BATCH ENZYMATIC HYDROLYSIS OF PRETREATED CORN STOVER AND

IMPROVEMENTS WITH COUNTERCURRENT SACCHARIFICATION

A Thesis

by

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ABSTRACT

Enzymatic saccharification of non-food biomass, such as lignocellulose, can produce sugars. Sugars are the common feedstock for bioethanol, which can be substituted for transportation fuel and address the shortage of fossil fuels. Traditional batch enzymatic saccharification usually wastes enzymes. An approach is countercurrent saccharification, which can make full use of enzymes and therefore reduce the enzyme loadings and lower the cost of sugar and biofuel production.

In this research, various types of enzymes, enzyme loadings, and pretreatments for corn stover have been studied in batch hydrolysis to determine the preferred reaction conditions for countercurrent saccharification. Based on the results, cellulase CTec3 shows better enzymatic saccharification performance than CTec2 for both raw and pretreated corn stover. For a given enzyme dose, lime pretreatment improves enzymatic digestibility of corn stover significantly. Shock treatment of lime-treated corn stover further increases substrate digestibility. At a CTec3 dose of 10 mg protein/g dry biomass, the glucose yield of lime + shock treated corn stover is close to 100%. In contrast, lime pretreated corn stover yields 85%, and raw corn stover yields only 25%. For lime + shock treated corn stover, adding additional HTec3 (1 mg protein/g dry biomass) improved both glucose and xylose yields nearly 30% compared to CTec3 (1 mg protein/g dry biomass). The effect of production inhibition on enzyme activity was

also tested. When 80 g/L glucose was initially added, glucose and xylose yields decrease 20% and 5%, respectively.

Countercurrent saccharification of lime + shock treated corn stover with enzyme CTec3 (1 mg protein/g dry biomass) and CTec3 (1 mg protein/g dry biomass) + HTec3 (1 mg protein/g dry biomass) was studied. When the systems reached steady state, the Slope Method was used to determine product yields and verify that steady state was achieved. For CTec3 (1 mg protein/g dry biomass), the glucose and xylose yields were 64% and 39%, respectively. For CTec3 (1 mg protein/g dry biomass) + HTec3 (1 mg protein/g dry biomass), glucose and xylose yields were 72% and 62%, respectively.

To reach a given glucose yield (64%), when only adding CTec3, countercurrent saccharification saves nearly 50% of the enzyme loading compared with batch saccharification. To reach a given glucose yield (72%), when adding CTec3 and HTec3 (50%:50%), countercurrent saccharification saves nearly 30% of the enzyme loading compared with batch saccharification.

It requires approximately two months to achieve steady-state countercurrent saccharification. In the future, simulation work is necessary to determine the optimal operating condition. Continuum Particle Distribution Modeling (CPDM) is a potential model to simulate countercurrent saccharification.

DEDICATION

This thesis is dedicated to my parents and my boyfriend, who have always been there for me. I am forever grateful.

ACKNOWLEDGEMENTS

Finally, I am grateful to my parents Huiqing Liang and Ronghui Zhou for their professional instruction, unconditional love, and support. Thanks also go to my boyfriend, Chao Gu, for his forever accompany. He is my gift from God.

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CHAPTER I

INTRODUCTION

Currently, fossil fuels such as natural gas, oil, and coal are the main sources of energy and chemicals. However, the shortage of fossil fuels and their impact on the environment are increasingly severe. Developing alternative energy resources is necessary and urgent. Biomass is a leading possible replacement for petroleum-derived liquid transportation fuels that captures solar energy and fixes carbon through photosynthesis (Klass, 2004). It is the only renewable energy resource that can be directly converted to liquid fuels and chemicals. Annually, photosynthesis is estimated to fix 2×10^{11} t of carbon, which contains nearly 3×10^{21} J of energy and is equivalent to 10 times the annual worldwide energy consumption (Schuck, 2006). Biomass is found all over the world with huge variety, such as agroforestry residues and municipal solid waste. Converting biomass into liquid fuels could efficiently relieve severe shortages of liquid fuels and reduce the dependence on fossil energy. Also, unlike petroleum-based fuels, burning bio-based fuels does not add greenhouse gases to the atmosphere, which will mitigate global warming.

Bioethanol is an important biofuel that is usually produced from corn. However, corn is a main food source for animals and human beings. To prevent food shortages, cellulosic ethanol is an attractive alternative. Lignocellulose accounts for nearly half of the world's biomass, and is mainly composed of cellulose, hemicellulose, and lignin

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(Lynch, 1987). Cellulose and hemicellulose are the most abundant components of biomass and can be converted into ethanol, but they are underutilized.

To produce cellulosic bioethanol, lignocellulose is first hydrolyzed to sugars by enzymes. Batch process are widely used in enzymatic saccharification; however, failure to make full use of enzymes in batch processes significantly increases the cost of biofuels. Countercurrent systems are widely used in liquid-liquid extraction (Martin and Synge, 1941), heat exchange (Uozu et al., 1989), and other systems. It also has great potential to improve enzymatic saccharification by fully utilizing enzymes resulting in higher sugar yields than batch saccharification thus reducing the cost of sugar and biofuel production. This is accomplished for the following reasons:

- Enzymes continue to be used longer than batch.

- Inhibition is less where the biomass is less reactive.

- High sugar concentrations can be produced where biomass is less digested and still reactive.

Previous studies of countercurrent saccharification used model compound α-cellulose and Solka-Floc as substrate (Zentay, 2014; Jeffries and Schartman, 1999). In this study, many batch results are presented to determine promising reaction conditions for countercurrent saccharification, such as substrate, enzyme type, and enzyme loading. This study focuses on a real-world substrate for the countercurrent saccharification to determine the improvement over batch saccharification.

CHAPTER II

PRETREATMENT METHODS

2.1 Introduction

Lignocellulose, such as corn stover, mainly consists of cellulose, hemicellulose, and lignin. Cellulose is a linear polysaccharide of glucose residues linked by β-1,4 glycosidic bonds (Holtzapple, 2003a). Hemicellulose is composed of xylose, mannose, galactose, rhamnose, and arabinose with xylose present in the highest amount. (Holtzapple, 2003b). Lignin consists of highly cross-linked phenylpropylene polymer, which resists to microbial attack (Holtzapple, 2003c).

For lignocellulose to be a biological feedstock, the hydrolysis of lignocellulose to glucose and xylose is very important; lignocellulose resists biodegradation. The main reason is the intimate association of lignin with cellulose and hemicellulose. Also, cellulose has a high degree of polymerization and crystallinity (Zhu et al., 2008). To remove lignin and lower the crystallinity, pretreatment is necessary to achieve effective enzymatic saccharification.

Pretreatment methods are usually categorized into physical (milling, grinding), chemical (acids, alkalines, wet oxidation, green solvents), and biological (fungi) methods (Brodeur et al., 2011). In this study, oxidative lime pretreatment and shock pretreatment are used.

2.2 Pretreatment Methods

2.2.1 Raw Biomass

In the United States, corn stover is the most abundant agricultural residue; approximately 80 million dry tons are produced every year (Kadam and McMillan, 2003). In this experiment, unwashed, Champion-milled, 2012 field corn stover is used as the substrate.

2.2.2 Lime Pretreatment

The literature shows that alkaline pretreatment removes lignin and acetyl groups from hemicellulose (Chang and Holtzapple, 2000). Compared to other basic pretreatments, lime pretreatment has many advantages: inexpensive, safe, and simple to recover (Chang et al., 1998). Lime pretreatment has been thoroughly investigated on corn stover (Kim, 2005).

In this study, lime pretreatment procedures mainly followed oxidative long-term pretreatment method (Sierra et al., 2009). Raw corn stover, $Ca(OH)_2$ and water were placed in the pretreatment vessel with the following conditions: 10 kg water/kg dry biomass and 0.15 kg $Ca(OH)_2$/kg dry biomass. The pretreatment time was 30 days with temperature 50 °C. Then the pretreated corn stover was washed, dried, and used as the substrate for enzymatic hydrolysis. The lime pretreatment experiment was performed by Mr. Austin Bond.

2.2.3 Shock Pretreatment

Generally, physical pretreatments such as ball milling effectively lower cellulose crystallinity (Bertran and Dale, 1985). However, all current mechanical treatments are impractical in industry because of high cost, high maintenance, or high energy assumption. Although its mechanism is unclear, shock pretreatment is a new physical pretreatment that has the following advantages: the cost is lower (<$5/tonne) than other mechanical methods and it has the potential to scale up.

Combining lime pretreatment with mechanical pretreatment dramatically improves enzymatic digestibility (Falls and Holtzapple, 2011). In this study, the material for shock treatment was lime-treated corn stover. The shock experiment was performed in a 20-L vessel along with a conical section and a run-up tube (Figure 2-1). The shock vessel was first loaded with 1.4 kg dry corn stover and 14 L water (including water in biomass). H_2 and O_2 were added to the head space of the apparatus and then ignited. Detonation caused a rapid pressure increase within 19 μs. The resulting shock wave is transferred to biomass through the water and breaks open the structure of corn stover. Lastly, the shock-treated biomass slurry was dried and used as a substrate for enzyme essay. The shock treatment experiment was performed by Mr. Austin Bond.
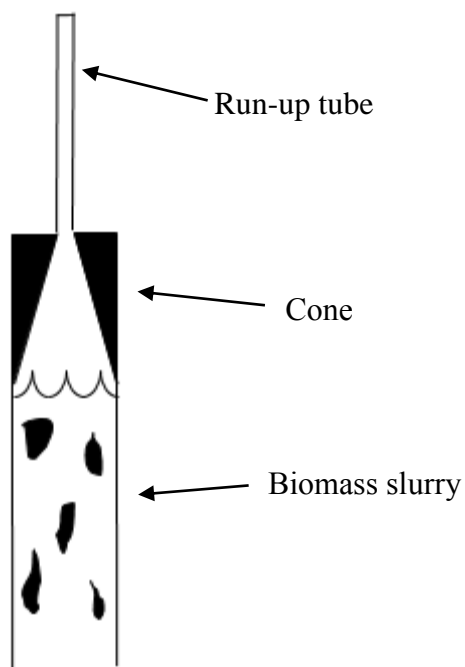
Figure 2-1 Schematic of shock apparatus.

2.3 Results and Discussion

The compositions of raw, lime pretreated, and lime + shock treated corn stover are shown in Table 2-1. The composition analysis was based on NREL analysis method (Hames et al., 2008). According to composition analysis results, glucan, xylan, and lignin (AIL + ASL) are the major components in corn stover. After lime pretreatment, the percentage of glucan increased from 32.6% to 45.3%, whereas lignin decreases from 11.7% to 8.6%, which indicates that lime pretreatment efficiently remove lignin. Shock treatment had only a minor impact on substrate composition.

Table 2-1 Compositions of raw, lime treated, and lime + shock treated corn stover.

Composition	Raw Corn Stover	Lime Treated Corn Stover	Lime + Shock Treated Corn Stover (Batch 1)[3]	Lime + Shock Treated Corn Stover (Batch 2)[3]	Lime + Shock Treated Corn Stover (Batch 3)[3]
Glucan (%)	32.6	45.3	44.3	43.72	42.87
Xylan (%)	19.3	18.1	19.6	20.4	20.4
Galactan (%)	1.2	N/A	0.8	N/A	N/A
Arabinan (%)	2.5	2.4	1.3	N/A	N/A
Mannan (%)	N/A	N/A	N/A	N/A	N/A
AIL[1] (%)	10.1	7.5	9.3	N/A	N/A
ASL[2] (%)	1.6	1.1	1.1	N/A	N/A
Ash (%)	6.3	2.9	3.2	N/A	N/A
Water Extractive (%)	20.1	10.8	11.8	N/A	N/A
Ethanol Extractive (%)	1.9	8.6	6.5	N/A	N/A
Total Extractives (%)	22.0	19.4	18.3	N/A	N/A
Closure (%)	95.6	96.6	97.0	N/A	N/A

([1]AIL: acid insoluble lignin; [2]ASL: acid soluble lignin; [3]The three batches followed same lime and shock pretreatment methods.)

2.4 Conclusion

In this chapter, lime and shock pretreatment methods are introduced. Detailed pretreatment processes are described. The lime treated and lime + shock treated corn stover are used as substrates for enzymatic hydrolysis.

CHAPTER III

BATCH ENZYMATIC HYDROLYSIS OF PRETREATED CORN STOVER

3.1 Introduction

Enzymatic saccharification is usually performed batchwise, which is simple and versatile. Zentay (2014) has tested different reaction conditions, such as reaction time, variety of enzymes, type of substrate, and enzyme loading. This chapter uses batch saccharification to explore different pretreatment methods, various types of cellulases, and enzyme loadings, along with effect of hemicellulase and production inhibition.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Substrate

The substrates studied in this chapter are raw, lime treated, and lime + shock treated corn stover. Detailed methods for lime and shock pretreatment are described in Chapter II. In these batch enzymatic saccharification experiments, 10% solid concentration is selected, which is neither too dilute nor too concentrated.

3.2.1.2 Citrate Buffer

Optimal performance of cellulase CTec3, cellulase CTec2, and hemicellulase HTec3 occur at pH 4.75–5.25, pH 5.0–5.5 and pH 4.8–5.2, respectively (Novozymes, 2010; Novozymes, 2012a; Novozymes 2012b). In this research, pH 4.8, 0.1-M citrate buffer was utilized to maintain relatively high enzyme activity. To prepare the buffer,

citric acid monohydrate and trisodium citrate dihydrate were added to deionized (DI) water. Detailed procedures are provided in Appendix B.

3.2.1.3 Antibiotics

To prevent growth of microorganisms that could consume produced sugars, an antibiotic cocktail was added to every sample. The cocktail was composed of tetracycline and cycloheximide solutions. Preparation methods for these solutions are provided in Appendix C.

3.2.1.4 Enzyme Solutions

Three enzymes – Novozymes Cellic® CTec2, CTec3, and HTec3 – are involved in this study. CTec2 is a blend of aggressive cellulases with high levels of β-glucosidases and hemicellulases that degrade lignocellulose into sugars (Novozymes 2010). CTec3 is Novozymes's newest commercial enzyme product for effective hydrolysis of cellulose. It contains proficient cellulase components boosted by proprietary enzyme activities and a new array of hemicellulase activities (Novozymes 2012a). HTec3 is the newest commercial enzyme product from Novozymes for effective hydrolysis of insoluble and soluble hemicelluloses (Novozymes 2012b).

Protein concentrations of diluted CTec2, CTec3, and HTec3 solutions were determined by Pierce^{TM} BCA protein assay, which was a formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. Proteins reduce Cu^{2+} to Cu^{1+}; 1 mole of reduced Cu^{1+} reacts with 2 moles of BCA to generate a purple color. The purple-colored product of this assay has a strong absorbance

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at 562 nm that increases nearly linearly with increasing protein concentrations over a broad working range (125–1000 µg/mL) (Fisher Scientific, 2013).

In this research, bovine serum albumin (BSA) was used as the standard to determine the protein concentration of diluted CTec2, CTec3, and HTec3 (Figure 3-1). More details about protein concentration assay are provided in Appendix D.

Based on Figure 3-1, the protein concentration of 1000-fold diluted CTec2, CTec3, and HTec3 solutions are 310, 326, and 243 µg/mL, respectively.

The enzyme solutions involved in the batch experiments are diluted 10 times. Detailed preparation methods of these solutions are provided in Appendix A.
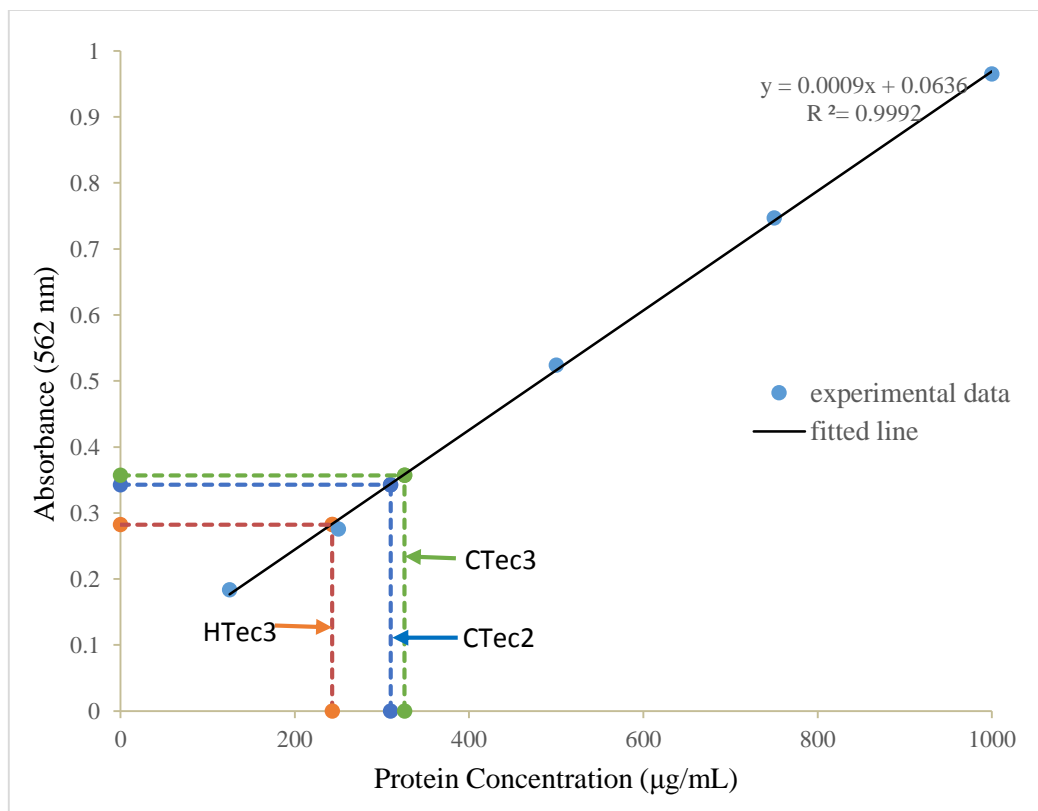
Figure 3-1 Protein concentration of diluted CTec2, CTec3, and HTec3.

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3.2.1.5 Incubator

Optimal performance of CTec2, CTec3, and HTec3 occur at temperatures of 45–50 ℃, 50–55 ℃, and 40–45 ℃, respectively. In this study, a standing incubator cabinet was utilized with temperature setting 50 ℃. The incubator had roller bottles with an axial rotation of 2 rpm, which ensured good mixing.

3.2.2 Calculation Methods

Before adding substrate to reactors, the target air-dry mass was determined by testing moisture content. In this experiment, a Denver Instruments IR 120 device was utilized. The target air-dry mass (M_t) was calculated by the following equation:

$$M_t = \frac{M_d}{1 - \text{MC}} \tag{3-1}$$

where,

M_t = target air-dry mass (g)

M_d = dry mass (g)

MC = moisture content (g H_2O/g air-dry mass)

To calculate the yields of glucose and xylose, product concentration, substrate composition, and mass as well as total reaction volume are required. Product concentrations are analyzed by HPLC, substrate compositions are shown in Table 2-1, substrate mass is 1 g dry biomass, and total reaction volume is 0.01 L. When glucan and xylan are hydrolyzed into glucose and xylose, the mass increases by factors of 1.111 and 1.136, respectively.

The yields of glucose and xylose are calculated by the equations below:

$$\text{Yield}_{\text{glucose}} = \frac{\text{Conc}_{\text{glucose}} \times \text{Vol}_{\text{reaction}}}{\text{Mass}_{\text{substrate}} \times \text{Frac}_{\text{glucan}} \times f_{\text{glucose}}} \tag{3-2}$$

$$\text{Yield}_{\text{xylose}} = \frac{\text{Conc}_{\text{xylose}} \times \text{Vol}_{\text{reaction}}}{\text{Mass}_{\text{substrate}} \times \text{Frac}_{\text{xylan}} \times f_{\text{xylose}}} \tag{3-3}$$

where,

$\text{Yield}_{\text{glucose}}$ = yield of glucose (g glucose/g potential glucose in biomass)

$\text{Yield}_{\text{xylose}}$ = yield of xylose (g xylose/g potential xylose in biomass)

$\text{Conc}_{\text{glucose}}$ = glucose concentration of test samples, enzyme, and substrate blanks subtracted (g/L)

$\text{Conc}_{\text{xylose}}$ = xylose concentration of test samples, enzyme, and substrate blanks subtracted (g/L)

$\text{Vol}_{\text{reaction}}$ = total reaction volume (0.01 L)

$\text{Mass}_{\text{substrate}}$ = dry mass of substrate loaded in the tubes (g)

$\text{Frac}_{\text{glucan}}$ = glucan fraction in dry biomass (g glucan/g dry biomass)

$\text{Frac}_{\text{xylan}}$ = xylan fraction in dry biomass (g xylan/g dry biomass)

f_{glucose} = correction factor due to hydrolysis of glucan (1.111)

f_{xylose} = correction factor due to hydrolysis of xylan (1.136)

In Equations 3-2 and 3-3, the numerator represents the mass of produced glucose or xylose whereas the denominator shows the equivalent glucose or xylose mass in substrate.

To determine the sugar yields, three types of samples (test sample, substrate blank sample and enzyme blank sample) are required for each reaction condition. The

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glucose and xylose hydrolyzed from cellulose and hemicellulose in corn stover are

calculated by the equations below:

$$\text{Vol}_{\text{reaction}} \times \text{Conc}_{\text{glucose}}$$

$$= \text{Vol}_{\text{reaction}} \times \left(\text{Conc}_{\text{gluc, test}} - \text{Conc}_{\text{gluc, subs}} - \text{Conc}_{\text{gluc, enzy}} \right) \qquad (3\text{-}4)$$

$$\text{Vol}_{\text{reaction}} \times \text{Conc}_{\text{xylose}}$$

$$= \text{Vol}_{\text{reaction}} \times \left(\text{Conc}_{\text{xylose, test}} - \text{Conc}_{\text{xylose, subs}} - \text{Conc}_{\text{xylose, enzy}} \right) \quad (3\text{-}5)$$

where,

$\text{Conc}_{\text{glucose}}$ = glucose concentration of test samples, enzyme, and substrate blanks

subtracted (g/L)

$\text{Vol}_{\text{reaction}}$ = total reaction volume (0.01 L)

$\text{Conc}_{\text{gluc, test}}$ = glucose concentration of test samples (g/L)

$\text{Conc}_{\text{gluc, subs}}$ = glucose concentration of substrate blank samples (g/L)

$\text{Conc}_{\text{gluc, enzy}}$ = glucose concentration of enzyme blank samples (g/L)

$\text{Conc}_{\text{xylose}}$= xylose concentration of test samples, enzyme, and substrate blanks

subtracted (g/L)

$\text{Conc}_{\text{xylose, test}}$= xylose concentration of test samples (g/L)

$\text{Conc}_{\text{xylose, subs}}$= xylose concentration of substrate blank samples (g/L)

$\text{Conc}_{\text{xylose, enzy}}$= xylose concentration of enzyme blank samples (g/L)

Test samples contain pre-weighed raw or pretreated corn stover 1 g (dry

biomass), 5 mL 0.1-M citrate buffer, antibiotic cocktail, and diluted enzymes. DI water

is also added to the tubes to ensure the total volume is 10 mL. Each test sample is repeated in triplicate.

Substrate blank samples are used to determine the mass of free sugars in biomass, which contains pre-weighed biomass 1 g (dry mass), 5 mL 0.1-M citrate buffer, an antibiotic cocktail, and DI water. The enzymes are replaced by additional DI water. Each substrate blank sample is repeated in duplicate.

Enzyme blank samples can be utilized to determine the amount of free sugars in enzyme solutions, which include DI water, 5 mL 0.1-M citrate buffer, antibiotic cocktail, and enzymes. The substrates are replaced by additional DI water. Each enzyme blank sample is repeated in duplicate.

Because of the presence of small amount of free sugars in the substrate and enzyme solutions, correction calculations are necessary. The measured glucose or xylose in test samples must be subtracted from sugars in substrate blanks and enzyme blanks to determine the glucose or xylose hydrolyzed from cellulose or hemicellulose in corn stover.

3.3 Experimental Design

For each batch enzymatic saccharification, substrate, buffer, water, antibiotic cocktail, and enzymes were added to a 50-mL centrifuge tube in the listed order (Figure 3-2).
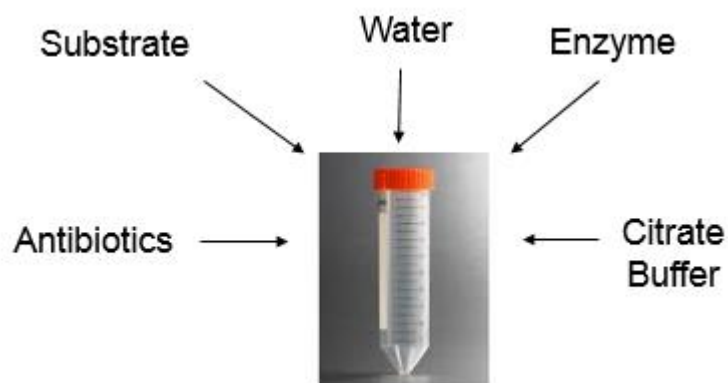
Figure 3-2 Schematic of batch saccharification experiment.

After five days reaction (Selig et al., 2008), the experiments were terminated and samples were analyzed by HPLC. Detailed preparation and termination procedures are provided in Appendix F. The HPLC analysis procedure is provided in Appendix E.

In this chapter, raw, lime treated, and lime + shock treated corn stover are investigated as substrates. Two kinds of cellulases (CTec2 and CTec3) and enzyme loadings (1, 2, 5, 10, 15 mg protein/g dry biomass) were studied. Because corn stover is nearly 20% xylan, to fully utilize the substrate, hemicellulase HTec3 was added to increase the xylose yield. High glucose concentrations inhibit cellulase activity (Hsieh, 2014). In this research, initial glucose concentrations 20, 40, 60, 80 g/L were added to the tubes along with pretreated corn stover to test the enzyme activity under high glucose concentrations.

3.4 Results and Discussion

3.4.1 The Effect of Pretreatment Methods on Enzymatic Hydrolysis of Corn Stover

In corn stover, pretreatment processes help break the complex structure of lignocellulose, thus significantly increasing the contact area between cellulase and cellulose. According to Figure 3-3, the glucose yield of lime-treated corn stover is much higher than that of raw corn stover at enzyme loading 1–25 mg protein/g dry biomass, which indicates lime pretreatment is very efficient. Compared with lime-treated corn stover, hydrolyzing lime + shock treated corn stover gives much higher glucose yield. When using CTec2 as cellulase (5–25 mg protein/g dry biomass), the difference in glucose yields between lime only and lime + shock treated corn stover is about 10%. When using CTec3 (10–25 mg protein/g dry biomass), the difference increases to 15%; therefore, CTec3 enhances the benefit of shock treatment. At CTec3 loading of 10 mg protein/g dry biomass, the glucose yield of lime + shock treated corn stover reaches close to 100%.
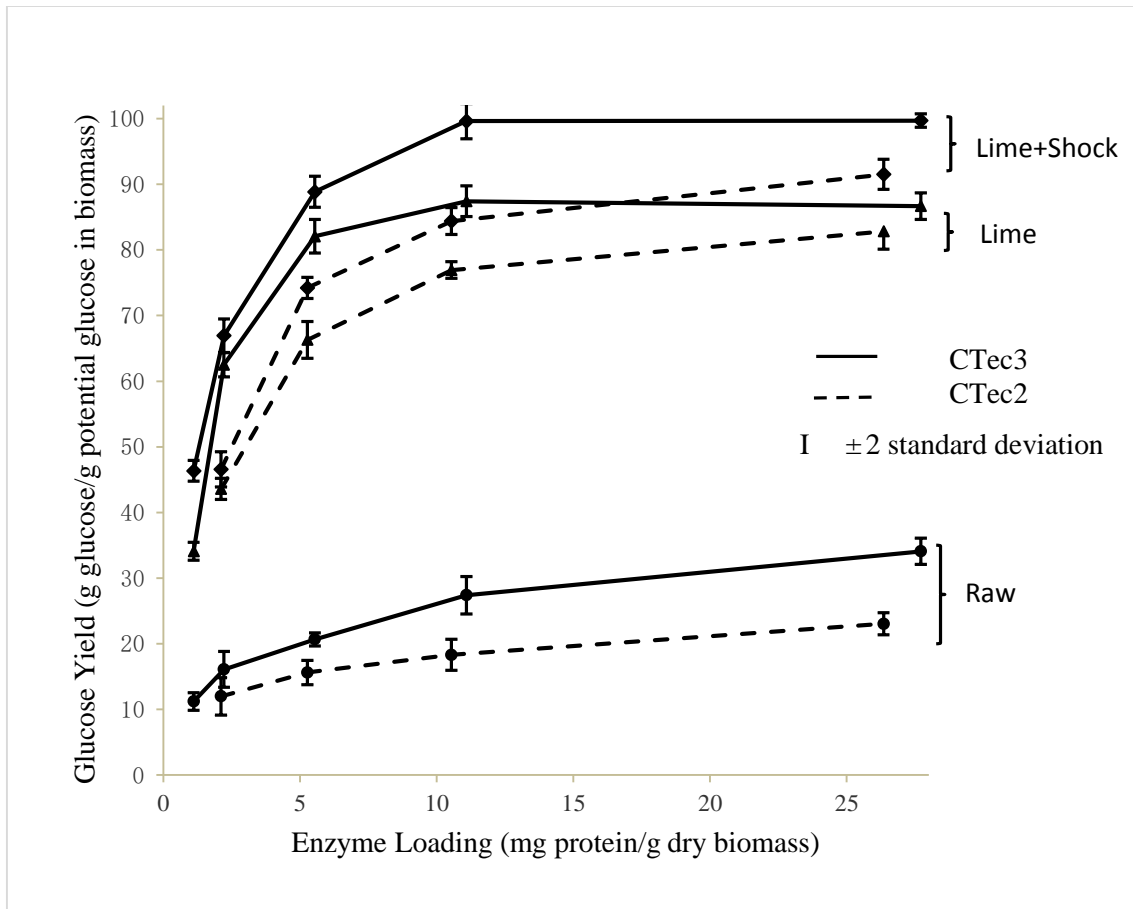
Figure 3-3 Effect of pretreatment methods, enzyme type, and loadings on glucose yield. ([1]Lime represents substrate is lime treated corn stover (Batch 1). [2]Lime + Shock represents substrate is lime + shock treated corn stover (Batch 1). [3]Raw represents substrate is raw corn stover.)

3.4.2 The Effect of Various Cellulases and Enzyme Loadings on Enzymatic Hydrolysis of Pretreated Corn Stover

Novozymes reports that CTec3 has at least 1.5 times higher conversion efficiency than that of CTec2 (Novozymes, 2012a). In this research, for lime + shock

treated corn stover to reach the same glucose yield (~46%), the loading of CTec2 is nearly double that of CTec3. For pretreated corn stover, at the same enzyme loadings (1– 10 mg protein/g dry biomass), the yield of glucose catalyzed by CTec3 is nearly 15% higher than that of CTec2. As enzyme loading increases, the difference of glucose concentration between CTec3 and CTec2 becomes smaller. For raw corn stover, when enzyme loading increases (>10 mg protein/g dry biomass), the difference of glucose yield between CTec3 and CTec2 remains unchanged.

Additionally, according to Figure 3-3, as enzyme loading increases from 1 to 10 mg protein/g dry biomass, the glucose yields of both raw and pretreated corn stover improve significantly. With enzyme loadings of 10–25 mg protein/g dry biomass, there is no obvious increase of glucose concentration for pretreated corn stover.

3.4.3 The Effect of Hemicellulase on Enzymatic Hydrolysis of Pretreated Corn Stover

Both raw and pretreated corn stover contain about 20% hemicellulose. To fully utilize the biomass, hemicellulase HTec3 was added to hydrolyze the hemicellulose. In this study, the effect of different HTec3 loadings on glucose and xylose yields was tested using a CTec3 loading of 1 mg protein/g dry biomass. The substrate is lime + shock treated corn stover (Batch 3); the results are shown in Figure 3-4. When the HTec3 loading increases from 0–1 mg protein/g dry biomass, both glucose and xylose yields improve nearly 30%. Glucose yields increase from adding hemicellulase because it further breaks down lignocellulose structure resulting in further exposure of cellulose in corn stover. When only adding HTec3 of 1 mg protein/g dry biomass, the yields of

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xylose and glucose are 48% and 5%, respectively, indicating that HTec3 is fairly

selective for hemicellulose and has little cross reactivity with cellulose.
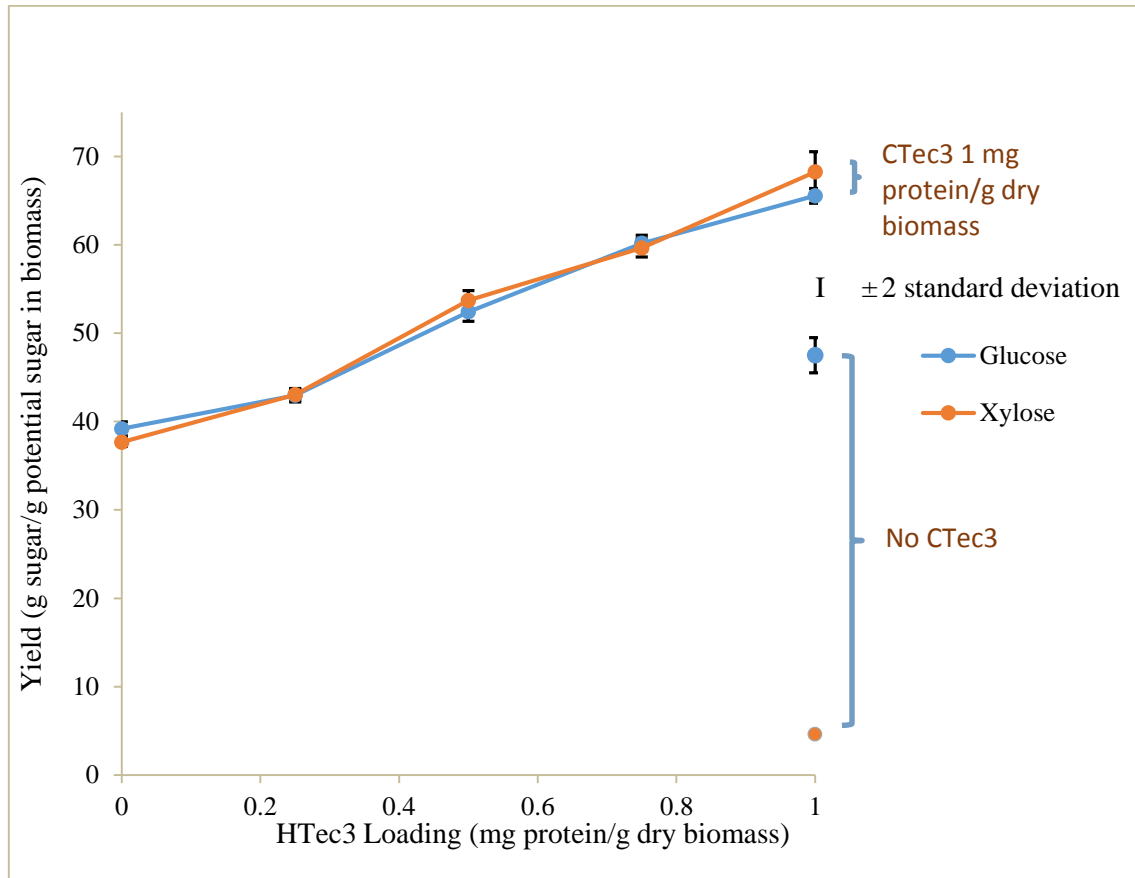
Figure 3-4 Effect of HTec3 loading on glucose and xylose yields.

3.4.4 The Effect of Product Inhibition on Enzymatic Hydrolysis of Pretreated Corn Stover

The literature reports that glucose binds with cellulase, which reduces

enzyme/substrate complex and therefore lowers substrate conversion (Holtzapple, 1984).

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This experiment was designed to verify that conclusion. Lime + shock treated corn stover (Batch 3) was chosen as substrate. Different glucose concentrations were initially added to the tubes along with pretreated corn stover, other reaction conditions remain unchanged. When initial glucose concentration increased from 20 to 80 g/L, both glucose and xylose yields show obvious decreases, whereas glucose yield decreases much faster (Figure 3-5). The decrease of xylose yield might be caused by the inhibition of hemicellulase activity in CTec3.
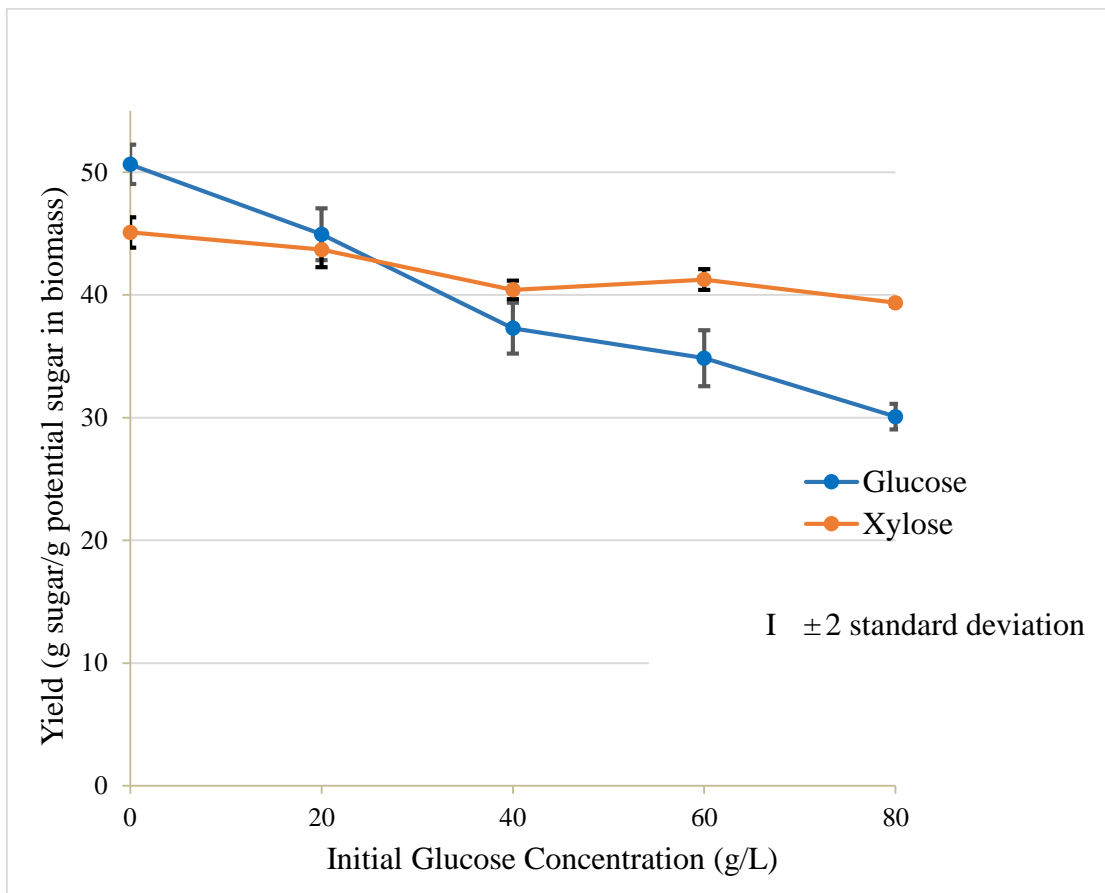
Figure 3-5 Effect of initial glucose concentration on glucose and xylose yields.

(CTec3 loading = 1 mg protein/g dry biomass)

3.5 Conclusion

This study shows that CTec3 performs better than CTec2 for both raw and pretreated corn stover. When increasing enzyme loadings (from 1 to 10 mg protein/g dry biomass), biomass conversion improved significantly. At a given enzyme dose, lime pretreatment enhances enzymatic digestibility of corn stover significantly. Shock treatment of lime-treated corn stover further increases substrate digestibility. At CTec3 doses of 10 mg protein/g dry biomass, the glucose yields of lime + shock, lime-treated only, and raw corn stover are ~100, 85, and only 25%, respectively. Using CTec3 (1 mg protein/g dry biomass), adding HTec3 (1 mg protein/g dry biomass) improves both glucose and xylose yields nearly 30%. When 80 g/L glucose was initially added, glucose and xylose yields decreases 20% and 5%, respectively, which indicates strong product inhibition.

CHAPTER IV

COUNTERCURRENT SACCHARIFICATION OF LIME + SHOCK TREATED

CORN STOVER

4.1 Introduction

Batch enzymatic saccharification shows many advantages, such as simplicity and versatility; however it has drawbacks as well. In batch analysis, high enzyme loadings are usually required to achieve high biomass conversion. As the biomass digests, it becomes less reactive while the enzymes become increasingly inhibited by accumulated product. In contrast, in countercurrent saccharification, the least reactive biomass contacts the lowest glucose concentration thus reducing the inhibition of product. Fresh liquid is added to the most digested biomass and product is removed continuously from the saccharification system (Zentay, 2014; Fu, 2007).

Countercurrent saccharification of a lignocellulose model compound (α-cellulose) has been studied by Zentay (2014). He used Novozymes CTec2 to perform multi-stage semicontinuous countercurrent saccharification. Compared to standard batch saccharification, to reach a given product yield, countercurrent saccharification reduced enzyme loadings by 5–11 times for glucan and 11–32 times for xylan. Jeffries and Schartman (1999) used Solka-Floc as substrate and an enzyme loading of 5.56 FPU/g to perform three-stage pseudo-countercurrent saccharification. Compared to batch saccharification, the countercurrent saccharification improved glucose yields by 1.39 and 1.46 times at solid concentrations of 4% and 8%, respectively.

In this study, countercurrent saccharification is tested with corn stover a "real-world" lignocellulose substrate to determine the improvement of countercurrent over batch enzymatic saccharification.

4.2 Materials and Methods

4.2.1 Materials

4.2.1.1 Substrate

As shown in Chapter Ⅲ, at a given enzyme loading, hydrolyzing lime + shock treated corn stover produces more sugars than only lime-treated corn stover. Considering the huge time costs to perform countercurrent experiments, only lime + shock treated corn stover is selected as substrate in this study.

4.2.1.2 Citrate Buffer

Similar to batch analysis, 0.1-M citrate buffer with a pH of 4.8 is used to maintain high enzyme activity. A large amount of citrate buffer is needed for this countercurrent experiment. The preparation method is provided in Appendix B.

4.2.1.3 Antibiotics

The time needed for countercurrent saccharification experiment trains to reach steady state is much longer than the typical five days of batch enzymatic saccharifications. Adding antibiotics to the reaction system is especially necessary to avoid microbial growth. Like batch saccharification, antibiotic solutions consist of tetracycline and cycloheximide. The preparation methods are provided in Appendix C.

4.2.1.4 Enzyme Solutions

In the countercurrent saccharifications, CTec3 is selected because of its much better performance than CTec2 (Chapter III). To increase the total sugar yield, HTec3 can also be added to enhance the hydrolysis of hemicellulose in pretreated corn stover. Detailed preparation procedures are provided in Appendix A.

4.2.2 Experimental Design

The selections of reaction conditions for the countercurrent experiment are based on batch analysis results. To save the cost of enzymes and ensure relatively high sugar yields, CTec3 is utilized with loading 1 mg protein/g dry biomass. To increase the xylose yield, HTec3 (1 mg protein/g dry biomass) is added to the system after the first steady state is reached.

Figure 4-1 shows the schematic of the countercurrent experiment. Selecting the number of bottles in the countercurrent train is critical. A train with 16 bottles obtains higher sugar yields than one with 8 bottles at identical reaction conditions (Zentay, 2014); therefore, a 16-bottle train was utilized in this study.
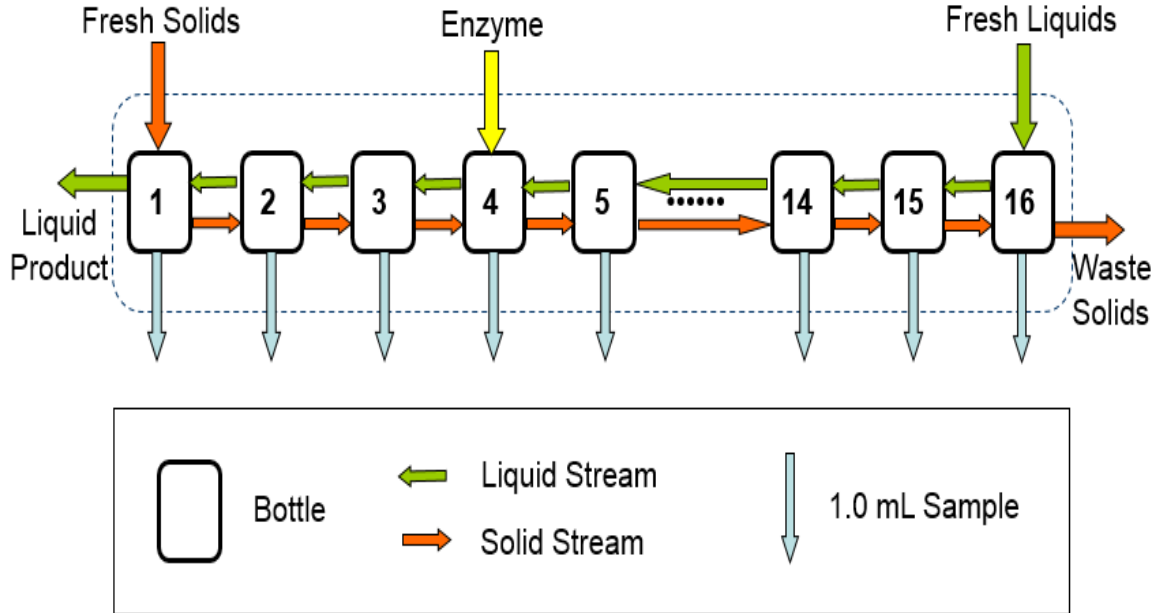
Figure 4-1 System diagram of countercurrent saccharification.

The initial loadings (Day 0) of countercurrent experiment are summarized in Table 4-1. All 16 bottles had the same initial loadings. The total volume of each bottle was 250 mL, the solid concentration was 10%, and given amount of antibiotics cocktail and enzymes were also added. Then transfers were performed every other day (48 hours), which gives relatively enough time for enzymes to hydrolyze the substrate.

Table 4-1 Initial loadings of countercurrent saccharification experiment.

	Citrate Buffer (mL)	Water (mL)	Substrate (g)	Tetracycline (mL)
Bottles 1 – 16	125	95.063	27.42	1
	Cycloheximide (mL)	CTec3 1 mg protein/ g dry biomass (mL)	Total Volume (mL)	
	0.75	0.767	250	

In every transfer, each bottle was centrifuged to achieve phase separation of liquid and solid wet cake (70–80% moisture content). The volume and mass of liquid and weight of wet cake for every bottle were recorded. The pH of the liquid was measured to ensure it was compatible with the enzymes. Liquid samples (1 mL) were taken from every bottle and analyzed by HPLC to determine when the system reached steady state. When the sugar concentrations from each bottle did not show significant change over a relatively long time (e.g., 15 days), the system was determined to reach steady state.

The liquid was transferred from "back" to "front" while sufficient wet cake was moved in the opposite direction to maintain a target wet weight in the bottle (90 g) (Figure 4-1). All the liquid was transferred from its current bottle to the previous bottle. The solid concentration of the total system was about to 10%, similar to the batch hydrolysis. The solid wet cake moved from the current bottle to the adjacent bottle was the current wet cake weight plus moved cake from previous bottle minus target wet cake (90 g). After the transfer procedure, 10 g dry biomass was loaded in Bottle 1 and 90 mL

liquid consisting of 50 mL citrate buffer and 40 mL DI water was added to Bottle 16.

Given amounts of antibiotic cocktail were also introduced to every bottle. The enzymes

tend to combine with the solid substrate, thus the enzyme addition location should be

close to the place where fresh solids were added during each transfer (Jeffries and

Schartman, 1999). Therefore, enzymes were added to Bottle 4. The detailed transfer

procedures are provided in Appendix G.

4.2.3 Calculation Methods

To calculate the glucose and xylose yields, the sugars entering and exiting the

countercurrent system must be determined. In every transfer, 10 g dry lime + shock

treated corn stover was added to Bottle 1, which means 4.85 g equivalent glucose and

2.32 g equivalent xylose entered system. The sugars exiting the system are the

summation of sugars exiting from Bottles 1 and 16, and sugars in liquid samples from all

16 bottles. Glucose yield is calculated by Equations 4-1 to 4-6. Calculation of xylose

yield is similar to glucose yield.

$$\text{Yield}_{\text{glucose}} = \frac{\text{Mass}_{\text{glucose, out}}}{\text{Mass}_{\text{glucose, in}}} \times 100\% \tag{4-1}$$

$$\begin{aligned}
\text{Mass}_{\text{glucose, out}} \\
&= \text{Mass}_{\text{glucose, Bottle 1}} + \text{Mass}_{\text{glucose, Bottle 16}} \\
&+ \text{Mass}_{\text{glucose, sum samples}}
\end{aligned} \tag{4-2}$$

$$\text{Mass}_{\text{glucose, Bottle 1}} = \text{Vol}_{\text{liq, 1}} \times \text{Conc}_{\text{glucose, 1}} \tag{4-3}$$

$$\text{Mass}_{\text{glucose, Bottle 16}} = \text{Mass}_{\text{cake, 16}} \times \text{MC}_{16} \times \text{Conc}_{\text{glucose, 16}} \tag{4-4}$$

$$\text{Mass}_{\text{glucose, samples}} = \sum \left(\text{Conc}_{\text{glucose},i} \times \text{Vol}_{\text{samples},i} \right), i = 1 \text{ to } 16 \qquad (4\text{-}5)$$

$$\text{Mass}_{\text{glucose, in}} = \text{Mass}_{\text{air-dry biomass}} \times (1 - \text{MC}_1) \times \text{Frac}_{\text{glucose}}$$

$$\times f_{\text{glucose}} \qquad (4\text{-}6)$$

where,

$\text{Yield}_{\text{glucose}}$ = glucose yield (g glucose/g potential glucose in biomass)

$\text{Mass}_{\text{glucose, in}}$ = total glucose entering the system in every transfer (g)

$\text{Mass}_{\text{glucose, out}}$ = total glucose out of system in every transfer (g)

$\text{Mass}_{\text{glucose, Bottle 1}}$ = glucose in liquid product exiting from Bottle 1 (g)

$\text{Mass}_{\text{glucose, Bottle 16}}$ = glucose in wet cake exiting from Bottle 16 (g)

$\text{Mass}_{\text{glucose, sum samples}}$ = summation of glucose in all liquid samples (g)

$\text{Mass}_{\text{cake, 16}}$ = mass of wet cake exiting from Bottle 16 (g)

$\text{Mass}_{\text{air-dry biomass}}$ = mass of substrate entering in Bottle 1 in every transfer (g)

$\text{Frac}_{\text{glucose}}$ = fraction of glucose in lime + shock treated corn stover

$\text{Conc}_{\text{glucose},i}$ = the glucose concentration of Bottle i (i =1 to 16) (g/L)

$\text{Vol}_{\text{liq, 1}}$ = liquid product exiting from Bottle 1 (L)

MC_1 = moisture content of substrate entering Bottle 1

MC_{16} = moisture content of wet cake exiting from Bottle 16

$\text{Vol}_{\text{samples},i}$ = the sample volume exiting from every bottle in every transfer (i =1 to 16) (0.001 L)

f_{glucose} = correction factor due to hydrolysis of glucan (1.111)

4.3 Results and Discussion

4.3.1 Section 1 – Addition of CTec3

In Section 1 (Days 0 to 126), cellulase CTec3 with loading 1 mg protein/g dry biomass was added to Bottle 4 at the end of every transfer. Glucose and xylose concentrations as a function of time and bottle number are shown in Figures 4-2 and 4-3. Between Days 0 and 65, glucose and xylose concentrations of Bottles 1 to 4 increased gradually, whereas the concentrations dropped slowly in Bottles 5 to 16. Based on Figures 4-2 and 4-3, between Days 78 and 126, both glucose and xylose concentrations of every bottle have stabilized. Regions between Days 78 and 96 are highlighted and shown in Figures 4-4 and 4-5, which clearly show the system reached steady state.
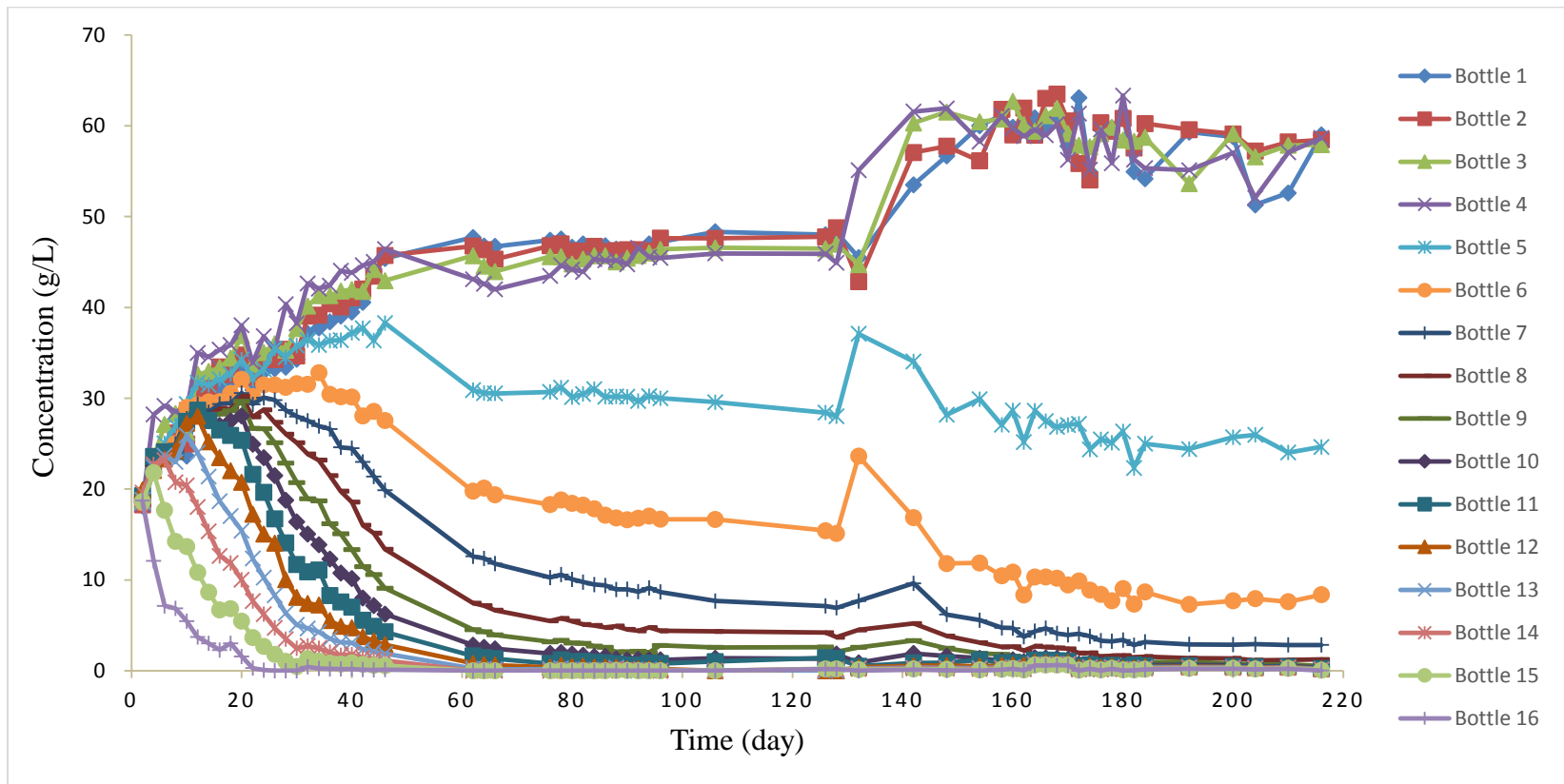
Figure 4-2 Glucose concentration as a function of bottle number and time between Days 0 and 216.

(From Days 0 to 126, substrate is lime + shock treated corn stover Batch 2; From Days 128 to 216, substrate is lime + shock treated corn stover Batch 3. Composition data are shown in Table 2-1.)
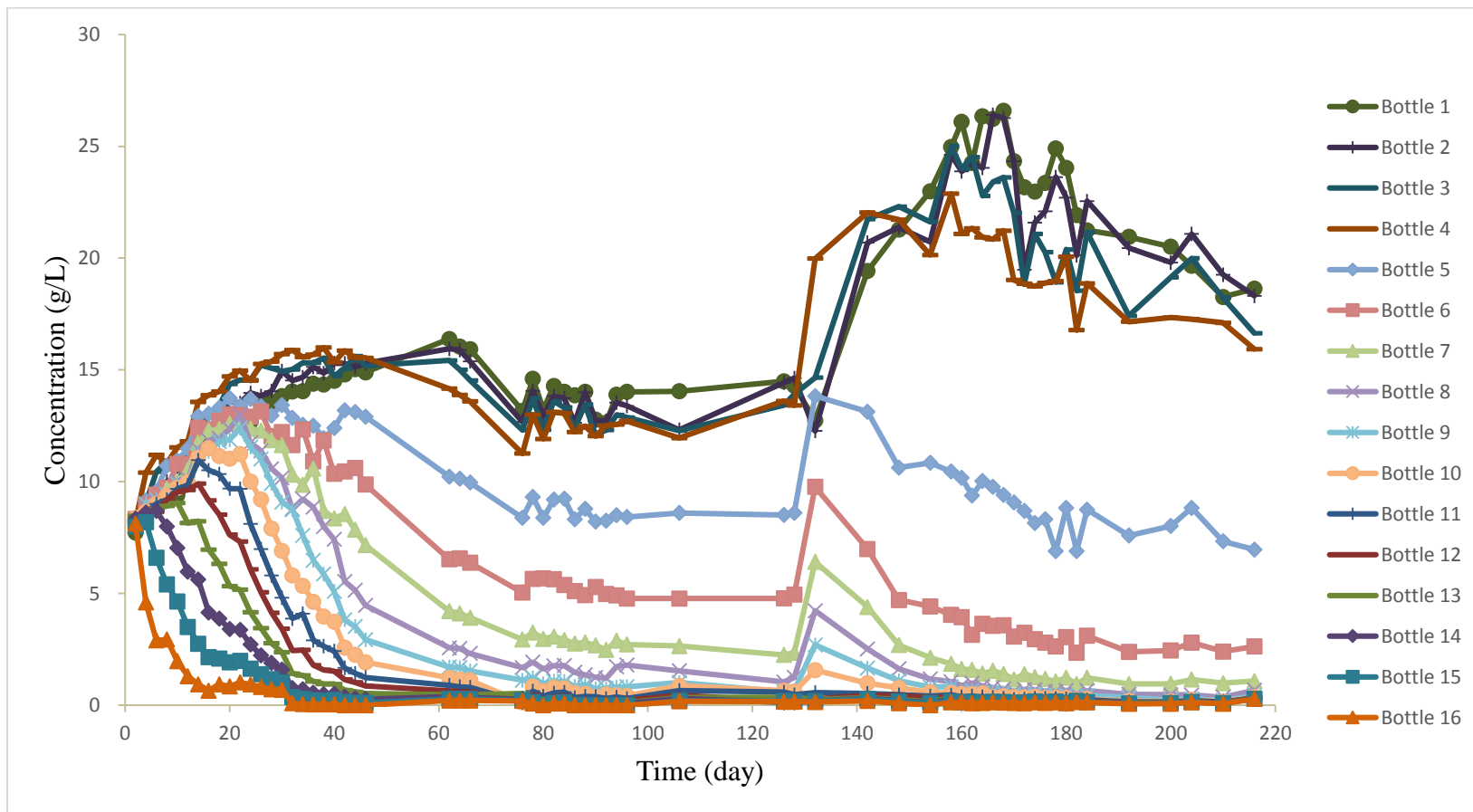
Figure 4-3 Xylose concentration as a function of bottle number and time between Days 0 to 216.

(From Days 0 to 126, substrate is lime + shock treated corn stover Batch 2; From Days 128 to 216, substrate is lime + shock treated corn stover Batch 3. Composition data are shown in Table 2-1.)
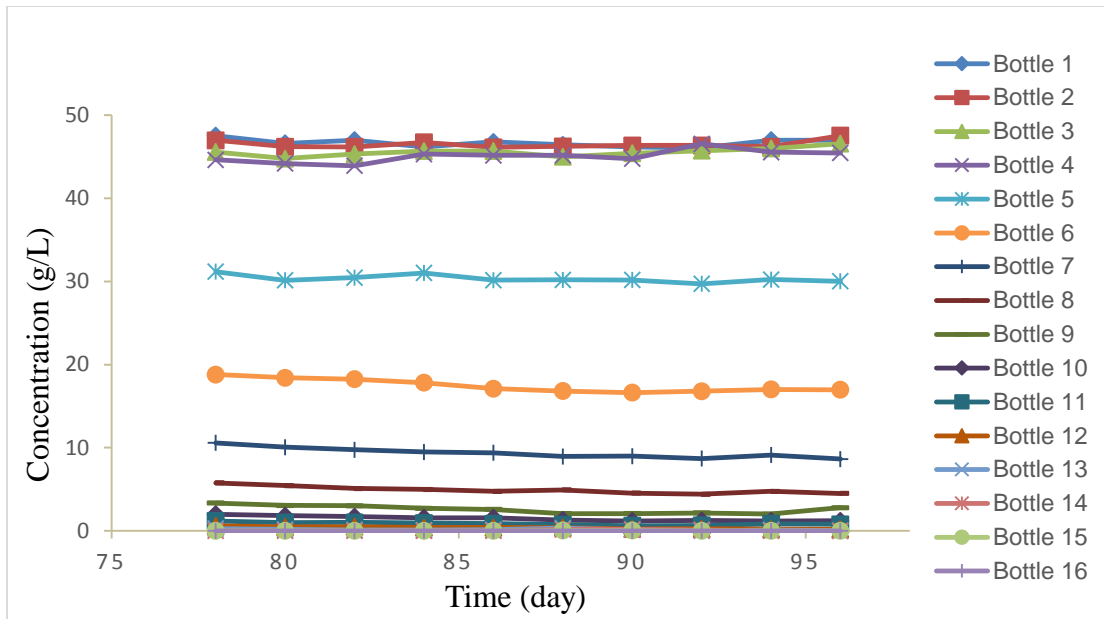
Figure 4-4 Glucose concentration as a function of time and bottle number between Days 78 and 96.
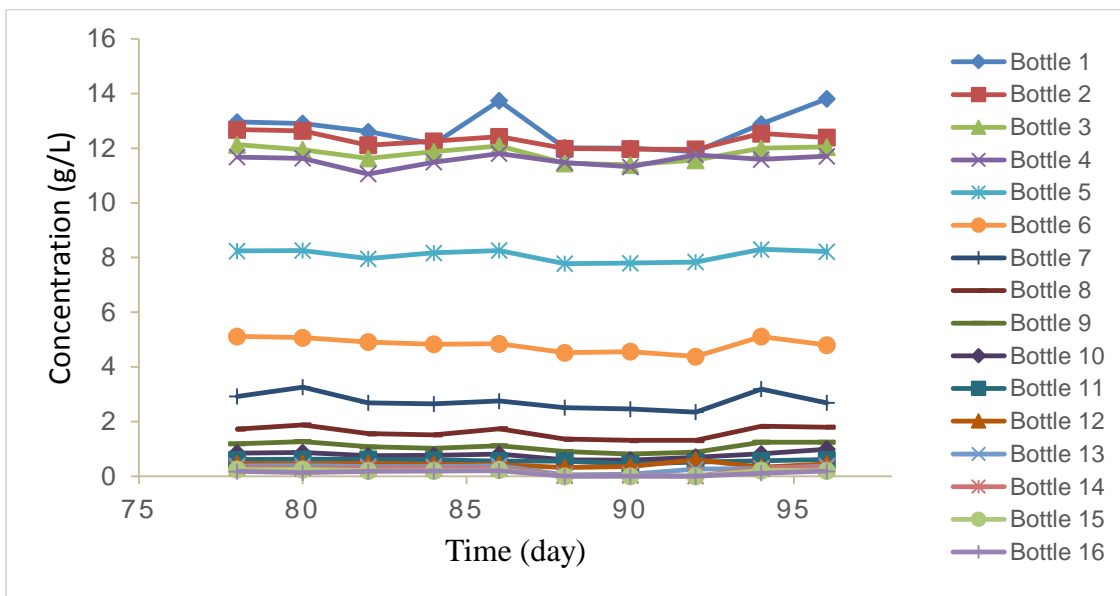
Figure 4-5 Xylose concentration as a function of time and bottle number between Days 78 and 96.

Figures 4-6 and 4-7 show the glucose and xylose concentrations as a function of bottle number on Day 82. The increase of sugar concentrations from Bottles 4 to 1 is very slight, whereas the concentration decreases dramatically from Bottles 4 to 10. In the transfer procedure, the liquid is transferred from "back" to "front" whereas the wet cake solid is moved in opposite direction. This experiment results verify that the enzymes have affinity to the substrate instead of existing in liquid phase. From Bottles 11 to 16, nearly no sugars can be detected, which indicates the enzymes are fully utilized.

Figure 4-6 Glucose concentration as a function of bottle number and comparison between two sections.
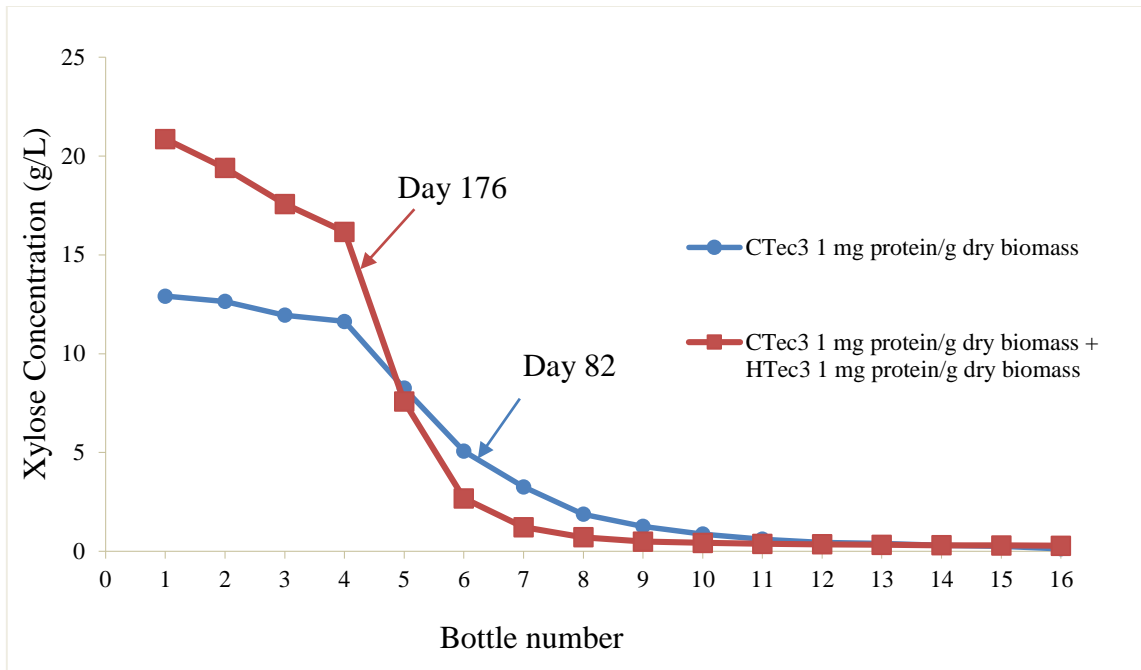
Figure 4-7 Xylose concentration as a function of bottle number and comparison between two sections.

The Slope Method verifies the system is at steady state while also getting reliable sugar yields (Smith, 2011). The samples taken during steady-state region Days 78 to 96 are analyzed with Slope Method. Table 4-2 lists the cumulative data of glucose, xylose, and total sugar in and out of the system and corresponding yields during this region. The sugar yields in the table fluctuate only in very narrow range. After 20 days, glucose, xylose, and total yields are 64%, 39%, and 56%, respectively.

Figure 4-8 shows cumulative glucose, xylose and total sugar mass out of the system along with cumulative sugar entering the system and their dependence on time are drawn and linear regression lines are added in Figure 4-8. Excellent fit and very low deviation of lines validate that steady state occurred after Day 78.

Table 4-2 Cumulative sugar data for Section 1.

Time (day)	Out (g)			In (g)				Yield		
	Cumulative Glucose	Cumulative Xylose	Cumulative Total Sugars	Dry Biomass	Biomass Equivalent	Glucose Equivalent	Xylose Equivalent	Total Sugar	Glucose	Xylose
78	3.17	0.98	4.15	10	7.17	4.85	2.32	0.58	0.65	0.43
80	6.54	1.94	8.49	20	14.34	9.71	4.63	0.59	0.67	0.42
82	10.23	3.06	13.29	30	21.51	14.56	6.95	0.62	0.70	0.44
84	13.25	3.97	17.22	40	28.68	19.41	9.27	0.60	0.68	0.43
86	16.12	4.81	20.94	50	35.85	24.26	11.59	0.58	0.66	0.42
88	19.23	5.74	24.97	60	43.02	29.12	13.90	0.58	0.66	0.41
90	22.13	6.55	28.68	70	50.19	33.97	16.22	0.57	0.65	0.40
92	24.68	7.25	31.93	80	57.36	38.82	18.54	0.56	0.64	0.39
94	27.83	8.11	35.94	90	64.53	43.68	20.85	0.56	0.64	0.39
96	30.85	8.95	39.80	100	71.70	48.53	23.17	0.56	0.64	0.39
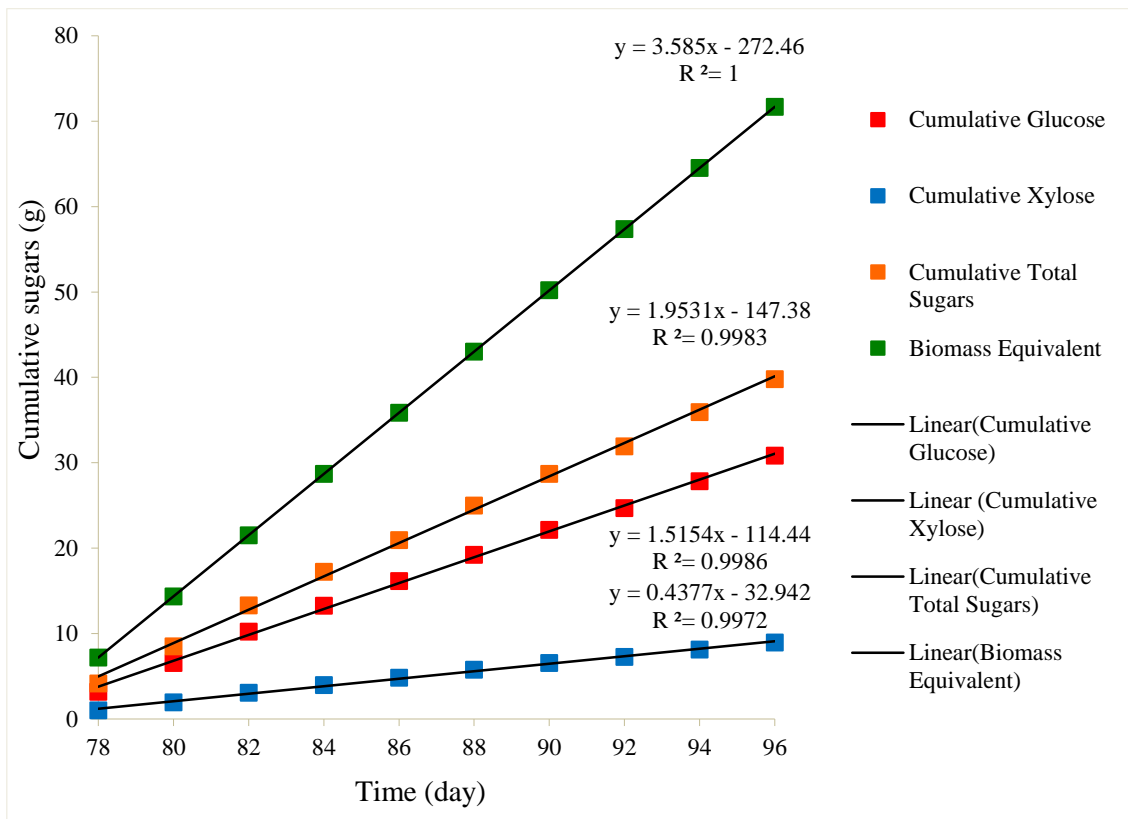
Figure 4-8 Sugar in and out of countercurrent system between Days 78 and 96.

4.3.2 Section 2 – Addition of CTec3 and HTec3

To fully utilize hemicellulose in corn stover, at the end of every transfer following Day 126, HTec3 (1 mg protein/g dry biomass) was added to Bottle 4 along with CTec3 (1 mg protein/g dry biomass). The other reaction conditions are identical with those of Section 1. After adding HTec3, both glucose and xylose yields in Bottles 1 to 4 show significant increases, as expected. As with Section 1, the steady state of Section 2 is determined by analyzing sugar concentrations of every bottle. Figures 4-2 and 4-3 show

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that after Day 156, the sugar concentrations of every bottle tend to stabilize, with only slight change and fluctuation. Just like Section 1, Days 158 to 180 are highlighted and shown in Figures 4-9 and 4-10.

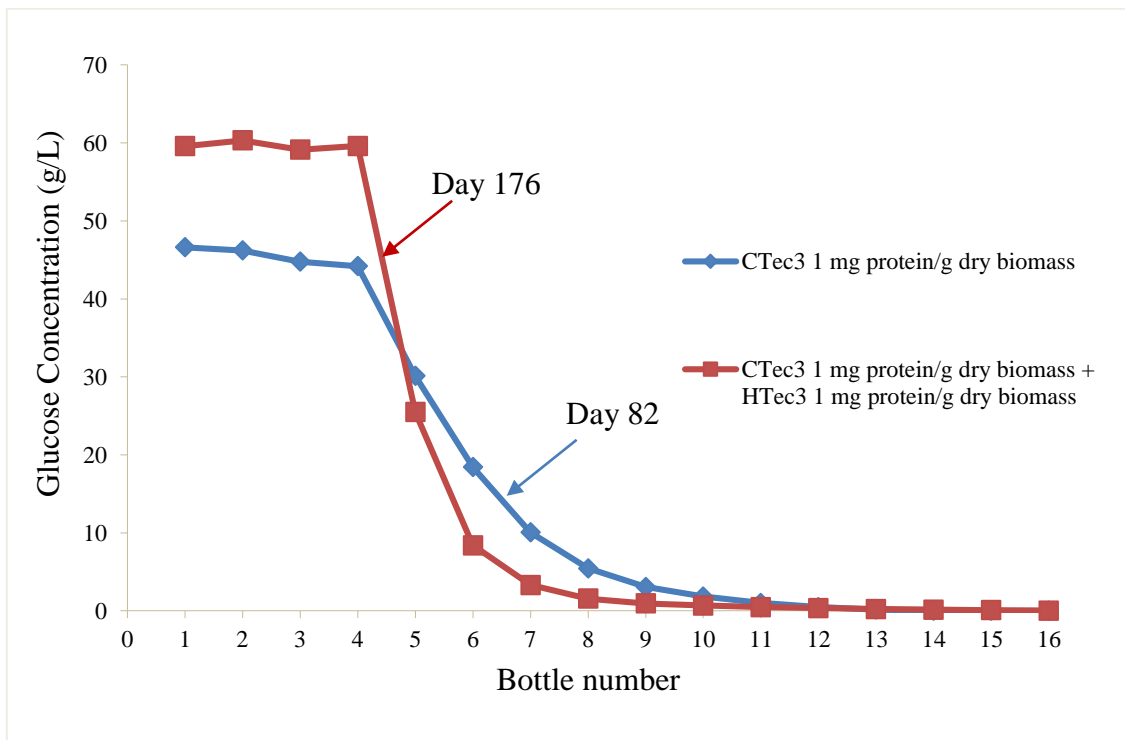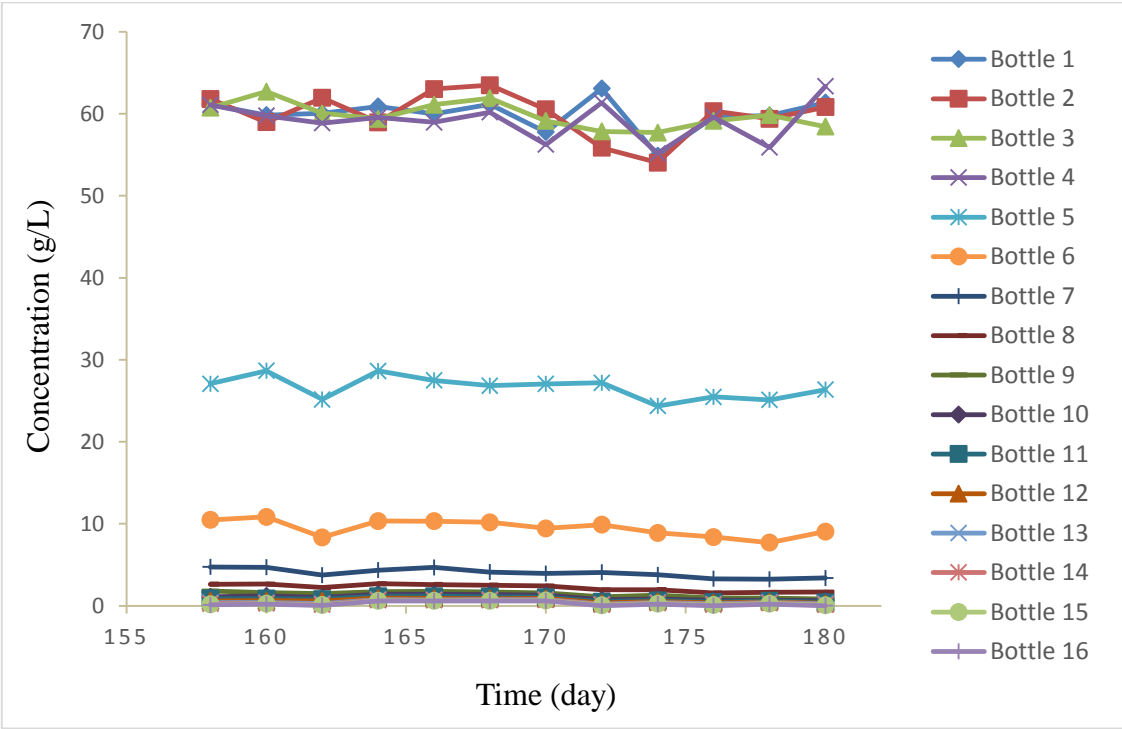
Figure 4-9 Glucose concentration as a function of bottle number and time between Days 158 and 180.
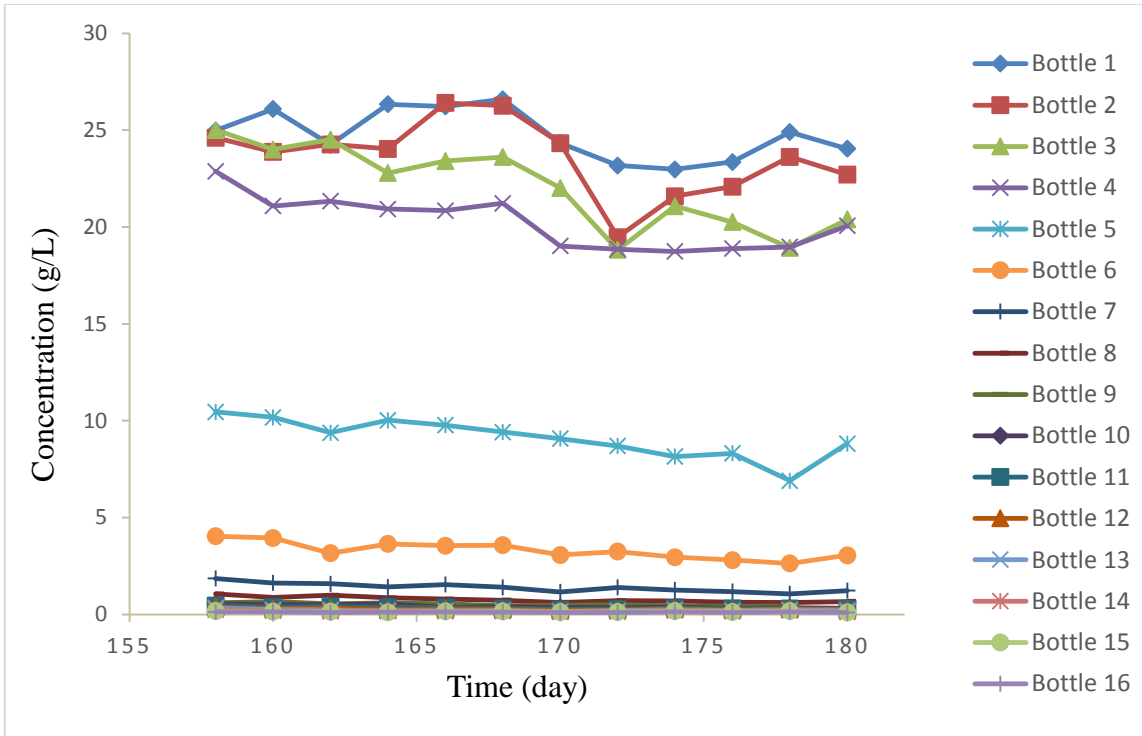
Figure 4-10 Xylose concentration as a function of bottle number and time between Days 158 and 180.

Figures 4-6 and 4-7 show the sugar concentrations as a function of bottle number on Day 176. Again, in this section, enzymes CTec3 and HTec3 were added to Bottle 4. The increase of glucose concentration from Bottles 4 to 1 is very slight just like Section 1. However, xylose concentration shows significant increase, which indicates hemicellulase HTec3 does not bind with substrate as tightly as cellulase CTec3. Significant decreases of sugar concentrations are also present from Bottles 4 to 10 in this section, and compared with Section 1, the decrease is sharper. Bottles 8 to 16 contribute very little to enzyme utilization, whereas in Section 1, the mark of no detected sugar begins with Bottle 10. This might be because more substrate is consumed in the two-day reaction in Bottle 4 and less solid wet cake is moved from Bottle 4 to latter bottles after adding HTec3.

Table 4-3 presents accumulative data in the steady-state region between Days 158 and 178. After 22 days accumulation, glucose, xylose and total sugar yields are 72%, 62%, and 69%, respectively. Figure 4-11 shows the corresponding plot; the fit is excellent and steady state is verified.

Table 4-3 Cumulative sugar data for Section 2.

Time (days)	Out			In				Yield		
	Cumulative Glucose	Cumulative Xylose	Cumulative Total Sugars	Dry Biomass	Biomass Equivalent	Glucose Equivalent	Xylose Equivalent	Total Sugar	Glucose	Xylose
158	3.58	1.58	5.16	10	7.08	4.76	2.32	0.73	0.75	0.68
160	6.93	2.95	9.88	20	14.15	9.52	4.63	0.70	0.73	0.64
162	9.90	4.25	14.15	30	21.23	14.28	6.95	0.67	0.69	0.61
164	13.38	5.78	19.15	40	28.31	19.04	9.27	0.68	0.70	0.62
166	16.67	7.22	23.89	50	35.39	23.80	11.59	0.68	0.70	0.62
168	19.96	8.62	28.58	60	42.46	28.56	13.90	0.67	0.70	0.62
170	23.71	10.01	33.72	70	49.54	33.32	16.22	0.68	0.71	0.62
172	27.33	11.53	38.86	80	56.62	38.08	18.54	0.69	0.72	0.62
174	30.77	12.90	43.66	90	63.70	42.84	20.86	0.69	0.72	0.62
176	34.33	14.39	48.72	100	70.77	47.60	23.17	0.69	0.72	0.62
178	37.87	15.79	53.66	110	77.85	52.36	25.49	0.69	0.72	0.62
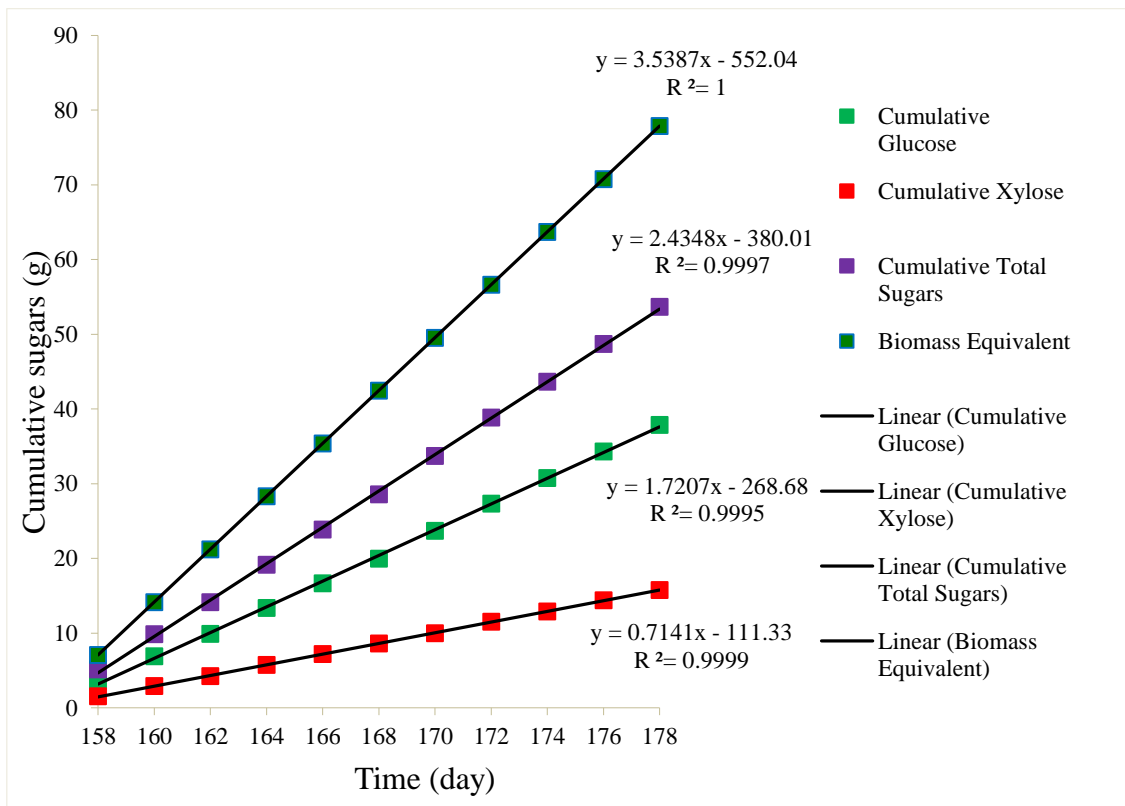
Figure 4-11 Sugar in and out of countercurrent system between Days 158 and 178.

4.3.3 Comparison of Countercurrent Saccharification with Batch Saccharification

To evaluate the efficiency of countercurrent saccharification, countercurrent results are compared with batch saccharification analysis. The results from a 5-day batch reaction are considered as "ultimate results" because no further improvement of sugar yields could be gained after 5 days. When using a CTec3 loading of 1 mg protein/g dry biomass in countercurrent saccharification, the glucose yield is 64%. To reach same glucose yield (64%), 1.9 times CTec3 loading is needed in batch saccharification (Figure

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4-12). When the enzyme loading is CTec3 (1 mg protein/g dry biomass) + HTec3 (1 mg protein/g dry biomass), the glucose yield is 72%. To achieve the same glucose yield (72%), 1.4 times CTec3 + HTec3 (50%:50%) loading is required in batch saccharification (Figure 4-13). In both sections, for the countercurrent saccharification, there were no benefits over batch saccharification on xylose yield.
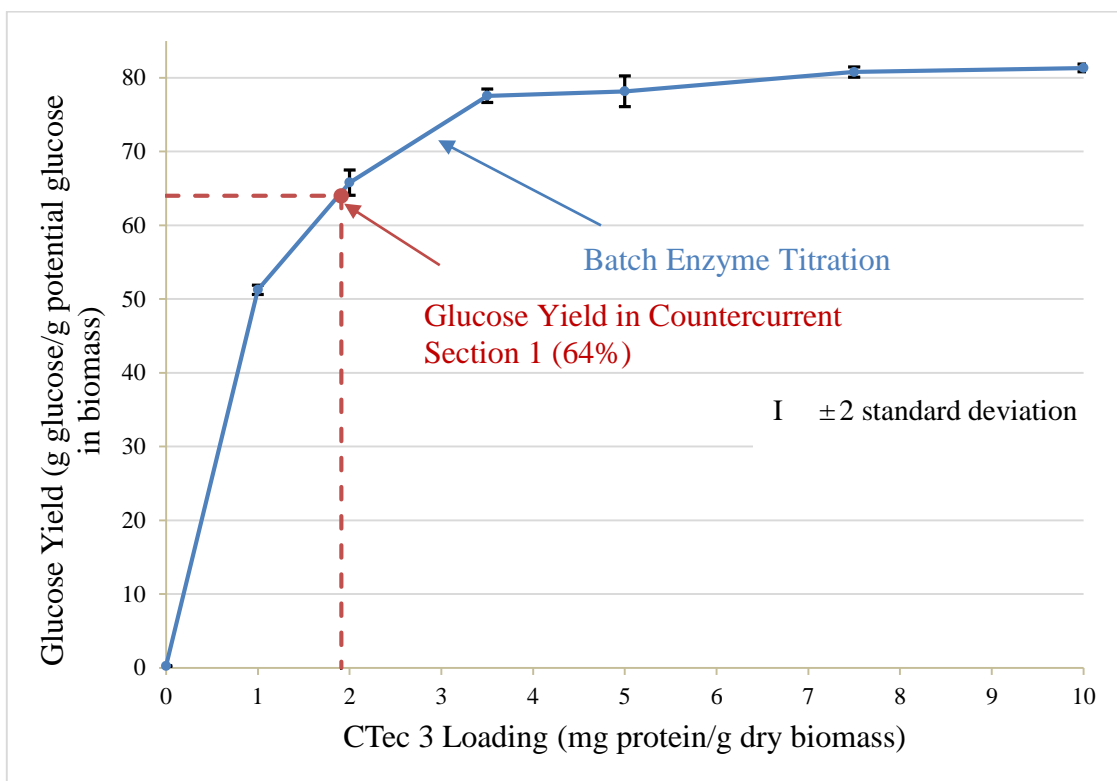
Figure 4-12 Comparison of glucose yield between batch and countercurrent experiment with only CTec3 loading.

(Substrate of batch enzyme titration was lime + shock treated corn stover (Batch 2).)

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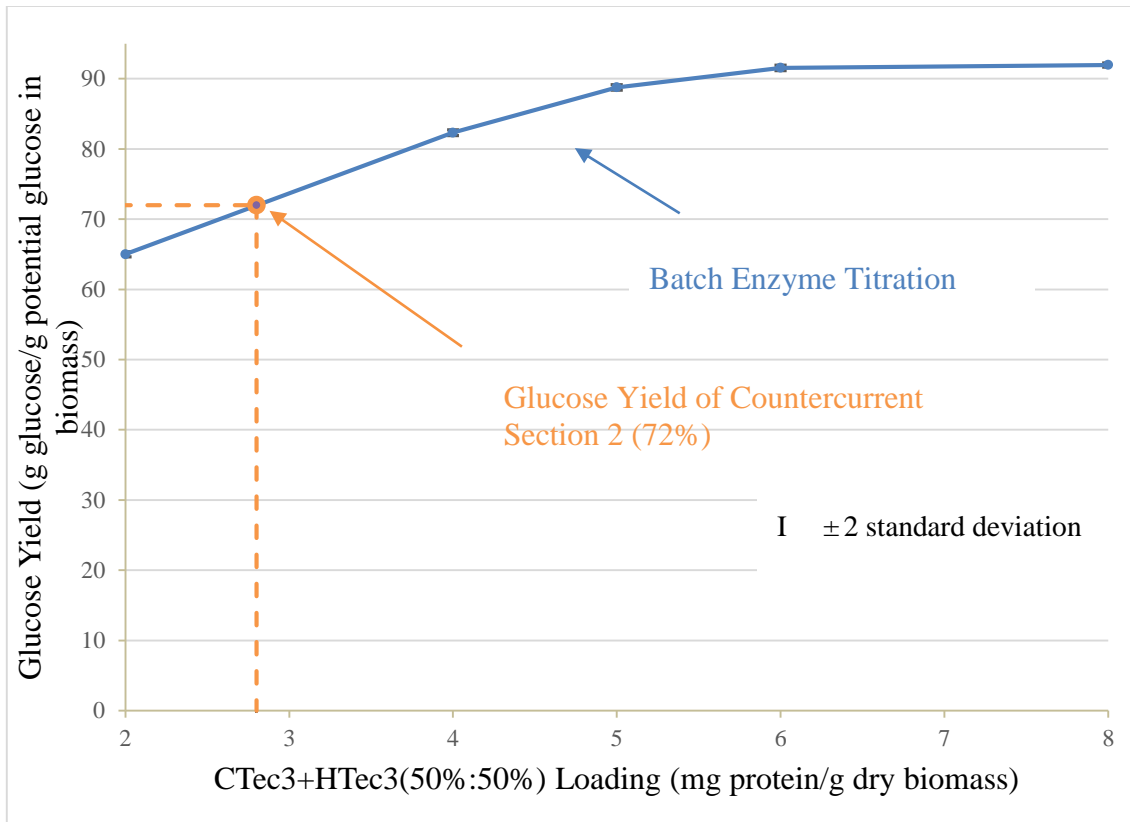
Figure 4-13 Comparison of glucose yield between batch and countercurrent experiment

with CTec3 + HTec3 (50%:50%) loading.

(Substrate of batch enzyme titration was lime + shock treated corn stover (Batch 3).)

4.4 Conclusion

In this chapter, countercurrent saccharification of lime + shock treated corn stover with enzyme loadings CTec3 (1 mg protein/g dry biomass) and CTec3 (1 mg protein/g dry biomass) + HTec3 (1 mg protein/g dry biomass) are studied. Both runs were identical except for the enzyme loadings.

When the systems reach steady state, the Slope Method was used to determine product yields and verify steady state. Using CTec3 (1 mg protein/g dry biomass), the glucose and xylose yields were 64% and 39%, respectively. Using CTec3 (1 mg protein/g dry biomass) + HTec3 (1 mg protein/g dry biomass), the glucose and xylose yields were 72% and 62%, respectively.

To reach a given glucose yield (64%), when only adding CTec3, countercurrent saccharification reduces the enzyme loading by a factor of 50% compared with batch saccharification. To reach a given glucose yield (72%), when only adding CTec3 and HTec3 (50%:50%), countercurrent saccharification reduces the enzyme loading by a factor of 30% compared with batch saccharification.

CHAPTER V

CONCLUSION AND FUTURE WORK

In this work, pretreated corn stover was used as a "real-world" lignocellulose substrate for countercurrent saccharification. The benefits of countercurrent saccharification over batch enzymatic saccharification have been shown.

Besides the two sections described in Chapter Ⅳ, more countercurrent work can be performed. To determine the maximum yield this system can reach, an enzyme loading of CTec3 (2 mg protein/g dry biomass) + HTec3 (2 mg protein/g dry biomass) or higher could be tested. Also, as shown in Figure 4-7, the xylose concentration significantly increases from Bottle 4 to Bottle 1, which indicates a considerable amount of free hemicellulase exists in the liquid phase. To fully utilize hemicellulase, HTec3 could be added to a latter bottle, such as Bottle 8 instead of Bottle 4.

At enzyme loading of CTec3 (1 mg protein/g dry biomass) + HTec3 (1 mg protein/g dry biomass), Bottles 8 to 16 barely contribute to sugar yields. As a result, the number of bottles used could be reduced.

Antibiotics involved in this study are tetracycline and cycloheximide, which are toxic and non-volatile. If this countercurrent technique is applied in the food industry, inexpensive, safe, and volatile antibiotics should be used.

In countercurrent saccharification, reaching steady state requires a very long time. To save costs, simulation is necessary to test various reaction conditions and determine the optimal operation condition. This countercurrent system can be simulated by the

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Continuum Particle Distribution Modeling (CPDM), which has successfully simulated

countercurrent fermentation (Thanakoses et al., 2003).

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ENZYME DILUTION

Materials:

      Novozymes CTec2 solution

      Novozymes CTec3 solution

      Novozymes HTec3 solution

      DI water

Apparatus:

      50-mL volumetric flask

      Kimwipes

      1000–5000 μL auto-pipette

      Pipette tips

      50-mL centrifuge tubes

Procedure:

1. Fill 50-mL volumetric flask with approximately 20–25 mL of DI water.

2. Take enzyme (CTec2, CTec3, or HTec3) out of refrigerator and shake well.

3. Take 5 mL enzyme solution with auto pipette.

4. Clean the enzyme residue that sticks on the outside of the pipette tip with Kimwipes.

5. Empty pipette into 50-mL volumetric flask. Keep the tip in the flask and remove it

from auto pipette.

6. Rinse the inside of tip several times with DI water.

7. Add DI water to the flask to 50 mL mark and shake well.

8. Pour the diluted enzyme into 50-mL centrifuge tubes and store in 4 ℃ refrigerator.

APPENDIX B

CITRATE BUFFER PREPARATION

Materials:

    Citric acid monohydrate

    Citric acid, trisodium salt dihydrate

    DI water

Apparatus:

    1-L volumetric flask

    pH meter

    Analytic balance with 0.0001-g precision

    Weighing boat

    Weighing spatula

Procedure:

1. Fill a 1-L glass volumetric flask with approximately 800 mL of DI water.

2. Weigh 8.4000 ± 0.0005 g of citric acid monohydrate and 17.6500 ± 0.0005 g trisodium citrate dihydrate and add to 1-L volumetric flask.

3. Shake to dissolve the solids well.

4. Fill water to the 1-L mark and shake well.

5. Measure pH of the citrate buffer; it should be 4.8 ± 0.02.

6. Store the solution in 4 ℃ refrigerator.

APPENDIX C

ANTIBIOTIC PREPARATION

Materials:

Tetracycline hydrochloride powder

Cycloheximide powder

DI water

Ethanol (190 proof)

Apparatus:

50-mL centrifuge tubes

100-mL volumetric flask

Analytic balance with 0.0001-g precision

Weighing paper

Weighing spatula

Procedure:

Procedure for 10 g/L tetracycline solution preparation:

1. Weigh 1.0000 $\pm$ 0.0005 g of tetracycline hydrochloride powder on weighing paper and add to 100-mL volumetric flask.

2. Add 70 mL 190-proof ethanol to flask and mix well.

3. Fill DI water to 100-mL mark and shake well.

4. Pour the tetracycline solution on 50-mL centrifuge tubes and store in the freezer.

Procedure for 10 g/L cycloheximide solution preparation:

1. Weigh 1.0000 ± 0.0005 g of cycloheximide powder on weighing paper add to 100-mL

volumetric flask.

2. Fill DI water to 100-mL mark and mix well.

3. Pour the cycloheximide solution on 50-mL centrifuge tubes and store in refrigerator.

APPENDIX D

PROTEIN CONCENTRATION ASSAY

This procedure is adapted from "Instruction Pierce^{TM} BCA Protein Assay" (Fisher

Scientific, 2013)

Materials:

BCA Reagent A (containing sodium carbonate, sodium bicarbonate,

bicinchoninic acid and sodium tartrate in 0.1-M sodium hydroxide)

BCA Reagent B (containing 4% cupric sulfate)

Albumin Standard Ampules (containing bovine serum albumin (BSA) at

2 mg/mL in 0.9% saline and 0.05% sodium azide)

Novozymes CTec2

Novozymes CTec3

Novozymes HTec3

DI water

Apparatus:

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

25-mL centrifuge tubes

2-mL microcentrifuge tubes

Pipette tips

Procedure:

Preparation of standards:

Use Table D-1 to prepare a set of protein standards.

Table D-1 Preparation of Diluted Albumin (BSA) Standards

Vial	Volume of Diluent (µL)	Volume and Source of BSA (µL)	Final BSA Concentration (µg/mL)
A	0	300	2000
B	125	375	1500
C	325g	325	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0

Dilute the contents of one Bovine Serum Albumin standard (BSA) ampule into several 2-mL microcentrifuge tubes clean vials.

Preparation of the BCA Working Reagent (WR):

Use the following formula to determine the total volume of WR required:

There are total nine standards and one unknown (CTec2, or CTec3, or HTec3) and two replicates of each sample, 2 mL of the WR is required for each sample:

(9 standards + 1 unknown) $\times$ (2 replicates) $\times$ (2 mL) = 40 mL WR required.

3. Combine 50 mL of Reagent A with 1 mL of Reagent B to yield a clear, green WR.

4. Dilute solutions of enzymes 1000 times.

Test procedures:

1. Pipette 0.1 mL of each standard and diluted enzyme sample replicate into a 25-mL

labeled test tube.

2. Add 2 mL of the WR to each tube and mix well.

3. Incubate tubes at room temperature for 2 h.

4. Set spectrophotometer to 562 nm, measure the absorbance of all the samples within

10 min.

5. Prepare a standard curve by plotting the average absorbance at 562 nm for each BSA

standard vs. concentration (μg/mL). Use the standard curve to determine the protein

concentration of each unknown sample.

Note: Because of the limitations in the spectrophotometer, results are valid only from

125 to 1000 μg/mL.

APPENDIX E

HPLC SAMPLE PREPARATION AND TESTING

Materials:

Samples from batch or countercurrent saccharification experiments

HPLC water

Glucose powder

Xylose powder

DI water

Apparatus:

1-mL syringe

0.2-µm syringe filter

2-mL microcentrifuge tubes

Autosampler snap-it vial

HPLC equipped with refractive index detector, autosampler, a pair of de-ashing guard columns (Bio-Rad Micro-Gurad de-ashing cartridges, 30 mm $\times$ 4.6 mm), and a HPLC carbohydrate analysis column (BioRad Aminex HPX-87P, 300 mm $\times$ 7.8 mm).

Procedure:

Procedures of HPLC samples preparation:

1. Prepare sugar standards (1, 3, 5, 10, 25, 50, 75 g/L glucose concentration, with a 2:1 ratio of glucose:xylose) and use a 50 g/L glucose concentration sample as a control verification standard (CVS).

2. Take 0.5-mL liquid samples from batch or countercurrent saccharification experiments with 1-mL syringe, attach a 0.2-μm filter, filter all liquid to a labeled vial and cover a vial cap.

3. Analyze samples with HPLC, the mobile phase is HPLC water with a flow rate of 0.6 mL/min, the assay time is 21 min per sample.

APPENDIX F

BATCH PROCEDURE

This procedure is adapted from NREL procedure "Enzymatic Saccharification of

Lignocellulosic Biomass" (Selig et al., 2008).

Materials:

Raw corn stover, lime pretreated corn stover, lime + shock treated corn stover

Diluted CTec2, Diluted CTec3, Diluted HTec3 (Appendix A)

Citrate buffer (Appendix B)

Tetracycline solution, cycloheximide solution (Appendix C)

DI water

Apparatus:

Incubator capable of agitation at ~2 rpm

50-mL centrifuge tubes

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

Moisture content analyzer (Denver Instruments IR 120)

Analytic balance with 0.0001-g precision

100-mL beakers or flasks

2-mL microcentrifuge tubes

Vortex Mixer

Procedure:

1. Measure the moisture content of substrate with moisture content analyzer.

2. Calculate the target air-dry substrate mass for 1 g dry biomass.

3. Measure protein concentration of CTec2, CTec3, and HTec3.

Procedures of test samples preparation:

1. Calculate required enzyme volume.

2. Calculate required DI water volume to make sure total reaction volume is 10 mL (assume substrate density ≈1 g/cm^3).

3. Weigh the target air-dry biomass of each sample and add to labeled tubes.

4. Add 5 mL citrate buffer, required water volume, 0.08 mL tetracycline solution, 0.06 mL cycloheximide solution in sequence to each tube and mix well with mixer.

5. Add required amount of enzyme to each tube, record the time and mix well.

6. Put the tubes in the incubator at 50 ℃ and axial rotation speed 2 rpm for exact 5 days.

Procedures of substrate blank samples preparation:

1. Calculated required DI water volume to make sure total reaction volume 10 mL (assume substrate density ≈1 g/cm^3).

2. Weigh the target air-dry biomass of each sample and add to labeled tubes.

3. Add 5 mL citrate buffer, required water volume, 0.08 mL tetracycline solution, 0.06 mL cycloheximide solution in sequence to each tube and mix well with mixer.

4. Put the tubes in the incubator together with test samples at 50 ℃ and axial rotation speed 2 rpm for exact 5 days.

Procedures of enzyme blank samples preparation:

1. Calculate required enzyme volume.

2. Calculated required DI water volume to make sure total reaction volume 10 mL.

3. Add 5 mL citrate buffer, required water volume, 0.08 mL tetracycline solution, 0.06 mL cycloheximide solution in sequence to each tube and mix well with mixer.

4. Put the tubes in the incubator together with test samples at 50 ℃ and axial rotation speed 2 rpm for exact 5 days.

Termination procedures:

1. After exactly five days, remove the tubes from the incubator and place them in boiling water for 20 min to deactivate the enzymes.

2. When the samples cool to room temperature, pour nearly 1.5 mL of liquid into 2-mL microcentrifuge tubes and store in freezer.

Note: Every test sample should accompany with its corresponding substrate blank and enzyme blank samples.

Test samples are repeated in triplicate.

Substrate and enzyme blank samples are repeated in duplicate.

APPENDIX G

TRANSFER PROCEDURE

This procedure is adapted from "Countercurrent Enzymatic Saccharification of

Lignocellulosic Biomass and Improvements over Batch Operation" (Zentay, 2014)

Materials:

Tetracycline solution (Appendix C)

Cycloheximide solution (Appendix C)

Diluted Novozymes CTec3 (Appendix A)

Diluted Novozymes HTec3 (Appendix A)

Citrate buffer (Appendix B)

DI water

Lime + shock treated corn stover

Apparatus:

Weighing boats

50-mL centrifuge tubes

2-mL microcentrifuge tubes

250-mL graduated cylinder

50-mL graduated cylinder

Citrate buffer (prepared, pH 4.8, 0.1-M)

Auto pipette (100–1000 μL)

Pipette tips

Weighing spatula

Centrifuge

pH meter

Procedure:

Preparation work:

1. Calibrate the pH meter with 1.68, 4.01 and 7.00 buffer solutions.

2. Measure 10 g dry lime + shock treated corn stover with weigh boat.

3. Remove all 16 bottles out of incubator.

4. Weigh all bottles and record the weight of bottles.

5. Balance Bottles 1-2, 3-4, 5-6, 7-8, 9-10, 11-12, 13-14, 15-16.

Transfer procedure:

Bottle 1:

1. Centrifuge Bottles 1, 2, 3, 4 at 3000 rpm for 5 min.

2. Remove the Bottles 1 and 2 from centrifuge.

3. Pour the liquid of Bottle 1 into a 250-mL cylinder and record liquid mass and volume.

4. Measure pH of the liquid and take 1-mL sample with auto pipette into 2 mL centrifuge tube.

5. Save nearly 45 mL liquid to a 50 mL tube.

6. Weigh bottle (without cap) + wet cake, and calculated the weight of wet cake.

7. Calculate the move target: wet cake + pre-weighed dry biomass - target weight (90 g).

8. Remove move target from the bottle and add pre-weighed dry biomass to Bottle 1.

9. Weigh the bottle (without cap) and calculate the wet cake again to ensure its weight is close to 90 g.

Bottle 2 – 15

1. Pour liquid from bottle to 250-mL cylinder slowly, record the liquid mass and volume.

2. Measure pH of liquid fraction, and take 1-mL sample with auto pipette into 2-mL microcentrifuge tubes.

5. Pour liquid to previous bottle.

6. Measure the bottle without cap and calculate wet cake weight.

7. Calculate move target: wet cake weight + moved weight from previous bottle - target weight (90 g).

8. Remove move target from the bottle and add wet cake removed from previous bottle.

9. Measure bottle weight without cap and calculate the wet cake weight.
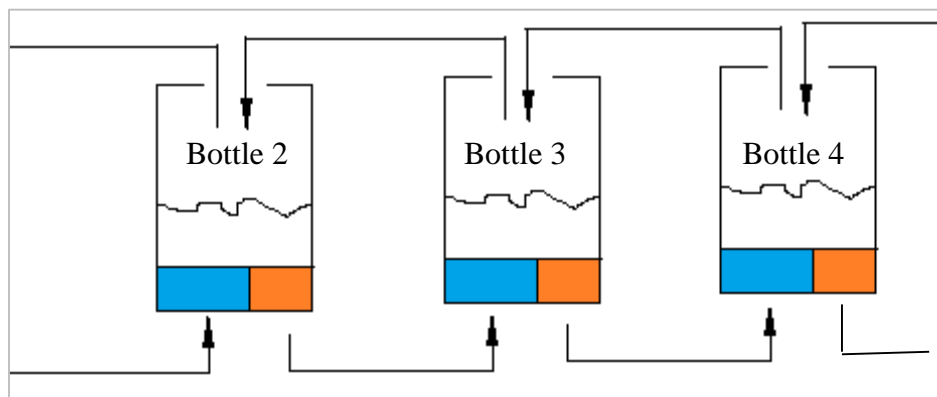
10. Repeat Steps 1–9 for next bottle.

Figure G-1 Schematic of Countercurrent saccharification.

(Use Bottles 2, 3, and 4 as examples)

Note: Before transfer to Bottle 5, centrifuge Bottles 5, 6, 7, 8 at 3000 rpm for 5 min;

Before transfer to Bottle 9, centrifuge Bottles 9, 10, 11, 12 at 3000 rpm for 5 min;

Before transfer to Bottle 13, centrifuge Bottles 13, 14, 15, 16 at 3000 rpm for 5 min.

Bottle 16

1. Pour liquid from Bottle 16 to 250-mL cylinder slowly, record the liquid mass and volume.

2. Measure pH of liquid fraction, and take 1-mL sample with auto pipette into 2-mL microcentrifuge tubes.

5. Pour liquid to previous bottle.

6. Measure the bottle without cap and calculate wet cake weight.

7. Calculate move target: wet cake weight + move weight from previous bottle - target weight (90 g).

8. Remove move target from the bottle and take nearly 0.5 g moved wet cake to test moisture content.

9. Place the rest moved wet cake in 4 °C refrigerator.

10. Add 50 mL of citrate buffer and 40 mL of DI water to Bottle 16.

Post-transfer procedure:

1. Add 0.4 mL of tetracycline solution and 0.3 mL of cycloheximide solution to every bottle.

2. Add required enzyme dose (CTec3 or CTec3 + HTec3) to Bottle 4.

3. Record final weight of each bottle with cap.

4. Close every bottle very tightly and shake to homogenize slurry.

5. Put the 16 bottles back to rolling incubator setting with 50 ℃ and axial rotation 2 rpm.