as well as various stabilizing compounds, proteins, and small amounts of sugar. The experimental solution was prepared using a 10:1 dilution of the stock solution in DI H_2O. In this step, care was taken to ensure accuracy and repeatability of dilution (Appendix G). This solution was prepared fresh for each experiment.

3.2.5 Incubator Design

In small batches of samples, 20×150 mm borosilicate glass, screw-top culture tubes (VWR, part # 9825-20X) were used. Sealant tape (Fisher, cat # 11-865-28) was added to ensure liquid-tight seals. In the case of enzyme blanks and samples with low solids concentrations, polytetraflouroethylene (PTFE or Teflon) thread tape was added to

the glass threads to guard against leaks. To allow for larger volumes, higher solids concentrations, and more space for mixing, 50-mL polypropylene conical centrifuge tubes with CentriStar caps (Corning, model # 430828) were used instead of standard 30-mL glass test tubes. The standard size of batch experiments was approximately 40–60 samples plus substrate and enzyme blanks. This number of samples, when using glass tubes, occupied the entire shake-table incubator. Because other researchers needed the same equipment, and were frequently performing studies of higher priority, an alternative incubator was sought. To facilitate large batches of samples for testing various parameters, an existing rolling incubator was modified using 4-in-inner-diameter PVC pipes. To hold the larger 50-mL centrifuge tubes, batches were secured inside of polystyrene trays, bundled together using rubber bands, and fit tightly into the PVC pipe sections (see Figures 3-1 and 3-2).

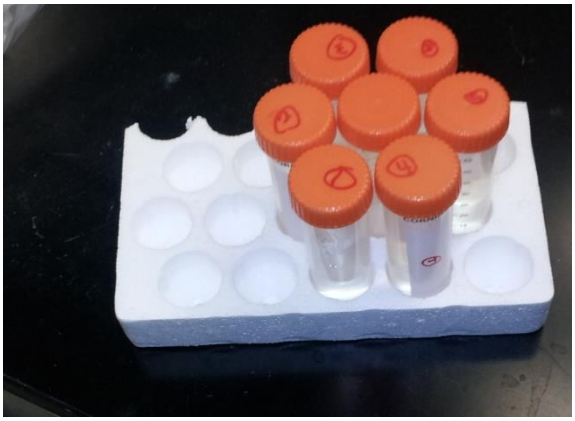

Figure 3-1 Bundle of reaction tubes.

Figure 3-2 Incubator loaded with trays full of reaction tubes.

Once final testing conditions were determined and batch size was substantially reduced, further studies of enzyme loading and reaction time were performed in a Lab-Line Instruments Orbital Environ Shaker operating at 50°C and with an agitation rate of 150 rpm. This equipment allowed use of 30-mL glass test tubes once again.

3.2.6 Batch Preparation

Each batch contained three sample type: test samples, enzyme blanks, and substrate blanks. Test samples contained pre-weighed substrate with a target dry mass. So that the dry mass remained constant throughout the experiment, the substrate moisture content was measured the same day as weighing occurred, and was used to correct the total mass placed in each tube. Test samples were run in triplicate; the results were more consistent if the reaction mixture containing the liquid components was prepared prior to addition to each tube. This reaction mixture contained 0.1-M citrate buffer, antibiotics, diluted CTec2 enzyme, and DI H_2O. The DI H_2O was varied so the

total mass of reactants in each tube totaled 20 g, with the additional assumption that the reaction slurries all had a density of approximately 1 g/cm^3 once well mixed.

Enzyme blank samples contained DI H_2O, citrate buffer solution, antibiotics, and a varying dosage of diluted CTec2. These samples were prepared alongside each batch with enzyme loading (mg protein/g digestible material) determined by the substrate and the desired amount of enzyme. Conventionally, the *digestible material* is defined as glucan, so the enzyme loading is expressed as mg protein/g glucan. During the initial phases of this work, the substrate composition was unknown; therefore, the enzyme loading was expressed as mg protein/g biomass, henceforth shortened to mg/g.

Substrate blank samples contain the prescribed mass of dry solids, DI H_2O, citrate buffer, and antibiotics. These samples are prepared similar to test samples, replacing the volume of enzyme solution with additional DI H_2O. The substrate blanks are used to subtract free sugars that may be present in the substrate resulting from pretreatment, foreign matter, or chemicals present that may contaminate the results.

The enzyme and substrate blanks were necessary to correct for free sugars the enzyme solution and substrate introduced to the system. In each test sample, these sugars had to be subtracted from the measured sugars to accurately determine sugar production.

3.2.7 Assumptions and Calculations

Each tube contained one of three sample types: substrate blank, enzyme blank, or test sample. In yield calculations, it was assumed that the water of hydrolysis was negligible and would not significantly affect measured reaction volumes. Based on saccharification stoichiometry, the maximum mass of water consumed in hydrolysis was

approximately 0.2 g. This amounted to a change in volume no greater than 1%, a systematic error that was considered small when compared to random error associated with the experiments. Although the water of hydrolysis had negligible impact on liquid volumes, it significantly impacts the mass of sugars. When glucan and galactan are converted to glucose and galactose, respectively, the mass increases by 11.1%. When xylan is hydrolyzed to xylose, the mass increases by 13.6%.

Adding the target dry mass for each sample required a simple correction for moisture content. Using a Denver Instruments IR 120 device, the moisture content of a substrate was determined in triplicate. These values were averaged and labeled as total solids (TS). Using this, the target weight was calculated using Equation 3-1

$$m_t = \frac{m_d}{\mathrm{TS}} \qquad \text{or} \qquad m_t = \frac{m_d}{(1-\mathrm{MC})} \tag{3-1}$$

where,

m_t = target air-dry mass (g)

m_d = dry mass desired (g)

TS = dry solids in air-dry biomass (g dry solid/g air-dry solid)

MC = H_2O in air-dry biomass (g H_2O/g air-dry solid)

3.3 Experimental Design

In this study, the variables of interest were solids concentration, enzyme loading, substrate, and reaction time. Temperature was fixed at 50°C, the optimum for enzymatic hydrolysis (Selig et al., 2008). Solids concentration is the biomass loaded relative to the total reaction volume, expressed as percent. The investigated solids concentrations were

5, 10, and 15%. The 15% concentration had so little free liquid that accurate and repeatable testing were impossible. The 5% samples were well-mixed and easy to sample, but were not chosen because the concentration is too dilute to be economical at industrial scale. Therefore, 10% became the standard solids concentration for all future research.

A matrix of substrates and reaction times were investigated. The substrates included recycled office paper, clean shredded printer paper, Solka-Floc, and α-cellulose. The time points ranged from 1 to 5 days. To ensure accuracy, each sample point was repeated in triplicate.

The tubes were assumed to be water-tight. This was determined to be a sound assumption by filling six tubes with DI H_2O, three with 20−21 mL and three with 35−36 mL. The starting weights for each loaded tube were measured and they were placed in standard incubator conditions for approximately one month. Periodically, the tubes were removed, allowed to cool, and weighed to measure losses (Table 3-1). It was determined that negligible leakage occurred at the operating conditions.

Table 3-1 Leak test results

		Tube + water mass (g)			(Tube ~ 13.2g)		Final Change (g)
Tube #	Day 0	Day 1	Day 4	Day 5	Day 7	Day 34	
1	33.7	33.7	33.7	33.6	33.6	33.4	0.3
2	33.1	33.1	33.1	33.1	33.1	32.9	0.2
3	33.8	33.8	33.7	33.7	33.7	33.5	0.3
4	48.7	48.7	48.6	48.6	48.6	48.4	0.3
5	48.1	48.1	48.1	48.1	48.1	47.9	0.2
6	48.4	48.4	48.4	48.4	48.4	48.2	0.2

Once the parameters of solids concentration, temperature, and time were selected, the substrate was selected to standardize testing. This selection was made using standard procedures (Selig et al., 2008). The initial study variables are summarized in Table 3-2.

Table 3-2 Preliminary batch study summary

System Variable	Printer Paper	Recycled Paper	α-Cellulose Powder	Solka-Floc
Incubation Temperature (°C)	50	50	50	50
Reaction Time (days)	5	5	5	5
Solids Concentration (% by mass)	10	10	10	10
Dry Mass Loaded (g)	2	2	2	2
Enzyme Loading (mg protein/g biomass)	10	10	10	10

Based on several factors, including liquid absorption, extent of mixing, and free-sugar production, Solka-Floc and α-cellulose powder outperformed each paper substrate. Because it had similar performance and lower cost, Solka-Floc was selected for further batch studies.

To determine a digestibility baseline for Solka-Floc, a secondary study was performed by varying the enzyme loading and reaction time. As previously stated, 10% solids concentration exhibited good mixing during incubation and excellent separation after centrifugation, and therefore was the standard for this study. Reaction time points ranged from hours to many days (Table 3-3).

Table 3-3 Time course batch study summary

Target Dry Mass	2.0 g								
Time (h)	1	3	6	12	24	72	120	192	336
Enzyme Loading (mg protein/ g biomass)	1	1	1	1	1	1	1	–	–
	2	2	2	2	2	2	2	2	2
	5	5	5	5	5	5	5	5	5
	10	10	10	10	10	10	10	10	10
	–	–	–	–	–	–	–	25	25

This study produced curves showing final sugar concentration versus time and provided baseline digestibility for this substrate at various enzyme loadings, which is critical to accurately evaluate results from later experiments.

3.4 Results and Discussion

3.4.1 Substrate Selection Results and Decision

To select a suitable substrate for digestibility studies, candidates included shredded printer paper; shredded, used office paper; shredded Whatman ashless filter paper circles; and Solka-Floc. In previous fermentation experiments, shredded paper had been used previously as a carbon source (Golub, 2012).

For a substrate to be considered acceptable, a number of criteria had to be met. To ensure thorough mixing, the key to even distribution of enzyme and sugars, the substrate had to have free liquid remaining once it had reached moisture equilibrium with the reaction mixture. At 10% solids concentration, Solka-Floc had substantial free liquid, whereas shredded office paper and filter paper absorbed a much higher

percentage of liquid. At the end of the reaction, the same held true. Only some paper samples with high enzyme loadings produced appreciable free liquid for sampling.

An equally important criteria was a favorable rate of digestion at the enzyme loadings tested. Ideal substrates had to exhibit measurable digestion at the end of the reaction period, but not digest so rapidly that it would be impossible to differentiate gains from changes in system variables. In this regard, paper samples once again underperformed Solka-Floc. To explain the performance gap in substrates, the sample composition was determined (Table 3-4). Solka-Floc and office paper were the best and worst performers, respectively, in the mixing and free-liquid test.

Table 3-4 Substrate compositions

Substrate	Paper				Solka-Floc			
Replicate	1	2	**Average**	Std. Dev.	1	2	**Average**	Std. Dev.
Glucan (%)	55.44	55.31	**55.38**	0.09	77.33	77.37	**77.35**	0.03
Xylan (%)	11.25	11.21	**11.23**	0.03	14.02	14.26	**14.14**	0.17
Galactan (%)	0.74	0.67	**0.71**	0.05	0.62	0.62	**0.62**	0.00
Arabinan (%)	0.00	0.00	**0.00**	0.00	0.00	0.00	**0.00**	0.00
Mannan (%)	0.00	0.00	**0.00**	0.00	0.00	0.00	**0.00**	0.00
Acid-Insoluble Lignin (%)	6.11	6.52	**6.32**	0.29	0.00	0.00	**0.00**	0.00
Acid-Soluble Lignin (%)	0.67	0.65	**0.66**	0.01	0.00	0.00	**0.00**	0.00
Ash (%)	11.59	12.68	**12.14**	0.77	0.00	0.00	**0.00**	0.00
Water Extractives (%)	3.60	3.60	**3.60**	0.00	–0.09	–0.09	**–0.09**	0.00
Ethanol Extractives (%)	0.58	0.58	**0.58**	0.00	0.79	0.79	**0.79**	0.00
Total Extractives (%)	4.18	4.18	**4.18**	0.00	0.70	0.70	**0.70**	0.00
Closure (%)	89.98	91.22	**90.60**		92.67	92.95	**92.81**	

Not only did the Solka-Floc have a higher content of digestible material than shredded paper (92.1% versus 67.3%), it was also ash-free and pure. One likely reason for the

poor performance of shredded paper was the presence of inhibitory compounds such as printer toner, pen ink, and staple fragments.

3.4.2 Enzyme Load and Maximum Yield

Initially, the investigation was limited to four enzyme loadings with a 5-day reaction period, the standard reaction time in accepted procedures (Selig et al., 2008). This more limited set of variables was tested, and the resulting data were used to generate curves of product sugar concentration over time (Figure 3-3).

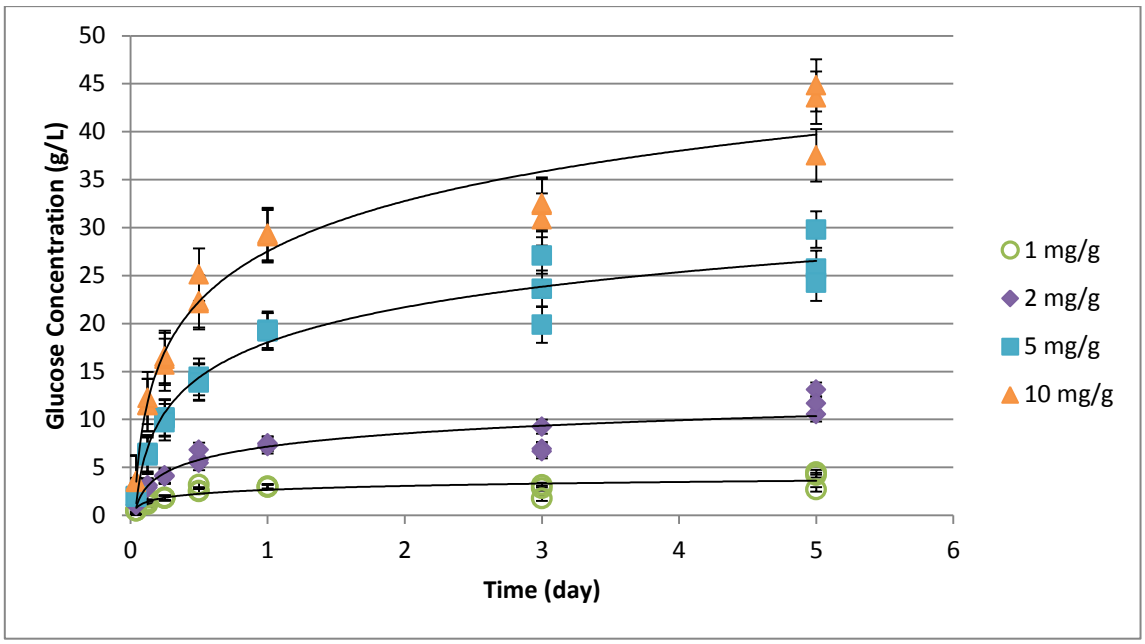

Figure 3-3 Initial batch study results for Solka-Floc. Error bars represent standard error within triplicate samples.

Although the hydrolysis curves have clearly begun to flatten at lower enzyme loadings (1 and 2 mg/g), there is still significant change occurring at higher enzyme loadings. Based on this observation, additional assays were conducted at longer reaction times and higher enzyme loadings (Figure 3-4).

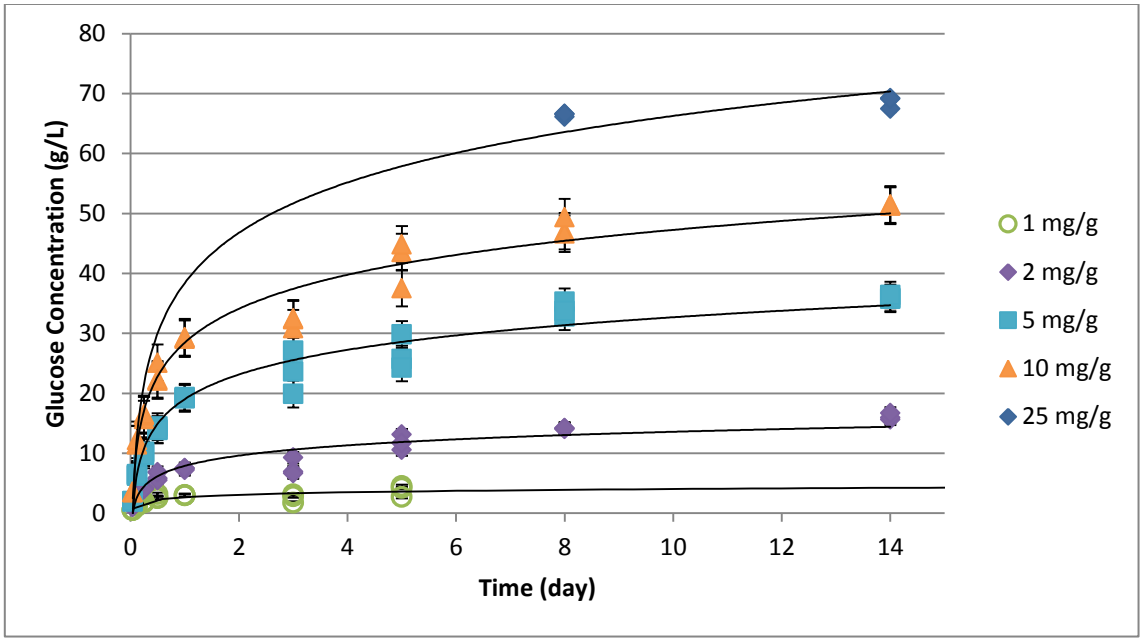

Figure 3-4 Batch study results with extended reaction times for Solka-Floc. Error bars represent standard error of triplicate samples.

With the additional points, sugar concentrations for 2 and 5 mg/g enzyme loadings have leveled off, indicating almost complete exhaustion of available enzyme activity after 14 days. To verify this observation, logarithmic fit lines were used to predict sugar concentrations if reactions continued an additional 14 days. The predicted values show absolute improvements of approximately 0.055% and 3.316% for 2 and 5 mg/g enzyme loadings, respectively.

Later studies utilized α-cellulose from Sigma Aldrich (C8002-5KG), assumed to perform similarly to Solka-Floc. To confirm this assumption, samples of α-cellulose underwent batch saccharification at reaction times from 1−12 hours. These data points were overlaid with the Solka-Floc results (Figure 3-5).

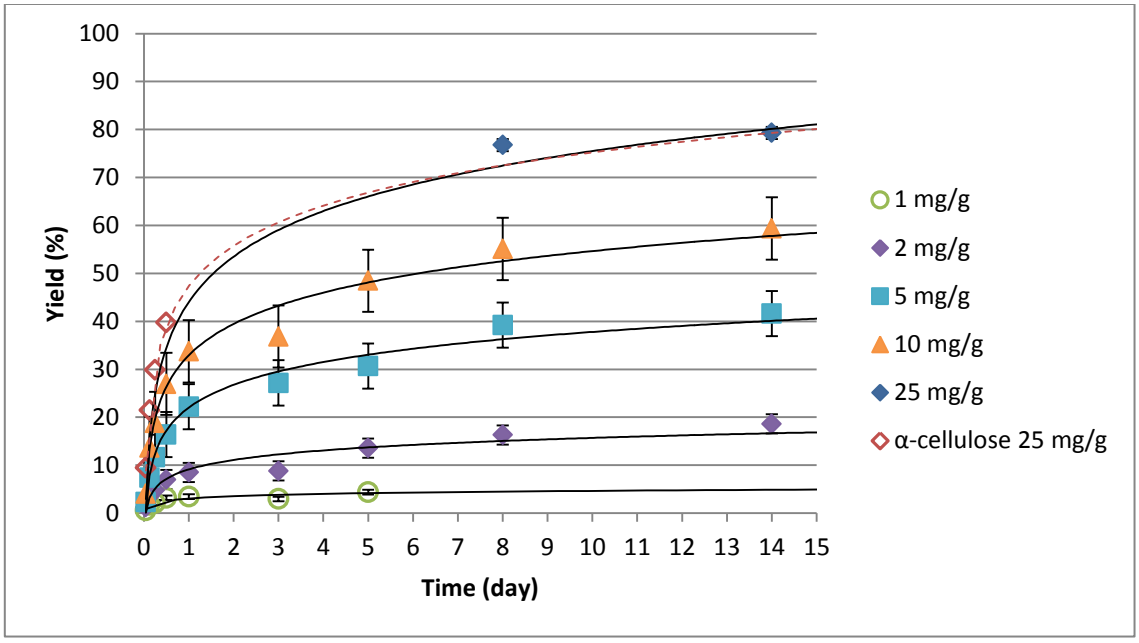

Figure 3-5 Batch saccharification yield for Solka-Floc (solid line) and α-cellulose (dashed line). Error bars represent standard error of averaged yield data.

The α-cellulose data points fit very closely to the curve for Solka-Floc at the same enzyme load (25 mg/g). Furthermore, the forecasted curve for α-cellulose is nearly identical to the same curve for Solka-Floc. Composition analysis of α-cellulose revealed further similarity to Solka-Floc (Table 3-5). From these data it was concluded that α-cellulose powder and Solka-Floc were sufficiently similar, and that α-cellulose would be used in countercurrent studies.

Table 3-5 Comparison of α-cellulose and Solka-Floc compositions

Substrate	α-cellulose		Solka-Floc			
Replicate	**Average**	Std. Dev.	1	2	**Average**	Std. Dev.
Glucan (%)	**78.51%**	0.02%	77.33%	77.37%	**77.35%**	0.03%
Xylan (%)	**14.38%**	0.25%	14.02%	14.26%	**14.14%**	0.17%
Galactan (%)	**1.37%**	0.06%	0.62%	0.62%	**0.62%**	0.00%
Arabinan (%)	**0.00%**	0.00%	0.00%	0.00%	**0.00%**	0.00%
Mannan (%))	**0.00%**	0.00%	0.00%	0.00%	**0.00%**	0.00%
Acid-Insoluble Lignin (%)	**0.00%**	0.00%	0.00%	0.00%	**0.00%**	0.00%
Acid-Soluble	**0.00%**	0.00%	0.00%	0.00%	**0.00%**	0.00%
Ash (%)	**0.00%**	0.00%	0.00%	0.00%	**0.00%**	0.00%
Water Extractives (%)	**–**	0.00%	-0.09%	-0.09%	**-0.09%**	0.00%
Ethanol Extractives (%)	**–**	0.00%	0.79%	0.79%	**0.79%**	0.00%
Total Extractives (%)	**0.79%**	0.00%	0.70%	0.70%	**0.70%**	0.00%
Closure (%)	**95.05%**		92.67%	92.95%	**92.19%**	

The sugar concentration data, along with compositional data and substrate concentration in each sample, were used to calculate the yield. During the initial batch saccharifications (0−5 day reaction time), the HPLC standards used only glucose, an oversight corrected in later (5−14 day reaction time) studies; therefore, the yields are reported as g glucose produced/g glucan fed. These values are calculated using Equations 3-2 and 3-3.

$$\text{Yield}_{\text{glucose}} = \frac{[\text{Glucose}]_{\text{sample}}}{[\text{Glucose}]_{\text{max}}} \tag{3-2}$$

$$[\text{Glucose}]_{\text{max}} = \frac{\left(\text{GluC}\cdot\text{Biomass}_{\text{dry}}\cdot\text{Hydro}_{glucan}\right)}{V_{reac}} \tag{3-3}$$

where,

$[Glucose_{sample}]$ = concentration of glucose in sample, blanks subtracted (g/L)

$[Glucose_{max}]$ = calculated max glucose concentration, assumed 100% digestion (g/L)

GluC = fraction of glucan content of dry biomass (g glucan/g dry biomass)

$Biomass_{dry}$ = dry biomass loaded in sample prior to reaction (g)

$Hydro_{glucan}$ = mass gain correction for water of hydrolysis of glucan (1.111)

V_{reac} = reaction volume of test tube (0.020 L in this study)

With these equations, glucose yields could be calculated averaging the yields across triplicate samples and including predicted longer-term yields, where applicable (Table 3-6).

Table 3-6 Summary of yield calculations and predictions for batch data

Time (h)	Label	Enzyme Loading (mg/g)				
		1	2	5	10	25
1	1H	0.58%	1.24%	2.29%	4.08%	–
3	3H	1.71%	3.53%	7.38%	13.65%	–
6	6H	2.11%	4.75%	11.63%	18.80%	–
12	12H	3.20%	7.02%	16.37%	26.95%	–
24	1D	3.45%	8.51%	22.21%	33.74%	42.53%
72	3D	2.97%	8.79%	27.17%	36.83%	57.08%
120	5D	4.38%	13.59%	30.69%	48.46%	63.85%
192	8D	–	16.31%	39.20%	55.10%	76.77%
336	14D	–	18.61%	41.62%	59.37%	79.29%
672	28D		18.66%	44.94%	64.48%	86.53%
Values predicted using exponential fit lines						

By the end of the reaction time studied, the 2 mg/g loading had run its course. In contrast, the 5 mg/g loading showed potential enzyme activity may remain even after 14 days. These data show that improvements to the sugar platform are possible by making full use of enzymes. Figure 3-6 shows the yields as a function of enzyme loading; this figure will be directly compared to future countercurrent data.

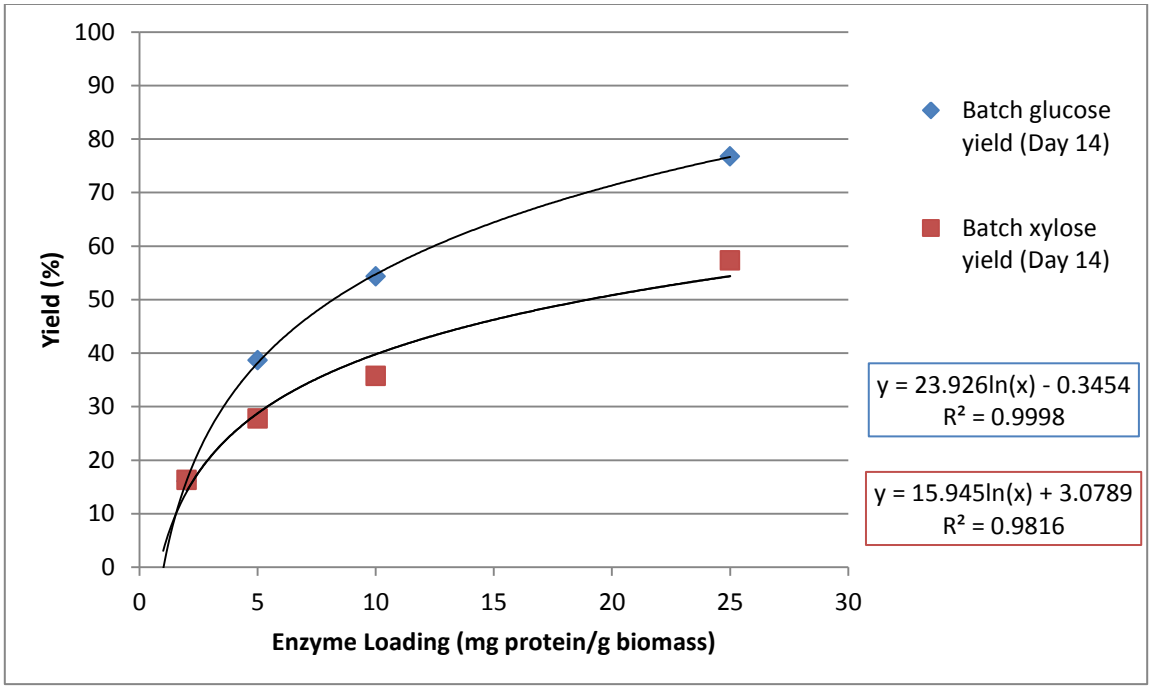

Figure 3-6 Glucose and xylose yields at 14 days as a function of enzyme loading. Smooth curve is a logarithmic fit.

3.5 Conclusion

Solka-Floc outperformed all tested alternatives in target liquid retention and digestibility, with much greater potential yields than paper. Because this material is widely used in the food industry and elsewhere, it is easy to secure large quantities at fairly low cost, which avoids problems with lot-to-lot variations.

To establish the baseline, the sugar yields at multiple reaction times and enzyme loadings were measured. There was remaining enzyme activity in all samples, except those with the lowest enzyme loading, which indicates that there is a substantial amount of enzyme activity that is discarded in batch reactions. Therefore, investigating how to more efficiently utilize the potential of these discarded enzymes seems prudent, which is

imperative to improving the economics of the sugar platform. The remaining studies presented here investigated one such operational improvement: countercurrent saccharification.

CHAPTER IV

COUNTERCURRENT ENZYMATIC SACCHARIFICATION

4.1 Introduction

In research, enzymatic saccharification of lignocellulose is used to quantify substrate digestibility under prescribed conditions, potentially as a measure of pretreatment efficacy. In industry, the same process is used to produce sugars from highly digestible biomass for use in ethanol fermentation and as nutritional supplements in foodstuffs and animal feed (a l sz , L sztity, 1 1). In both research and industry, enzymatic saccharification of lignocellulose is performed in batch operations, where biomass, buffer, water, enzymes, and sterilization agents are added simultaneously and allowed to react for a fixed time period. In the laboratory, the standard reaction time is 3–7 days, with 5 days being a common standard (Selig et al., 2008). In this batch mode of operation, the long time and high enzyme requirements can reduce the commercial viability of lignocellulose feedstocks because it requires multiple large reactor vessels be staged so that product is produced continuously by the plant. Further, leftover, active enzymes are discarded or destroyed in the subsequent treatment of the sugar solution product.

An attractive alternative to batch operation is a continuous reaction that produces product continuously. Frequently, countercurrent flow is the ideal case for systems that exchange energy or reactants and products, and this type of system offers advantages for enzymatic saccharification as well. During saccharification, biomass substrate becomes

recalcitrant towards further digestion because easy-to-digest components are hydrolyzed and enzymes are inhibited by high sugar concentrations. To counter these obstacles, the sugar-rich liquid phase and the enzyme-rich solid phase are contacted countercurrently, thereby increasing contact time between undigested biomass and enzyme. Also, sugars flow away from less digestible biomass, which decreases product inhibition.

For this study, the goals follow:

- Determine operating parameters to ensure consistent material transfers and to achieve steady-state conditions within a reasonable amount of time.
- Identify variables that can be easily controlled and their effect on system performance.
- Gauge the performance of a countercurrent system and how it compares to batch operation.
- Develop a platform on which future studies can be performed to investigate biomass treatment methods, operating conditions, and antibiotic alternatives.

4.2 Materials and Methods

4.2.1 Equipment

On a laboratory scale, it is difficult to replicate a commercial-scale countercurrent reactor vessel, so a series of small semi-continuous vessels are used to approximate the same conditions. To mimic the operating conditions of a large-scale vessel, smaller reaction vessels were placed within a custom-made standing incubator cabinet with rotating PVC pipe sleeves with a nominal ID of 4 inches. Within these sleeves, 1-L Nalgene centrifuge bottles from Fisher (catalog # 05-562-26) with a

nominal OD of 3.75 inches were housed allowing for continuous axial rotation at approximately 2 rpm. Axial rotation provided adequate mixing provided there was sufficient free volume and appropriate solid concentrations were used. Unlike MixAlco fermentations, no mixing bar was required to achieve good agitation.

4.2.2 Substrate

The substrate used for all countercurrent experiments was α-cellulose from Sigma Aldrich (C8002-5KG). A single bag, initially containing approximately 5 kg of cellulose powder, was sufficient for all studies in countercurrent operation, and later, in antibiotic studies. Compositional analysis of the substrate confirmed it was ash-free, lignin-free, and contained minimal non-digestible extractives. Table 4-1 gives the compositional data obtained, including deviation between replicates.

Table 4-1 Composition of α-cellulose

Replicate	**Average**	Std. Dev.
Glucan (%)	**78.51**	0.02
Xylan (%)	**14.38**	0.25
Galactan (%)	**1.37**	0.06
Arabinan (%)	**0.00**	0.00
Mannan (%)	**0.00**	0.00
Acid-Insoluble Lignin (%)	**0.00**	0.00
Acid-Soluble Lignin (%)	**0.00**	0.00
Ash (%)	**0.00**	0.00
Water Extractives (%)	**–**	0.00
Ethanol Extractives (%)	**–**	0.00
Total Extractives (%)	**0.79**	0.00
Closure (%)	**95.05**	

The α-cellulose has extremely similar composition to Solka-Floc (Table 3-5), and was selected to replace it on other merits. Solka-Floc tended to clump easily and was

susceptible to static charge when weighing samples into plastic or glass containers. This static cling caused some material to stick to the top or outside lip of a test tube or bottle. Once the charge dissipated, the material would fall from the container, resulting in mass losses. Besides producing less measurement errors, α-cellulose was perceived to be a more regulated and consistent substrate because lot information and material grade could be tracked through its manufacturer.

4.2.3 Citrate Buffer

To buffer the reaction mixture to the optimal pH range, a sodium citrate buffer solution was used. A 0.1-M solution with a pH of 4.8 was prepared in large quantities to be used throughout saccharification experiments (Appendix D).

4.2.4 Antibiotics

Antibiotic solutions were prepared from the pure, powder forms of tetracycline and cycloheximide. Great care was observed when handling either of the powders, or the solutions of these antibiotics, because they are both toxic. Tetracycline was dissolved in a mixture of ethanol and water, 70% ethanol by volume. The tetracycline solution was prepared at a concentration of 10 g powder/L. This solution was stored in the freezer. Cycloheximide was dissolved in DI H_2O, also at a concentration of 10 g powder/L. Cycloheximide was stored in the refrigerator to prevent freezing (Appendix E).

4.2.5 Enzyme Solution

The enzyme used in all experiments was Novozyme Ctec2 (lot # VCPI 0007), a blend of cellulases, hemicellulases, β-glucosidase, and other stabilizing proteins and sugars. The protein content of the stock enzyme solution was determined to be 294 mg

protein/mL using the Pierce BCA assay. Using the filter paper unit (FPU) assay, the filter paper activity of the CTec2 was measured as 225 ± 20 FPU/mL, equivalent to a specific activity of 765 FPU/g. To reliably provide equal dosage in experiments, the syrupy enzyme stock was diluted with DI H_2O by a factor of 10 prior to use (Appendix G).

4.2.6 1-Liter Bottles

Countercurrent studies and blanks used 1-L Nalgene centrifuge bottles as reaction vessels. To prevent leaks, the open bottles were covered with a single layer of Parafilm and then the screw-top lid was secured. The bottles were filled only 30–40% of their maximum volume, which provided excellent mixing without the need for a mixing bar commonly used in MixAlco fermentations. For clean separation, centrifuging at 3000 rpm for 5 min was sufficient. This low speed prevented bottle failure.

4.2.7 Transfer Procedures

Transfers consisted of moving liquid from "back" to "front" of each train, and moving a specified amount of solids in the opposite direction. First, bottles were weighed to ensure no significant losses had occurred since the last transfer and this mass was recorded. Then, bottles were centrifuged to separate the phases; liquid was poured out to measure liquid volume and mass. Solid mass was determined by subtracting the known empty bottle weights from the observed mass of bottle plus wet cake. At this stage, the pH of the separated liquid was measured to ensure each vessel was operating within the acceptable pH range for the enzyme. For Novozyme Ctec 2, the acceptable

p r ange is 4.5–5.1; therefore, corrective action was to be taken if samples were outside of 4.6–5.0.

At this point, transfers begin with the solid phase by targeting a wet cake mass in each vessel. For the first stage, 10 g of dry biomass was to be added during each transfer; therefore, the targeted amount of wet cake to be removed was the difference between the observed wet cake mass and the target plus the incoming solids. Wet cake was removed to a plastic weighing boat and set aside to be transferred to the next bottle. Once the target wet cake was achieved and verified, liquid from the following stage could be added (e.g., liquid from Bottle 3 transfers into Bottle 2). In the case of liquid, all free liquid was transferred each time and a 1-mL sample was taken from each stage for analysis by high performance liquid chromatography (HPLC). At the final stage, make-up liquid was added to maintain the same solids concentration of 10%. For 10 g of dry solids, this meant that 90 g of liquid (50 g of citrate buffer and 40 g of water) must be added (Appendix H).

4.2.8 Termination of Countercurrent Trains

When steady state was achieved and maintained for the desired period of time, trains were terminated following a modified version of transfer procedures. As in standard transfers, bottles were weighed to determine slurry mass and then centrifuged. In addition to normal 1-mL samples, a reserve liquid sample of 30–40 mL was stored in 50-mL conical bottomed centrifuge tubes and placed in the freezer. These large reserves allowed for future testing to verify results or composition. Additionally, instead of transferring solids as per transfer procedures, wet cake samples were taken and stored in

50-mL conical bottomed centrifuge tubes for moisture content testing for use in final mass balances. Once all reserve samples had been obtained, the 1-L Nalgene bottles were flushed of remaining substrate, rinsed repeatedly, and sterilized for use in future studies.

4.3 Experimental Design

4.3.1 Selection of Conditions

For countercurrent studies, operating conditions were chosen by analyzing the data obtained from previous batch studies. It was desired to operate at relatively low enzyme loadings and to reach steady state in a reasonable amount of time. Based on these constraints and the data available, the decision was made to simultaneously investigate two enzyme loadings: 2 and 5 mg of protein/g of dry biomass (mg/g). These loadings determined the initial dose of enzyme, as well as the amount to be added during each transfer.

Based on a preliminary study of the countercurrent system and transfer procedures, a target wet cake weight of 85 g and 80 g was selected for the 2 and 5 mg/g trains, respectively. In the 5 mg/g train, the target weight required slight adjustments to assure continuous solids and liquids transfer occurred.

4.3.2 Scale

In these countercurrent studies, an important consideration was the number of bottles required to fully utilize the potential of the CTec2 enzyme. The goal was to use a sufficiently large number of stages such that enzymes in the first and last stages of a given train had little remaining activity when they exited the system. This objective was

balanced against available man hours, resources, and space. To allow for multiple conditions to be tested simultaneously, a train of eight bottles was used.

As with batch studies, solids loading is also an important factor. There needs to be ample void volume to allow for mixing as well as a sufficiently large slurry volume to allow for clean separation of phases and accurate measurement of volumes and masses. To these ends, each bottle was initially loaded with 250 g of total materials (Tables 4-2 and 4-3).

Table 4-2 Train 1 Initial Loading

2 mg/g Initial Loading								
Bottle	B1	B2	B3	B4	B5	B6	B7	B8
0.1-M Citrate Buffer (mL)	125	125	125	125	125	125	125	125
Water (mL)	96	96	96	96	96	96	96	96
Substrate (g)	23.67	23.65	23.66	23.66	23.67	26.69	26.66	23.68
Tetracycline (mL)	2	2	2	2	2	2	2	2
Cycloheximide (mL)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Dilute (10:1) CTec2 (mL)	1.701	1.701	1.701	1.701	1.701	1.701	1.701	1.701

Table 4-3 Train 2 Initial Loading

5 mg/g Initial Loading								
Bottle	B1	B2	B3	B4	B5	B6	B7	B8
0.1-M Citrate Buffer (mL)	125	125	125	125	125	125	125	125
Water (mL)	93	93	93	93	93	93	93	93
Substrate (g)	24.25	24.25	24.25	24.25	24.25	24.25	24.25	24.25
Tetracycline (mL)	2	2	2	2	2	2	2	2
Cycloheximide (mL)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Dilute (10:1) CTec2 (mL)	4.253	4.253	4.253	4.253	4.253	4.253	4.253	4.253

4.3.3 Addition of Enzymes, Nutrients, and Antibiotics

To more quickly reach steady-state conditions in a given train, each bottle was loaded identically as indicated in Tables 4-2 and 4-3. This procedure allowed for sugar

concentrations to climb rapidly during the first three to five days of operation, leveling off as the initial dose of enzymes became thermally degraded. To reach steady state, additional substrate, liquid, and enzyme were added at appropriate feed locations. The additions of solids and liquids were straightforward, occurring at opposite ends of each train.

Based on literature, the enzymes have an affinity for the solid substrate; therefore, the enzyme addition location should be near the "front" of each train where fresh solids were added during each transfer (Jeffries, Schartman, 1999). Therefore, fresh CTec2 enzyme was introduced to Bottles 4 and 5 in the 2 and 5 mg/g trains, respectively. In future studies, this addition location is an important operating variable that can be changed to modify system performance.

For this study, an important consideration was to maintain sterility to ensure that neither product nor enzyme was lost to bacterial growth. The antibacterial cocktail was potent but, similar to the enzymes themselves, was subject to thermal degradation over time. To counteract this loss of potency, a booster dose of antibiotics was administered to each bottle on each transfer day. Ideally, the dose would exactly replace antibiotics lost to degradation each transfer, but this exact dosage was not known. Therefore, the goal was to operate between the minimum dose required to prevent microbe activity and the maximum dose that prevents enzyme inhibition. Based on observations during batch studies, as well as the retention time of liquid in the system, the booster dose was fixed as 20% of the initial dose, administered during each transfer.

4.3.4 Prediction of Steady State from Observations

Steady state can be determined with absolute confidence by analyzing concentration data provided by HPLC analysis; however, there were numerous surrogate parameters that could be observed during transfers that indicated the system was approaching or operating at steady state. Although simply suggestive, these trends were good indicators of when more immediate HPLC analysis of collected samples was prudent.

The first observable parameter was the color of sampled liquids. As the system moved towards steady state, the color gradient from start to end of a train became quite marked. A more precise indicator was pH measurements taken during transfers. As the sugar concentration began to level off in each bottle, the pH stabilized. Although not directly correlated with the sugar concentration in a liquid sample, the pH trend from Bottles 1 through 8 showed less variation as the system approached steady state. The final observation was an oscillation of mass transfer targets, which was initially believed to be an error. As systems began to operate under steady-state conditions, they frequently exhibited the behavior of variable, but predictable, wet-cake transfer targets. During a given transfer, the initial stage would require a large mass transfer (e.g., 45 g of wet cake), but during the subsequent transfer, the same stage would require a much smaller mass transfer (e.g., 10 g of wet cake). Although the exact cause of this oscillation is unknown, the phenomenon has been observed in countercurrent mixed-acid fermentations as well.

4.4 Results and Discussion

4.4.1 Analysis of Data

The countercurrent system provided a wealth of data useful for determining its performance, both observable parameters and sugar concentrations. When analyzing these data, the first step was to track observable parameters during transfer procedures. These trends helped predict when the system was approaching steady state and provided some insight into overall performance. During transfers, a spreadsheet was used to record parameters such as mass of each phase, liquid pH, solids transferred, and solids added. In addition to the directly observable values, the moisture content of exiting waste solids was determined for many samples. Liquid samples taken from each bottle during transfers were analyzed by HPLC to measure sugar concentrations. As data were gathered, each variable was plotted against time. To be considered steady state, certain variables must have stabilized, especially sugar concentration in each bottle. Prior to this determination, which was made using data from HPLC analysis, other indicators of system performance (e.g., pH profile, liquid mass transferred, solid mass transfer oscillation) were observed that indicated a train was approaching steady state.

Resources allowing, a 1-mL liquid sample was obtained from every stage during each transfer and labeled with the date, train number, and bottle number. These samples were stored in 2-mL homo-polymer microtubes and frozen in trays until a sufficient number of samples were collected to justify HPLC analysis, necessary to identify steady state. This number was typically between 48 and 80 samples, to which an array of sugar standards and a control verification standard (CVS) were added. Becasue the two, eight-

bottle trains were operated simultaneously, this equated to between three and five transfers, or 6–10 days. Train 3 transfers occurred on the alternate days from Trains 1 and 2, and likewise required 6–10 days to accumulate sufficient samples for analysis. The procedures for sample preparation and analysis methods previously detailed were followed for these samples. HPLC analysis gave data in the form of a retention time, peak area, and calculated concentration for each tested component. In the case of these studies, the final report included these values for glucose, xylose, and galactose. Using a template spreadsheet to streamline data processing, calculations were made for each sample and the results were accumulated and further processed to determine steady-state operation, absolute sugar production, and final system yield.

4.4.2 Calculations and Slope Method

Equations for calculating product sugar totals and yields were derived from an overall system mass balance, accounting for all entering and exiting streams of mass (Figure 4-1).

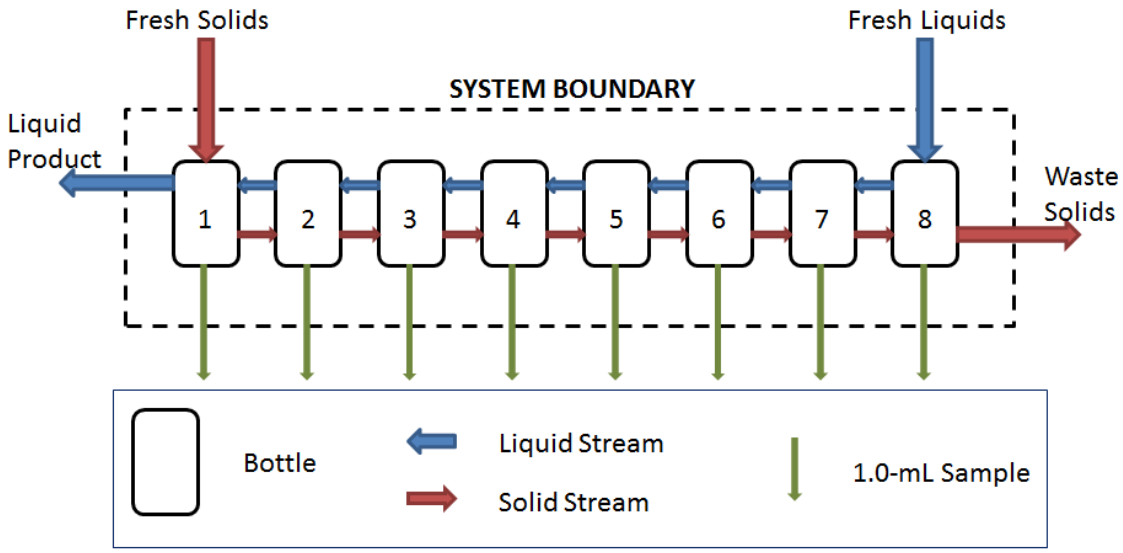

Figure 4-1 System diagram and boundaries for mass balance.

In this model, blue streams indicate liquid flow, red streams indicate solids flow, and green streams indicate liquid samples taken for analysis, with the dashed box indicating the system boundary.

The key values obtained from HPLC analysis were concentrations of various product sugars. By combining these data with parameters monitored during transfers, it was possible to calculate absolute sugar content using Equations 4-1 to 4-6

$$\text{Glucose}_{out} = \text{Glucose}_{L,out} + \text{Glucose}_{S,out} + \text{Glucose}_{samples} \quad (4\text{-}1)$$

$$\text{Glucose}_{L,out} = \left(V_{l,i}/1000\right)[\text{Glucose}_1] \quad (4\text{-}2)$$

$$\text{Glucose}_{S,out} = \left(\left(\frac{L_S}{\rho_L}\right)/1000\right)[\text{Glucose}_8] \quad (4\text{-}3)$$

$$\text{Glucose}_{samples} = \left(\sum[\text{Glucose}_i]\right)V_{sample} \quad (4\text{-}4)$$

$$L_S = (1 - \text{MC})\, S_{out} \quad (4\text{-}5)$$

$$\rho_L = \frac{M_{l,8}}{V_{l,8}} \tag{4-6}$$

where,

Glucose_{out} = Total glucose exiting system (g)

$\text{Glucose}_{L,out}$ = Total glucose exiting in liquid from Bottle 1 (g)

$\text{Glucose}_{S,out}$ = Glucose in wet cake exiting system (g)

$\text{Glucose}_{samples}$ = Glucose in liquid samples from all bottles (g)

$[\text{Glucose}_i]$ = Concentration of glucose in Bottle *i* (g/L)

$V_{l,i}$ = Volume of liquid in bottle *i* (mL)

$M_{l,i}$ = Liquid mass in Bottle *i* (g)

L_S = Liquid retained in exiting waste solids (g)

S_{out} = Waste solids exiting system (g)

MC = Moisture content of exiting waste solids (g dry solids/g wet solids)

ρ_L = Density of free liquid (g/mL)

V_{sample} = Volume of liquid samples (0.001 L)

From these equations, the total quantity of glucose exiting the system was calculated for each transfer day. The process was repeated to find xylose and galactose production. All three values were then combined to find the total sugar produced by the system on a given transfer day.

Analysis of steady-state data was performed using the Slope Method. This method has a significantly lower error than the Average Method, and is more informative than the Accumulation Method, especially when analyzing long-running

countercurrent systems (Smith 2011). Once steady state was reached, these systems required a quick turn-around, so additional studies could be performed while resources and labor were available. It was imperative that data be analyzed quickly after they were collected. To this end, the template for analysis was tailored to handle approximately one week of data at a time, providing a four-point snapshot of system performance that could then be pieced together as it was collected.

To validate the determination of steady state, sugar production data from the time period believed to be steady state were analyzed with regression formulas as outlined in Equations 4-7 to 4-12 (Skoog et al., 1996) to calculate the slope *m* and the standard deviation of the slope D_m.

$$m = \frac{S_{xy}}{S_{xx}} \tag{4-7}$$

$$D_m = \sqrt{\frac{D_r^2}{S_{xx}}} \tag{4-8}$$

where,

x_i = time data

y_i = moving-cumulative-sum data

N = number of (x_i, y_i) data points

$$S_{xy} = \sum x_i y_i - \frac{\sum x_i \sum y_i}{N} \tag{4-9}$$

$$S_{xx} = \sum x_i^2 - \frac{(\sum x_i)^2}{N} \tag{4-10}$$

$$S_{yy} = \sum y_i^2 - \frac{(\sum y_i)^2}{N} \tag{4-11}$$

$$D_r = \sqrt{\frac{S_{yy} - m^2 S_{xx}}{N-2}} \tag{4-12}$$

In Microsoft Excel, these equations can be written as follows using Equations 4-13 and 4-14 to calculate the slope m and standard deviation of the slope D_m, respectively.

$$m = \textbf{SLOPE}(Yi, Xi) \tag{4-13}$$

$$D_m = \textbf{SQRT}(\textbf{STEYX}(Yi, Xi)\text{^}2/((\textbf{COUNT}(Yi)\text{-}1)*\textbf{STDEV}(Xi)\text{^}2)) \tag{4-14}$$

where X_i and Y_i are the x_i and y_i data arrays, respectively, and Microsoft Excel function calls are in bold. It is important for time data to be in the form of experiment day, with $d = 0$ being the day on which the system was initialized.

4.4.3 Cumulative System Data and Performance Analysis

Gathering all collected data into a cumulative table allowed for direct comparison of trends within various parameters between trains. This cumulative results table also tracked instantaneous yield of the system, both in terms of glucose only and in terms of total sugar produced as calculated in Equations 4-14 to 4-20.

$$\text{Sugar}_{out} = \text{Glucose}_{out} + \text{Xylose}_{out} + \text{Galactose}_{out} \tag{4-14}$$

$$\text{Biomass}_{dry} = (1 - \text{MC})\ \text{Biomass}_{fed} \tag{4-15}$$

$$\text{Biomass}_{digestible} = \text{Biomass}_{dry}\ \text{DC} \tag{4-16}$$

$$Biomass_{equiv} = \text{Biomass}_{dry}\big((1.11\ \text{GluC}) + (1.36\ \text{XylC}) + (1.11\ \text{GalC})\big) \tag{4-17}$$

$$\text{DC} = \text{GluC} + \text{XylC} + \text{GalC} \tag{4-18}$$

$$\text{Yield}_{inst} = \frac{\text{Sugar}_{out}}{\text{Biomass}_{equiv}} \tag{4-19}$$

$$\text{Yield}_{total} = \frac{\sum \text{Sugar}_{out}}{\sum \text{Biomass}_{equiv}} \tag{4-20}$$

where,

Sugar_{out} = total sugar removed from system on given day (g)

Glucose_{out} = glucose removed from system on given day (from Eq 4-1) (g)

Xylose_{out} = xylose removed from system on given day (from Eq 4-1) (g)

Galactose_{out} = galactose removed from system on given day (from Eq 4-1) (g)

Biomass_{dry} = actual dry biomass fed to system on given day (g)

Biomass_{fed} = measured mass of biomass fed to system on given day (g)

$\text{Biomass}_{digestible}$ = digestible material fed to system on given day (g)

Biomass_{equiv} = digestible biomass including required water of hydrolysis (g)

DC = digestible content (g digestible material/g dry solid)

GluC = glucan content (g glucan/g dry solid)

XylC = xylan content (g xylan/g dry solid)

GalC = galactan content (g galactan/g dry solid)

Yield_{inst} = instantaneous yield for given day
(g sugar/g equivalent sugar in biomass)

Yield_{total} = yield over entire operational period
(g sugar/g equivalent sugar in biomass)

To measure the digestibility of any one biomass component, the above equations were modified. Glucose yield from glucan fed was an important parameter because only glucose production was measured in the first set (0−5 day reaction time) of batch saccharifications. These yields were calculated using Equations 4-21 and 4-22.

$$\text{Glucan}_{fed,equiv} = \text{GluC} \cdot \text{Biomass}_{dry} \cdot \text{Hydro}_{\text{glucan}} \quad (4\text{-}21)$$

$$\text{Yield}_{glucose} = \frac{\text{Glucose}_{out}}{\text{Glucan}_{fed,equiv}} \quad (4\text{-}22)$$

where,

$\text{Glucan}_{fed,equiv}$ = equivalent glucose in biomass fed on given day (g)

$\text{Hydro}_{\text{glucan}}$ = mass gain correction for water of hydrolysis of glucan (1.111)

$\text{Yield}_{glucose}$ = instantaneous glucose yield on given day

(g glucose/g equivalent glucose in biomass fed)

4.4.4 Train 1

Countercurrent Train 1 had eight bottles and operated with an enzyme concentration of 2 mg/g. Train 1 was incubated at 50°C for a total of 30 days, with transfers occurring on even-numbered days beginning on Day 2. At the end of each transfer, the target wet cake mass for each bottle was 85 g. Diluted CTec2 was introduced in Bottle 4, fresh liquids entered in Bottle 8, and fresh solids in Bottle 1.

Determination of the steady state was made by plotting concentrations of glucose, xylose, and galactose over time, and observing the change in these concentrations between transfers. Once the concentrations had stabilized and only slight, oscillating variations were observed, the system was operating at steady state. The plots of sugar concentration over time are shown in Figures 4-2 to 4-4.

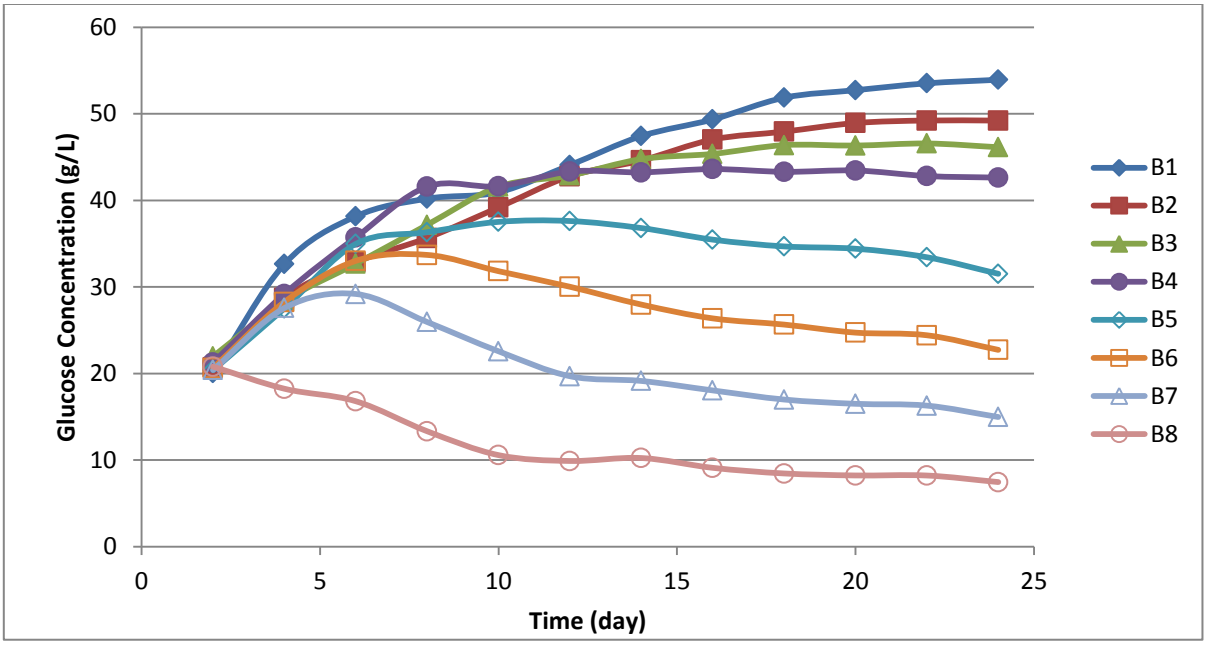

Figure 4-2 Train 1 glucose concentration as a function of time and bottle number.

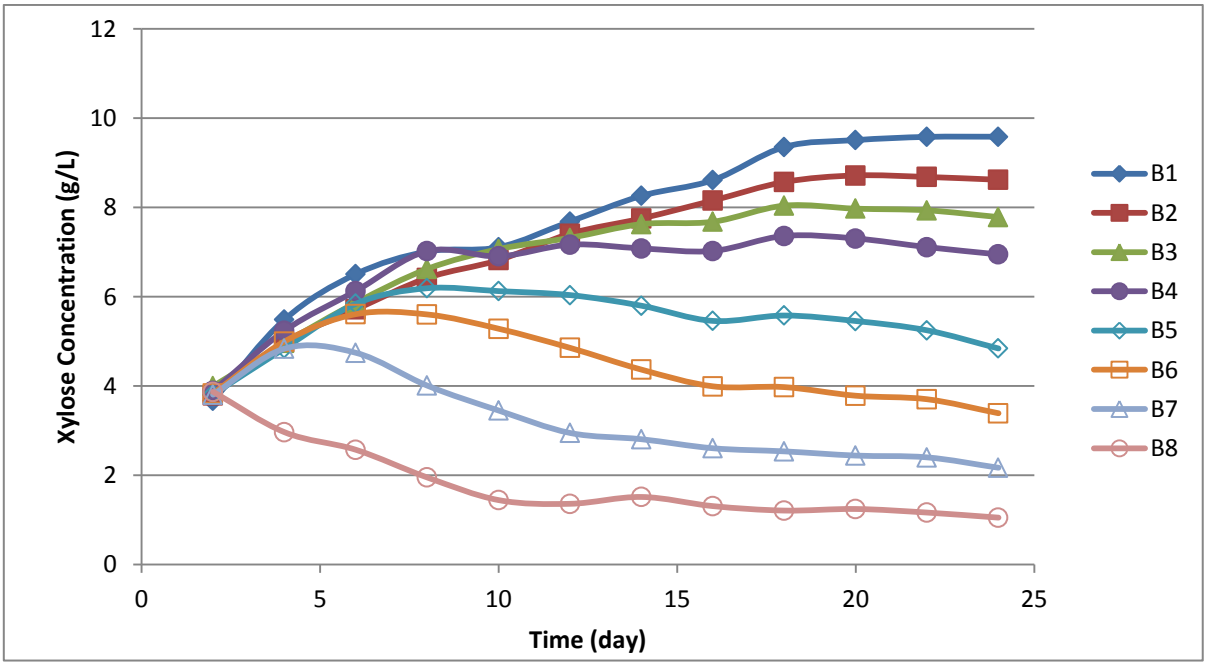

Figure 4-3 Train 1 xylose concentration as a function of time and bottle number.

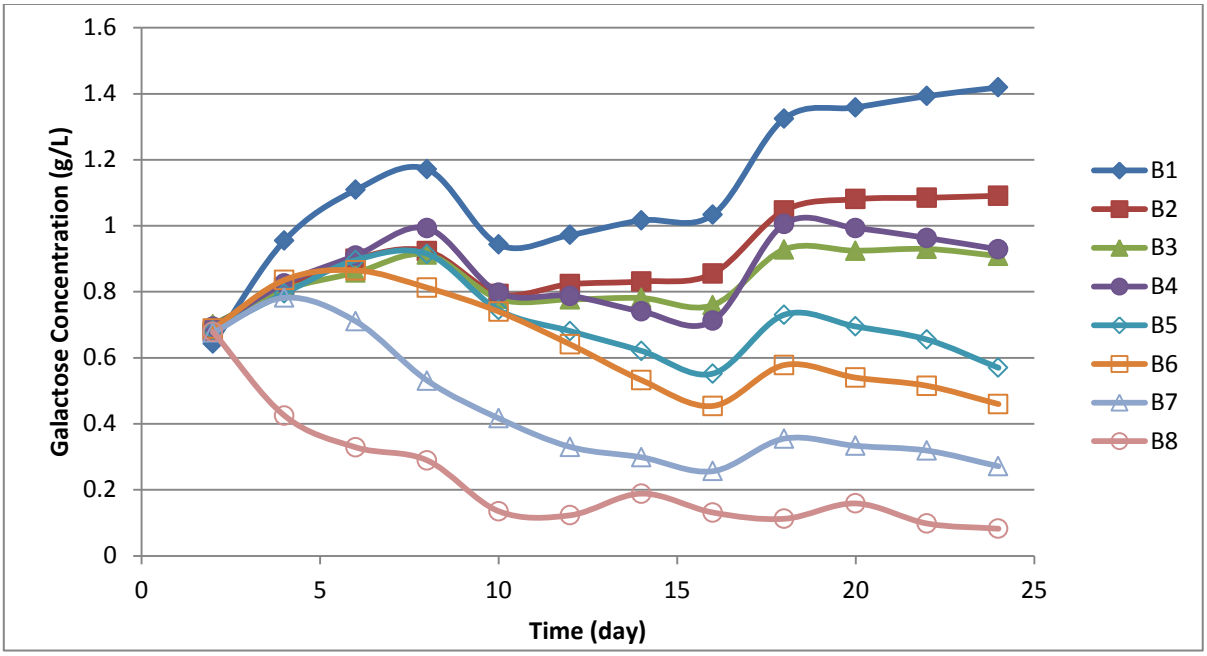

Figure 4-4 Train 1 galactose concentration as a function of time and bottle number.

Galactose concentrations were difficult to quantify reliably because of the preparation of HPLC standards and the detection threshold. The concentration of galactose in the sugar standard stock solution was significantly lower than either glucose or xylose, requiring only 0.115 g for a 50-mL stock solution. In addition to error introduced during preparation of standards, the concentration of galactose in liquid samples was significantly lower than those of glucose or xylose. The much smaller peak produced by galactose in the chromatograms was more affected by baseline detector noise than the peaks from other components. The lower concentrations within the center of this group of data resulted from differing galactose calibration curves during HPLC sequences, an issue that was observed with other trains as well.

Even before samples were analyzed by HPLC, there were indications that the system was approaching steady state. The most informative of these trends were the liquid mass transferred and the liquid phase pH as functions of time, presented in Figures 4-5 and 4-6, respectively.

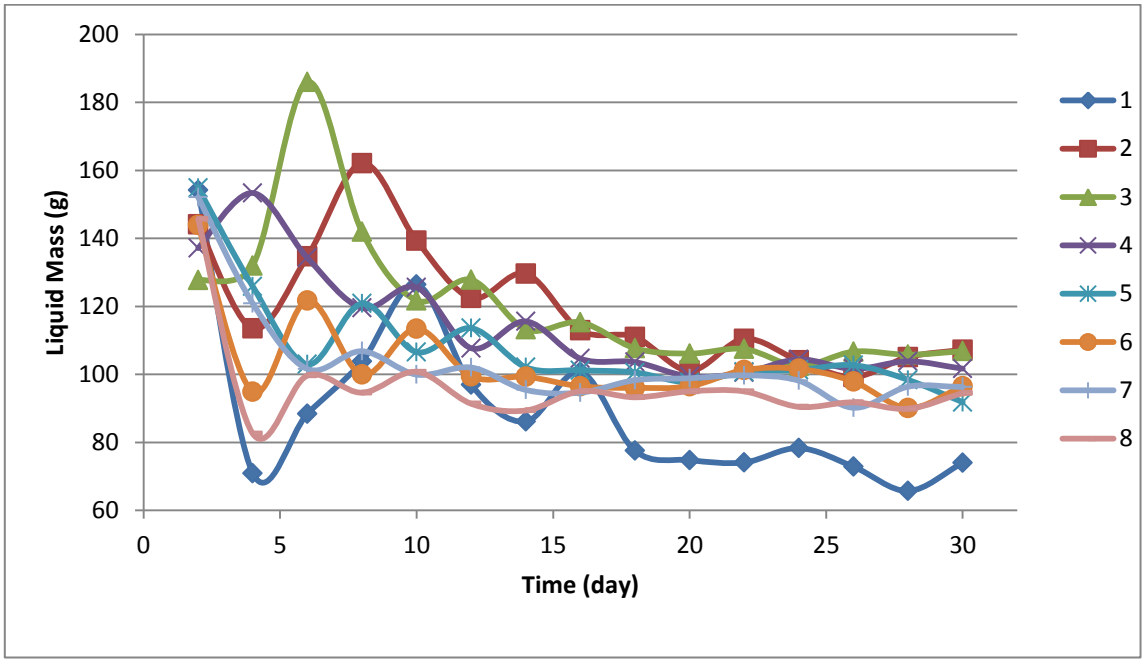

Figure 4-5 Liquid mass in each bottle during transfers in Train 1.

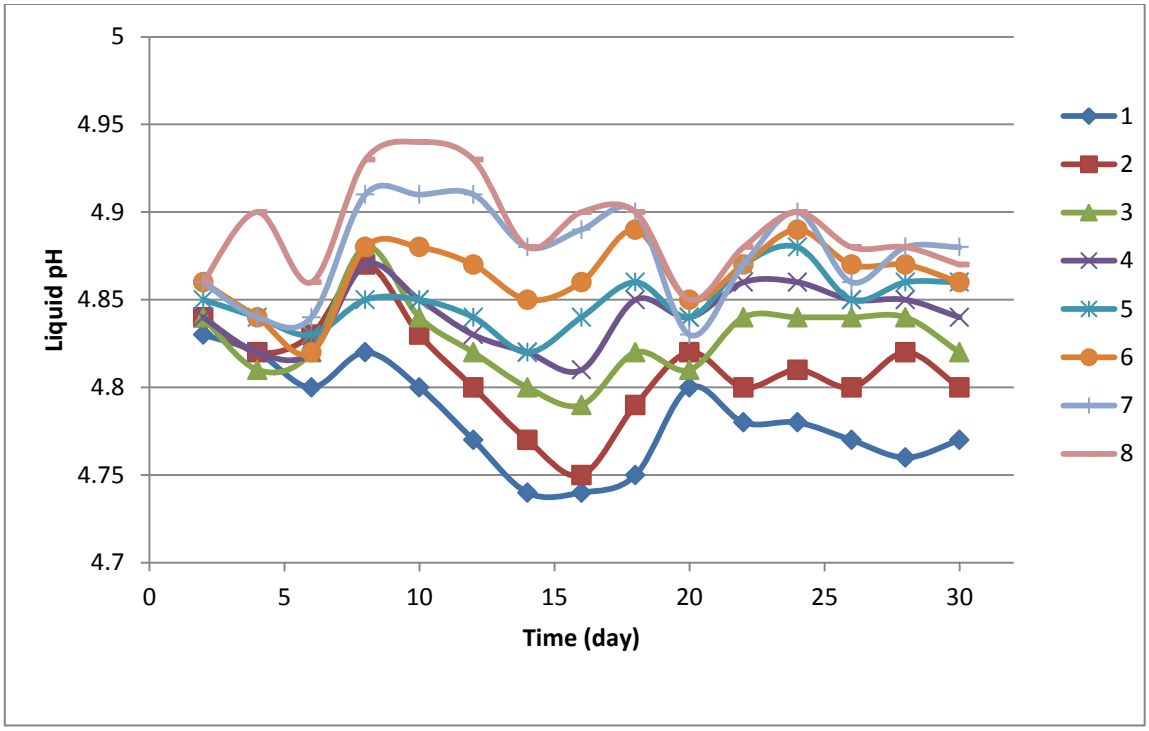

Figure 4-6 pH of liquid fraction in each bottle during transfers in Train 1.

It is important to recall that each stage was initially loaded as an individual batch system. As transfers proceeded, the total free liquid decreased during each transfer. This continued until excess volume had been transferred out of the system, and only the daily addition of 90 mL of fresh liquid plus sugars produced and dissolved into the liquid remained in each bottle. In this context, the initial downward trend followed by a stabilized volume is logical. During each transfer, Bottle 1 was where fresh, dry (93.936% TS) biomass was added. It absorbed a significant portion of contacting liquid until it reached the equilibrium moisture content of 22–25% TS, which is typical of wet cake throughout the system. Consistently, Bottle 1 had a lower liquid mass. These trends were observed for all countercurrent systems studied.

The pH trend is fairly straightforward as well. Initially, all bottles had approximately the same conditions and same pH. Over time, a gradient formed from Bottles 1 to 8. Discrepancies on Days 14 and 18 were discovered and corrected; improper calibration of the pH probe had resulted in a reading offset of 0.1 pH units.

Based on these observations and HPLC data, Train 1 was determined to have operated at steady state from Days 12 to 24. A new plot focusing on this steady-state region was generated for sugar concentration plotted against time (Figures 4-7) and for steady-state sugar concentration as a function of bottle position within the train (Figure 4-8).

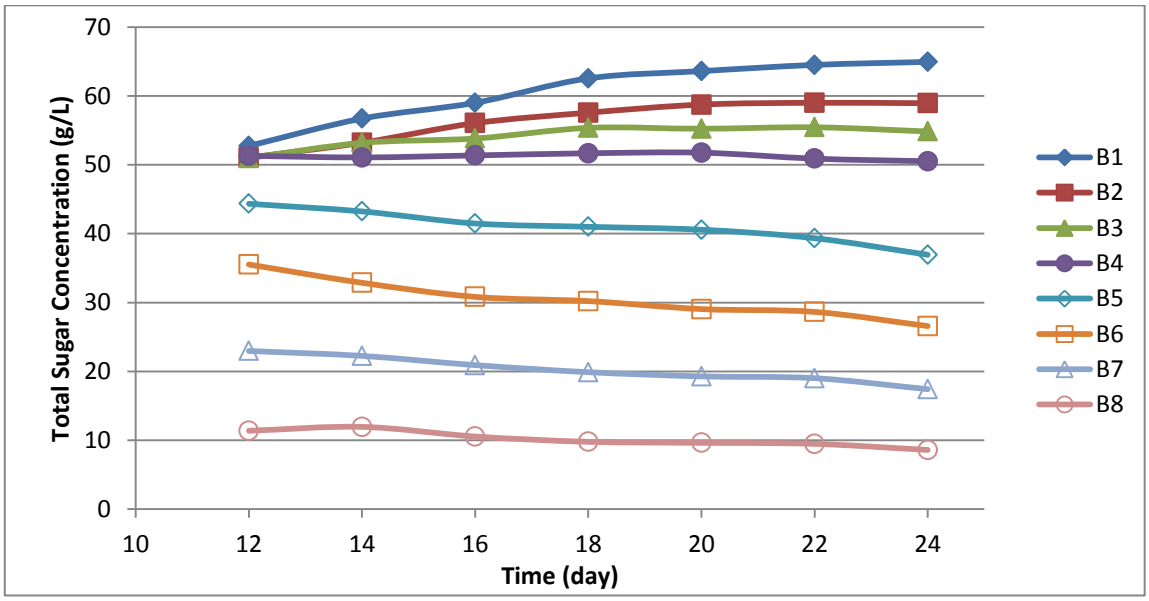

Figure 4-7 Total sugar concentration as a function of time and bottle number during steady-state operation of Train 1.

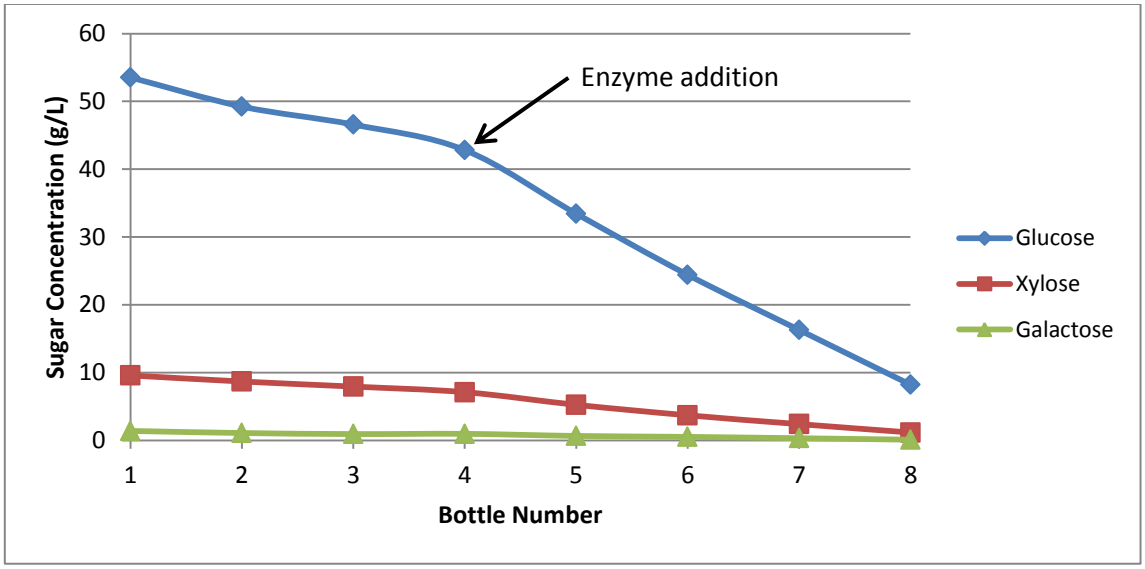

Figure 4-8 Sugar concentrations in Train 1 as a function of bottle number on Day 22, within steady-state region.

In Train 1, the enzyme addition point was Bottle 4, visible as the inflection point in Figure 4-8. A change in sugar concentration between bottles indicates remaining enzyme activity, with a larger change corresponding to a larger transfer of active enzyme between bottles. The change in sugar concentrations is smaller near the front of the train, presumably because there is more enzyme moving downstream with the solids than moving upstream with the liquid. This observation confirms that the enzyme prefers to stay bound to the solid substrate as opposed to remaining suspended in the liquid phase. The non-zero slope at the ends of the train indicate that sugar concentrations are still changing from stage-to-stage, and that more bottles are required to fully utilize remaining enzyme activity.

Data were accumulated over the entire reaction period and compiled into a table listing several performance parameters including: accumulated solids and liquids fed to

system, accumulated solids and liquid removed from system, glucose and total sugar removed daily, and instantaneous yields for both glucose and combined sugars. At this point, yields were corrected for mass changes from the water of hydrolysis for each component sugar. The hexose sugars (glucose and galactose) both capture an additional 11.1% mass during hydrolysis, whereas pentose sugars (xylose) increase by 13.6% (Wyman et al., 2005). Instantaneous yields were plotted against reaction day, with a dashed line representing average yield at steady state, calculated using the Slope Method (Figure 4-9).

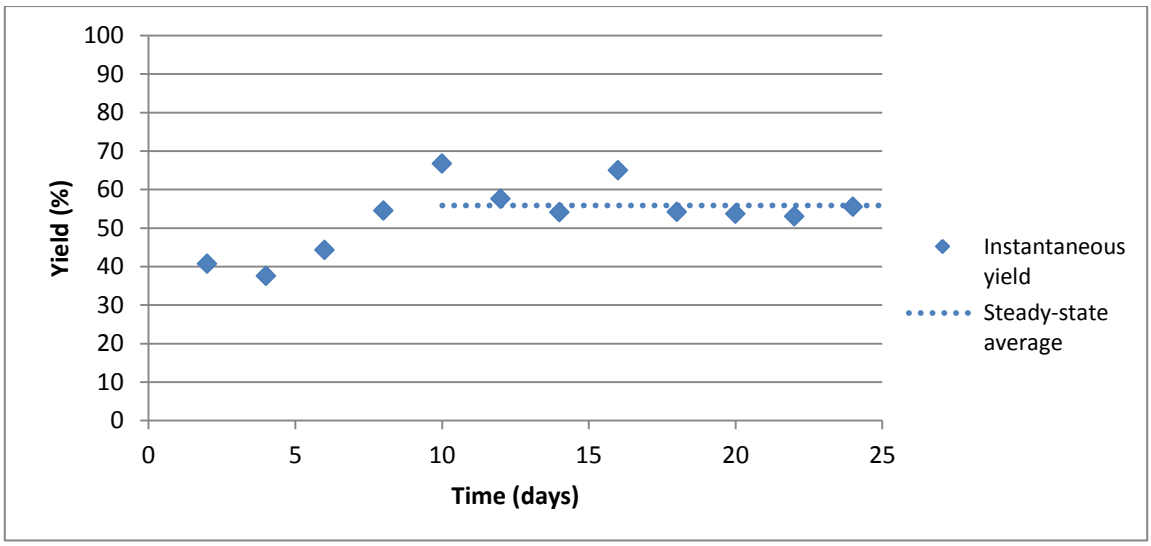

Figure 4-9 Train 1 instantaneous yields and average steady state yield. The average is based on the Slope Method.

Within the steady-state region, both combined sugar yield and glucose yield (g glucose produced/g equivalent glucose in biomass fed) were plotted against time (Figure 4-10). Once again, the average steady-state yields were calculated with the Slope Method.

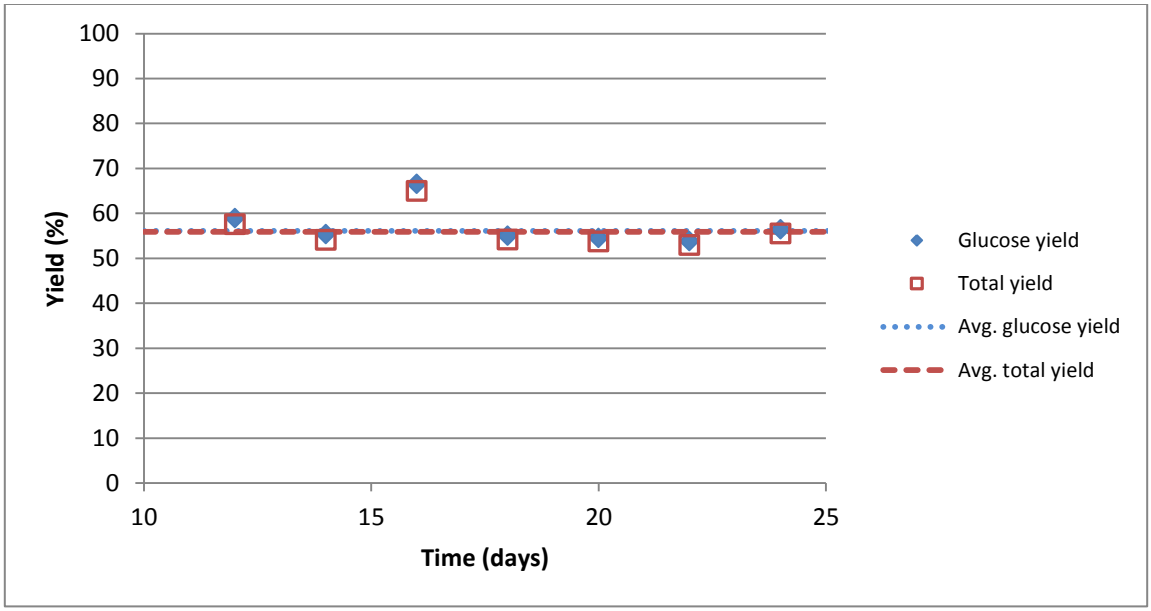

Figure 4-10 Instantaneous and average yields for glucose and total sugars during steady-state operation of Train 1. Averages are based on the Slope Method.

Within the steady-state region, there was very little day-to-day variance in yield. To confirm this stability, the Slope Method was used to analyze data of produced sugars and fed solids against time (Figure 4-11).

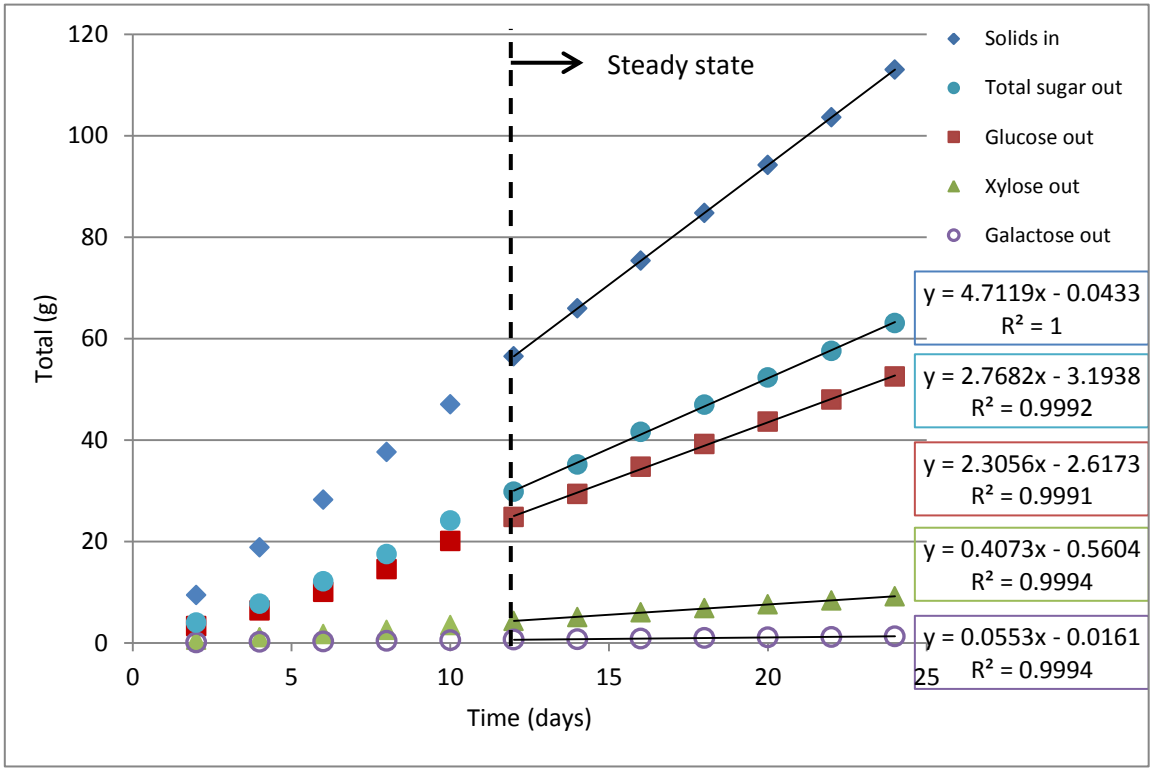

Figure 4-11 Solids in and sugar out of Train 1, fit lines represent steady-state region.

The excellent fit and low deviation of the slope support the conclusion that this region represents steady-state operation.

4.4.5 Train 2

Countercurrent Train 2 was an eight-bottle train, operated with an enzyme concentration of 5 mg/g. Train 2 was incubated at 50°C for a total of 46 days, with transfers occurring on even-numbered days beginning on Day 2. At the end of each transfer, the target wet cake mass for each bottle was 80 g. Diluted CTec2 was introduced in Bottle 5, fresh liquids entered in Bottle 8, and fresh solids in Bottle 1.

As with Train 1, the steady state determination was made by plotting concentrations of glucose, xylose, and galactose over time, and observing the change in these concentrations between transfers (Figures 4-12 to 4-14).

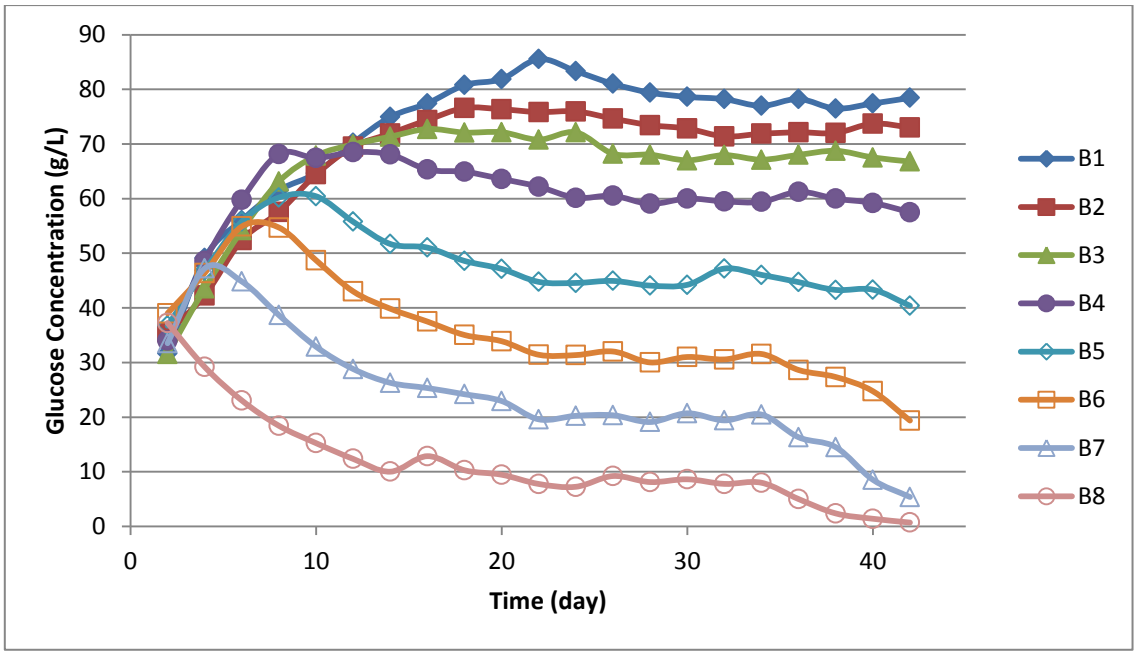

Figure 4-12 Train 2 glucose concentration as a function of time and bottle number.

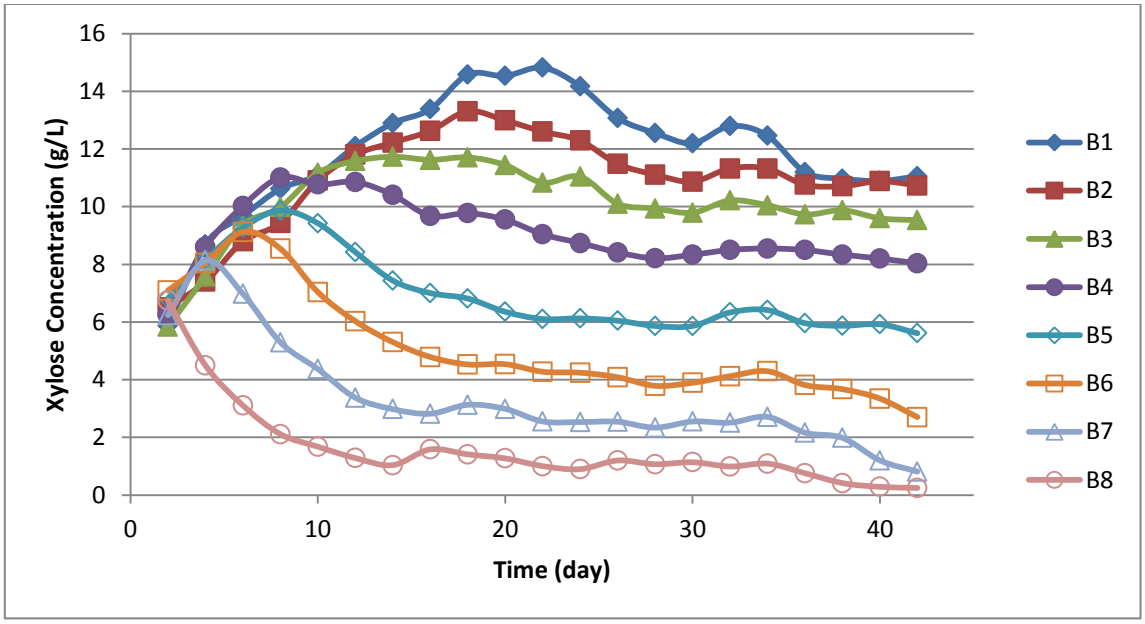

Figure 4-13 Train 2 xylose concentration as a function of time and bottle number.

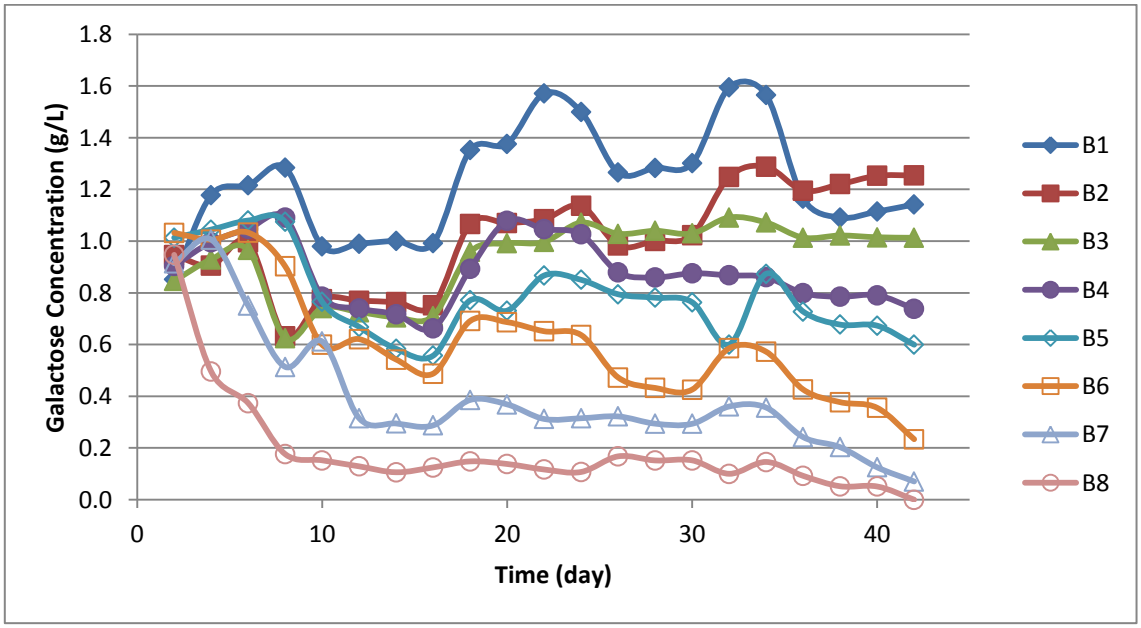

Figure 4-14 Train 2 galactose concentration as a function of time and bottle number.

Again, it was difficult to reliably quantify galactose concentrations because of very noisy data. Because the absolute amount of galactose was so low, and its contribution to total sugars was small, the error these noisy data introduce was ignored in later yield calculations.

The same trends were observed in Train 2 as in Train 1, and plots of liquid mass transferred and liquid phase pH as functions of time were generated (Figures 4-15 and 4-16).

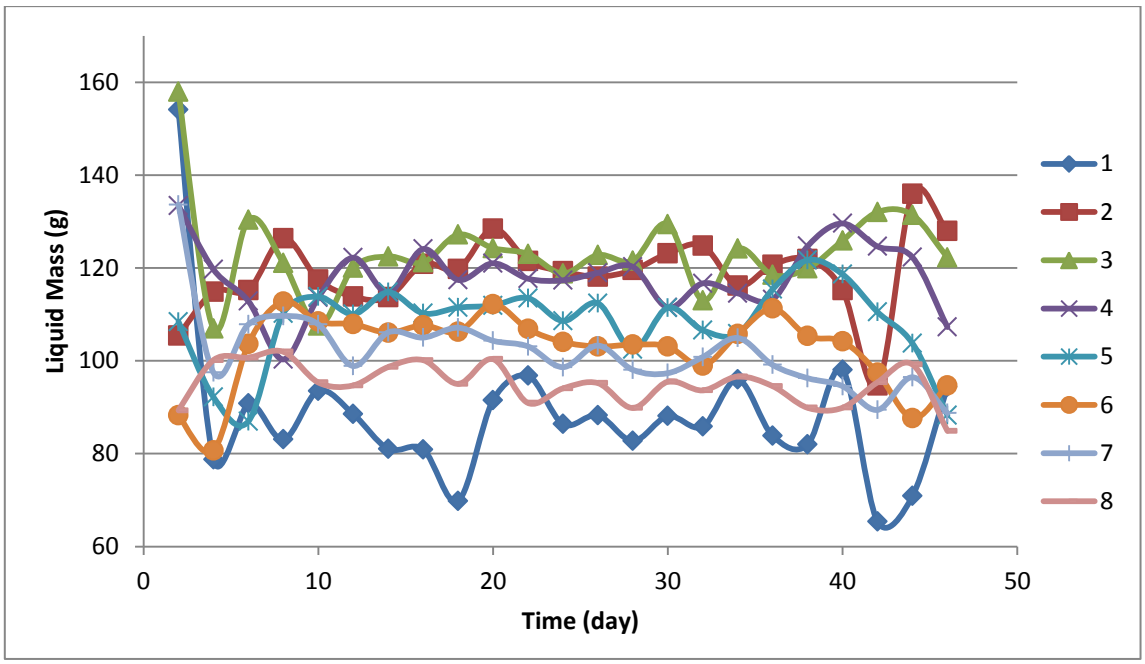

Figure 4-15 Liquid mass in each bottle during transfers in Train 2.

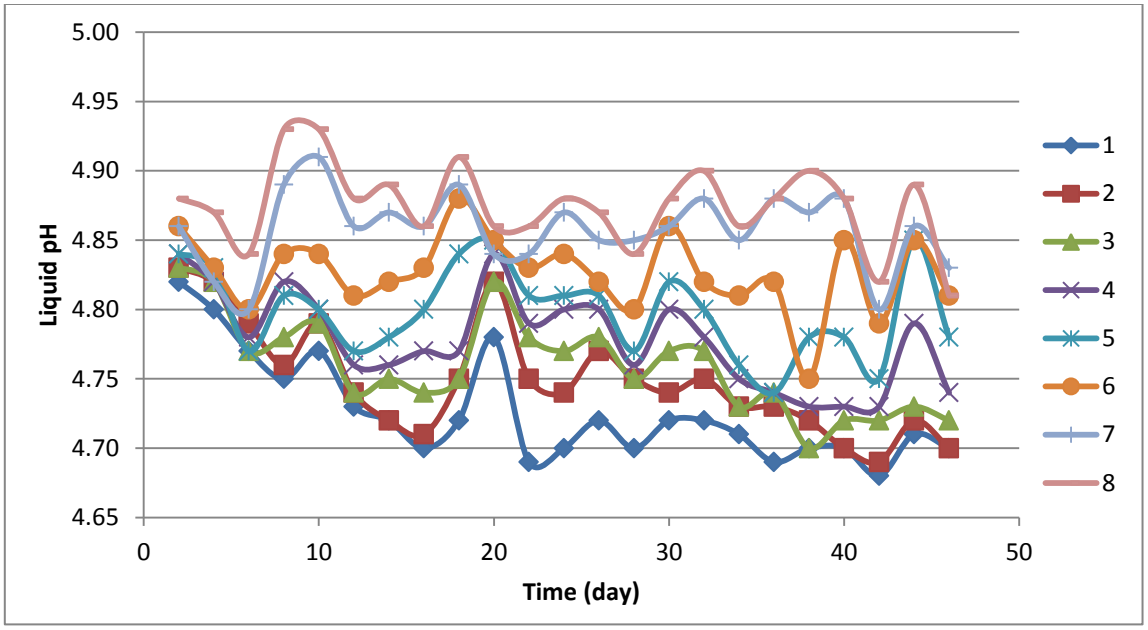

Figure 4-16 pH of liquid fraction in each bottle during transfers in Train 2.

The same trends regarding initially falling liquid levels were observed, although the trends were not as drastic as Train 1. This may have been because of a higher initial rate of sugar production, because of the higher enzyme concentration. Following the first several transfers in Train 2, Bottle 1 behaved similarly to Train 1, with a consistently lower liquid mass than other bottles.

In Train 2, the pH was more variable, possibly as a result of increased enzyme concentration and higher hydrolysis rate. The same poorly calibrated pH probe used to measure liquid in Train 1 was used in Train 2 on the same days, and appropriate corrections to pH measurements were again made for Days 14 and 18.

Based on observations and HPLC data, Train 2 operated at steady state from Days 24 through 40. Plots of this region were generated for sugar concentration against

time and for steady-state sugar concentration as a function of bottle position (Figures 4-17 and 4-18).

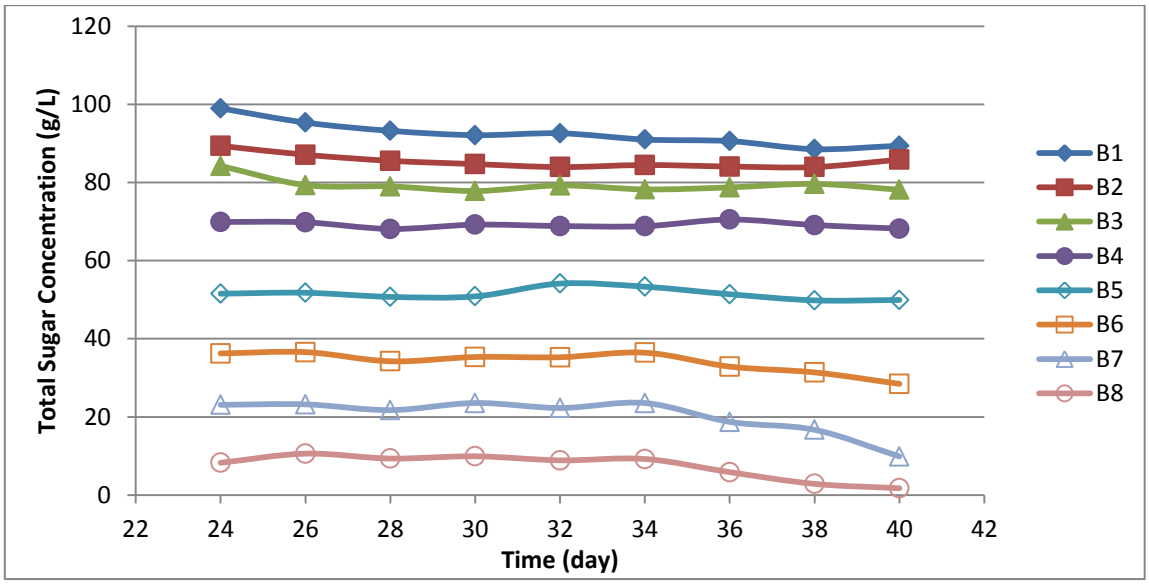

Figure 4-17 Total sugar concentration as a function of time and bottle number during steady-state operation of Train 2.

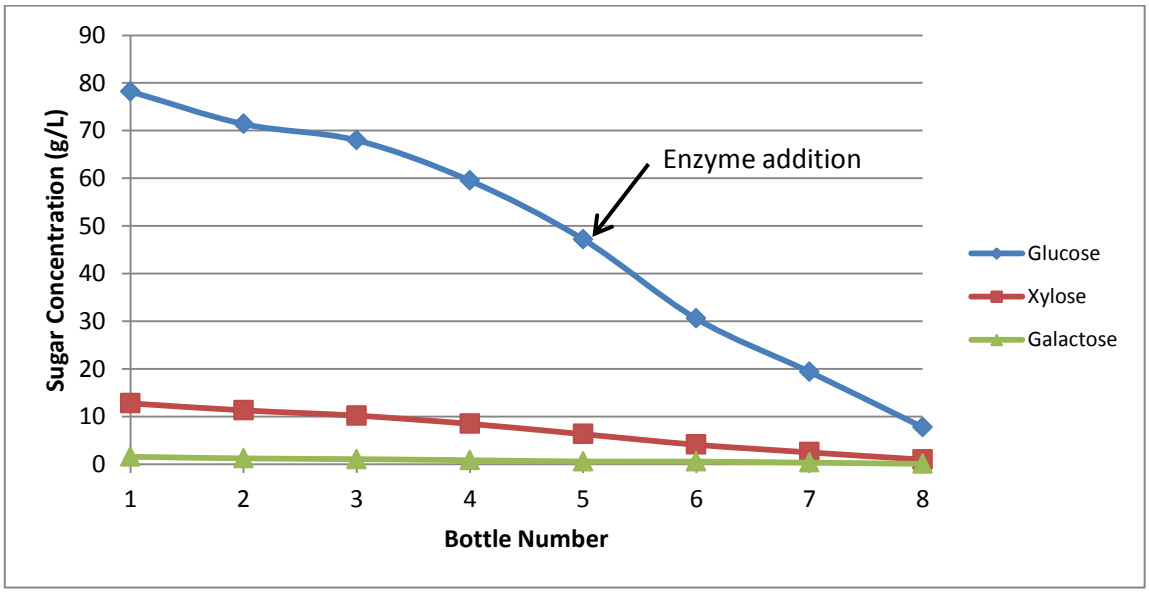

Figure 4-18 Sugar concentrations in Train 2 as a function of bottle number on Day 32, within steady-state region.

Similar to Train 1, there is remaining activity at both ends of Train 2. From these systems, it was clear that more stages are required to take full advantage of countercurrent operation.

Accumulated data was again used to plot instantaneous yields as well as glucose and total yields within the steady-state region (Figures 4-19 and 4-20).

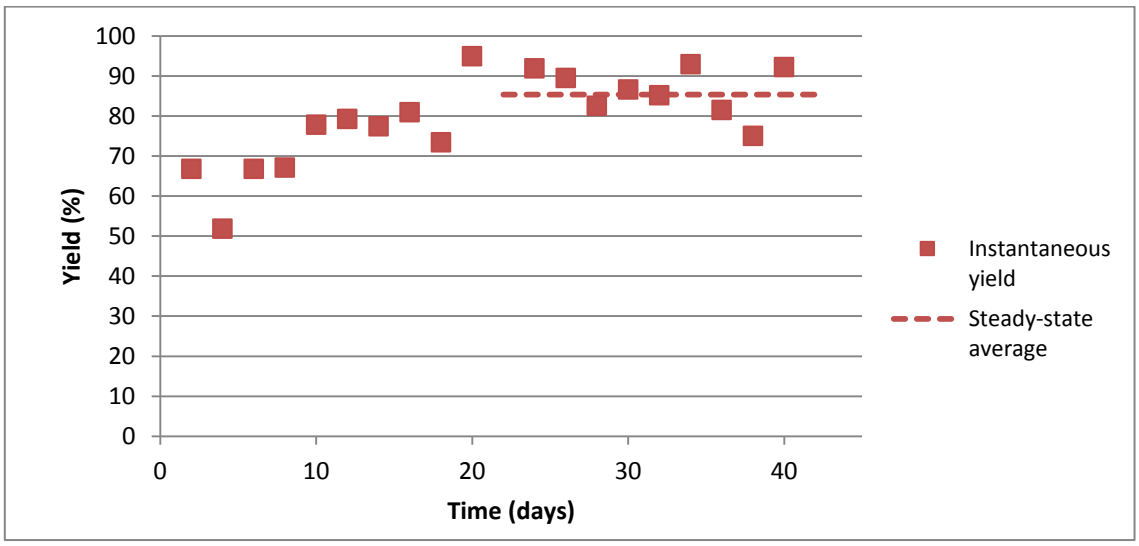

Figure 4-19 Train 2 instantaneous yields and average steady-state yield. Average is based on the Slope Method.

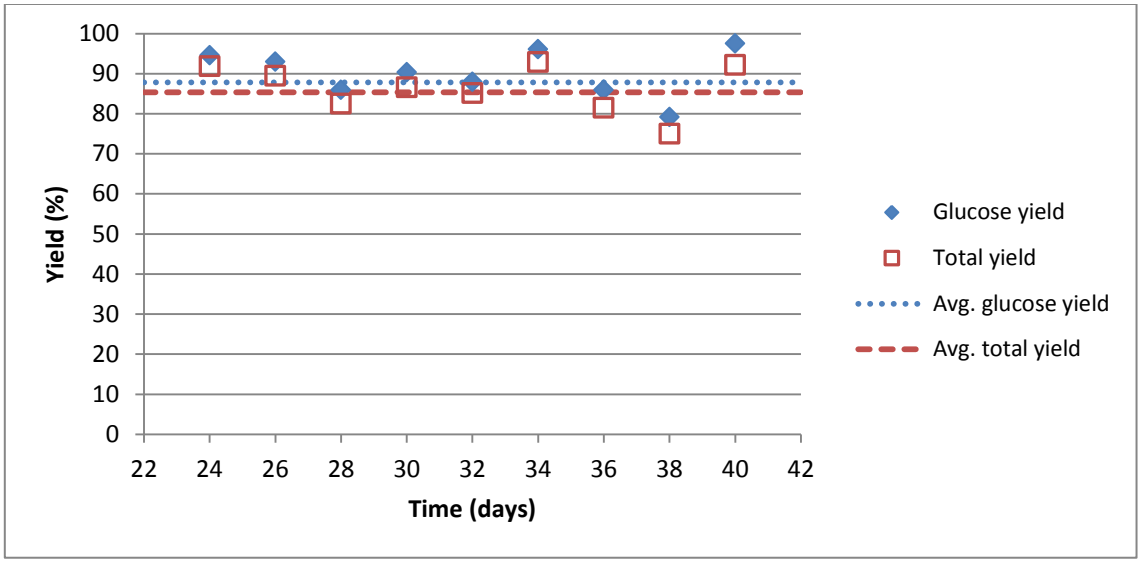

Figure 4-20 Instantaneous and average yields for glucose and total sugars during steady-state operation of Train 2. Averages are based on Slope Method.

Once again, the yield variance during steady-state operation was fairly low. To confirm, the Slope Method was again employed (Figure 4-21).

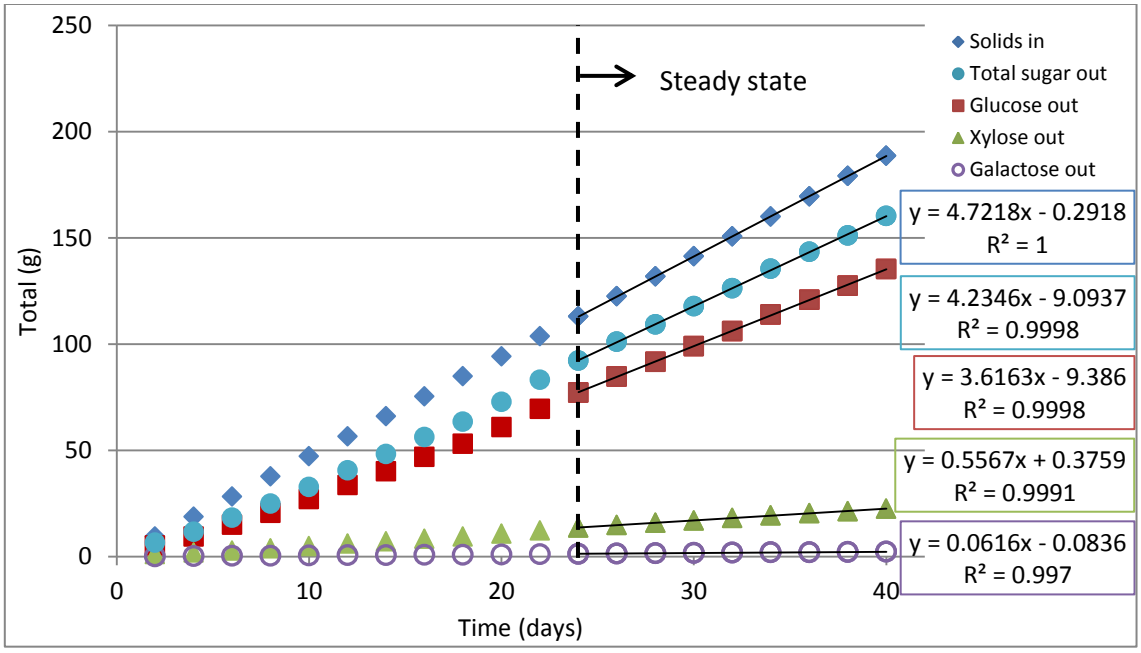

Figure 4-21 Solids in and sugar out of Train 2, fit lines represent steady-state region. A good fit parameter and low variance again support the conclusion of steady state.

4.4.6 Train 3

Countercurrent Train 3 was a 16-bottle train, operated with an enzyme concentration of 2 mg/g. Train 3 was incubated at 50°C for a total of 52 days, with transfers occurring on even-numbered days beginning on Day 2. At the end of each transfer, the target wet cake mass for each bottle was 85 g. Diluted CTec2 was introduced in Bottle 8, fresh liquids entered in Bottle 16, and fresh solids in Bottle 1.

With Train 3, it was more difficult to assess steady state because of an upset that occurred as the train began to enter the steady-state region. The most damaging error was an incorrectly set incubator temperature of 60°C, rather than 50°C, caused by failure to notice an adjustment made by a student worker. This error occurred on Day 34 and

was corrected within 24 hours, resulting in a short-term increase in sugar production followed by a decline in enzyme activity caused by thermal denaturation of the proteins. Plots of glucose, xylose, and galactose concentrations over time show the system nearing steady state just as the upsets occurred (Figures 4-22 to 4-24).

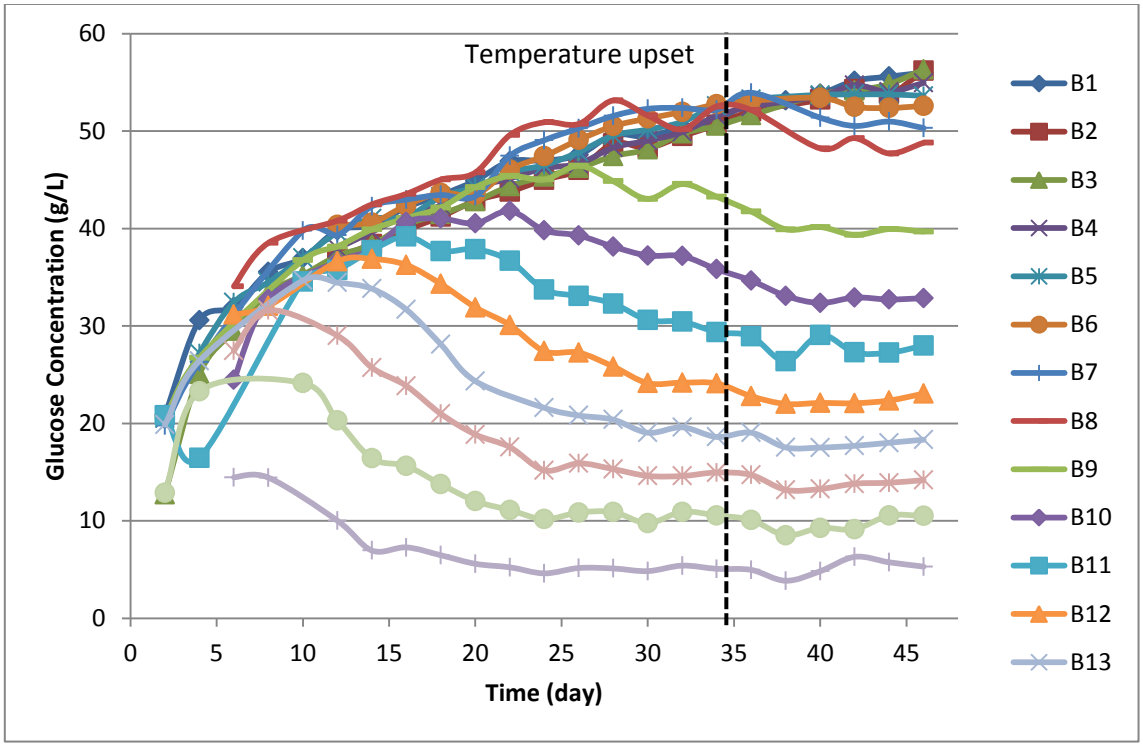

Figure 4-22 Train 3 glucose concentration as a function of time and bottle number.

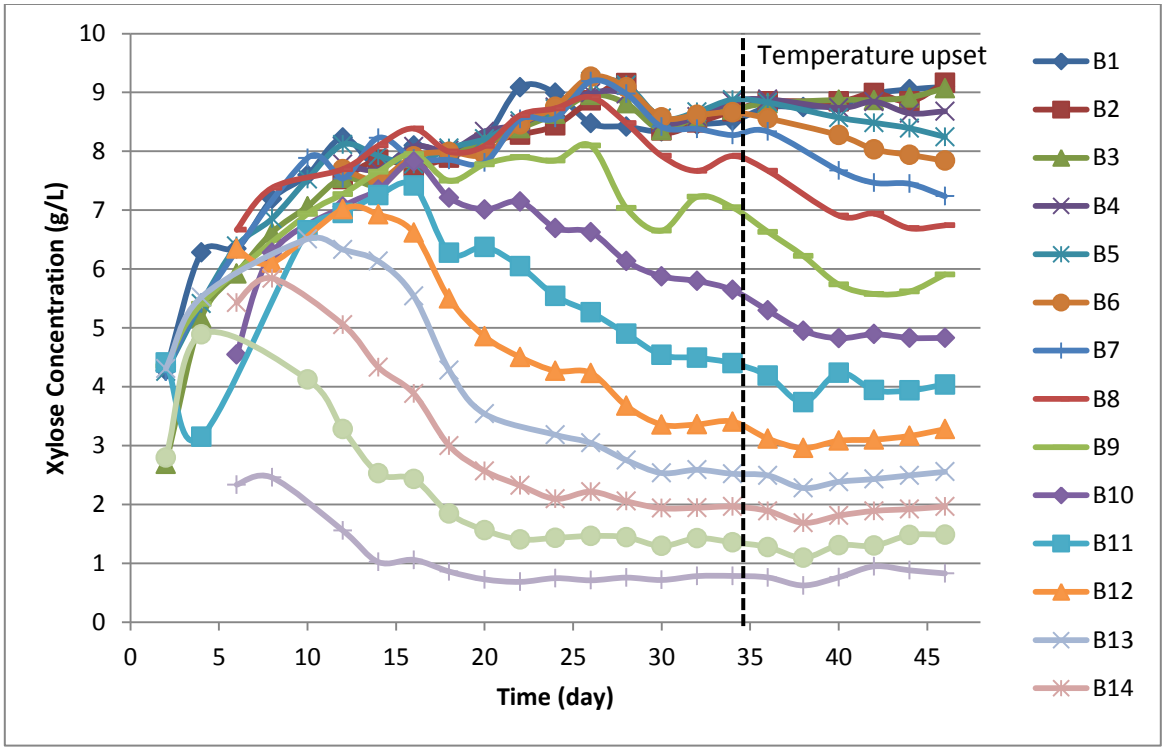

Figure 4-23 Train 3 xylose concentration as a function of time and bottle number.

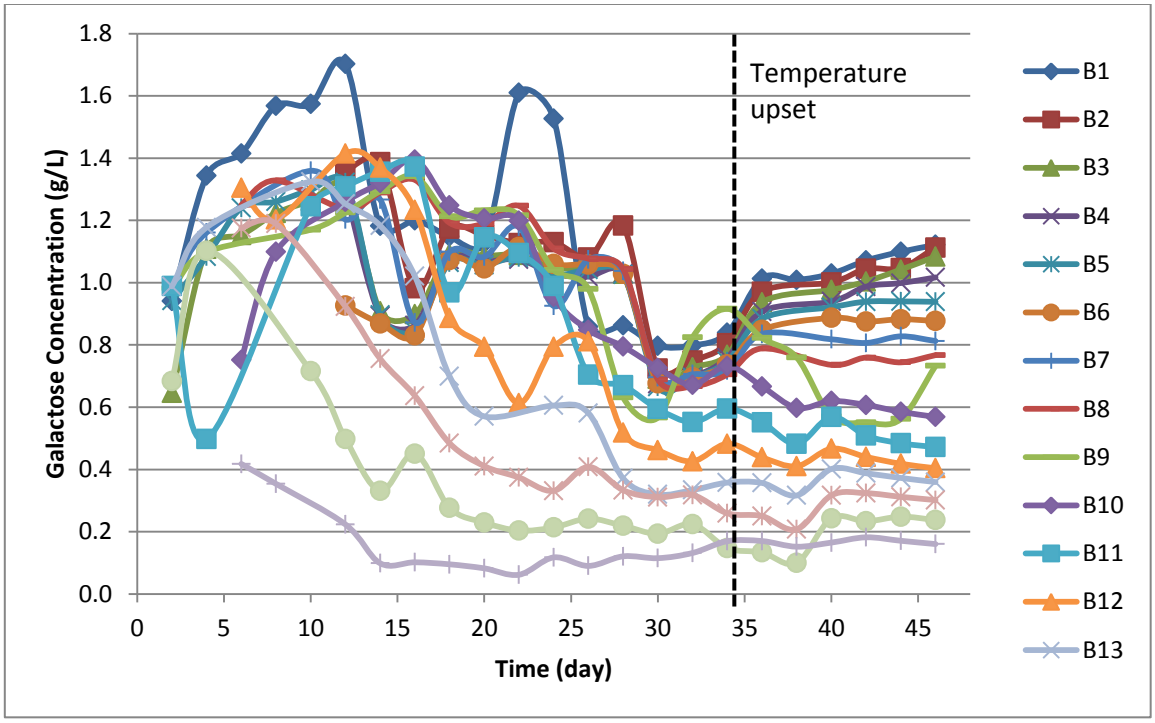

Figure 4-24 Train 3 galactose concentration as a function of time and bottle number.

To help assess when Train 3 was operating at steady state, trends in liquid mass and pH were compared to the steady-state region trends observed in Trains 1 and 2 (Figures 4-25 and 4-26).

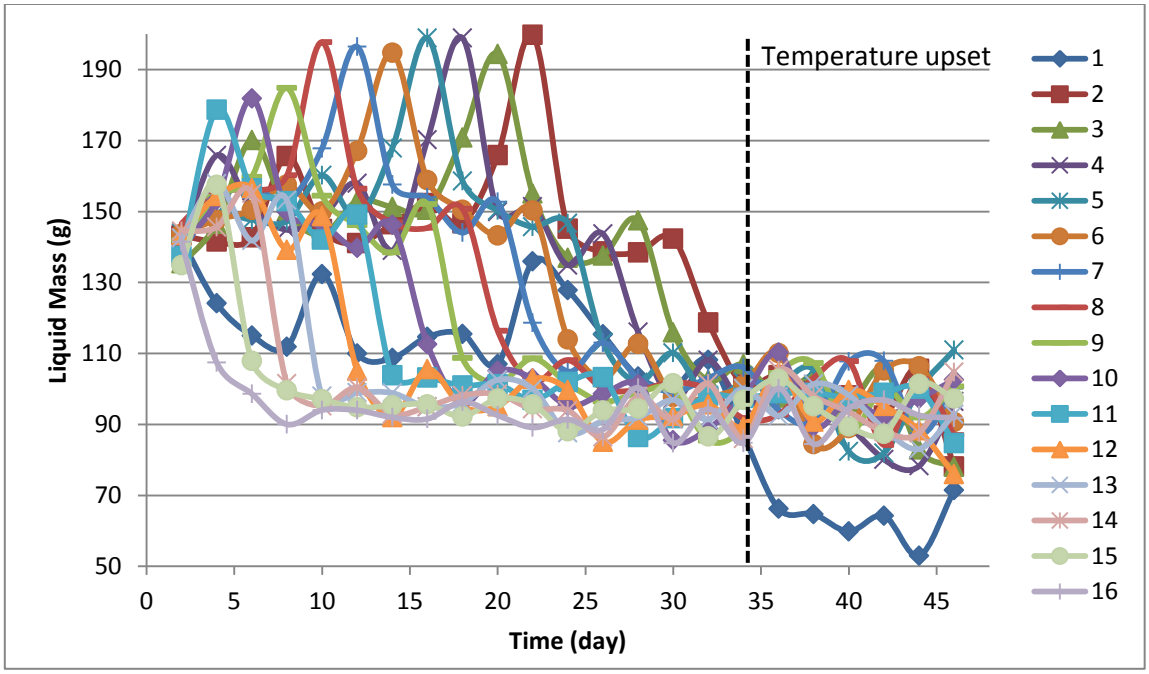

Figure 4-25 Liquid mass in each bottle during transfers in Train 3.

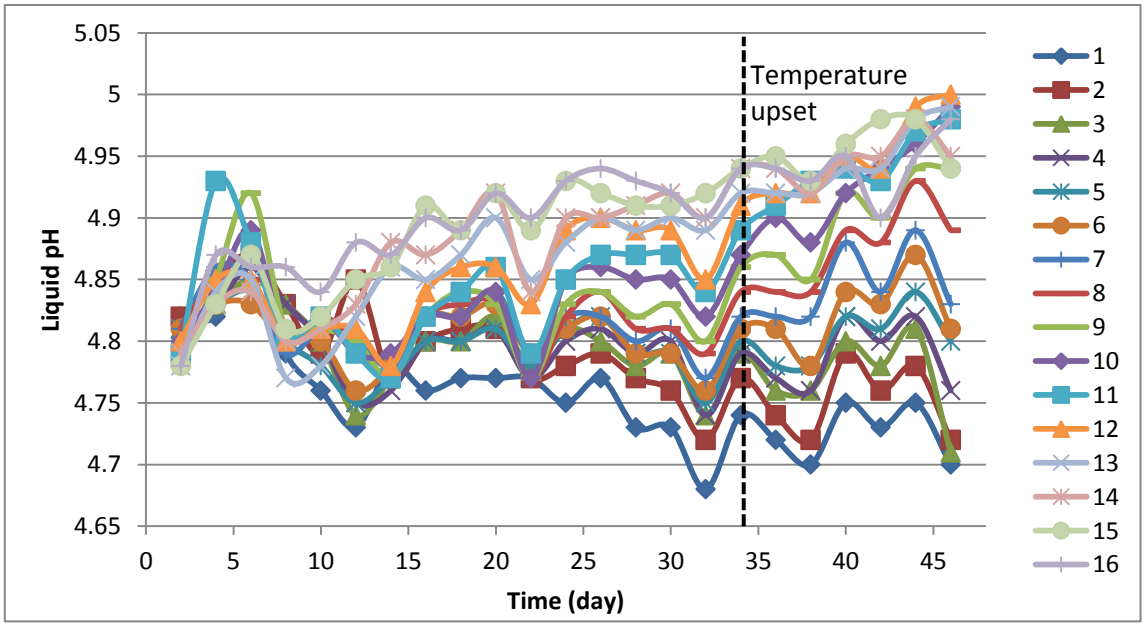

Figure 4-26 pH of liquid fraction in each bottle during transfers in Train 3.

Based on the trends in pH and liquid mass, as well as the measured sugar concentrations, the system appears to operate at steady state from Days 18–34. Plots of sugar concentration as a function of time and bottle number were generated (Figures 4-27 and 4-28).

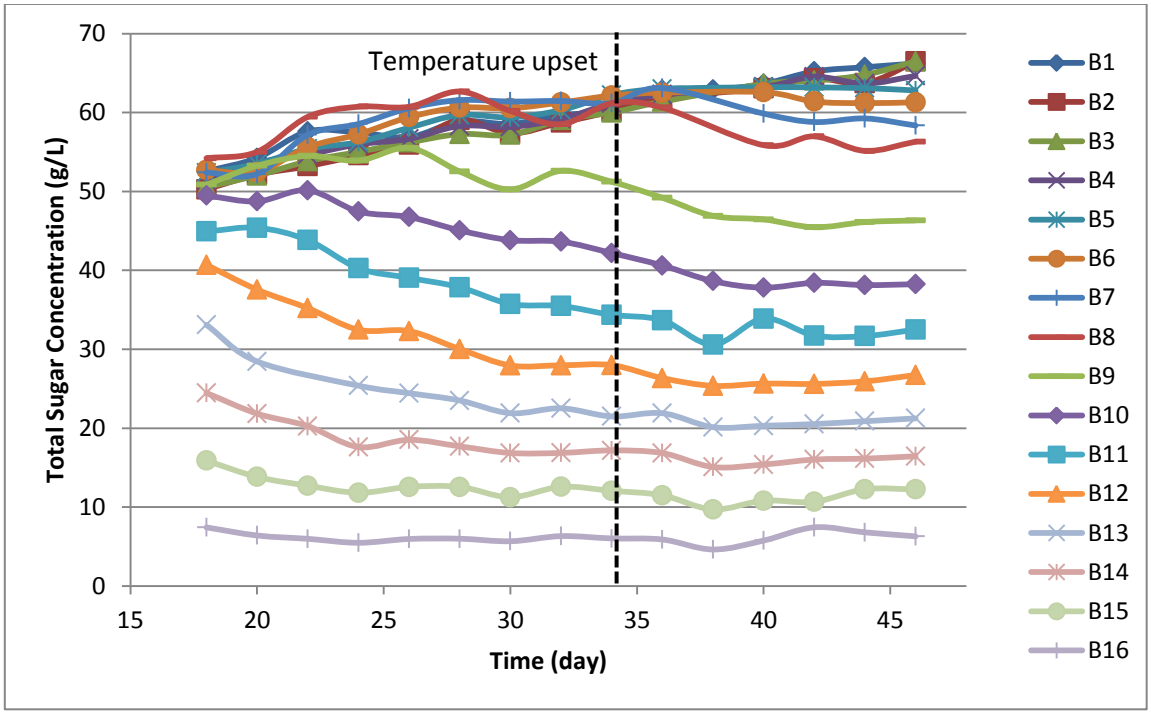

Figure 4-27 Total sugar concentration as a function of time and bottle number, Train 3.

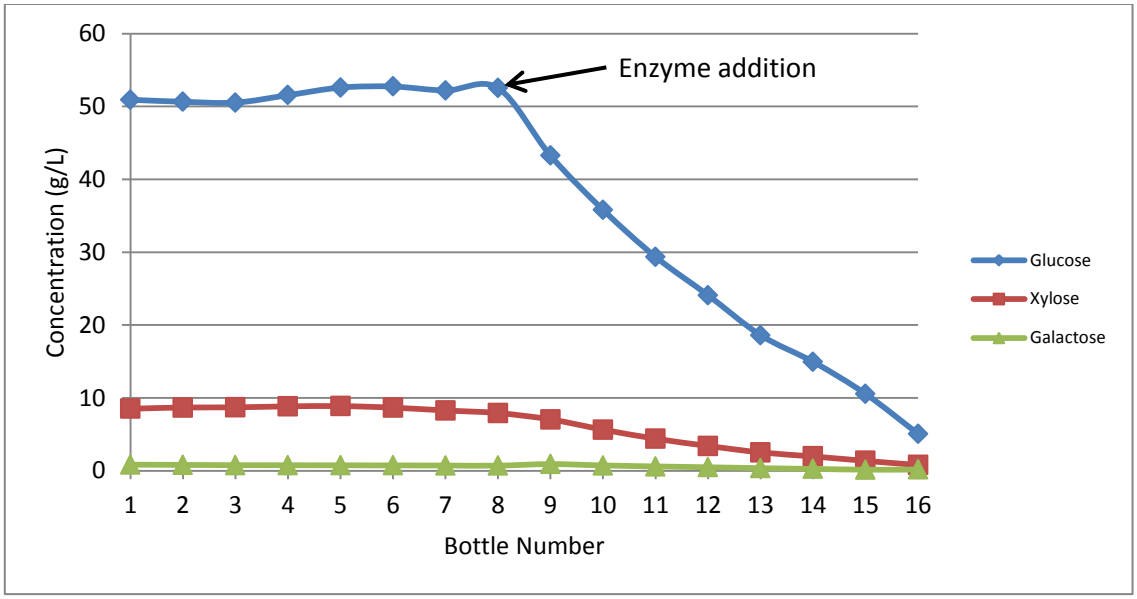

Figure 4-28 Sugar concentrations in Train 3 as a function of bottle number on Day 34, within steady-state region.

As shown in Figure 4-28, Train 3 had a flat concentration profile near the front, indicating no enzyme activity remains within the first few bottles. However, even with a 16-bottle train, there is remaining activity at the back. In the future, based on the concentration profile, the enzyme addition point should be moved from Bottle 8 forward 6. It may also be necessary to include additional stages at the back of the train to fully utilize remaining enzyme activity.

Accumulated data were used to generate a plot of instantaneous yield against time (Figure 4-29).

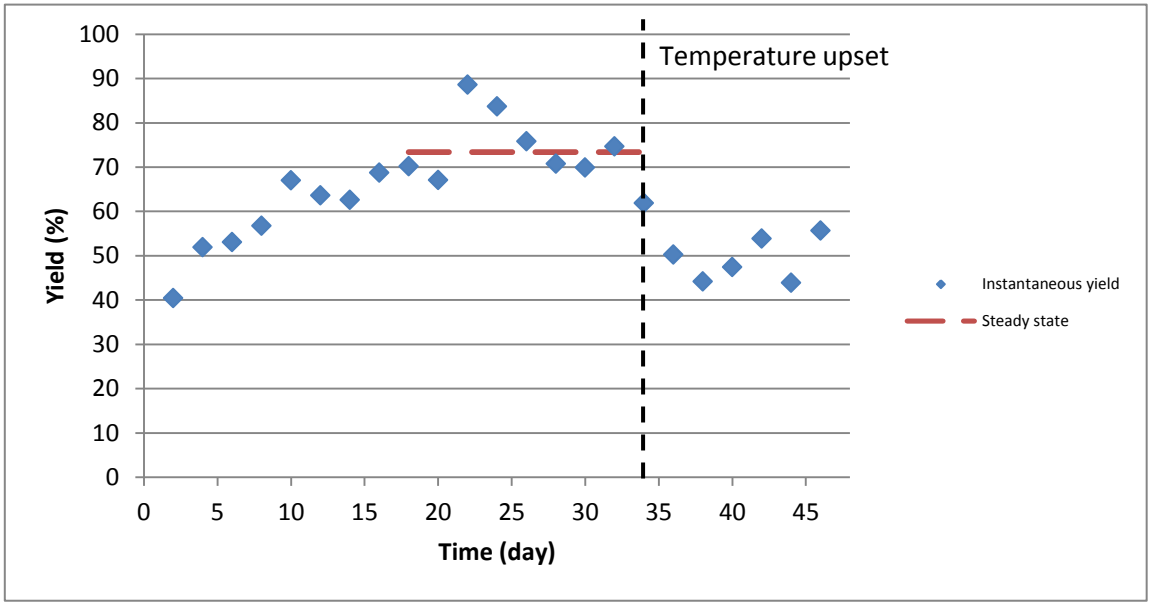

Figure 4-29 Instantaneous yields for Train 3. Average is based on Slope Method.

The set temperature error is very apparent in observed yields, with the yield dropping from approximately 74% just prior to the upset, to approximately 48% just after the upset. The reason this set point error so drastically affected yields, but not concentrations, may have been chance. Once enzyme activity decreased, less solid material was converted to soluble sugars. This in turn led to lower free liquid levels in Train 3, which negatively impacts calculated yield, and strongly depends on free liquid volume exiting Bottle 1. It appears that liquid volumes and produced sugars may have decreased in parallel, leading to very little change in sugar concentration.

To evaluate the performance of Train 3, the pre-upset region of steady-state operation was used. During steady state, Train 3 shows more variability in predicted yields, but has relatively flat sugar concentration and pH profiles. The Slope Method was

applied to the pre-upset sugar output data to evaluate the steady-state region (Figure 4-30).

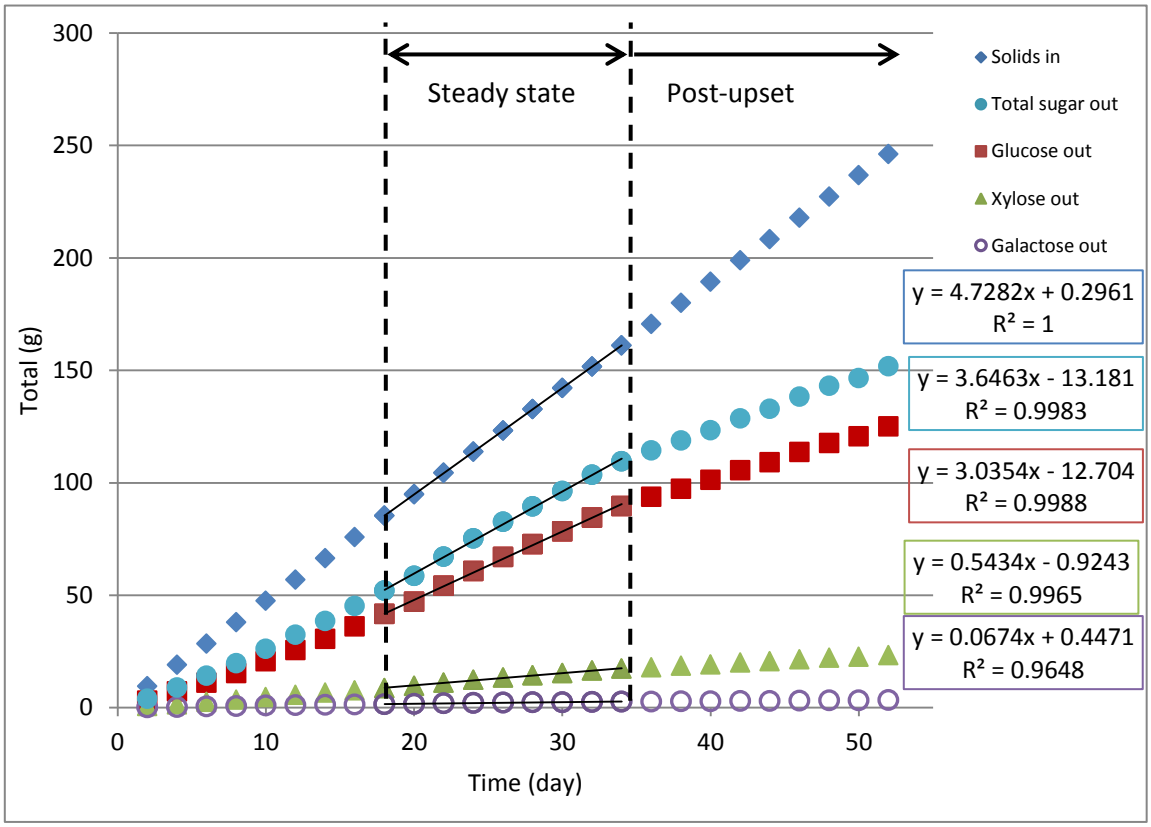

Figure 4-30 Solids in and sugar out of Train 3. Fit lines represent the steady-state region.

It is immediately obvious that the upset affected sugar production, indicated by the noticeably smaller slope in the post-upset region.

Even with limited steady-state data from pre-upset operation, Train 3 had better yields than Train 1. Table 4-4 succinctly summarizes the differences between all three trains.

Table 4-4 Summary of all trains

Train	Moisture content of solids (%TS)	Peak glucose conc. (g/L)	Peak xylose conc. (g/L)	Peak galactose conc. (g/L)	Slopes: Cummulative solids in (g dry biomass/day)	Cummulative sugars out (g sugar/day)	Cummulative glucose out (g glucose /day)	Cummulative xylose out (g xylose /day)	Cummulative galactose out (g galactose /day)
1	93.94	53.94	9.59	1.42	4.71	2.77	2.31	0.407	0.055
2	93.94	85.55	14.82	1.59	4.72	4.23	3.62	0.557	0.062
3	94.26	56.54	9.10	1.70	4.73	3.65	3.04	0.543	0.067

Train	Standard deviation of the slope for: solids in	total Sugar out	glucose out	xylose out	galactose out	Total sugar yield (sugar out/ equiv. sugars in)	Glucose yield (glucose out/ equiv. glucose in)	Xylose yield (xylose out/ equiv. xylose in)	Galactose yield (galactose out/ equiv. galactose in)
1	0.0023	0.0345	0.0304	0.0045	0.0006	0.559 ± 0.0345	0.561 ± 0.0304	0.529 ± 0.0045	0.771 ± 0.0006
2	0.0057	0.0237	0.0183	0.0064	0.0013	0.853 ± 0.0237	0.878 ± 0.0183	0.722 ± 0.0064	0.857 ± 0.0013
3	0.0012	0.0570	0.0405	0.0121	0.0049	0.734 ± 0.0570	0.736 ± 0.0405	0.704 ± 0.0121	0.937 ± 0.0049

Error on conversion is ± 1.0 standard deviation.

There are clear benefits gained from increasing the train size from 8 to 16 bottles; at 2 mg/g enzyme loading, the total sugar yield increased from 55.9% to 73.4%.

4.4.7 Comparison of Countercurrent Data to Batch Studies

To quantify the added value of countercurrent operation, the continuous data are compared to batch saccharification data. Earlier batch experiments were repeated using α-cellulose as the substrate, with a 5-day reaction period, typical in industry and therefore considered the "ultimate conversion" for industrial applications. Figure 4-31 shows the 5-day yield for the batch saccharifications of α-cellulose as a function of enzyme loading. Also, the yields are shown for countercurrent saccharifications as discreet data points.

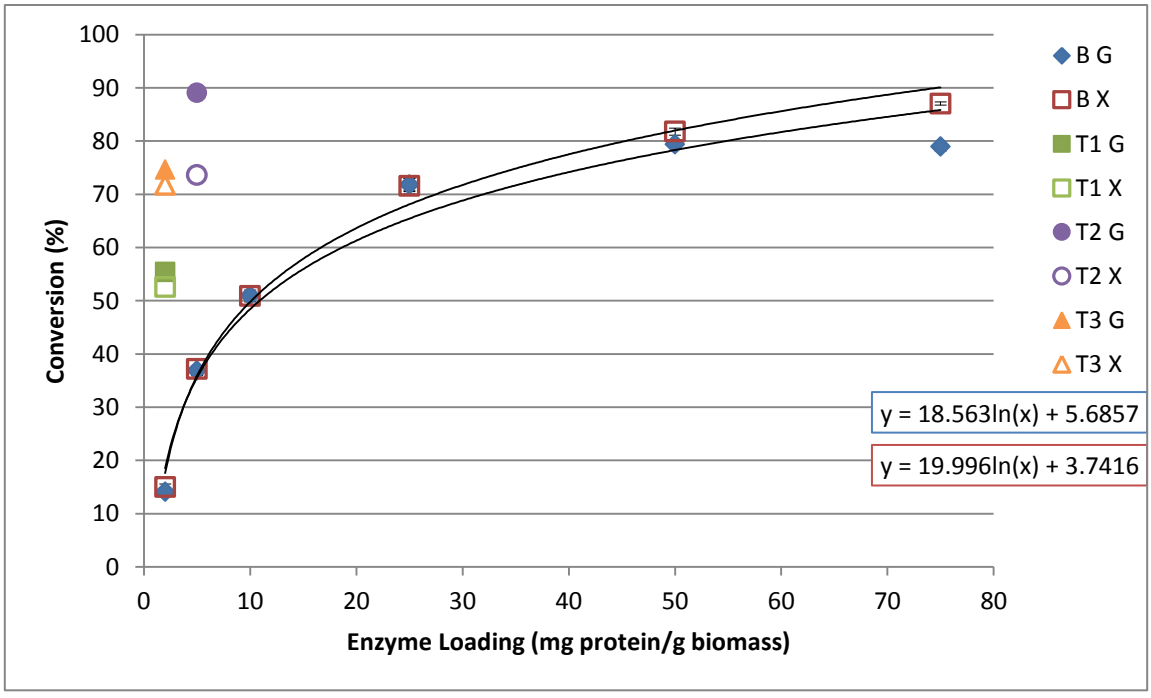

Figure 4-31 Continuous curves show 5-day yield for batch saccharification. Discrete data points are yields for continuous countercurrent saccharification. B G, batch glucose; B X, batch xylose; T1 G, Train 1 glucose; T1 X, Train 1 xylose; T2 G, Train 2 glucose; T2 X, Train 2 xylose; T3 G, Train 3 glucose; T3 X, Train 3 xylose.

Table 4-5 compares these 14-day batch saccharification yields to steady-state yields for each train, as determined by the Slope Method.

Table 4-5 Comparison of enzyme requirements for batch and countercurrent saccharification. Yields are from Slope Method.

	Train 1	Train 2	Train 3
Glucose conversion (%)	55.42	89.06	74.66
Enzyme loading (mg protein/g biomass)			
Continuous countercurrent	2	5	2
Batch	16	84*	41
Factor improvement	8	16.8	20.5
Xylose conversion (%)	52.56	73.61	71.75
Enzyme loading (mg protein/g glucan)			
Continuous countercurrent	2	5	2
Batch	12	34	31
Factor improvement	6	6.8	15.5

*Based on logarithmic fits from data presented in Figure 4-31.

To achieve the same yields, countercurrent saccharification reduces enzyme loading by 5–11 times for glucan and 11–32 times for xylan.

4.4.8 Economic Benefits

To illustrate the economic impact of the improvements in enzyme efficiency reported, Equation 4-23 was used to estimate the enzyme cost to produce one pound of sugar using commercial costs for enzyme (Kazi et al., 2010). Equation 4-23 was used to estimate this value.

$$\text{Cost per lb} = \left(\text{Cost}_{\text{enzyme}}\right)\left(\text{Load}_{\text{enzyme}}\right)\left(\frac{1000\text{ g biomass}}{\text{kg biomass}}\right)\left(\frac{\text{kg enzyme}}{1000000\text{ mg enzyme}}\right)$$

$$\left(\frac{1}{\text{Biomass}_{\text{digestible}}}\right)\left(\frac{1}{\text{Sugar}_{\text{equiv}}}\right)\left(\frac{1}{\text{Conversion}}\right)\left(\frac{\text{kg sugar}}{2.205\text{ lb sugar}}\right) \quad (4\text{-}23)$$

where,

Cost per lb = enzyme cost per pound of sugar produced ($/lb)

$Cost_{enzyme}$ = cost of enzyme per unit of protein content ($5.07/kg protein)

$Load_{enzyme}$ = enzyme loading (mg protein/g biomass)

$Biomass_{digestible}$ = digestible fraction of biomass

(glucan + xylan + galactan = 0.9426 kg digestible biomass/kg biomass)

$Sugar_{equiv}$ = equivalent sugar accounting for water of hydrolysis

(1.115 kg total sugar/kg digestible biomass)

Conversion = steady-state conversion of system

(kg digested/kg digestible material fed)

Table 4-6 shows the estimated enzyme costs for each train.

Table 4-6 Enzyme cost per pound of sugar produced at steady-state yields.

Train	Enzyme Loading (mg enzyme/g biomass)	Conversion (%)	Enzyme Cost ($/lb sugar)
1	2	55.9	0.00783
2	5	85.3	0.0128
3	2	73.4	0.00596

The current cost that the ethanol industry will accept for raw glucose is roughly $0.14−$0.16/lb (Lux 2013). Historically, enzyme costs have been one of the largest contributing factors to cellulosic sugar costs. In the countercurrent systems investigated, enzyme costs are only 3.7−9.1% of the target price for sugars. If sugar can be produced

from lignocellulose as inexpensively as this analysis suggests, ethanol from lignocellulose is a more viable endeavor.

It should be noted that the model substrate in these studies was ash- and lignin-free, with a digestible content >94%. In real-world biomass, lignin and ash content lower the total fraction of biomass that can be converted into sugar, and lignin may decrease the availability of digestible material to enzymes. Fortunately, lime pretreatment of corn stover substantially reduces lignin content, removing approximately half (reduced from 15.7% to 8.3%) of all lignin present in raw biomass (Holtzapple, Wales, et al., 2013).

4.5 Conclusion

Two studies were performed using multi-stage semi-continuous countercurrent saccharification to investigate the improvement in sugar yield as compared to batch saccharification. The first study consisted of two, eight-bottle trains with identical solids concentrations, solid and liquid feed rates, and antibiotic loads. The enzyme loading was maintained at 2 and 5 mg/g in Trains 1 and 2, respectively. Both trains were operated until they reached steady-state operation, at which time the train was terminated and samples were stored, should retesting be necessary. In the second study, a 16-bottle train (Train 3) with identical operating parameters as Train 1 (2 mg/g) operated until it approached steady state, at which point temperature upsets disturbed the steady-state conditions.

Although the countercurrent saccharifications significantly improved sugar yields compared to batch saccharification, there is still plenty of room for improvements:

- Rather than using nonvolatile tetracycline and cycloheximide, alternative volatile antibiotic compounds should be explored.
- Figures 4-7, 4-17, and 4-27 showed that there was still latent enzyme activity remaining at the back of the train, where waste solids were removed, even in Train 3. Figure 4-27 also shows that the front of Train 3 contained four stages where there was no significant increase in sugar concentration, indicating that these bottles added no value to the system. Therefore, adding more bottles and changing the enzyme addition point closer to the front of the train will optimize enzyme usage in countercurrent systems.
- Although the semi-continuous batch transfer system is appropriate for laboratory-scale experiments and exploring the performance of a countercurrent system, it will be necessary in the future to operate a truly continuous countercurrent system to demonstrate commercial scale-up and viability.

CHAPTER V

VOLATILE ANTIBIOTIC STUDY

5.1 Introduction

One of the most critical drawbacks to producing sugars for ethanol fermentation is the need for sterility. Although it introduces an extra process feature, and therefore more cost, it is not an insurmountable obstacle, even at commercial scale. At the laboratory scale, sterility is achieved using a cocktail of potent antimicrobial compounds: tetracycline and cycloheximide. An alternative may be to use volatile antimicrobial compounds that can be recovered during the sugar concentration step following countercurrent saccharification. The motivation for moving away from the laboratory compounds follows: (1) cost, (2) safety, and (3) the need to ensure potency of antimicrobials throughout the residence time of the compound.

Tetracycline and cycloheximide both degrade slowly at the operating conditions used in saccharification. To counteract this, booster doses were added during countercurrent studies. In long-term commercial operation, this may not be viable, so it is desired to find an alternative method of preventing microbial growth. Because these antibiotics are nonvolatile, they cannot be easily recovered after saccharification. Although cycloheximide is rendered harmless at pH >7.0, tetracycline and its degradation products may remain toxic and relatively stable. This poses concerns if sugar products are to be used to feed yeast, animals, or humans. Cost also plays a part. If a recoverable, reusable antimicrobial is selected, it can nearly eliminate the costs

associated with antibiotic addition once the system is operating at steady state. By recycling recovered antibiotics, and feeding enough fresh material to make up for losses caused by thermal degradation or entrainment within waste solids, the day-to-day volatile antibiotic costs are reduced to a fraction of the costs associated with current antibiotics.

5.2 Materials and Methods

5.2.1 Study Design

Under constraints of time and resources, the only study run on volatile antibiotic alternatives was conducted in a single, large batch assay. Each alternative antibiotic was tested at several concentrations believed to be effective at preventing microbial growth, alongside a control sample containing the standard antibiotic cocktail. The assay followed the batch methods already outlined (see Appendix F), with the exception that in test samples, the standard antibiotic cocktail was completely replaced with one of the alternative solutions. To complete the study using the fewest saccharification assays, the samples were limited to 10-mL volumes and the assay was performed using 20×150 mm Durex culture tubes with screw caps from VWR. This allowed for a single, large assay, which would limit variability associated with individual preparations of any reagent.

5.2.2 Ethanol

Ethanol is a relatively safe, low-toxicity alternative to current antibiotics, with a health hazard rating of 2 according to the National Fire Protection Agency (NFPA) diamond. Highly effective disinfection requires concentrations over 70% by volume;

however, high ethanol concentrations adversely affect enzyme activity, especially xylanase. Therefore, the concentrations tested ranged from 5–20% by volume.

5.2.3 Chloroform

Chloroform shares the fairly low toxicity of ethanol, also rated a 2 in health hazard level. Chloroform is commonly used to maintain sterility in microbiology labs, because it substantially inhibits enzyme activity at concentrations as low as 1% by volume. In this study, the chloroform concentrations were 0.05–1.0% by volume.

5.3 Antibiotic Selection

Several factors were considered when selecting volatile antibiotic alternatives. The primary concern was efficacy in preventing microbial growth, a subject that has been studied in some detail for a large variety of compounds. Based on these studies, several potential compounds were selected for future investigation, including both commercially produced chemicals and naturally sourced organic compounds (Dorman, Deans, 2000).

One of the selected alternative antibiotics was ethanol, which is commonly used as a disinfectant and is the end product of fermentation. By using ethanol, it eliminates the need for outside chemicals, creating a more sustainable system.

Chloroform was selected because it is both easy to acquire and use. At room temperature, it exhibits very low solubility in the aqueous phase. At the operating conditions of 50°C, it exerts a high vapor pressure. At 50°C, pure chloroform has a vapor pressure of 530 mm Hg.

5.4 Results and Discussion

5.4.1 Observations and Results

Even before samples were analyzed, it was immediately clear that some samples had suffered substantial inhibition of enzyme activity. By simply allowing particulates to settle, it was clear that high ethanol and chloroform concentrations inhibited sugar production. To quantify this inhibition, samples were tested using high performance liquid chromatography (HPLC) (Appendix C).

To gauge performance, extent of digestion was used to compare each volatile antibiotic sample to the controls, which were prepared according to standard batch saccharification procedure using tetracycline and cycloheximide as antimicrobial agents. To calculate the extent of digestion, Equations 5-1 to 5-8 were used.

$$V = \frac{[\text{inositol}]_{\text{initial}}}{[\text{inositol}]_{\text{final}}} V_{\text{inositol}} \tag{5-1}$$

$$\text{Glu} = [\text{Glu}]\ V/1000 \tag{5-2}$$

$$\text{Xyl} = [\text{Xyl}]\, V/1000 \tag{5-3}$$

$$\text{Gal} = [\text{Gal}]\ V/1000 \tag{5-4}$$

$$\text{Sugar}_{\text{total}} = \text{Glu} + \text{Xyl} + \text{Gal} \tag{5-5}$$

$$\text{Biomass}_{\text{dry}} = \text{Biomass}(1 - \text{MC}) \tag{5-6}$$

$$\text{Biomass}_{\text{equiv}} = \text{Biomass}_{\text{dry}}\big((1.11\ \text{GluC}) + (1.36\ \text{XylC}) + (1.11\ \text{GalC})\big) \tag{5-7}$$

$$\text{Digestibility} = \frac{\text{Sugar}_{\text{total}}}{\text{Biomass}_{\text{equiv}}} \tag{5-8}$$

where,

V = volume of sample (mL)

$[\text{inositol}]_{\text{initial}}$ = concentration of inositol standard (60 g/L)

$[\text{inositol}]_{\text{final}}$ = final concentration of inositol detected in sample (g/L)

V_{inositol} = volume of inositol standard added (0.4 mL)

Glu = mass of glucose in sample (g)

[Glu] = concentration of glucose detected in sample (g/L)

GluC = fraction of glucan content of dry biomass (g glucan/g dry biomass)

Xyl = mass of xylose in sample (g)

[Xyl] = concentration of xylose in sample (g/L)

XylC = fraction of xylan content of dry biomass (g xylan/g dry biomass)

Gal = mass of galactose in sample (g)

[Gal] = concentration of galactose in sample (g/L)

GalC = fraction of galactan content of dry biomass (g galactan/g dry biomass)

$\text{Sugar}_{\text{total}}$ = total sugar in sample (g)

Biomass = air-dry biomass loaded in sample tube (g)

$\text{Biomass}_{\text{dry}}$ = dry biomass loaded in sample tube (g)

$\text{Biomass}_{\text{equiv}}$ = digestible biomass including required water of hydrolysis (g)

MC = moisture content of substrate (g H_2O/g air-dry biomass)

Using these equations, the extent of digestion was calculated for each sample and control, and antibiotic test samples were compared to the control using a simple ratio of digestion (Table 5-1).

Table 5-1 Antibiotic study results summarized**.**

Antibiotic Load		Biomass (g)	Dry Biomass (g)	Digestible Biomass equiv. (g)	Total Sugar (g)	Digestibility (g sugar/ g $biomass_{equiv}$)	Average Digestibility (g sugar/g $biomass_{equiv}$)	Digestibility Ratio (%) Sample:Control
Chloroform	1	2.1292	2.0001	2.1017	1.348	**64.15%**		
0.05%	2	2.1291	2.0000	2.1016	1.359	**64.65%**	**65.13%**	97.84%
	3	2.1292	2.0001	2.1017	1.399	**66.57%**		
Chloroform	1	2.1291	2.0000	2.1016	1.237	**58.85%**		
0.10%	2	2.129	1.9999	2.1015	1.192	**56.73%**	**56.11%**	84.30%
	3	2.1292	2.0001	2.1017	1.109	**52.76%**		
Chloroform	1	2.1293	2.0002	2.1018	0.707	**33.64%**		
0.50%	2	2.1291	2.0000	2.1016	0.736	**35.03%**	**34.83%**	52.32%
	3	2.129	1.9999	2.1015	0.752	**35.80%**		
Chloroform	1	2.1292	2.0001	2.1017	0.460	**21.89%**		
1%	2	2.1292	2.0001	2.1017	0.414	**19.71%**	**19.62%**	29.47%
	3	2.1292	2.0001	2.1017	0.362	**17.25%**		
Ethanol	1	2.1291	2.0000	2.1016	1.121	**53.35%**		
5%	2	2.1294	2.0003	2.1019	1.145	**54.48%**	**53.65%**	80.60%
	3	2.129	1.9999	2.1015	1.116	**53.11%**		
Ethanol	1	2.1295	2.0004	2.1020	0.693	**32.97%**		
10%	2	2.1294	2.0003	2.1019	0.729	**34.68%**	**34.46%**	51.77%
	3	2.1291	2.0000	2.1016	0.751	**35.72%**		
Ethanol	1	2.1295	2.0004	2.1020	0.173	**8.25%**		
20%	2	2.1295	2.0004	2.1020	0.165	**7.87%**	**7.95%**	11.94%
	3	2.1291	2.0000	2.1016	0.163	**7.74%**		
	1	2.1291	2.0000	2.1016	1.397	**66.47%**		
Control	2	2.1292	2.0001	2.1017	1.407	**66.92%**	**66.56%**	100.00%
	3	2.1293	2.0002	2.1018	1.393	**66.29%**		
Substrate	1	2.1291	2.0000	2.1016	0	**0.00%**		
Blank	2	2.1291	2.0000	2.1016	0	**0.00%**		

The results are promising for low doses of chloroform, should future studies confirm antimicrobial activity at such low levels. Ethanol appears to be a poor choice, because it is very inhibitory.

5.4.2 Future Studies

Future studies should include an investigation of other antibiotic compounds, such as essential plant oils. Also, it is necessary to verify efficacy by observing

antimicrobial effects over longer reaction times than the five-day period studied. While performing these additional studies, the control should include standard antibiotic treatments to verify results. Potential alternative antimicrobials derived from essential plant oils are of special interest. At industrial scale, the parent plant could be added directly to the biomass feed and would only be added in the amount required to make-up for losses after recovered oils are recycled. Preliminary research has resulted in several candidate oils to be considered in future testing. Compounds of interest include citral (also known as lemonal), geranyl acetate, carvacrol, and eugenol. These oils can be derived primarily from lemon myrtle or lemongrass, citronella, oregano or thyme, and clove, respectively. Simplifying the process by feeding the source plants to the system mixed with biomass reduces pre-processing costs, and therefore product costs.

5.5 Conclusion and Future Work

The idea of using a recoverable antibiotic serves multiple purposes, including lowering operating costs and increasing quality of the product stream by removing impurities associated with degradation products from current antibiotics. The toxic nature of tetracycline and cycloheximide, and their degradation products, make a strong case for replacement with a less dangerous alternative.

There are several additional studies that must be concluded before any alternatives can be used at a commercial scale. To find the best candidate, additional studies investigating other alternative compounds in a rigorous series of experiments are needed. Because of the long residence time of the liquid phase, it is necessary to prove these compounds are effective at operating conditions for several days up to one month.

Finally, it must be shown that any candidate compound can be readily separated from the sugar product stream by evaporation to eliminate additional recovery costs. Once these conditions are satisfied, volatile antibiotics can become a valuable addition to the countercurrent saccharification platform and allow it to be scaled-up to industrial levels of production.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

The main goal of this work was to investigate countercurrent saccharification as a more efficient alternative to batch saccharification. By improving efficiency of enzyme utilization, the lignocellulose-to-sugar platform can be made more commercially viable. To approximate lignocellulose within the studied systems, model substrates (Solka-Floc and α-cellulose) were selected which would behave similarly to biomass such as corn stover. Figure 6-1 shows that the model substrate (α-cellulose) exhibits similar reactivity to lime-treated corn stover (Holtzapple, Zentay, 2013).

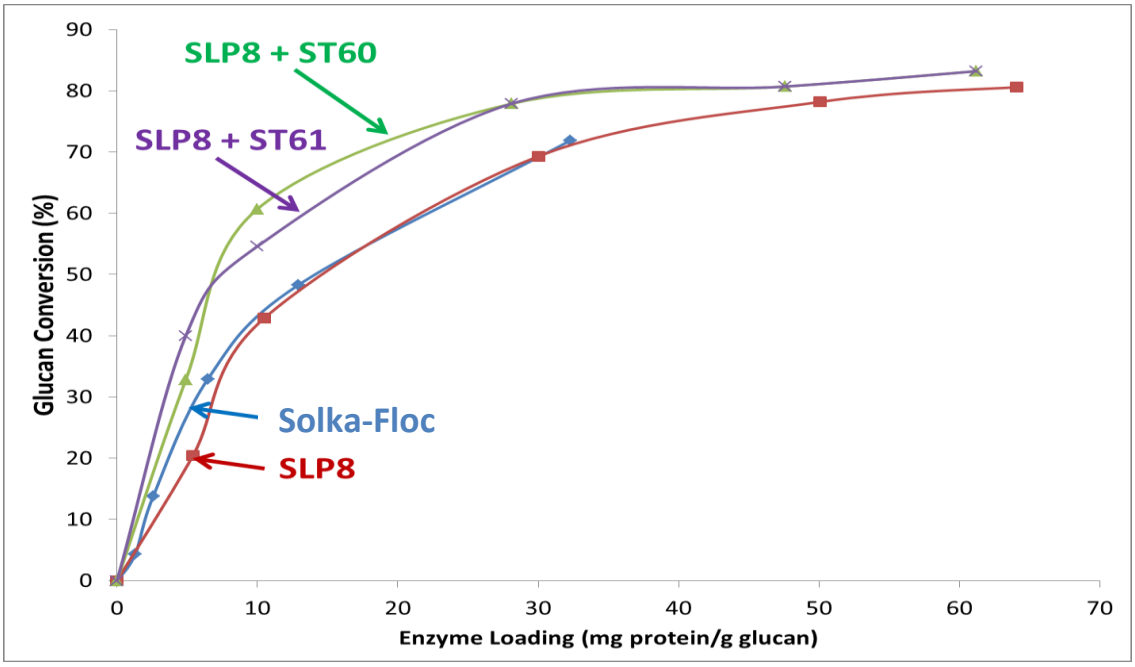

Figure 6-1 Comparison of α-cellulose and lime-treated corn stover (SLP8). Also shown, shock-treated corn stover (SLP8+ST60 and SLP8+ST61).

It should be noted that shock treatment makes lime-treated corn stover significantly more reactive, particularly at low enzyme loadings such as those employed in countercurrent saccharification.

Studies were performed to establish baseline batch digestibility and then to measure improvement using countercurrent saccharification. Finally, alternative antibiotics were briefly investigated to improve safety and recoverability in future endeavors.

In Chapter II, two distinct analysis methods were compared. The DNS method proved unwieldy and inconsistent when applied to the conditions of this study and was therefore abandoned. HPLC was determined to be a feasible analysis method once component costs were mitigated and rigorous maintenance and operation procedures were implemented.

Several substrates were tested to find a biomass analog that could be used in conditions most suitable for commercial scale-up. After several studies, Solk-Floc was chosen for batch saccharification studies. Solk-Floc allowed for a solids concentration of 10%, key to commercial viability. Using Solka-Floc, curves of sugar concentration and yield as functions of time were generated. These curves served as a baseline for comparison of future countercurrent work. A later comparison study established the similar reactivity of Solka-Floc and α-cellulose. Because of this similarity − and its purity, consistency, and ease of handling − α-cellulose was selected for countercurrent studies.

Studies of countercurrent systems investigated three multi-stage trains with varying system variables. Train 1 (2 mg/g enzyme loading, 8 bottles) operated with a

steady-state total sugar yield of 55.9%. Train 2 (5 mg/g enzyme loading, 8 bottles) reached 85.3%, and Train 3 (2 mg/g enzyme loading, 16 bottles) obtained 73.4% yield before system upsets occurred. The countercurrent systems showed a substantial decrease in the amount of enzyme required. To reach a specified digestibility, the ratio of the enzyme load for batch versus countercurrent saccharification was 5−11 (glucose) and 11−37 (xylose).

A final study was conducted to investigate volatile alternatives for antimicrobial agents. The current cocktail is both non-volatile (difficult to recover) and a health hazard. In this initial study of alternatives, chloroform and ethanol were used to completely replace the tetracycline and cycloheximide cocktail that was standard. Except at very low concentrations, these compounds proved to be strong inhibitors of enzyme activity. Of the two, chloroform appears to be the best alternative studied. Many questions remain about volatile antimicrobials that must be answered.

Future work should include the following:

- Optimize bottle count and enzyme addition location in countercurrent trains.
- Modify the countercurrent system to be a truly continuous countercurrent system to demonstrate commercial viability.
- Prove long-term antimicrobial properties of the tested alternative antibiotics.
- Investigate other volatile antimicrobial alternatives including plant essential oils.
- Investigate shock-treated biomass in countercurrent systems to potentially further improve yields.

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APPENDIX A

DNS Reagent

This procedure is adapted from NREL procedure "Measurement of Cellulase Activities" (Adney, Baker, 1996).

Reagents and equipment needed:

Deionized distilled water (DI H_2O)	3,5 dinitrosalicylic acid
Sodium hydroxide	Sodium potassium tartrate
Sodium metabisulfite	Phenol
Hot plate	Ventilated hood
Assorted glassware	

Procedure:

1. Place all needed reagents and equipment in vent hood and perform all steps within well ventilated space.
2. Measure 1416 mL of DI H_2O into a large container to use in solution preparation.
3. Pour 1 L of DI H_2O into a flask or bottle that can safely hold at least 1.5 L.
4. To this water, at 10.6 g of 3,5 dinitrosalicylic (DNS) acid and stir to dissolve.
5. Once dissolved add 19.8 g sodium hydroxide (NaOH) and stir to dissolve.
6. To this solution add 306 g sodium potassium tartrate (Rochelle salts).
7. Add 8.3 g sodium metabisulfite and mix until dissolved.
8. Use remaining water to rinse out any of the above reagents that remained in weigh boats and add to solution.

9. Melt pure phenol crystals in a test tube using a water bath maintained at 50°C. Add 7.6 mL of phenol to DNS reagent solution.
10. Titrate a 3 mL sample with 0.1-N HCl to the phenolphthalein endpoint. It should require 5–6 mL of Cl . Add NaO if needed and repeat titration.
11. Wrap container in foil and store DNS reagent in refrigerator for up to two weeks.

This solution can be prepared in any volume so long as all components are added in the prescribed ratios.

WARNING:

Several components are corrosive, caustic, or toxic, handle with care. DNS acid and the final DNS reagent will stain skin and clothing, take care to avoid contact.

APPENDIX B

DNS Method and Testing

This procedure is adapted from NREL procedure "Measurement of Cellulase Activities" (Adney, Baker, 1996).

Termination of samples and development of color:

1. Remove all samples from incubator.
2. From each test sample, pipette a 1.5-mL aliquot of liquid into an empty, labeled screw-top tube.
3. Add 3.0 mL of DNS reagent to each 1.5-mL aliquot and mix to terminate enzyme activity.
4. Prepare sugar standard solutions to cover the sugar concentration range of interest, typically 1 to 10 mg/mL.
5. Dilute each sugar standard by adding 0.5 mL of the standard to 1.0 mL of 0.1-M citrate buffer.
6. To each diluted sugar standard, add 3.0 mL of DNS reagent and mix well.
7. Cap all tubes tightly.
8. Place all samples, blanks, and standards in vigorously boiling water bath for exactly 5.0 minutes to develop color. Ensure that water level covers the portion of tubes containing liquid.
9. After 5.0 minutes have elapsed, move all samples to an ice-water bath to quench the color development reaction.

Testing of samples:

1. Once any solids have settled, or after briefly centrifuging samples, dilute each sample by adding 0.200 mL of color developed liquid to 2.5 mL of DI H_2O.
2. Using a UV-vis-NIR spectrophotometer, with DI H_2O as a background reference sample, determine the absorbance of each sample at 540 nm.
3. Using the sugar standards to construct a linear calibration curve, calculate the sugar content of each test sample.

APPENDIX C

HPLC Sample Preparation and Testing

1. If samples are frozen, allow to thaw completely.
2. Vortex for 10–15 seconds.
3. Place 1.5–2 mL of liquid into labeled 2-mL Eppendorf tube.
4. Centrifuge Eppendorf tubes in a micro centrifuge at 13000 rpm for 10 minutes.
5. Using a 1 mL disposable syringe, extract free liquid from Eppendorf tubes.
6. Attach a 0.2-μm cellulose acetate filter (VWR, cat. # 28145-477) unit and filter liquid sample into labeled autosampler 12×32 mm snap-it vial (Thermo Scientific, C4011-5).
7. Secure vial caps (Thermo Scientific, cat. # C4011-51) to vials.
8. Prepare sugar standards (1, 2, 5, 10, 25, 50, 75, and 100 g/L glucose concentration, with a 100:18:8 ratio of glucose:xylose:galactose) using analytic grade sugars.
9. Prepare a separate control verification standard (CVS) at 25 g/L glucose concentration (with the same sugar ratios).
10. Analyze samples using an HPLC equipped with refractive index detector, auto-sampler, a pair of de-ashing guard columns (Bio-Rad Micro-Gurad de-ashing cartridges, 30 mm × 4.6 mm), and a HPLC carbohydrate analysis column (Bio-Rad Aminex HPX-87P, 300 mm × 7.8 mm), using HPLC water as a carrier

phase. Maintain analytic column temperature at 85°C, with a water flow rate of 0.6 mL/min, setting assay time to 21 minutes per sample.

APPENDIX D

Citrate Buffer Preparation

This procedure is adapted from NREL procedure "Measurement of Cellulase Activities" (Adney, Baker, 1996).

Reagents and equipment needed:

1 L glass bottle or flask	(2)	Deionized distilled water (DI H_2O)
Glass stir rod	(1)	Citric acid monohydrate
Sodium hydroxide (NaOH)		pH probe and meter
1-L volumetric flask		0.1-N hydrochloric acid (HCl)

Procedure for 1 M stock solution:

1. Fill a 1-L glass volumetric flask with approximately 800 mL of DI H_2O.
2. Weigh 210 g of citric acid monohydrate and add to volumetric flask.
3. Once dissolved, weigh out approximately 35–40 g of NaO and add to flask.
4. Stir to dissolve and measure pH of solution.
5. If pH is near 4.4, skip to Step 7.
6. Take the appropriate action below based on measured pH.
    a. If pH is below 4.4, add a small amount of extra NaOH. Return to Step 4.
    b. If pH is above 4.4, add a small amount of 0.1-N HCl. Return to Step 4.
7. Once pH is 4.4 ± .05, dilute solution to approximately 950 mL and retest pH.
8. Following the same procedure as Step 6, target a pH of 4.48 ± 0.01.
9. Dilute to 1-L mark and test final pH.
10. Store 1-M stock solution of sodium citrate in flask or 1-L bottle in refrigerator.

Procedure for 0.1-M working solution:

1. Add exactly 100 mL of 1-M sodium citrate stock solution into a 1-L volumetric flask.
2. Dilute to approximately 800 mL with DI H_2O.
3. Stir to mix and measure pH of solution.
4. If pH is near 4.7, skip to Step 7.
5. Take the appropriate action below based on measured pH.
    a. If pH is below 4.7, add a small amount of extra NaOH. Return to Step 4.
    b. If pH is above 4.7, add a small amount of 0.1-N HCl. Return to Step 4.
6. Once pH is 4.7 ± .05, dilute solution to approximately 950 mL and retest pH.
7. Following the same procedure as Step 6, target a pH of 4.78 ± 0.01.
8. Dilute to 1-L mark and test final pH.
9. Store 0.1-M working solution of sodium citrate in flask or 1-L bottle in refrigerator.

APPENDIX E

Antibiotic Preparation

Reagents and equipment needed:

Analytic balance w/ 0.1 mg precision	Weighing papers
Weighing spatula	Gloves (2 pairs per antibiotic)
Dust mask or respirator	100-mL volumetric flask (1 per antibiotic)
Deionized distilled water (DI H_2O)	Ethanol (200 proof)
Tetracycline hydrochloride	Cycloheximide

Procedure for tetracycline solution (10 mg/mL) preparation:

1. Put on protective dust mask and two pairs of gloves.
2. Working in a ventilated area or hood, weigh 1.000 ± 0.005 g of tetracycline hydrochloride powder on weighing paper.
3. Carefully funnel into a 100-mL volumetric flask.
4. Immediately store tetracycline powder and discard outer layer of gloves.
5. Add 70 mL of 200-proof ethanol to flask and gently mix to dissolve powder.
6. Fill to mark with DI H_2O and mix well.
7. Store solution in sealed containers in freezer at –10°C for up to three months.

Procedure for cycloheximide solution (10 mg/mL) preparation:

1. Put on protective dust mask and two pairs of gloves.
2. Working in a ventilated area or hood, weigh 1.000 ± 0.005 g of cycloheximide powder on weighing paper.

3. Carefully funnel into a 100-mL volumetric flask.
4. Immediately store cycloheximide powder and discard outer layer of gloves.
5. Add 70 mL of DI H_2O to flask and gently mix to dissolve powder.
6. Fill to mark with DI H_2O and mix well.
7. Store solution in sealed containers in refrigerator for up to three months.

WARNING:

Tetracycline hydrochloride and cycloheximide both have proven developmental toxicity. Both are toxic to the reproductive system and liver. Cycloheximide is also toxic to the nervous system.

Cycloheximide is an ACUTE toxin, exhibiting an LD50 of 2 mg/kg in rats (arsenic has an LD50 in rats of 763 mg/kg), great care should be exercised when handling. To decontaminate a surface of cycloheximide, use an alkali solution such as soap.

APPENDIX F

Batch Procedure

This procedure is adapted from NREL procedure "Enzymatic Saccharification of Lignocellulosic Biomass" (Selig et al., 2008).

Reagents and equipment needed:

Incubator capable of agitation at ~150 rpm

Glass culture tubes (20×150 mm) with screw-caps (VWR, part # 9825-20X)

Auto-pipettes (20–200-µL, 100–1000-µL, and 500–5000-µL)

Moisture content analyzer (Denver Instruments IR 120)

50-mL polypropylene centrifuge tubes (Corning, model # 430828)

Analytic balance w/ 0.1 mg precision

Teflon thread tape

Sealant tape (Fisher, cat # 11-865-28)

100-mL beakers or flasks (1 per sample set)

Substrate

0.1-M sodium citrate buffer (Appendix D)

Dilute CTec2 enzyme (Appendix G)

Deionized distilled water (DI H_2O)

Tetracycline solution (Appendix E)

Cycloheximide solutions (Appendix E)

Determination of samples required:

1. For every combination of solids concentration, enzyme load, and reaction time being tested, three sample replicates are required.
2. For every substrate being tested, two substrate blanks replicates are required.
3. For every enzyme load tested, two enzyme blank replicates are required.

Substrate weighing and preparation:

1. One day prior to the start of batch saccharification, obtain the moisture content of desired substrate in triplicate.
2. Using the average of all three results, calculate required air-dry biomass per sample to 4 decimal places.
3. Weigh out this target mass ± 0.0003g for each sample into labeled glass tubes.

Calculating required enzyme load:

1. Determine protein content of enzyme being used (294 mg/mL for CTec2).
2. Calculate required volume of diluted enzyme (10:1) for 2.000 g dry sample of substrate so that target enzyme load (mg protein/g glucan) is met.

Calculating reaction mixture composition (20-mL reaction volume):

1. Determine number of replicates per sample (triplicate for test samples, duplicate for substrate blanks in this research).
2. Each test sample will contain 10 mL of citrate buffer, 0.160 mL tetracycline, and 0.120 mL cycloheximide.
3. Add the volumes of substrate (assume $\rho \approx 1$ g/cm^3), citrate buffer, tetracycline, cycloheximide, and required dilute enzyme (see above).
4. Subtract these values from the total reaction volume of 20 mL to determine water required.

Reaction mixture preparation (for 20-mL reaction volume):

1. Label a beaker or flask for each triplicate sample set and duplicate substrate blank set being tested.

2. To ensure sufficient liquid for accurate pipetting, multiply the values by 3.5 for test sample sets. Add this amount of each component to the appropriate beaker and mix.
3. To ensure sufficient liquid for accurate pipetting, multiply the values by 2.5 for substrate blank sets. Add this amount of each component to the appropriate beaker and mix.
4. Just prior to the start of saccharification, add the correct amount of enzyme to each reaction mixture.
5. Pipette the exact amount of reaction mixture required to reach a final slurry volume of 20.0 mL (assuming $\rho = 1$ g/cm^3). Record the time that reaction mixture addition begins for each sample.

Enzyme blank preparation (for 20-mL reaction volume):

1. Prepare each enzyme blank (duplicates per enzyme loading) individually.
2. To each test tube add 10.0 mL of 0.1-M citrate buffer.
3. To each test tube add 0.160 mL of tetracycline solution.
4. To each test tube add 0.120 mL of cycloheximide solution.
5. To each test tube add the same volume of dilute CTec2 as was added to test samples.
6. To each test tube add the volume of water required to total exactly 20.0 mL.

Incubation and termination procedures:

1. Place samples into wire rack pairs and zip-tie the racks together to prevent test tubes from moving during incubation.

2. Place racks in incubator, oriented so that tubes are parallel to direction of agitation.
3. Secure racks to incubator table if necessary using wire or zip-ties.
4. Set incubator to operate at 50°C and use an agitation rate of 150 rpm.
5. Allow samples to incubate at these conditions for duration of experiment (5-day reaction was standard).
6. At the end of incubation period remove samples from racks 5 minutes prior to termination.
7. If a volume standard is being used to calculate final sugar content, add it at this point.
8. Place samples in boiling water for 20 min, ensuring boiling water covers entire reaction volume.
9. After boiling, allow samples to cool before transferring to storage vessels or proceeding to testing preparation.
10. If not testing immediately, centrifuge terminated samples and store liquid fraction in freezer.

APPENDIX G

Enzyme Dilution

Reagents and equipment needed:

50-mL volumetric flask	Small beaker
Deionized distilled water (DI H_2O)	Novozyme CTec2
500–5000-µL auto pipette	Kim wipes

Procedure:

1. Fill 50-mL volumetric flask with approximately 20–25 mL of DI H_2O.
2. Remove CTec2 from refrigerator and shake well.
3. Pour a small volume (7–10 mL) of CTec2 stock solution into small beaker.
4. Using auto pipette, draw exactly 5.000 mL of enzyme solution into pipette tip.
5. Carefully wipe sides of pipette tip with Kim wipe, being careful not to touch opening of pipette tip.
6. Slowly empty pipette into volumetric flask, avoiding bubble production.
7. Keeping the tip inside the neck of the flask, remove pipette tip from auto pipette and rinse inside of tip 3–5 times with DI H_2O.
8. Fill carefully to mark, again avoiding bubble production.

NOTE: If larger quantities of dilute CTec2 are desired, it is preferable to make multiple 50-mL batches and homogenize them. This will avoid errors in adding the correct volume of enzyme, as the stock solution is very dense and tends to drip from larger pipette tips.

APPENDIX H

Transfer Procedures for Eight-Bottle Trains

Preparation

Gather the following for each transfer day:

- weigh boats (nine per train, or wash and re-use)
- 50-mL tubes for liquid reserve (one per train)
- 2.0-mL plastic microtubes for sampling (one per bottle per train)
- graduated cylinder (250 mL)
- graduated cylinder (50 mL)
- citrate buffer (prepared, pH 4.8, 0.1-M)
- 1000-µL pipette
- 1000-µL pipette tips (11 per train)
- Diluted CTec2 enzyme
- Tetracycline solution
- Cycloheximide solution
- Metal spatula

Takedown and calibration

1. Tare a weigh boat, measure 10 g of dry cellulose powder for each train, set aside.
2. Remove bottles from incubator and place on paper towels on counter to cool.
3. Calibrate pH probe using 1.68, 4.01, and 7.00 pH buffer solutions.
4. Open Bottles 1–4 and remove Parafilm from lids and threads.

5. Weigh Bottles 1–4, lids removed, record initial weight.
6. Balance Bottles 1–2, 3–4 for centrifuge with lids on.
7. Open Bottles 5–8, remove parafilm from lids and threads.
8. Weigh Bottles 5–8, lids remove, record initial weight.
9. Balance Bottles 5–6, 7–8 for centrifuge with lids on.

Centrifuge Settings

1. Turn centrifuge on.
2. Centrifuge should be using Rotor 5.2.
3. Place balanced bottles into cups and set speed to 3000 rpm for 5 minutes.

Transfer Procedure

Calculate the slurry weight for each bottle = (initial weight) – (bottle weight)

NOTE: To ensure maximum separation of solid and liquid phases during transfers remove only two bottles from centrifuge to begin transfers. Leave remaining two bottles in centrifuge opposite each other and run centrifuge at 3000 rpm for 2 minutes just prior to removing said bottles for transfer.

First Bottle

1. CAREFULLY remove bottle from centrifuge, avoiding sudden movements and jostling.
2. Tare balance with 250-mL graduated cylinder.
3. Slowly pour liquid from bottle 1 into cylinder, record liquid mass and volume.
4. Measure pH of liquid in cylinder.

5. Take 1-mL sample of liquid and pipette into the appropriate micro-centrifuge tube.
6. Dispose of pipette tip.
7. Save 45 mL of liquid from Bottle 1in the labeled 50-mL tube and discard excess.
8. Record wet cake weight, calculate move target

   (wet cake) + (pre-weighed dry cellulose) – (target weight)
9. Remove move target from first bottle and set aside.
10. Add pre-weighed dry cellulose to Bottle 1, record wet cake after transfer.

Bottles 2−7

1. Tare 250-mL graduated cylinder; carefully transfer liquid from bottle to cylinder, record mass and volume of liquid.
2. Measure pH of liquid fraction.
3. Take 1-mL sample and store in appropriately labeled tube.
4. Dispose of pipette tip.
5. Pour liquid remaining into previous bottle, set aside previous bottle
6. Record wet cake weight.
7. Calculate move target

   (wet cake weight) + (weight set aside from previous bottle) – (target weight)
8. Remove move target and set aside.
9. Add solids from previous bottle to current bottle, record wet cake weight after transfer.
10. Repeat Steps 1– for next bottle.

Bottle 8

1. Tare 250-mL graduated cylinder; carefully transfer liquid from bottle to cylinder, record mass and volume of liquid.
2. Measure pH of liquid fraction.
3. Take 1-mL sample and store in appropriately labeled tube.
4. Dispose of pipette tip.
5. Pour liquid remaining into previous bottle, set aside previous bottle.
6. Record wet cake weight.
7. Calculate move target

   (wet cake weight) + (weight set aside from previous bottle) – (target weight)
8. Remove move target and set aside (for moisture content testing).
9. Add solids from previous bottle to current bottle, record wet cake weight after transfer.
10. Add 50 mL of citrate buffer solution and 40 mL of deionized water to Bottle 8.

Ending Transfers

WARNING:

Antibiotics are highly concentrated and pose a moderate-severe health hazard. Both antibiotic solutions are teratogens; avoid skin, mucous membrane, and eye contact. Double glove during handling and immediately dispose of outer layer of gloves after antibiotic additions.

1. To every bottle add 0.4 mL of tetracycline.
2. To every bottle add 0.3 mL of cycloheximide.

3. To appropriate bottle add prescribed enzyme dose (refer to transfer sheet for location and dose for each train).
4. Record final weight of each bottle, without lid.
5. Place Parafilm squares securely over each bottle and tighten lids on appropriate bottles.
6. Place upright in shake table incubator set to 50°C with no agitation for 30 minutes.
7. Tighten lids again to ensure leak-proof seal.
8. Gently shake in a side-to-side motion to homogenize slurry.
9. Place back into rolling incubator in original location.
10. Check that incubator door is securely locked.

Clean-up

- Place 1-mL sample tray back into freezer.
- Place liquid reserve samples in appropriate bags in same freezer.
- Return antibiotics and enzyme solution to storage in appropriate freezer or refrigerator.
- Return citrate buffer to refrigerator.
- Clean and dry weigh boats.
- Clean spatula and return to drying oven.
- Wipe down counters first with water and then with 70% ethanol solution.
- Clean the balance.