ASSESSMENT OF SHOCK PRETREATMENT OF CORN STOVER USING THE CARBOXYLATE PLATFORM

A Dissertation

by

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ABSTRACT

Fuels and chemicals from lignocellulosic biomass, a renewable energy source, is an attractive solution to meet ever-increasing global energy needs and reduce global climate change. In the biochemical conversion of lignocellulose, the first and the most expensive step is pretreatment. This study focuses on the efficacy of shock pretreatment, a mechanical process that uses a shockwave to alter the biomass structure.

Corn stover was pretreated with lime and shock. The two pretreatments (lime-only and lime + shock) were evaluated using enzymatic hydrolysis, batch mixed-culture fermentations, and continuous countercurrent mixed-culture fermentation. In a 120-h enzymatic hydrolysis, shock pretreatment increased the glucan digestibility of SLP (submerged lime pretreatment) corn stover by 3.5% and OLP (oxidative lime pretreatment) corn stover by 2.5%. The continuum particle distribution model (CPDM) was used to simulate a four-stage continuous countercurrent mixed-culture fermentation using empirical rate models obtained from simple batch experiments. The CPDM model determined that lime + shock pretreatment increased the total carboxylic acids yield by 28.5% over lime-only pretreatment in a countercurrent fermentation with a VSLR (volatile solids loading rate) of 12 g/(L·day) and LRT (liquid retention time) of 30 days. In a semi-continuous countercurrent fermentation performed in the laboratory for 112 days with a VSLR of 1.875 g/(L·day) and LRT of 16 days, lime + shock pretreatment increased the total carboxylic acids yield by 14.8%. The experimental results matched

closely with CPDM models predictions (4.05% error).

Calcium carbonate and magnesium carbonate were compared as buffers for mixed-culture fermentations of lime and lime + shock pretreated corn stover. Batch fermentations at five different substrate loadings of lime and lime + shock pretreated corn stover were performed with $MgCO_3$ and $CaCO_3$ buffer. In batch fermentations with 100 g/L substrate, the carboxylic acid production more than doubled (2.7 times for lime and 2.6 times for lime + shock corn stover) when $MgCO_3$ buffer was used. In addition, CPDM was used to simulate and predict the performance of a four-stage countercurrent fermentation using $MgCO_3$ and $CaCO_3$ buffer. CPDM predicts that in a four-stage countercurrent fermentation with a high volatile solids loading rate (VSLR 12 g/(L·day)) and low liquid residence time (LRT 10 day), using $MgCO_3$ buffer will yield a carboxylic acid concentration of 26.1 g/L, a 22.5% increase over $CaCO_3$ buffer. Adding shock to lime pretreatment increased the yields at all substrate loadings in both batch fermentations and CPDM model predictions.

The effect of hydrogen and carbon dioxide gas concentrations in the headspace of mixed-culture fermentations was studied. Using H_2:CO_2 (1:1) at 1 atm in the fermenter headspace increased the total carboxylic acids by 37%. Using CO_2-only in the headspace reduced the total acids by 4%, but shifted the acid spectrum toward high-molecular-weight acids.

This work is dedicated to my parents, Anjali and Jayant Darvekar, for always encouraging and believing in me. I will be forever grateful for their support.

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1. INTRODUCTION

There is a huge discrepancy between the rate of discovery of new oil reserves and the rate of oil consumption; this will eventually lead to an energy crisis [1]. Also, burning fossil fuels increases atmospheric carbon dioxide concentration, a greenhouse gas that leads to global warming [2, 3]. Today, the transportation sector is almost entirely dependent on petroleum-based fuels and accounts for 70% of global carbon monoxide emissions and 19% of global carbon dioxide emissions [4]. In the last 100 years, the average temperature of the earth's surface has increased by 0.7˚C and model predictions anticipate an additional warming of 1.1 to 6.4˚C by the end of 21st century [3]. Biofuels is an attractive solution to these problems because it is renewable and carbon neutral.

First-generation processes produce liquid biofuels from food crops such as cereals, sugar crops, and oilseeds; however, they have a major limitation because they compete for land and water used to produce food [5]. Second-generation biofuels use lignocellulose, the world's fourth largest energy source behind oil, coal, and natural gas, respectively. Large quantities of lignocellulose are available as crop residues and it has the potential for high crop yields per acre [6]. Lignocellulose can be converted into liquid fuels using three major platforms: thermochemical platform (gasify biomass to syngas, which is catalytically transformed into fuels), sugar platform (enzymes convert biomass into simple sugars, which are fermented to ethanol), and the carboxylate platform (a mixed-culture transforms biomass to carboxylate salts, which are chemically

converted to fuels). The MixAlco™ (Fig. 1) process is an example of the carboxylate platform [7]. The carboxylate platform has the highest product yields in literature [8]. Compared to ethanol and other alcohols, carboxylic acids are thermodynamically favored provided methane production is inhibited; therefore, sterile conditions are not required. Also, a mixed culture utilizes all biomass components (e.g., lignocellulose, starch, protein, and fats), which increases yield. The ability to utilize varied biomass components under non-sterile conditions – and still achieve high product yields – makes the carboxylate platform economically attractive.

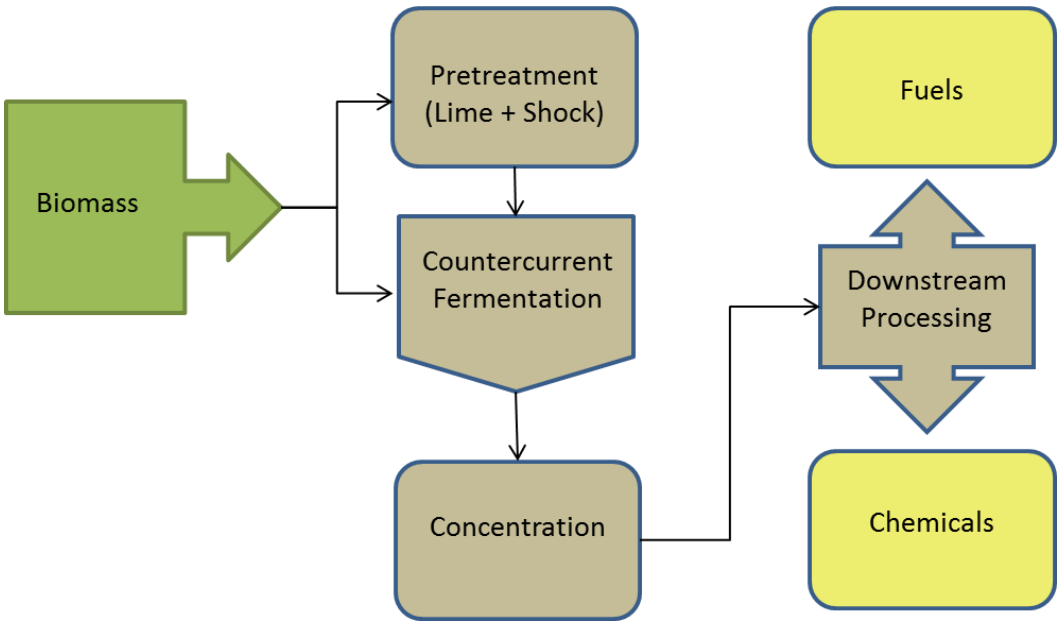

Figure 1.1 Schematic of MixAlco process.

In the biochemical conversion of lignocellulose, the first and the most expensive step is pretreatment [9]. Lignocellulosic biomass consists of three primary components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are both carbohydrate polymers and comprise up to 60−80% of lignocellulose. Using enzymes or bacteria, they can be converted to sugars or carboxylic acids, which can be further transformed into fuels and chemicals. Hemicellulose is covalently bound together by lignin, a polymer of phenyl propane units linked primarily by ether bonds. In its native form, lignocellulose highly resists biochemical conversion because of its structural characteristics, such as high lignin content [10, 11], low accessible surface area [12], and high cellulose crystallinity [11, 13, 14]. Hence, prior to fermentation or enzymatic hydrolysis, pretreatment is required to reduce lignin content and thereby render lignocellulose more digestible. There are many chemical pretreatments, each with their own advantages and disadvantages [15]. The lime pretreatment process effectively and economically removes lignin while preserving vital hemicellulose sugars [15, 16], which are lost in acid pretreatment technologies. The delignification of biomass using lime pretreatment highly depends on temperature, availability of oxygen, and reaction time [17]. Using this principle, two equally effective lime pretreatment methods have been developed. The OLP (oxidative lime pretreatment) uses pure oxygen at a pressure of 6.9 bar and high temperature (110 °C) for 4 h, whereas SLP (submerged lime pretreatment) uses atmospheric-pressure air as the oxidizing agent along with low temperature (50 °C) for 28 days.

In addition to chemical pretreatments, certain mechanical pretreatments (e.g., ball-milling, acoustic cavitation and hydrodynamic cavitation) enhance biomass digestibility [18-20]. However, because of excessive energy requirements, these are not feasible for large-scale industrial applications. A recently developed mechanical pretreatment called shock pretreatment uses a shockwave to render biomass more amenable to enzymatic digestion [21]. Shock pretreatment has low energy requirements and has the potential for commercial use as a pretreatment that enhances existing chemical pretreatments. The estimated cost of shock pretreatment is about $5/tonne [22].

Following pretreatment, biomass is fermented using a mixed culture of microorganisms to produce carboxylate salts. A four-stage countercurrent fermentation is used to minimize the effects of product inhibition, which improves yields [23, 24]. By selecting appropriate downstream processing steps, these carboxylate salts can be further converted into a wide variety of chemicals or fuel. Historically, these mixed-culture fermentations have used calcium carbonate as a buffer to neutralize the carboxylic acids. It is inexpensive and is readily calcined to lime, which can be used as a pretreatment agent. Because it is poorly soluble, an excess amount can be added at the start of the fermentation; it need not be added every day [25]. This “auto buffering” eliminates the need for a sophisticated pH controller; as more carboxylic acids are produced, more calcium carbonate dissolves into the fermentation broth. The primary disadvantage of calcium carbonate is that it does not control to pH~7. At 1-atm CO_2 pressure, it buffers around pH of 5.6–6. Neutral pH is important because it affects fermentation rates, products, and yields [26, 27]. Lowering the pH from 7 to 6 greatly reduces cellulose and

hemicellulose hydrolysis [28]; hence, a different buffer is needed to control the fermentation pH near 7.

To make hydrocarbon fuels (gasoline, diesel, or jet fuel), the carboxylate salts are concentrated and thermally converted to ketones, hydrogenated to alcohols, and then catalytically converted to hydrocarbons [29]. Divalent carboxylate salts (e.g., calcium carboxylates) begin thermally decomposing at much lower temperatures (170–180°C) than do monovalent ions, like sodium carboxylate salts (410–420°C) [30]. Also, the average ketone yield for sodium salts was less than half that for calcium salts. Considering these factors, magnesium carbonate was chosen as the buffer for mixed-culture fermentations.

The purpose of this research is to study the efficacy of adding shock to enhance lime pretreatment digestibility of corn stover using enzymatic hydrolysis. In addition, to evaluate its performance in mixed-acid fermentations, batch fermentations were performed. The data from these batch fermentations were used to model a four-stage countercurrent fermentation using Continuum Particle Distribution Modeling (CPDM) and the preditions were compared with experimental values.

This dissertation also evaluates the effect of magnesium carbonate as a buffer for mixed-culture fermentations in the MixAlco™ process. Calcium carbonate and magnesium carbonate buffers were compared in mixed-culture fermentations of lime and lime + shock pretreated corn stover. CPDM was used to simulate and predict its performance in a four-stage countercurrent fermentation.

The effect of hydrogen and carbon dioxide compositions in the headspace of the mixed-acid fermentations is also studied for its impact on the total carboxylic acid production, conversions, and yields.

2. ASSESSMENT OF SHOCK PRETREATMENT OF CORN STOVER[*]

This study evaluated the efficiency of adding shock pretreatment, a mechanical process that uses a shockwave to alter the biomass structure. Two pretreatments (lime-only and lime + shock) were evaluated using enzymatic hydrolysis, batch mixed-culture fermentations, and continuous countercurrent mixed-culture fermentation. In a 120-h enzymatic hydrolysis, shock pretreatment increased the glucan digestibility of SLP (submerged lime pretreatment) corn stover by 3.5% and OLP (oxidative lime pretreatment) corn stover by 2.5%. The continuum particle distribution model (CPDM) was used to simulate a four-stage continuous countercurrent mixed-culture fermentation using empirical rate models obtained from simple batch experiments. The CPDM model determined that lime + shock pretreatment increased the total carboxylic acids yield by 28.5% over lime-only pretreatment in a countercurrent fermentation with a VSLR (volatile solids loading rate) of 12 g/(L·day) and LRT (liquid retention time) of 30 days. In a semi-continuous countercurrent fermentation performed in the laboratory for 112 days with a VSLR of 1.875 g/(L·day) and LRT of 16 days, lime + shock pretreatment increased the total carboxylic acids yield by 14.8%. The experimental results matched closely with CPDM models predictions (4.05% error).

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2.1. Introduction

Combusting fossil fuels increases the concentration of atmospheric carbon dioxide, a greenhouse gas that leads to global warming [3]. Today, the transportation sector almost entirely depends on petroleum-based fuels and accounts for 19% of global carbon dioxide emissions [4]. Biofuels address this problem because they are carbon neutral. First-generation processes produce liquid biofuels from food crops such as cereals, sugar crops, and oilseeds; however, they have a major limitation because they compete for land and water used to produce food [5]. Second-generation biofuels use lignocellulose, the world's fourth largest energy source behind oil, coal, and natural gas, respectively. Large quantities of lignocellulose are available as crop residues and it has the potential for high crop yields per acre [6].

Lignocellulose can be converted to biofuels by thermochemical processes, such as gasification which produces syngas that is further transformed to fuels using catalysts. Alternatively, biochemical processes use enzymes or microorganisms to convert biomass into fuels [1]. Biochemical processes have higher potential yields of fuel (gal/ton dry biomass) because a lot of energy is lost to heat in thermochemical processes [7, 8]. However, most biochemical processes require use of extracellular enzymes and sterile conditions to grow a particular bacteria or fungi, which makes them expensive and difficult to control. An alternative approach is the MixAlco^{TM} process, which uses a mixed-culture of microorganisms rather than a monoculture. The MixAlco^{TM} process is an example of the carboxylate platform, which converts biomass to a mixture of

carboxylic acid salts that are chemically converted into fuels and chemicals. The carboxylate platform has the highest product yields in literature [31]. Compared to ethanol and other alcohols, carboxylic acids are thermodynamically favored; hence, no sterile conditions are required.

In the biochemical conversion of lignocellulose, the first and the most expensive step is pretreatment [9]. Lignocellulosic biomass consists of three primary components: cellulose, hemicellulose, and lignin. Cellulose and hemicellulose are both carbohydrate polymers and comprise up to 60−80% of lignocellulose. Using enzymes or bacteria, they can be converted to sugars or carboxylic acids, which can be further transformed into fuels and chemicals. Hemicellulose is covalently bound together by lignin, a polymer of phenyl propane units linked primarily by ether bonds. In its native form, lignocellulose highly resists biochemical conversion because of its structural characteristics, such as high lignin content [10, 11], low accessible surface area [12], and high cellulose crystallinity [11, 13, 14]. Hence, prior to fermentation or enzymatic hydrolysis, pretreatment is required to reduce lignin content and thereby render lignocellulose more digestible. There are many chemical pretreatments, each with their own advantages and disadvantages [15]. The lime pretreatment process effectively and economically removes lignin while preserving vital hemicellulose sugars [15, 16], which are lost in acid pretreatment technologies. The delignification of biomass using lime pretreatment highly depends on temperature, availability of oxygen, and reaction time [17]. Using this principle, two equally effective lime pretreatment methods have been developed. The OLP (oxidative lime pretreatment) uses pure oxygen at a pressure of 6.9 bar and

high temperature (110 °C) for 4 h, whereas SLP (submerged lime pretreatment) uses atmospheric-pressure air as the oxidizing agent along with low temperature (50 °C) for 28 days.

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2.2. Methods

2.2.1. Raw substrates

Corn stover was provided by Texas A&M AgriLife. It was air dried to a moisture content of about 10% and stored in air-tight bins in the laboratory to maintain constant

moisture and to reduce contact with air. Chicken manure, which served as a nutrient source in fermentations, was obtained from Feather Crest Farms Inc. (Bryan, TX). It was dried in the oven at 105 °C for 48 h to a moisture content of 4% and homogenized to obtain a nutritionally consistent substrate. The inoculum used for fermentations was a mixed-culture of marine microorganisms collected from beach sediment in Galveston Island, TX. This inoculum was first adapted to the fermentation substrate (80% corn stover/20% chicken manure) by batch fermentation (7 days). The liquid from this fermentation was used to inoculate all subsequent batch and countercurrent fermentations.

2.2.2. Fermentor configuration

Batch and countercurrent fermentations were performed in 1-L polypropylene centrifuge bottles capped with a rubber stopper with a hole drilled in the middle (Fig. 2.1). A glass tube is inserted through the hole and sealed using a rubber septum, which allowed gas sampling and venting. The ¼-inch stainless steel pipe rods inserted in the rubber stopper enhanced mixing of the slurry. The fermentors were placed in a Wheaton Modular Cell Production Roller Apparatus (Fisher Scientific, Pittsburgh, PA) and were rotated at 2 rpm at 40˚C.

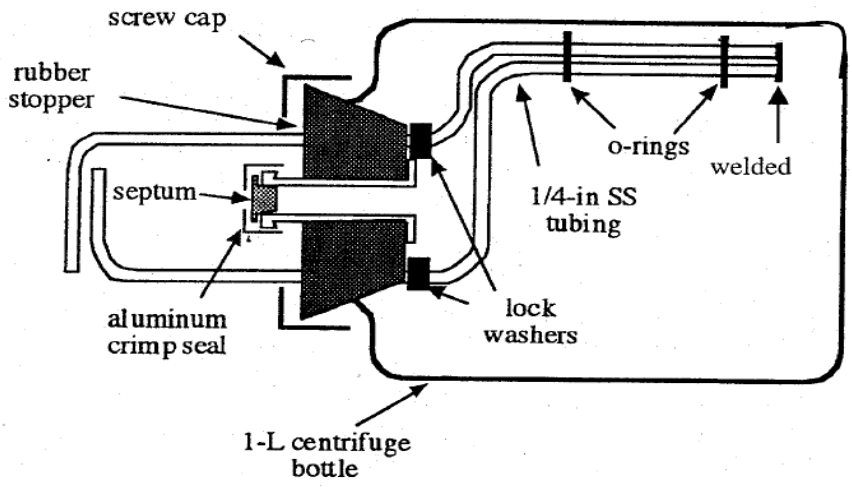

Figure 2.1 Schematic of a plastic rotary fermentor.

2.2.3. Methanogen inhibition

Iodoform (CHI_3) was used to inhibit methane production. Iodoform solution (20 g CHI_3/L 190-proof ethanol) was added to each fermentor (60 µL once at the beginning in batch fermentations, 30 µL every other day in countercurrent fermentations). Because iodoform is light, air, and temperature sensitive, to prevent degradation the solution was kept in amber-colored glass bottles wrapped in foil, stored at –20 °C, and special care was taken to replace the cap immediately after use [32].

2.2.4. Pretreatment

2.2.4.1. SLP (submerged lime pretreatment)

SLP (submerged lime pretreatment) was conducted at 50 °C for 4 weeks (28 days) with lime loading of 0.15 g $Ca(OH)_2$/g dry biomass in a 60-L jacketed vessel (Fig.

2.2a). The vessel was loaded with corn stover (3.5 kg dry weight), water (31.5 kg), and $Ca(OH)_2$ (0.525 kg). CO_2-free air was slowly bubbled (1 L/min) from the bottom of the vessel, which provided oxygen. CO_2 was removed by scrubbing air through a column of NaOH solution (96 g/L), which prevented the loss of lime to calcium carbonate. Hot water in the vessel jacket was maintained at 50 °C by recycling through an electric water heater. The pH remained at 11.5 throughout the duration (28 days). Upon completing the desired reaction time, to remove excess lime the biomass slurry was neutralized using 5-N HCl (1.2 L) to a pH of 4.5, washed thrice with distilled water, and air dried at room temperature to a moisture content of approximately 10%.

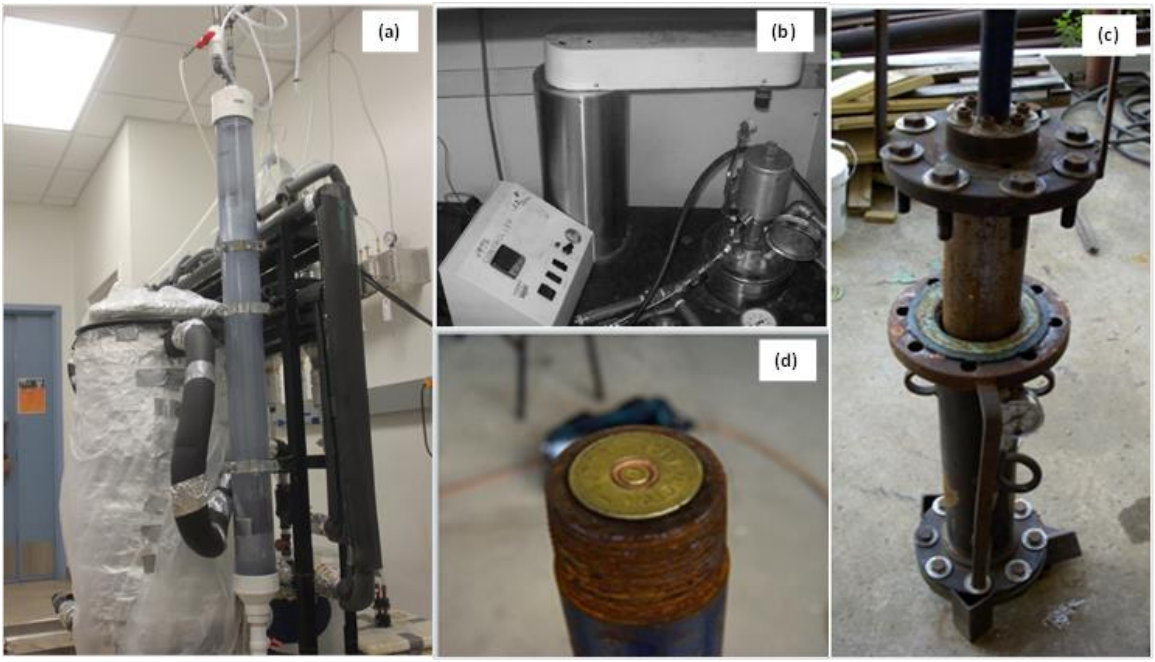

Figure 2.2 Pretreatment apparatus. (a) SLP (submerged lime pretreatment). (b) OLP (oxidative lime pretreatment). (c) Shock pretreatment. (d) Shotgun shell loaded in the barrel.

2.2.4.2. OLP (oxidative lime pretreatment)

OLP (oxidative lime pretreatment) employs harsher reaction conditions and is much shorter (only 4 h) than SLP pretreatment. It was conducted in a high-pressure 8-L Parr reactor (Fig. 2.2b). The reactor was loaded with 310 g corn stover, 155 g lime (0.5 g $Ca(OH)_2$/g dry biomass), and 4.5 L of water. Pure oxygen (6.9 bar) was applied and the pretreatment was performed at 110 °C for 4 hours. To remove excess lime, the biomass slurry was then neutralized with 5-N HCl to a pH of 4.5, washed thrice with distilled water, and air dried to a moisture content of 10%.

2.2.4.3. Shock pretreatment

The shock pretreatment was performed in a 3-L steel shock tube (4-in Sch. 40) with circular metal flanges welded onto each end (Fig. 2.2c). The upper metal flange has a 27.5-in-long steel barrel (1-in Sch.40) welded onto it and a shotgun shell fits inside the open top end. The firing mechanism consists of a spring-loaded firing pin that strikes the shotgun shell. Threading at the top of the barrel allows the firing mechanism to be securely fastened. A 10% biomass slurry (200 g dry pretreated biomass and 1.8 L distilled water) was poured into the shock tube and a gasket was placed between the top flange of the shock tube and the metal flange attached to the barrel. Then, the eight nuts and bolts around the flanges were tightened. The shotgun shell (Winchester Expert High Velocity 3-½-in, 1-3/8-oz steel BB shot) was place at the top of the barrel, the firing mechanism was screwed on, and the shotgun shell was discharged. The upper flange was then unbolted and the slurry was poured onto steel trays and air dried at room temperature to a moisture content of 10%. Detailed information is presented elsewhere [21, 33].

2.2.5. Compositional analysis

Compositional analysis of raw (not pretreated) corn stover and pretreated corn stover was performed using the NREL laboratory analytical procedure for the determination of structural carbohydrates and lignin in biomass NREL/TP-510-42618 [34]. Ash content was determined by heating the samples in a furnace at 575 °C for 24 h.

2.2.6. Enzymatic hydrolysis

The enzymatic hydrolysis of corn stover treated with lime and lime + shock was performed using the enzymatic saccharification procedure NREL/TP-510-42629 [35] with a modified biomass loading. Instead of 1% cellulose concentration, 10% biomass loading was used to assess the pretreatments at industrially relevant concentrations. The enzyme used was Novozymes Cellic Ctec2 with a protein content of 294 mg protein/mL determined using the Pierce BCA assay. The reaction mixture consisted of 1 g biomass sample (dry wt.), 5.0 mL sodium citrate buffer (0.1 M, pH 4.8), 0.04 mL tetracycline solution (10 mg/mL in 70% ethanol), and 0.03 mL cycloheximide solution (10 mg/mL in distilled water). Distilled water was added to bring the total volume to 10.00 mL including the enzyme solution. The enzyme loading was 48 mg protein/g glucan and the reaction tubes were incubated at 50 °C for 120 h. All samples were hydrolyzed in triplicate.

2.2.7. Mixed-culture fermentations

2.2.7.1. Batch fermentations

Batch fermentations at five different substrate loadings were performed with corn stover treated with lime and lime + shock to obtain the required data for the Continuum Particle Distribution Model (CPDM) [23, 36]. The substrate consisted of 80% pretreated corn stover and 20% chicken manure. The desired amount of substrate was added in a 1-L polypropylene rotary fermenter along with 3 g of calcium carbonate buffer and 20 mL of adapted marine inoculum. To prevent loss of carbon to methane, 120 µL of iodoform solution (20 g CHI_3/L acetone) was added to inhibit methanogens. Deoxygenated water

was added to bring the volume to 200 mL in each fermentor and incubated at 40 °C for 35 days. The fermentors were rotated horizontally at 2 rpm in a Wheaton Modular Cell Production Roller Apparatus located in an incubator (Fig. 2.3). Every day, all fermentors were removed from the incubator and fermentation gases were released and a liquid sample was taken every other day to be analyzed for carboxylic acid concentrations via gas chromatography. The five different substrate concentrations used were 20, 40, 70, 100, and 100+ g dry substrate/L liquid. The 100+ fermentor had the same substrate loading as the 100 g/L, but an additional 20 g carboxylic acids/L (16 g/L acetic acid, 1 g/L propionic acid, and 3 g/L butyric acid) were added to capture any inhibitory effects of initially present product.

Figure 2.3 Fermentation incubator.

2.2.7.2. Countercurrent fermentations

Four-stage semi-continuous countercurrent fermentation (Fig. 2.4) was performed for 112 days with the OLP corn stover and the OLP + shock pretreated corn stover. For the first two weeks, the fermentations were started and monitored like batch fermentations, which allowed the culture to establish. Thereafter, biomass transfers were conducted every other day. The solids and the liquids were transferred in opposite directions [37]. During transfers, calcium carbonate buffer (1 g) was added to maintain

the pH around 5.6 to each fermentor. The detailed procedure is given elsewhere [23, 38]. The most important operating parameters for a countercurrent fermentation are liquid residence time (LRT) and volatile solids loading rate (VSLR).

$$\text{Liquid residence time (LRT)} = \frac{\text{Total liquid in all fermentors}}{\text{Flow rate of liquid out of the train}}$$

$$\text{Volatile solids loading rate (VSLR)} = \frac{\text{Volatile solids fed to the system}}{\text{Total liquid in all fermentors} \times \text{time}}$$

$$\text{Conversion} = \frac{\text{VS digested}}{\text{VS fed}}$$

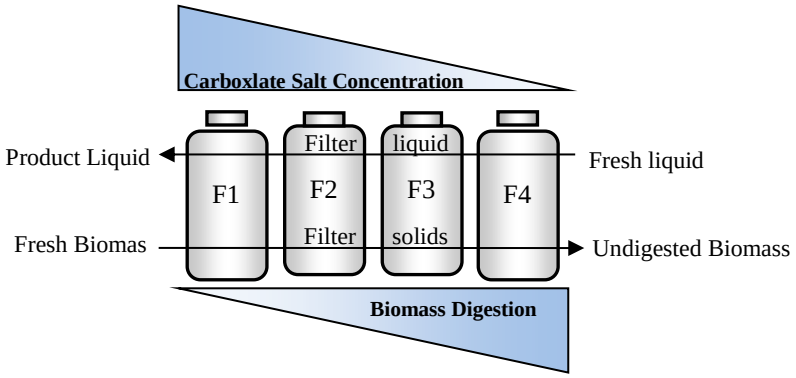

Figure 2.4 Four-stage semi-continuous countercurrent fermentation.

2.2.8. Continuum particle distribution model (CPDM)

CPDM is a powerful tool to simulate fermentation performance in many reactor configurations (e.g., continuous stirred tank reactor CSTR, plug flow reactor PFR, countercurrent and cocurrent CSTR cascades) using empirical rate models obtained from simple batch experiments [36]. It is reasonably accurate (within 10%), and saves thousands of man-hours in elaborate countercurrent fermentation experiments, which require 3 to 4 months to acquire a single steady-state data point [36]. A continuum particle (CP) is defined as a collection of biomass particles that equals 1 g volatile solids (VS) at time zero and is representative of the entire feedstock entering the fermentation [24]. A distribution function is used to express the number of CPs left in a particular interval of conversion from 0 to 1 at a particular time in the fermentation process. The governing empirical rate equation is obtained from a set of batch fermentations with varying initial substrate concentrations, some with externally added product to capture the product inhibition effects.

$$r_{pred} = \frac{e(1-x)^f}{1+g(\phi \cdot \mathrm{Aceq})^h}$$

where:

x = conversion of VS (dimensionless)

e, f, g, and h = empirical constants

ϕ = acid equivalence (total grams acid/gram Aceq)

r_{pred} = predicted reaction rate (g of acetic acid equivalent generated/(time·g VS))

The mixed-acid concentration can be expressed as molar acetic acid equivalents (α), which is the reducing potential of an equivalent amount of acetic acid [39]:

$$\alpha = 1.0\times \text{acetic(mol/L)} + 1.75\times \text{propionic(mol/L)} + 2.5\times \text{butyric(mol/L)} + 3.25\times \text{valeric(mol/L)} + 4.0\times \text{caproic(mol/L)} + 4.75\times \text{heptanoic(mol/L)}$$

The acetic acid equivalent (Aceq) can be expressed on a mass basis as:

$$\text{Aceq (Acetic acid equivalents g/L)} = 60.05\ (\text{g/mol}) \times \alpha$$

The conversion is given by

$$\text{conversion} = x(t) = \frac{\text{Aceq}(t) - \text{Aceq}(t=0)}{S_0 \sigma}$$

where, S_0 is the initial amount of substrate (g VS/L) and σ is the selectivity (g Aceq produced/g VS digested), which is assumed to be constant throughout each batch fermentation. The σ value used for all CPDM calculations in this study was 0.87, which was obtained from the countercurrent fermentation experiment.

Once all the acid concentrations and conversions are obtained from the batch experiments, they are fit to Equation 1 by the method of least squares in Microsoft Excel to get the empirical constants (*e, f, g,* and *h*) for that specific system. This rate equation was then used to simulate a four-stage countercurrent fermentation with varying VSLRs and LRTs in MATLAB.

2.2.9. Analytical methods

Sugars were analyzed using HPLC (Bio-Rad Aminex HPX-87P column). The mobile phase was HPLC-grade water flowing at 0.6 mL/min and the column temperature was 85 °C. Carboxylic acids in the fermentations were measured using a gas chromatograph (Agilent 7890A) with a flame ionization detector (FID). The fermentation liquid was centrifuged at 13,000 rpm for 10 min and mixed with equal parts of internal standard (1.162 g/L 4-methyl-*n*-valeric acid) and 3-M phosphoric acid. The column used was Agilent J&W HP-5 (model# 19091J-413) with helium as the carrier gas.

2.3. Results and discussion

2.3.1. SLP (submerged lime pretreatment) and OLP (oxidative lime pretreatment)

Table 2.1 shows the compositional analysis of all the biomass feedstocks used in this study. The raw corn stover (no pretreatment) had a total lignin content of 0.14 g lignin/g dry biomass whereas the SLP corn stover had a total lignin content of 0.09 g lignin/g dry biomass and OLP corn stover had 0.08 g lignin/g dry biomass. The glucan content was also very similar for the SLP corn stover (0.44 g glucan/g dry biomass) and OLP corn stover (0.45 g glucan/g dry biomass).

Table 2.1 Compositional analysis of biomass feedstocks used in this study[a].

Biomass	Glucan (%)	Xylan (%)	Galactan (%)	Arabinan (%)	Acid-insoluble lignin (%)	Acid-soluble lignin (%)	Ash (%)	Total extractives (%)	Total closure (%)
Raw corn stover	36.06 ± 0.14	23.08 ± 0.11	1.48 ± 0.06	3.34 ± 0.02	12.76 ± 0.71	1.30 ± 0.11	1.63 ± 0.13	12.11 ± 0.05	91.76 ± 0.66
SLP corn stover	44.21 ± 0.30	20.80 ± 0.10	0.4 ± 0.30	1.79 ± 0.40	8.18 ± 1.30	0.98 ± 0.01	4.10 ± 0.60	17.40 ± 0.30	96.10 ± 4.20
SLP + shock corn stover	48.30 ± 0.20	20.80 ± 0.10	1.2 ± 0.10	2.50 ± 0.10	8.40 ± 0.20	1.10 ± 0.01	2.30 ± 0.90	13.50 ± 0.09	96.98 ± 1.50
OLP corn stover	45.60 ± 0.50	18.2 ± 0.20	0.8 ± 0.20	1.9 ± 0.30	6.40 ± 0.10	1.10 ± 0.04	7.01 ± 0.05	15.10 ± 0.07	95.30 ± 0.80
OLP + shock corn stover	50.50 ± 0.60	20.2 ± 0.80	1.2 ± 0.20	2.0 ± 0.01	5.40 ± 0.50	1.20 ± 0.01	5.35 ± 0.18	6.9 ± 0.05	91.60 ± 0.10

[a]Values are average of two samples and error is ± ½ range.

The enzymatic hydrolysis results in Table 2.2 show that for SLP corn stover after 120 h, the glucan digestibility was 81.8% and the overall digestibility was 80.7%. For OLP corn stover, the glucan digestibility was 83.6% and the overall digestibility was 80.7%. Hence, both the pretreatments are very effective and give similar results. SLP uses very mild conditions (50°C, 1-atm air) making it inexpensive and suitable for industrial pretreatment. However, because SLP takes 28 days to complete, OLP was used in the laboratory to rapidly generate the large quantity of pretreated corn stover required for the countercurrent fermentation experiment.

Table 2.2 Summary of enzymatic hydrolysis results[a].

Biomass	Biomass loading	Glucan digestibility (%)	Xylan digestibility (%)	Overall digestibility (%)
SLP corn stover	10%	81.88 ± 0.25	73.81 ± 4.06	80.75 ± 0.22
SLP + shock corn stover	10%	85.45 ± 0.61	72.85 ± 2.69	81.88 ± 1.00
OLP corn stover	10%	83.61 ± 0.01	73.63 ± 0.02	80.70 ± 0.01
OLP + shock corn stover	10%	86.19 ± 0.01	74.98 ± 0.01	82.92 ± 0.01

[a]Values are average of two samples and error is ± ½ range.

2.3.2. Enzymatic hydrolysis of shock pretreated corn stover

Shock pretreatment uses a shockwave (rapid pressurization) to render biomass more amenable to biological and enzymatic digestion [21]. Table 2.2 summarizes the results of 120-h enzymatic hydrolysis with an enzyme loading of 48 mg protein/g glucan

on SLP, SLP + shock, OLP, and OLP + shock pretreated corn stover. The added shock pretreatment increased glucan digestibility of SLP corn stover by 3.5% and overall digestibility by 1.1%. With OLP corn stover, adding shock pretreatment step resulted in a 2.5% increase in glucan digestibility and a 2.2% increase in the overall digestibility.

2.3.3. CPDM modeling of shock pretreated corn stover

The first step in CPDM modeling is to obtain the empirical rate parameters from a set of batch fermentations at different substrate loadings [40]. Table 2.3 shows the experimental set-up of the batch fermentations performed in this study using five different substrate loadings. These batch fermentations can be used to quickly assess biomass for use in the MixAlco™ process (carboxylate platform) [41, 42]. Fig. 2.5 shows the Aceq concentrations for the batch fermentations. At low substrate concentrations (20 g/L and 40 g/L), SLP + shock corn stover does significantly better than SLP; however, at higher substrate concentrations (70, 100, 100+ g/L) both substrates (SLP and SLP + shock corn stover) have similar Aceq concentration, which could be caused by higher product inhibition. At 20 g/L, SLP and SLP + shock had Aceq of 4.3 and 5.15 g/L, respectively, an increase of 17.6%. The respective conversions were 0.52 and 0.55 g VS digested/g VS fed. At 40 g/L, SLP and SLP + shock had Aceq of 12.5 and 15.4 g/L, respectively, an increase of 23.4%. The respective conversions were 0.45 and 0.47 g VS digested/g VS fed. At higher substrate concentrations of 70, 100 and 100+ g/L, the biomass conversions decreased as expected (0.39, 0.42, 0.39 for SLP corn stover and 0.45, 0.46, 0.44 for SLP + shock corn stover) and both the substrates had similar Aceq concentrations (Fig. 2.5).

Table 2.3 CPDM batch fermentations set-up.

	Solid Loading 20 g/L		**Solid Loading 40 g/L**		**Solid Loading 70 g/L**		**Solid Loading 100 g/L**		**Solid Loading 100+ g/L**	
	SLP	SHOCK	SLP	SHOCK	SLP	SHOCK	SLP	SHOCK	SLP	SHOCK
Total corn stover (g)	3.55	3.51	7.09	7.02	12.41	12.28	17.73	17.54	17.73	17.54
Chicken manure (g)	0.85	0.85	1.71	1.71	2.99	2.99	4.27	4.27	4.27	4.27
Calcium carbonate (g)	3	3	3	3	3	3	3	3	3	3
Urea (g)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Inocula (mL)	20	20	20	20	20	20	20	20	20	20
Deoxygenated water (mL)	180	180	180	180	180	180	180	180	174.8	174.8
Iodoform (µL)	60	60	60	60	60	60	60	60	60	60
100+ salts Ca-acetate (g)									3.2	3.2
100+ salts Ca-propionate (g)									0.2	0.2
100+ salts Ca-butyrate (g)*									0.6	0.6
Total liquid (mL)	200	200	200	200	200	200	200	200	200	200

* Ca-butyrate was added as a solution (Amount = 2.56 mL, Concentration = 0.096 g/mL)
Iodoform solution (20 g CHI_3/L 190-proof ethanol)

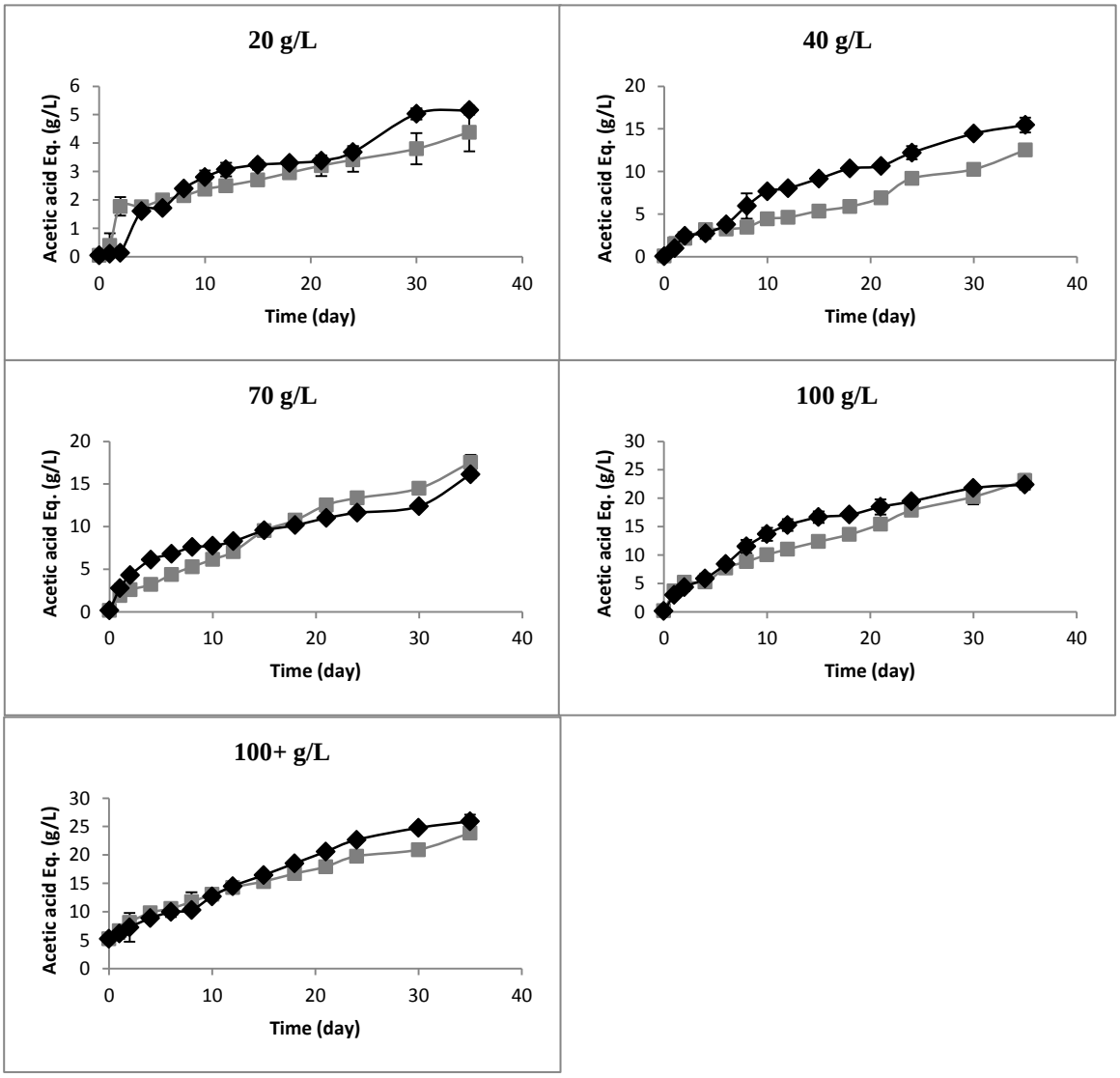

Figure 2.5 Acetic acid equivalents concentration profiles (SLP + shock pretreated corn stover (♦), SLP pretreated corn stover (■)) for CPDM batch fermentations.

Using least-square regression, an empirical rate model (Equation 1) was fit to these Aceq concentration profiles. The governing rate equation for SLP corn stover is

$$r_{pred} = \frac{0.012(1-x)^{1.63}}{1+4.01\times10^{-5}(0.77\cdot \text{Aceq})^{2.99}}$$

The governing rate equation for SLP + shock corn stover is

$$r_{pred} = \frac{0.018(1-x)^{3.10}}{1+2.98\times10^{-6}(0.77\cdot \text{Aceq})^{2.93}}$$

Using MATLAB, these rate equations were used to simulate four-stage countercurrent fermentations with varying LRTs and VSLRs. Fig. 2.6 shows a CPDM map of SLP + shock corn stover superimposed over the CPDM map of SLP corn stover. For every LRT and VSLR in the map SLP + shock corn stover has a higher carboxylic acid concentration and conversion. The benefit is more pronounced at high VSLRs. At low VSLR (4 g/(L·day)), the carboxylic acid concentrations and conversions are very similar and the both maps coincide. At higher VSLR, the SLP + shock map shifts up and towards the right giving higher conversions and carboxylic acid concentrations. At a very high VSLR (12 g/(L·day)) and LRT (30 day), which is suitable for large-scale fermentations, the carboxylic acid concentrations for SLP + shock and SLP corn stover are 36 and 28 g/L respectively, a 28.5% increase.

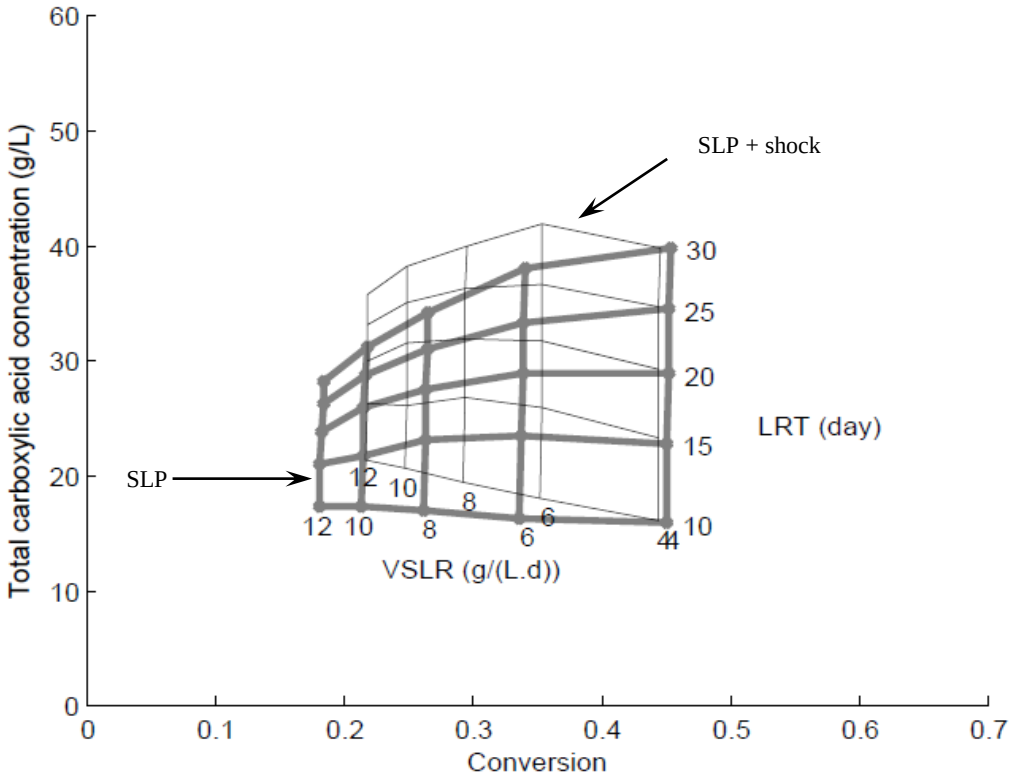

Figure 2.6 Predicted "CPDM map" generated using MATLAB for countercurrent fermentations with 10% total solids (100 g solids/L slurry) using SLP and SLP + shock pretreated corn stover (80%) and chicken manure (20%).

2.3.4 Countercurrent fermentations

To assess the benefit of shock pretreatment in countercurrent fermentations and to verify the accuracy of CPDM, a semi-continuous countercurrent fermentation was performed for 112 days with OLP and OLP + shock corn stover. Instead of SLP, OLP was used to rapidly generate pretreated biomass because it is much faster (4 h) and the performance is identical to SLP, as shown earlier. Table 2.4 shows the operating

parameters and key results of the countercurrent fermentations. Fig. 2.7 shows the carboxylic acid profile for the two trains (OLP and OLP + shock corn stover). For both the trains, the VSLR was 1.875 g/(L·day) and LRT was 16 days. For the OLP + shock corn stover, the steady-state carboxylic acid concentration was 16.3 g/L, 14.8% higher than OLP corn stover (14.2 g/L). The steady state was calculated by taking the average concentration of all values after Day 78. The experimental results matched closely with the CPDM model predictions for acid concentrations with an error of 3.38% for OLP corn stover train and 4.05% for OLP + shock corn stover train.

Table 2.4 Operating parameters and key results of countercurrent fermentations.

4-Stage fermentation train	**OLP corn stover**	**OLP+Shock corn stover**
LRT (days)	16	16
VSLR (g VS/(L liquid·day))	1.875	1.875
Transfer frequency	every 2 days	every 2 days
Total solids	10%	10%
Calcium carbonate added in all four fermentors	1 g every transfer	1 g every transfer
Incubation temperature	40°C	40°C
Steady-state carboxylic acid concentration (experimental)	14.19 g/L	16.29 g/L
CPDM predicted steady-state carboxylic acid concentration	14.67 g/L	16.95 g/L
CPDM error (%)	3.38%	4.05%
Conversion (experimental)	0.49	0.47
CPDM predicted conversion	0.50	0.51

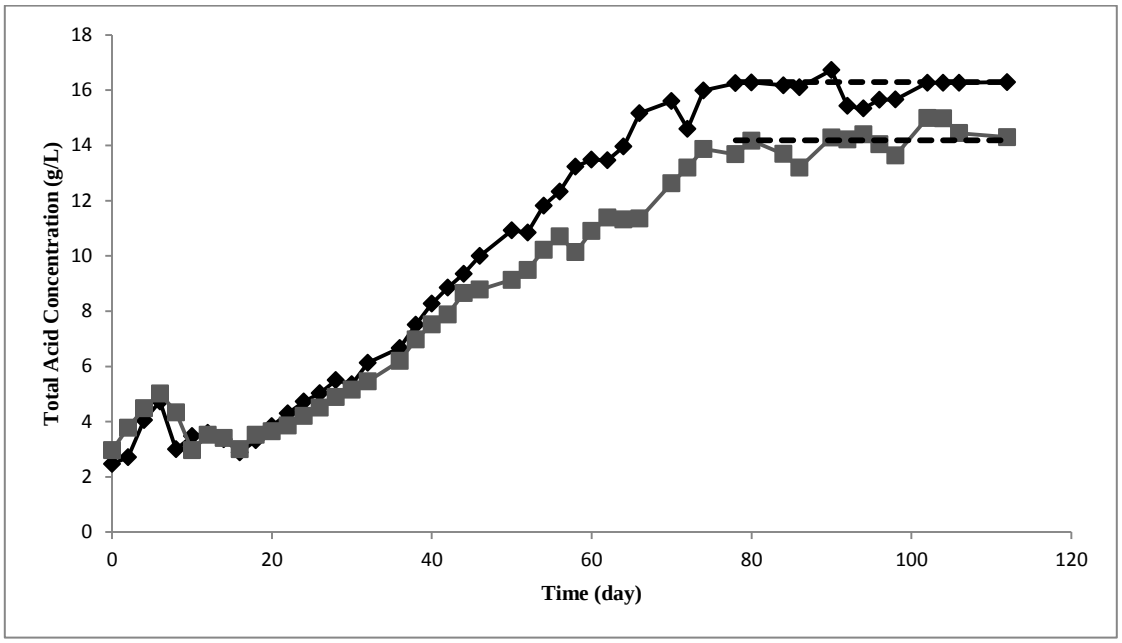

Figure 2.7 Carboxylic acid concentration profile (OLP + shock pretreated corn stover (♦), OLP pretreated corn stover (■)) for four-stage countercurrent fermentation.

2.4. Conclusions

Shock pretreatment increases the digestibility of lignocellulosic biomass and can be used as added step to enhance chemical pretreatments. Shock pretreatment performed better than lime-only pretreatment in enzymatic saccharification as well as in mixed-acid fermentations (batch and countercurrent). CPDM predicts that at high VSLR rate of 12 g/(L·day) and LRT 30 days, adding shock pretreatment to lime pretreatment (SLP) increased the total carboxylic acid yields by 28.5%. A laboratory-scale countercurrent fermentation matched closely with CPDM predictions (4.05% error), establishing the

benefit of shock pretreatment in enhancing product yields for biochemical conversions like mixed-acid fermentations. Short residence times (30 to 90 s) and small vessels ensure that shock pretreatment is economically feasible. Total cost of shock pretreatment is estimated to be $5/tonne [22], which is very small compared to conventional chemical pretreatments (~$45/tonne) [43].

3. EFFECT OF MAGNESIUM CARBONATE BUFFER ON MIXED-CULTURE FERMENTATIONS

In this study, calcium carbonate and magnesium carbonate were compared as buffers for mixed-culture fermentations of lime and lime + shock pretreated corn stover. Batch fermentations at five different substrate loadings of lime and lime + shock pretreated corn stover were performed with $MgCO_3$ and $CaCO_3$ buffer. In batch fermentations with 100 g/L substrate, the carboxylic acid production more than doubled (2.7 times for lime and 2.6 times for lime + shock corn stover) when $MgCO_3$ buffer was used. In addition, the Continuum Particle Distribution Model (CPDM) was used to simulate and predict the performance of a four-stage countercurrent fermentation using $MgCO_3$ and $CaCO_3$ buffer. CPDM predicts that in a four-stage countercurrent fermentation with a high volatile solids loading rate (VSLR 12 g/(L·day)) and low liquid residence time (LRT 10 day), using $MgCO_3$ buffer will yield a carboxylic acid concentration of 26.1 g/L, a 22.5% increase over $CaCO_3$ buffer. Adding shock to lime pretreatment increased the yields at all substrate loadings in both batch fermentations and CPDM model predictions.

3.1. Introduction

Today, a vast majority of global energy needs are met by combusting fossil fuels, which causes pollution, acid rain, and global warming [4]. In the last 100 years, the average temperature of the earth's surface has increased by 0.7˚C and model predictions anticipate an additional warming of 1.1 to 6.4˚C by the end of 21st century [3]. This will increase sea levels, change weather patterns, and acidify the oceans. Biofuels are carbon neutral and can help reduce dependence on fossil fuels [44]. Second-generation biofuels produced from lignocellulosic biomass do not compete with food crops for land or water [5] and have potential for high yields of gasoline equivalents produced per acre [45].

Lignocellulose can be converted into liquid fuels using three major platforms: thermochemical platform (gasify biomass to syngas, which is catalytically transformed into fuels), sugar platform (enzymes convert biomass into simple sugars, which are fermented to ethanol), and the carboxylate platform (a mixed-culture transforms biomass to carboxylate salts, which are chemically converted to fuels). The MixAlco™ process is an example of the carboxylate platform [7]. The carboxylate platform has the highest product yields in the literature [8]. Compared to ethanol and other alcohols, carboxylic acids are thermodynamically favored provided methane production is inhibited; therefore, sterile conditions are not required. Also, a mixed culture utilizes all biomass components (e.g., lignocellulose, starch, protein, and fats), which increases yield. The ability to utilize varied biomass components under non-sterile conditions – and still achieve high product yields – makes the carboxylate platform economically attractive.

In its natural state, lignocellulose is poorly digestible to enzymes and microorganisms and needs pretreatment prior to fermentation [10, 11]. Lime pretreatment is an effective and inexpensive way to remove lignin while preserving hemicellulose, which is solubilized in acid pretreatments [15, 16]. Submerged lime pretreatment (SLP), which uses atmospheric-pressure air as the oxidizing agent at a low temperature (50˚C) for 28 days, was used to pretreat raw corn stover. In addition, the biomass was further pretreated with a recently developed shock pretreatment, which uses a shockwave to render biomass more amenable to digestion [21].

Following pretreatment, biomass is fermented using a mixed culture of microorganisms to produce carboxylate salts. A four-stage countercurrent fermentation is used to minimize the effects of product inhibition, which improves yields [23, 24]. By selecting appropriate downstream processing steps, these carboxylate salts can be further converted into a wide variety of chemicals or fuel. Historically, these mixed-culture fermentations have used calcium carbonate as a buffer to neutralize the carboxylic acids. It is inexpensive and is readily calcined to lime, which can be used as a pretreatment agent. Because it is poorly soluble, an excess amount can be added at the start of the fermentation; it need not be added every day [25]. This “auto buffering” eliminates the need for a sophisticated pH controller; as more carboxylic acids are produced, more calcium carbonate dissolves into the fermentation broth. The primary disadvantage of calcium carbonate is that it does not control to pH~7. At 1-atm CO_2 pressure, it buffers around pH of 5.6–6. Neutral pH is important because it affects fermentation rates, products, and yields [26, 27]. Lowering the pH from 7 to 6 greatly reduces cellulose and

hemicellulose hydrolysis [28]; hence, a different buffer is needed to control the fermentation pH near 7.

To make hydrocarbon fuels (gasoline, diesel, or jet fuel), the carboxylate salts are concentrated and thermally converted to ketones, hydrogenated to alcohols, and then catalytically converted to hydrocarbons [29]. Divalent carboxylate salts (e.g., calcium carboxylates) begin thermally decomposing at much lower temperatures (170–180°C) than do monovalent ions, like sodium carboxylate salts (410–420°C) [30]. Also, the average ketone yield for sodium salts was less than half that for calcium salts. Considering these factors, magnesium carbonate was chosen as the buffer for mixed-culture fermentations.

The main purpose of this study is to determine the effect of magnesium carbonate as a buffer for mixed-culture fermentations in the MixAlco™ process. Calcium carbonate and magnesium carbonate buffers were compared in mixed-culture fermentations of lime and lime + shock pretreated corn stover. In addition, Continuum Particle Distribution Model (CPDM) was used to simulate and predict its performance in a four-stage countercurrent fermentation.

3.2. Methods

3.2.1. Raw substrates

Both the countercurrent and batch fermentations used 80% corn stover/20% chicken manure. Corn stover, the energy source, was generously provided by Texas A&M AgriLife, the agriculture college at Texas A&M University. It was air dried to a

moisture content of about 10% and stored in air-tight bins in the laboratory to maintain constant moisture and to reduce contact with air. Chicken manure, the nutrient source, was obtained from Feather Crest Farms Inc. (Bryan, TX). It was oven dried at 105°C for 48 h to 4% moisture and homogenized to obtain a nutritionally consistent substrate.

3.2.2. Inoculum

The mixed-culture inoculum source affects the yield and final acid concentration. Marine inoculum performs better than terrestrial inoculum and rumen fluid [46]. In this study, the inoculum was from beach sediment in Galveston Island, TX. First, this inoculum was adapted to the fermentation substrate (corn stover/chicken manure) by batch fermentation (7 days) using two different buffers (calcium carbonate and magnesium carbonate). Then, the liquid from this fermentation was used to inoculate batch fermentations.

3.2.3. Fermentor

Batch fermentations were performed in 1-L polypropylene centrifuge bottles capped with a rubber stopper with a hole drilled in the middle. A glass tube is inserted through the hole and sealed using a rubber septum, which allowed gas sampling and venting. The ¼-inch stainless steel pipe rods inserted in the rubber stopper enhanced mixing of the slurry. The fermentors were placed in a Wheaton Modular Cell Production Roller Apparatus (Fisher Scientific, Pittsburgh, PA) and were rotated at 2 rpm at 40˚C.

3.2.4. pH control

The two buffers were calcium carbonate ($CaCO_3$) and magnesium carbonate ($MgCO_3$). At the beginning of the fermentation, all $CaCO_3$ (6 g) was added at once. $CaCO_3$ is poorly soluble and slowly dissolves in the fermentation slurry as more carboxylic acids are produced. In contrast, $MgCO_3$ is highly soluble and cannot be added all at once. Instead, it was added as a solid powder every day for the first seven days and every two days thereafter to control the pH near neutrality (7.00–7.10).

3.2.5. Pretreatment

3.2.5.1. SLP (submerged lime pretreatment)

SLP (submerged lime pretreatment) was conducted at 50 °C for 4 weeks (28 days) with lime loading of 0.15 g $Ca(OH)_2$/g dry biomass in a 60-L jacketed vessel. The vessel was loaded with corn stover (3.5 kg dry weight), water (31.5 kg), and $Ca(OH)_2$ (0.525 kg). CO_2-free air was slowly bubbled (1 L/min) from the bottom of the vessel, which provided the oxygen source. To prevent formation of calcium carbonate by the reaction of lime with CO_2, and hence reduce the loss of lime, CO_2 was removed by scrubbing air through a column of NaOH solution (96 g/L). Hot water in the vessel jacket was maintained at 50 °C by recycling through an electric water heater. The pH remained at 11.5 throughout the duration (28 days). This pretreatment operated at 10% solids concentration, which is suitable in a large industrial setting. Upon completing the desired reaction time, the biomass slurry was neutralized using 5-N HCl (1.2 L) to a pH of 4.5, washed thrice with distilled water, and air dried at room temperature to a moisture content of approximately 10%.

3.2.5.2. Shock pretreatment

The shock pretreatment was performed in a 20-in-long, 4-in-diameter steel shock tube (Sch. 40) with circular metal flanges welded onto each end. The upper metal flange has a 27.5-in-long steel barrel (1-in Sch. 40) welded onto it and a shotgun shell fits inside the open top end. The firing mechanism consists of a spring-loaded firing pin, which strikes the shotgun shell. Threading at the top of the barrel allows the firing mechanism to be securely fastened. When the apparatus is bolted shut, the total volume is 3.02 L. Biomass slurry (200 g dry pretreated biomass and 1.8 L distilled water) was poured into the shock tube and a gasket was installed in the top flange and the eight nuts and bolts were tightened to seal the flanges. The shotgun shell (Winchester Expert High Velocity 3 ½-in, 1 3/8-oz steel BB shot) was place on the top of the barrel, the firing mechanism was screwed on, and the shotgun shell was discharged. The upper flange was then unbolted and the slurry was poured onto steel trays and air dried at room temperature to a moisture content of 10% [21, 33].

3.2.6. Mixed-culture batch fermentations

Batch fermentations at five different substrate loadings with two different buffers ($CaCO_3$ and $MgCO_3$) were performed with lime treated and lime + shock treated corn stover (Table 3.1). The data obtained from these batch fermentations were used to simulate a four-stage countercurrent fermentation using the Continuum Particle Distribution Model (CPDM)[23, 36]. The substrate was 80% pretreated corn stover and 20% chicken manure. The desired amount of substrate was added in a 1-L polypropylene rotary fermenter along with 40 mL of adapted marine inoculum. To inhibit methanogens,

30 µL of iodoform solution (20 g CHI_3/L acetone) was added. Deoxygenated water was added to bring the volume to 400 mL in each fermentor and incubated at 40 °C for 43 days. Every day, all fermentors were removed from the incubator to release fermentation gases. Every other day, liquid samples were taken to be analyzed for carboxylic acid concentrations in the gas chromatograph. In addition, 30 µL of iodoform solution was added. The five different substrate concentrations used were 20, 40, 70, 100, and 100+ g dry substrate/L liquid. The 100+ fermentor had the same substrate loading as the 100 g/L, but an additional 20 g carboxylic acids/L (80% acetate, 5% propionate, 15% butyrate) to capture any inhibitory effects of initially present product. In the fermentations with $CaCO_3$ buffer, 6 g of $CaCO_3$ was added at the beginning of the fermentation. In the fermentations with $MgCO_3$ buffer, $MgCO_3$ was added every day for the first seven days and every two days after that until the pH was close to neutral (7.00–7.10). The performance of batch fermentation was evaluated using the total carboxylic acid concentration, yield, conversion, and selectivity parameters which are defined as follows:

$$\text{Yield} = \frac{\text{Total carboxylic acids produced (g)}}{\text{VS fed (g)}}$$

$$\text{Conversion} = \frac{\text{VS digested (g)}}{\text{VS fed (g)}}$$

$$\text{Selectivity} = \frac{\text{Total carboxylic acids produced (g)}}{\text{VS digested (g)}}$$

Table 3.1 Experimental set-up for batch fermentations using $CaCO_3$ and $MgCO_3$ as buffers[a].

Solid Concentration (g/L)	**20**		**40**		**70**		**100**		**100+**	
	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK
Total corn stover (g)	6.8	7.1	13.7	14.2	24.0	24.9	34.3	35.6	34.3	35.6
Chicken manure (g)	1.7	1.7	3.4	3.4	5.9	5.9	8.5	8.5	8.5	8.5
Urea (g)	0.2	0.2	0.4	0.4	0.7	0.7	1	1	1	1
Inocula (mL)	40	40	40	40	40	40	40	40	40	40
Deoxygenated water (mL)	359	359	359	358	358	357	357	356	357	356
Iodoform (µL)[b]	30	30	30	30	30	30	30	30	30	30
100+ salts Ca-acetate (g)	–	–	–	–	–	–	–	–	6.4	6.4
100+ salts Ca-propionate (g)	–	–	–	–	–	–	–	–	0.4	0.4
100+ salts Ca-butyrate (g)	–	–	–	–	–	–	–	–	1.2	1.2
Total liquid (mL)	400	400	400	400	400	400	400	400	400	400

[a]For fermentations with $CaCO_3$ as the buffer, 6 g of $CaCO_3$ was added to all fermentors at the beginning. For fermentations with $MgCO_3$ as the buffer, $MgCO_3$ was added every other day till the pH is neutral.
[b]Added every second day

3.2.7. Continuum particle distribution model (CPDM)

CPDM is a powerful tool to simulate fermentation performance in many reactor configurations (e.g., continuous stirred tank reactor CSTR, plug flow reactor PFR, countercurrent and cocurrent CSTR cascades) [36]. It uses simple batch experiments to obtain empirical rate models with reasonable accuracy (within 10%), thus saving thousands of man-hours conducting elaborate countercurrent fermentations which last for 3 to 4 months to obtain a single steady-state data point [47]. A continuum particle (CP) is defined as a collection of biomass particles that has 1 gram volatile solids (VS) at time zero and is representative of the entire feedstock entering the fermentation [24]. A distribution function is used to express the number of CPs remaining in a particular conversion interval from 0 to 1 after a particular time in the fermentation. The governing empirical rate equation is obtained from a set of batch fermentations with varying initial substrate concentrations (some with externally added product to capture the product inhibition effects).

$$r_{pred} = \frac{e(1-x)^{f}}{1+g(\phi \cdot \mathrm{Aceq})^{h}}$$

where:

x = conversion of VS

e, f, g, and h = empirical constants

ϕ = total grams acid/gram Aceq

Acetate equivalents (Aceq) are used to express the mixed acids on a common basis and are defined as follows:

$$\text{Aceq (Acetic acid equivalents g/L)} = 60.05\ (\text{g/mol}) \times \alpha$$

Where α is given by,

$$\alpha = 1.0\times \text{acetic(mol/L)} + 1.75\times \text{propionic(mol/L)} + 2.5\times \text{butyric(mol/L)} + 3.25\times \text{valeric(mol/L)} + 4.0\times \text{caproic(mol/L)} + 4.75\times \text{heptanoic(mol/L)}$$

The conversion is given by,

$$\text{conversion} = x(t) = \frac{\text{Aceq}(t) - \text{Aceq}(t=0)}{S_0\sigma}$$

where S_0 is the initial amount of substrate (g VS/L) and σ is the selectivity (g Aceq produced/g VS digested), which is assumed to be constant throughout each batch fermentation. From the batch experiments, all the acid data during 43 days and final conversions are obtained. The empirical constants (*e*, *f*, *g*, and *h*) in Equation 5 are fit to the data using the method of least squares in Microsoft Excel. This rate equation was then used to simulate a four-stage countercurrent fermentation with varying volatile solid loading rates (VSLRs) and liquid residence times (LRTs) in MATLAB.

$$\text{Liquid residence time (LRT)} = \frac{\text{Total liquid in all fermentors}}{\text{Flow rate of liquid out of the train}}$$

$$\text{Volatile solids loading rate (VSLR)} = \frac{\text{Volatile solids fed to the system}}{\text{Total liquid in all fermentors} \times \text{time}}$$

$$\text{Conversion} = \frac{\text{VS digested}}{\text{VS fed}}$$

3.2.8. Analytical methods

The carboxylic acids from the fermentations were measured using a gas chromatograph (Agilent 7890A) with a flame ionization detector (FID). The fermentation liquid was centrifuged at 13,000 rpm for 10 min and mixed with equal parts of internal standard (1.162 g/L 4-methyl-*n*-valeric acid) and 3-M phosphoric acid. The column used was Agilent J&W HP-5 (model# 19091J-413) with helium as the carrier gas. The pH of the slurry in fermentors was measured using Oakton (WD-35614) pH meter. The pH meter was calibrated every time before use. The moisture and ash content were measured using the standard NREL laboratory analytical procedures [48]. The VS content was calculated as

$$\text{Volatile Solids (VS)} = \frac{\text{Oven dry weight} - \text{Ash weight}}{\text{Oven dry weight}}$$

3.3. Results and discussion

3.3.1. Batch fermentations

Table 3.1 summarizes the set-up for the batch fermentation experiments that compare $MgCO_3$ to traditional $CaCO_3$ buffer. In batch fermentors with $CaCO_3$ buffer, excess solid $CaCO_3$ (6 g) was added at the beginning of the fermentations; the pH slowly dropped from 6.7 and stabilized around 5.6 (Fig. 3.1). In contrast, batch fermentors with

frequent addition of $MgCO_3$ buffer maintained pH 7.00–7.10. The fermentors with $MgCO_3$ buffer showed significantly improved yield, conversion, selectivity, and the total carboxylic acid production (Table 3.2).

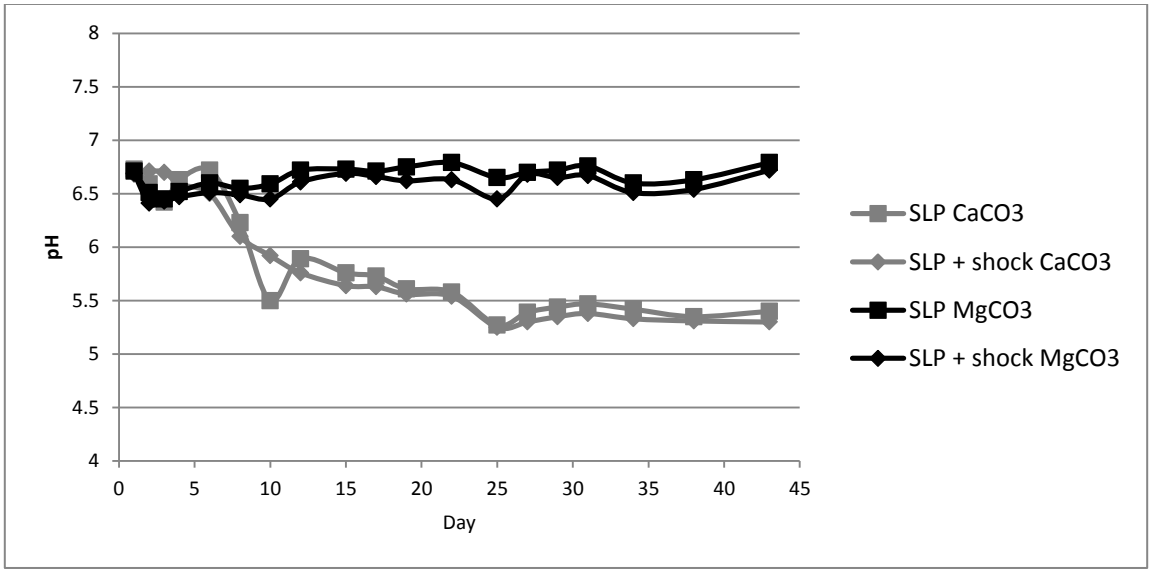

Figure 3.1 pH of the 100 g/L fermenters. $CaCO_3$ was added all at once in the beginning, whereas $MgCO_3$ was added every second day to bring the pH to 7.00–7.10.

Table 3.2a Fermentation results with $CaCO_3$ buffer.

Solid Concentration (g/L)	**20**		**40**		**70**		**100**		**100+**	
	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK
Total carboxylic acid conc. (g/L)	3.63	4.1	7.95	9.92	10.85	10.93	11.27	13.26	22.42	24.29
Yield (g total acids/g VS fed)	0.13	0.15	0.13	0.15	0.14	0.15	0.12	0.13	0.12	0.13
Conversion (g VS digested/g VS fed)	0.52	0.55	0.45	0.47	0.39	0.45	0.42	0.46	0.39	0.44
Selectivity (g total acids/g VS digested)	0.25	0.27	0.29	0.32	0.36	0.33	0.29	0.28	0.31	0.30

Table 3.2b Fermentation results with $MgCO_3$ buffer.

Solid Concentration (g/L)	**20**		**40**		**70**		**100**		**100+**	
	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK	SLP	SLP + SHOCK
Total carboxylic acid conc. (g/L)	9.06	9.49	14.13	19.22	19.18	24.67	30.41	34.52	42.22	50.17
Yield (g total acids/g VS fed)	0.44	0.43	0.35	0.43	0.27	0.34	0.29	0.34	0.42	0.51
Conversion (g VS digested/g VS fed)	0.6	0.66	0.58	0.6	0.53	0.58	0.54	0.58	0.52	0.56
Selectivity (g total acids/g VS digested)	0.73	0.65	0.60	0.72	0.51	0.59	0.54	0.59	0.81	0.91

At 100 g/L of SLP corn stover, which is suitable for large-scale production, $MgCO_3$ buffer had a total carboxylic acid concentration of 30.4 g/L, 2.7 times greater than $CaCO_3$ buffer (11.2 g/L). At 100 g/L of SLP + shock corn stover, $MgCO_3$ buffer had a total carboxylic acid concentration of 34.5 g/L, 2.6 times greater than $CaCO_3$ buffer (13.2 g/L). This clearly shows the benefit of replacing $CaCO_3$ buffer with $MgCO_3$ buffer, which can control the pH near neutrality (Fig. 3.1). Also, the benefit adding shock to lime pretreatment is clearly visible in both $MgCO_3$ and $CaCO_3$ buffers. At 100 g/L corn stover concentration using $MgCO_3$ buffer, adding shock pretreatment increased the total carboxylic acid concentration (34.5 g/L), a 13.5% increase over lime only pretreatment (30.4 g/L). Similarly, at 100 g/L corn stover concentration using $CaCO_3$ buffer, adding shock pretreatment increased the total carboxylic acid concentration (13.2 g/L), a 17.7% increase over lime only pretreatment (11.2 g/L).

For all other substrate concentrations (20, 40, 70, 100+ g/L), the $MgCO_3$ buffer performed better than $CaCO_3$ buffer using both SLP and SLP + shock corn stover. At all substrate loadings, adding a shock pretreatment to lime pretreatment (SLP) increased the carboxylic acid concentration, yield, conversion, and selectivity.

3.3.2. CPDM modeling

The data obtained from batch fermentations was used to simulate a four-stage countercurrent fermentation using CPDM [41, 42]. For five different substrate concentrations of SLP corn stover using $MgCO_3$ and $CaCO_3$ buffer, the Aceq profiles are shown for SLP (Fig. 3.2) and SLP + shock (Fig. 3.3). Using least-square regression in Microsoft Excel, the empirical rate model (Equation 5) was fit to these Aceq

concentration profiles to obtain the empirical rate constants *e*, *f*, *g*, and *h*. For a substrate under the same fermentation conditions, these constants are unique. Table 3.3 gives the values of *e*, *f*, *g*, and *h* for the four different conditions used in this study.

Table 3.3 CPDM parameters.

Parameter	**$CaCO_3$ buffer**		**$MgCO_3$ buffer**	
	SLP	SHOCK	SLP	SHOCK
e	0.012	0.018	0.021	0.021
f	1.63	3.1	0.232	0.293
g	4.01×10^{-5}	2.98×10^{-6}	0.059	0.021
h	2.99	2.93	0.954	1.010

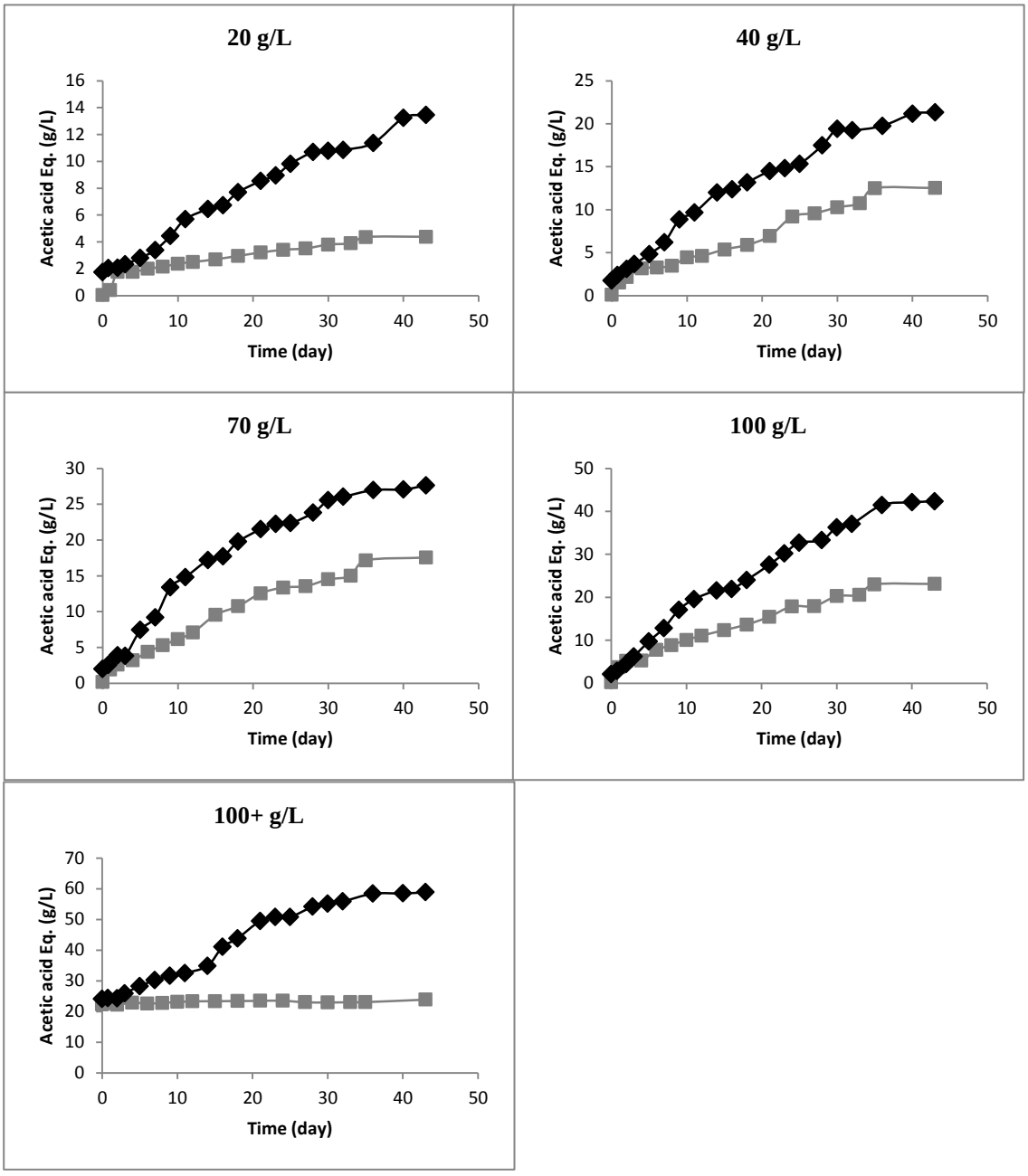

Figure 3.2 Acetic acid equivalents concentration profiles for SLP pretreated corn stover ($MgCO_3$ buffer (♦), $CaCO_3$ buffer (■)) for CPDM batch fermentations.

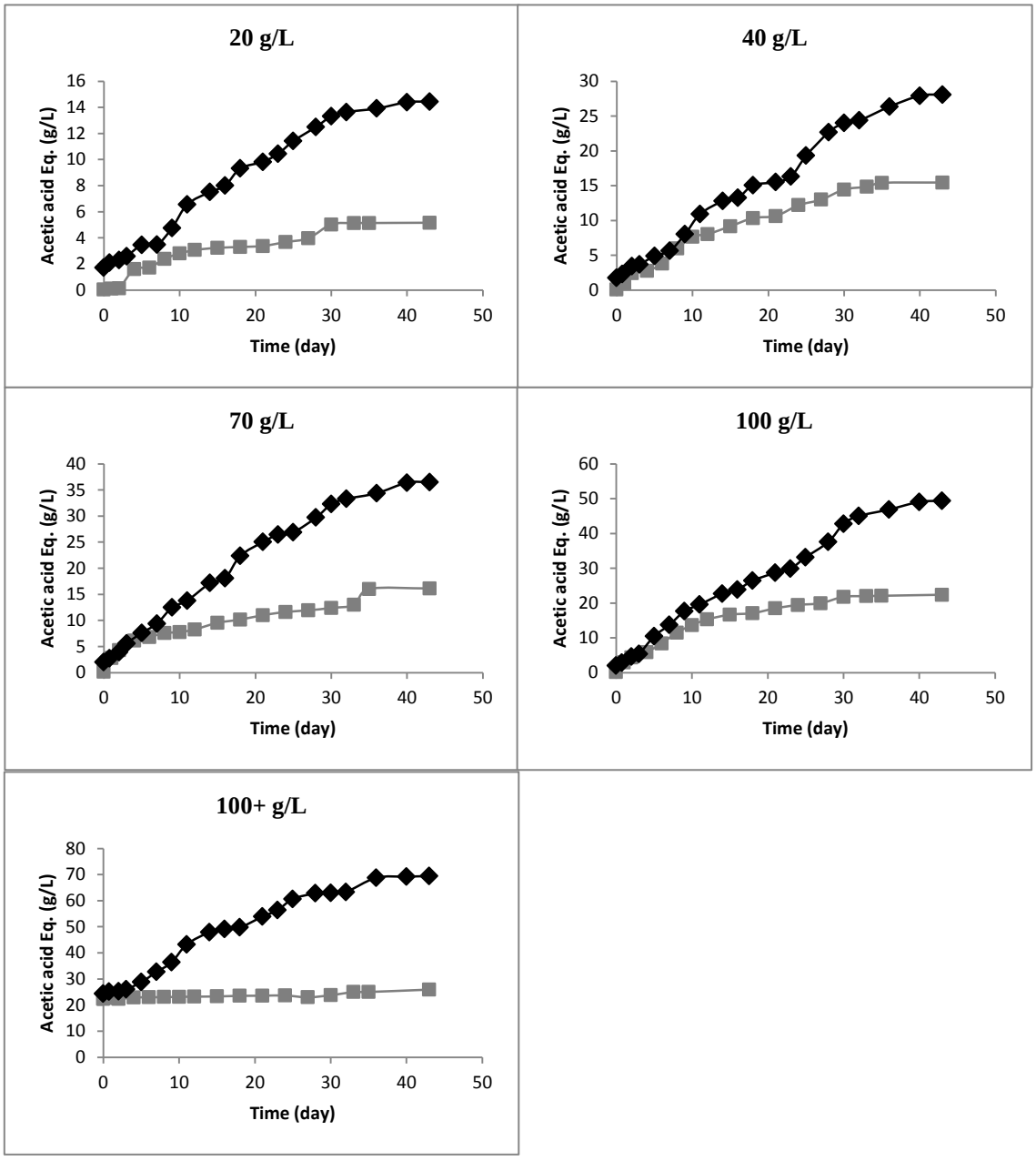

Figure 3.3 Acetic acid equivalents concentration profiles for SLP + shock pretreated corn stover ($MgCO_3$ buffer (♦), $CaCO_3$ buffer (■)) for CPDM batch fermentations.

Using MATLAB, these rate equations were used to simulate four-stage countercurrent fermentations with varying LRTs and VSLRs. Figure 3.4 shows a CPDM map of SLP + shock corn stover using both $MgCO_3$ and $CaCO_3$ buffers. For every LRT and VSLR, compared to $CaCO_3$, $MgCO_3$ buffer has a higher carboxylic acid concentration and conversion. At very high VSLR (12 g/(L·day)) and low LRT (10 day), the total carboxylic acid concentrations for $MgCO_3$ and $CaCO_3$ buffer are 26.1 and 21.3 g/L, respectively, a 22.5% increase. At low VSLR (4 g/(L·day)) and high LRT (30 day), the total carboxylic acid concentrations for $MgCO_3$ and $CaCO_3$ buffer are 50.9 and 39.7 g/L, respectively, a 28.1% increase. Using $MgCO_3$ buffer at VSLR 4 g/(L·day) and LRT 30 days, the maximum predicted carboxylic acid concentration was 50.9 g/L.

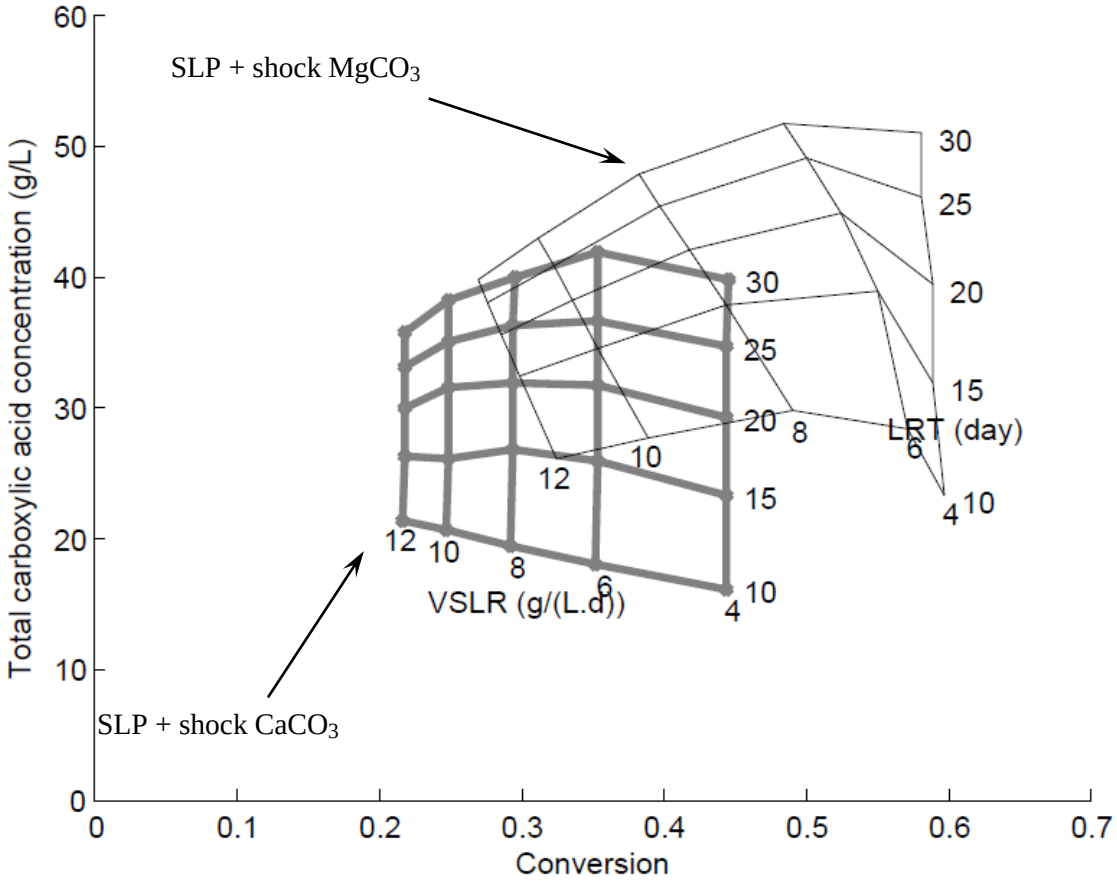

Figure 3.4 "CPDM map" for countercurrent fermentations with 100 g solids/L slurry using SLP + shock pretreated corn stover (80%) and chicken manure (20%) and $CaCO_3$ and $MgCO_3$ buffers.

Using $MgCO_3$ buffer, Figure 3.5 compares the SLP and SLP + shock corn stover and shows that adding shock pretreatment improved the performance at every VSLR and LRT. At very high VSLR (12 g/(L·day)) and low LRT (10 day), the carboxylic acid concentrations for SLP + shock and SLP corn stover are 26.1 and 20.5 g/L, respectively, a 27.1% increase. At low VSLR (4 g/(L·day)) and high LRT (30 day), the carboxylic acid concentrations for SLP + shock and SLP corn stover are 50.9 and 43.6 g/L respectively, a 16.7% increase.

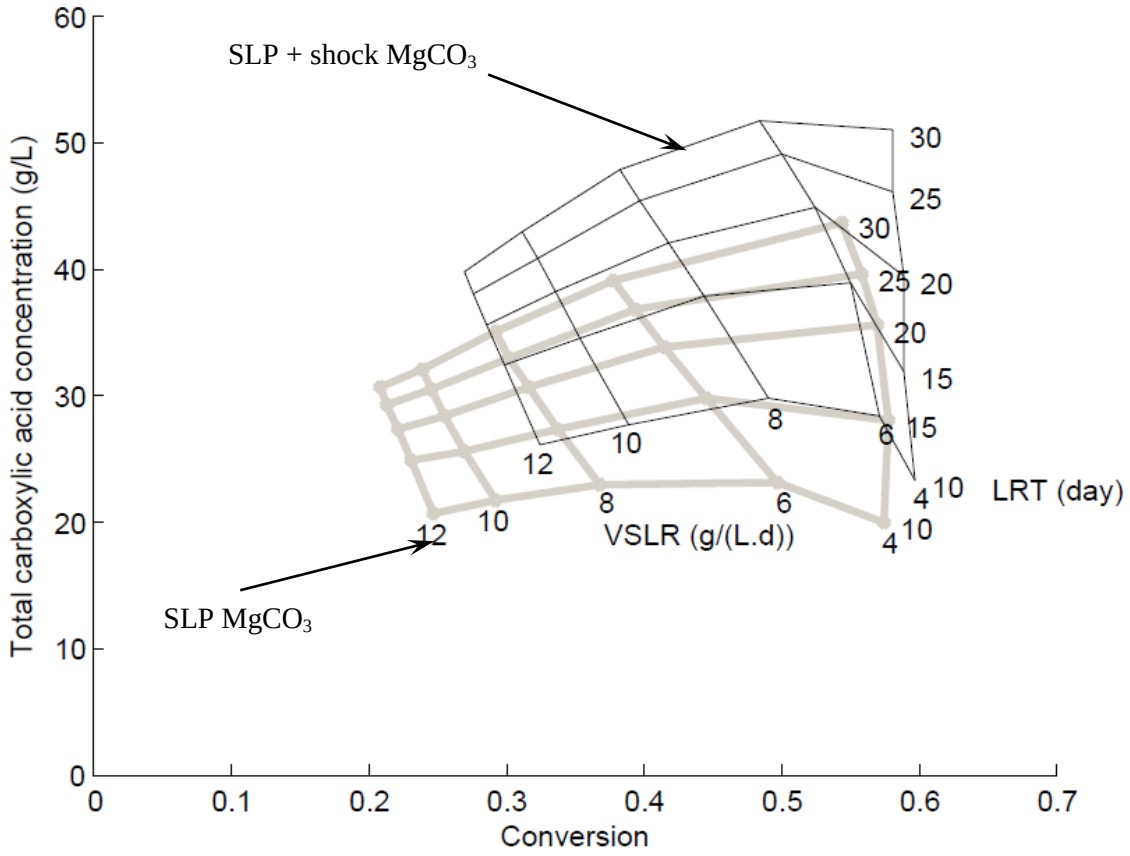

Figure 3.5 "CPDM map" for countercurrent fermentations with 100 g solids/L slurry using SLP and SLP + shock pretreated corn stover (80%) and chicken manure (20%) and $MgCO_3$ buffer.

3.4. Conclusions

$MgCO_3$ buffer controlled the pH near neutrality. Compared to traditional $CaCO_3$ buffer, $MgCO_3$ increased the carboxylic acid production, yield, conversion, and selectivity of mixed-culture fermentations. In batch fermentations with 100 g/L substrate, $MgCO_3$ increased carboxylic acid production (2.7 times for SLP and 2.6 times for SLP + shock corn stover). CPDM predicts that in a four-stage countercurrent

fermentation with high VSLR (12 g/(L·day)) and low LRT (10 day), using $MgCO_3$ buffer yields a carboxylic acid concentration of 26.1 g/L, a 22.5% increase over $CaCO_3$ buffer. Using $MgCO_3$ buffer at VSLR 4 g/(L·day) and LRT 30 days, the maximum predicted carboxylic acid concentration is 50.9 g/L. This study strongly suggests that in the MixAlco™ process $MgCO_3$ should replace $CaCO_3$ buffer. Also, at all substrate loadings in batch fermentations adding shock to lime pretreatment increased the yields. At high VSLR (12 g/(L·day)) and low LRT (10 day), CPDM predicts a carboxylic acid concentration of 26.1 g/L for SLP + shock corn stover, a 27.1% increase over SLP corn stover.

4. EFFECT OF GASEOUS COMPOSITION IN THE HEADSPACE OF MIXED-CULTURE FERMENTORS

4.1. Introduction

Anaerobic fermentations produce many different products, such as acetic, propionic, butyric, and caproic acids. They can be purified and used individually or as precursors to biofuels. These individual products are typically generated in dilute concentrations. Because the fermentation broth has high water content, a lot of energy is required to concentrate and purify the products, which increases production costs. Many factors (e.g., pH, temperature, type of feed, organic load, batch or continuous mode of operation) affect the production and concentration of these products. The effect of headspace gaseous composition is one such factor that can affect fermentation pathways. The literature indicates that headspace hydrogen and carbon dioxide concentrations can energetically suppress or favor certain pathways and alter product concentrations to favor a particular product [49, 50]; however, the effect of headspace composition on mixed-acid fermentations and the carboxylate platform is unknown.

The main objective of this study is to find the effect of hydrogen and carbon dioxide compositions in the headspace of the mixed-acid fermentations. The aim is to direct the mixed-culture fermentation toward high-molecular-weight carboxylic acids or toward one major compound. Batch fermentations of raw (no pretreatment) corn stover were performed in stainless steel fermentors with pure hydrogen, pure carbon dioxide,

mixture of hydrogen and carbon dioxide (1:1), and pure nitrogen (control) at 2.05×10^5 Pa (abs) in the headspace (Fig. 4.1).

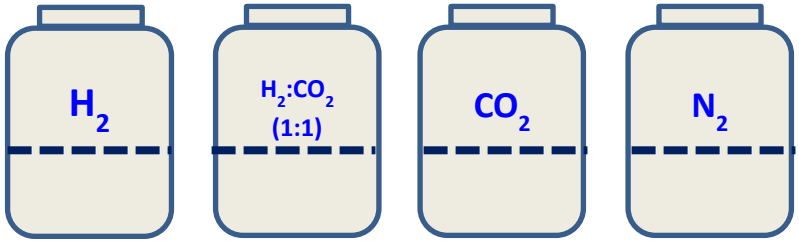

Figure 4.1 Batch fermentors with different headspace gas compositions.

4.2. Materials and methods

4.2.1. Substrates

Raw corn stover was provided by AgriLife, the agricultural college at Texas A&M University. It was harvested in 2010. The freshly harvested corn stover had a moisture content of 30%, it was then air dried in the lab to a moisture content of 10% (wet basis). This dry raw corn stover was wrapped in plastic and stored in bins in the dry chemical storage room in our laboratory.

4.2.2. Nutrients

Chicken manure (20% of the dry feed) supplied the trace nutrients and minerals source in all the fermentations. Wet chicken manure was supplied by Feather Crest Farms, Inc. It was air dried in the lab to a moisture content <10%. It was transferred to

Ziploc (re-sealable) air-tight plastic bags and stored in the dry chemical storage room of our laboratory.

4.2.3. Methanogen inhibitor

Iodoform (CHI_3) was used to inhibit methane production. Iodoform solution (50 µL, 20 g CHI_3/L 190-proof ethanol) was added to each fermentor at the beginning. Thereafter, it was only added if there was a methane peak observed in the gas chromatograph of the headspace gas sample. Because iodoform is sensitive to light, air, and temperature, the solution was kept in amber-colored glass bottles wrapped in foil and stored at –20 °C. Special care was taken to replace the cap immediately after use [51].

4.2.4. Inoculum

Fermentations were inoculated with a mixed culture of marine microorganisms obtained from beach sediment in Galveston Island, TX. A 3-ft-deep hole was dug at the waterline on the beach. Sediment was removed from the bottom of the hole and immediately placed in airtight plastic bottle filled with deoxygenated water, 0.275 g/L cysteine hydrochloride, and 0.275 g/L sodium sulfide. This was done to minimize microbial oxygen exposure. Anaerobic batch fermentation with raw corn stover were performed to allow the bacteria to adapt to this substrate and the conditions. Fermentation broth from this fermentation was used as adapted inoculum for the actual experiments. Use of adapted inoculum to particular substrates ensured uniform results every time.

4.2.5. Stainless steel fermentors

Plastic fermentors are ideal for studying biomass conversion and carboxylic acid production. The low-density plastic bottle, rubber stopper, and rubber septum are dense enough to prevent escape of carbon dioxide and methane gas and to prevent air from entering; however, they are not suitable for studying the headspace gas composition, because small molecules like hydrogen gas can pass through these barriers with ease.

Only stainless steel and glass are suitably dense to contain hydrogen gas for analysis. Of the two, stainless steel is the preferred material because glass is easily broken and the resulting shards are a safety hazard.

The 1-L stainless steel (SS) fermentors (Fig. 4.2) were made from a 6-inch length of 4-inch SCH 10 pipe with 1/8-inch thick stainless steel plate welded to either end. The top plate of the fermentor has a 2-inch hole where a quick-connect fitting is welded. A 2-inch gasket and cap are placed onto the fitting and held in place by a tightened clamp.

Figure 4.2 Stainless steel fermentor with a pressure guage.

Figure 4.3 Stainless steel fermentors being filled with desired gas and headspace pressure.

4.2.6. Fermentation headspace gas analysis

Biogas (mixture of H_2, CO_2, N_2, and CH_4) is produced as a fermentation proceeds and needs to be removed from the fermentors to relieve pressure buildup and prevent fermentor rupture. For the first two weeks, each fermentor was vented daily

because of the high initial digestion rate, and then each fermentor was vented every two days [52].

Biogas composition was measured and methane was monitored by manual injection of a 5-mL gas sample into an Agilent 7890A GC system with a thermal conductivity detector (TCD).

4.2.7. Fermentation acid analysis

Fermentation liquid samples (1 mL) were collected every day for the first three days and after that every other day for all experiments. Ultra-centrifuged (13,000 rpm, 10 min) fermentation liquid was mixed with equal parts of internal standard (1.162 g/L 4-methyl-*n*-valeric acid) and 3-M phosphoric acid (H_3PO_4), and then ultra-centrifuged again (13,000 rpm, 10 min). The H_3PO_4 ensures that the carboxylate salts are converted into carboxylic acid prior to analysis. The carboxylic acid concentration was measured using an Agilent 7890A Series Gas Chromatograph (GC) system equipped with a flame ionization detector (FID) and an Agilent 7683 automatic liquid sampler. A 30-m fused-silica capillary column (J&W Scientific Model # 123-3232) was used. The column head pressure was maintained at 2 atm (abs). After each sample injection, the GC temperature program raised the temperature from 40 to 200 °C at 20 °C/min. The temperature was subsequently held at 200 °C for 2 min. The total run time per sample was 16 minutes. Helium was the carrier gas.

4.2.8. pH control

$MgCO_3$ was used to control the pH of the fermentations near neutrality. $MgCO_3$ is highly soluble and cannot be added all at once. Instead, it was added as a solid powder every day for the first seven days and every two days thereafter to control the pH near neutrality (7.00–7.10).

4.2.9. Mixed-culture batch fermentations

Batch fermentations were performed with four different gaseous compositions in the headspace at 2.05×10^5 Pa in duplicates. The different gaseous compositions used in the headspace were pure H_2, pure CO_2, H_2:CO_2 (1:1), and pure N_2. Table 4.1 shows the experimental apparatus for batch fermentations. The total fermentation broth volume was 400 mL in the 1-L stainless steel fermentors. The substrate loading in each fermentor was 100 g solids/L slurry with 80% corn stover and 20% chicken manure. $MgCO_3$ was added every other day to maintain pH near neutrality. To inhibit methanogens, 50 µL of iodoform solution (20 g CHI_3/L ethanol) was added. Deoxygenated water was added to bring the volume to 400 mL in each fermentor and incubated at 40 °C for 35 days in a rotary incubator at 2 rpm. Every other day, all fermentors were removed from the incubator to release fermentation gases. Every other day, liquid samples were taken to be analyzed for carboxylic acid concentrations in the gas chromatograph. The respective headspace gases were filled again to 2.05×10^5 Pa (abs) before returning them to the incubator. The performance of batch fermentation was evaluated using the total carboxylic acid concentration, yield, conversion, and selectivity parameters, which are defined as follows:

$$\text{Yield} = \frac{\text{Total carboxylic acids produced (g)}}{\text{VS fed (g)}}$$

$$\text{Conversion} = \frac{\text{VS digested (g)}}{\text{VS fed (g)}}$$

$$\text{Selectivity} = \frac{\text{Total carboxylic acids produced (g)}}{\text{VS digested (g)}}$$

Table 4.1 Batch fermentations experimental set-up with different headspace gas compositions.

Headspace Gas	**H_2**	**CO_2**	**H_2:CO_2 (1:1)**	**N_2**
Solids loading (g/L)	100	100	100	100
Total corn stover (g)	35.6	35.6	35.6	35.6
Chicken manure (g)	8.5	8.5	8.5	8.5
Urea (g)	1	1	1	1
Inocula (mL)	40	40	40	40
Deoxygenated water (mL)	356	356	356	356
Iodoform (μL)	50	50	50	50
Total liquid (mL)	400	400	400	400

4.3. Results and discussion

4.3.1. Batch fermentations

Figure 4.4 shows the acid concentrations after 35 days in the fermentations different gas compositions. The fermentor with N_2 (control) in the headspace had a total acetic acid equivalent of 28.6 g/L. The fermentor with H_2 + CO_2 in the headspace had the highest total acetic acid equivalent of 36.8 g/L, which is 37% more than the N_2 fermentor.

Table 4.2 shows the percent change in different carboxylic acids (acetic, propionic, butyric, caproic, and total acetic equivalents) in the H_2, H_2 + CO_2, and CO_2 fermentors as compared to N_2 fermentor.

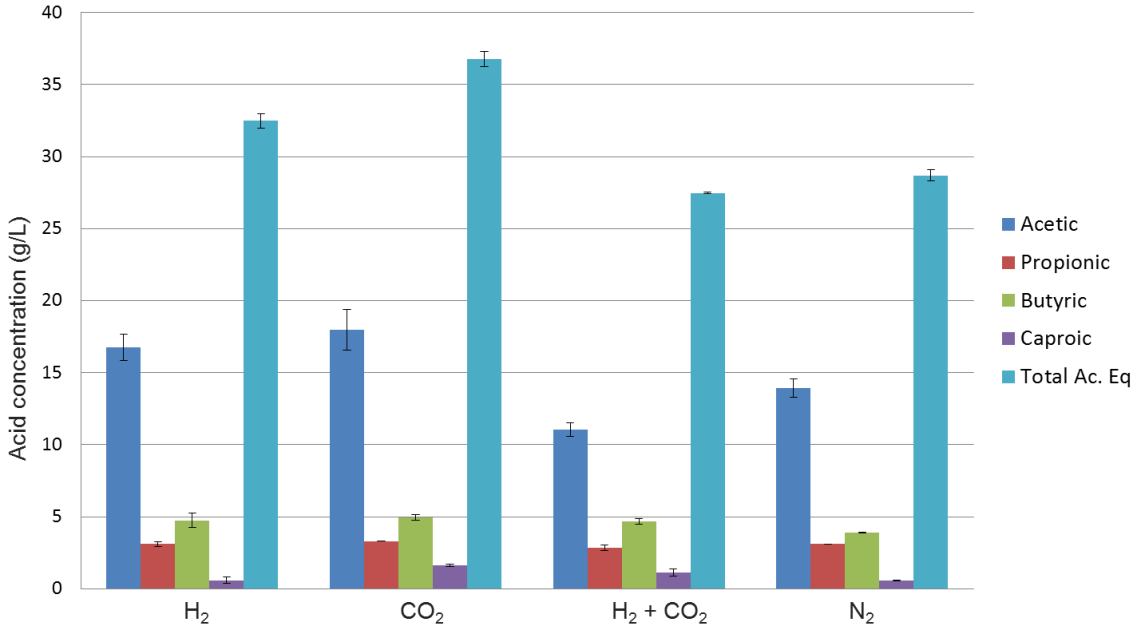

Figure 4.4 Acid data for different gas compositions.

Table 4.2 Change in acid production (% change compared to N2 fermentor).

	Acetic C2	Propionic C3	Butyric C4	Caproic C6	Total Ac. Eq.
H_2	20.3	–0.2	21.9	5.2	13.2
$H_2 + CO_2$	29.2	6.4	27.4	187.1	37.2
CO_2	–20.6	–8.4	20	98.7	–4.2

The fermentor with $H_2 + CO_2$ showed the highest total acetic acid equivalents production with 1.6 g/L of caproic (C6) acid, which is a 187% increase compared to the N_2 fermentor. The CO_2 fermentor showed a modest decrease (4%) in the total acetic equivalents produced, but had 20% higher butyric (C4) and 99% higher caproic (C6) production. Thus, adding 2.05×10^5 Pa (abs) pressure of CO_2 in the headspace shifted the carboxylic acid spectrum toward higher molecular weight acids (C4 and C6). Because CO_2 is inexpensive and readily available, it can be used to direct the fermentation toward higher molecular weight acids.

Adding 2.05×10^5 Pa (abs) of hydrogen in the headspace increased the overall acetic equivalents production by 13%, but did not change the product spectrum considerably. Table 4.3 summarizes the fermentation results.

Table 4.3 Fermentation results.

Fermentor	**H_2**	**CO_2**	**H_2:CO_2 (1:1)**	**N_2**
Total carboxylic acid conc. (g/L)	26.2	20.7	28.9	21.3
Yield (g total acids/g VS fed)	0.1932	0.1664	0.2499	0.1722
Conversion (g VS digested/g VS fed)	0.42	0.32	0.51	0.42
Selectivity (g total acids/g VS digested)	0.46	0.52	0.49	0.41

4.4. Conclusions

This study shows that the headspace gas composition of mixed-culture fermentation can affect the total acid production as well as the product distribution. Using H_2:CO_2 (1:1) at 2.05×10^5 Pa (abs) in the fermenter headspace increased the total acetic equivalents by 37%. Using CO_2 at 2.05×10^5 Pa (abs) in the fermenter headspace reduced the total acids by 4%, but shifted the acid spectrum toward higher molecular weight acids.

5. VOLATILE ANTIMICROBIALS FOR COUNTERCURRENT SACCHARIFICATION

5.1. Introduction

In the sugar platform, lignocellulosic biomass is converted into sugars via enzymatic saccharification using commercial enzyme blends. This is usually done as a batch process with reaction times of 3–7 days. At the end of a batch enzymatic saccharification, some enzyme activity remains, but leftover enzymes are usually discarded. Countercurrent systems are generally more efficient in chemical processes and are widely used in liquid-liquid extraction, heat exchange, and fermentations [53, 54]. It also has great potential to improve enzymatic saccharification by more fully utilizing enzymes resulting in higher sugar yields than batch saccharification.

Using α-cellulose, Zentay [55] reported that to achieve a given glucan conversion, countercurrent saccharification reduces enzyme loadings by 8 to 20.5 times compared to batch saccharification. At an enzyme loading of 10 FPU/g cellulose (~20 mg enzyme/g cellulose), enzymes cost contribute $0.68 to $1.47/gal ethanol, when the cost of cellulose enzyme is $10/kg [56]. More efficient use of enzymes using countercurrent saccharification has the potential to significantly reduce the cost of biofuels.

A major challenge in using countercurrent saccharification to produce sugars is maintaining a sterile environment. Countercurrent saccharification is a continuous

process with liquid residence times ranging from a few weeks to months. At laboratory scale, a potent mixture of antimicrobials (cycloheximide and tetracycline) is used to prevent the growth of microbes and maintain sterility. These antimicrobials are expensive and toxic for human or animal use. They are non-volatile and cannot be recovered after the process. This makes the sugars produced unfit for yeast, animal, or human use; hence, there is a need to find alternative volatile antimicrobials that can be used safely at commercial scale. Volatile antimicrobials can be recovered and re-used during the sugar concentration step following countercurrent saccharification, thereby reducing costs and making the process safe (Figure 5.1).

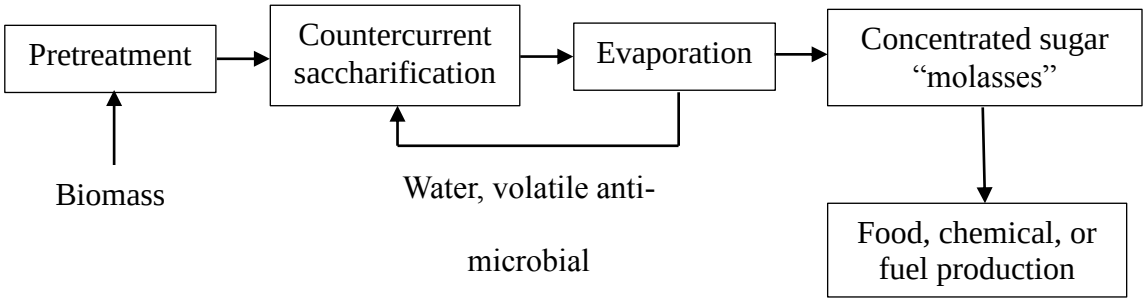

Figure 5.1 Production of food, chemicals, and fuels from lignocellulosic sugars.

Chloroform has been shown to be an effective volatile antimicrobial for use in countercurrent saccharification [55]. It is easily available and is commonly used to maintain sterility in microbiology labs. At commercial scale, the drawback of using chloroform is that it substantially inhibits enzyme activity at higher concentrations (>1%). It is also toxic to human beings and can cause death due to respiratory and cardiac arrhythmia with EEGL (emergency exposure guidance level) of 100 ppm (for 1 hour) [57].

Various volatile plant oils have been shown to be effective antimicrobials against a wide spectrum of gram-negative and gram-positive microorganisms [58]. These could be used as volatile antimicrobials in countercurrent saccharification. At industrial scale, the parent plant could be added directly to the biomass feed instead of using refined volatile oils thereby reducing costs. Thyme oil and oregano oil have the widest spectrum antimicrobial activity [58] and hence were selected in this study.

The purpose of this study is to test thyme oil and oregano oil as volatile antimicrobials for use in countercurrent enzymatic saccharification. Batch enzymatic saccharification studies were performed with lime-pretreated corn stover with CTec2 enzyme and thyme oil, oregano oil, and chloroform as volatile antimicrobials. These were compared with the standard antimicrobial cocktail of cycloheximide and tetracycline. An active inoculum of marine mixed-culture microorganisms was introduced in all batch reactors to further challenge these antibiotics and test if they can withstand external contamination.

5.2. Materials and methods

5.2.1. Substrate

Submerged lime pretreated (SLP) corn stover was used as the substrate. For the detailed SLP procedure refer to Appendix B.

5.2.2. Citrate buffer

Citrate buffer was used to maintain an optimum pH (~4.8) for highest cellulase enzyme activity. 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 was 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 was diluted to 0.1 M and pH was adjusted again to 4.8 using NaOH and hydrochloric acid (HCl).

5.2.3. Standard antibiotics

The standard antibiotic cocktail (non-volatile) recommended by NREL was used as a control to test volatile antibiotics. 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 N). Relevant safety information is also provided in Appendix N.

5.2.4. Volatile antibiotics

Chloroform, thyme oil and oregano oil were tested as volatile antibiotics. Chloroform was obtained from Fisher Scientific (Acros Organics, 99+% pure). Thyme oil and oregano oil were obtained from NOW Foods (Codes 7635 and 7577, respectively).

5.2.5. 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. Using the Pierce BCA assay, the protein content for CTec2 was 294 mg/mL. The enzyme loading was 5 mg protein/g dry biomass. 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 O). This solution was prepared fresh for each experiment.

Please note that Novozyme CTec3 contains its own antimicrobials whereas CTec2 does not; therefore, CTec2 was selected for this study.

5.2.6. Incubator

Optimal performance of CTec2 occurs at temperatures of 45–50°C. In this study, a standing incubator cabinet was utilized with temperature setting 50°C. The incubator had roller bottles with an axial rotation of 2 rpm, which ensured good mixing.

5.2.7. Batch reactor

Batch saccharifications were performed in 1-L plastic centrifuge bottles (Thermo Fisher Schientific, catalog# 05-562-25). The total volume of the reaction mixture was 200 mL.

5.2.8. Experimental design

Batch enzymatic hydrolysis were performed with SLP corn stover as the substrate, Novozyme CTec2, and five different antibiotic conditions including one that had no antibiotics (Table 5.1). All batch hydrolyses were performed in duplicates. Initial experiments showed that sometimes even the batch reactor with no antibiotics did not show any growth of microorganisms and the sugar levels remained constant after 3 weeks. This could have been possible because of the relatively clean atmosphere of the laboratory setting. Some reactors never got contaminated and maintained sterility. Hence an external inoculum of live microorganisms was introduced in all reactors to ensure there was contamination and test the antibiotics further. An active mixed culture of microorganisms from a marine environment was used as the inoculum. On Days 5, 10, and 15, 2 mL of marine mixed-culture inoculum was added and sugar concentrations were tested using HPLC. The batch saccharifications lasted for 20 days. The final concentration of chloroform, thyme oil, and oregano oil in the batch reactor was 0.2% (volume basis).

Table 5.1 Experimental set-up of batch hydrolysis**.**

Antibiotic	**Wet biomass (g)**	**0.1 M Citrate buffer (mL)**	**Dilute enzyme (1:10) (mL)**	**Antibiotic (mL)**	**DI water (mL)**	**Total (mL)**
Chloroform	5.51	100.00	1.53	0.40	92.56	200.00
Oregano	5.51	100.00	1.53	0.40	92.56	200.00
Thyme	5.51	100.00	1.53	0.40	92.56	200.00
Std. (Tetracycline/ cylcoheximide)	5.51	100.00	1.53	2.80	90.16	200.00
No antibiotic	5.51	100.00	1.53	0.00	92.96	200.00

5.3. Results and discussions

Table 5.2 shows the sugar concentrations of all the batch reactors on Days 0, 5, and 20. On Days 0 and 5, all the batch reactors have similar sugar concentrations, including the batch reactors with no antibiotics. On Day 20, all the reactors with antibiotics have total sugars in the range 17−19 g/L. In contrast, the reactors without antibiotics have total sugar concentrations ~8 g/L. These results demonstrate that all reactors with antibiotics (chloroform, thyme oil, oregano oil, and the standard antibiotic cocktail) could stop the growth of added microorganisms. In contrast, the reactors without antibiotics had microbial growth, which is evident from the low sugar concentrations. Chloroform and the essential plant oils were as effective against added microorganisms as the standard antibiotic cocktail of tetracycline and cycloheximide. These volatile plant oils can be used as antimicrobials for enzymatic saccharifications with long residence times, such as countercurrent saccharification.

Table 5.2 Effect of volatile antibiotics in batch saccharifications.

	Day 0			Day 5			Day 20		
Antibiotic	Glucose (g/L)	Xylose (g/L)	**Total**	Glucose (g/L)	Xylose (g/L)	**Total**	Glucose (g/L)	Xylose (g/L)	**Total**
Chloroform 0.2%	0	0.125	0.125	13.647	5.051	18.698	13.380	4.938	18.318
	0	0.161	0.161	13.908	5.346	19.254	13.624	5.150	18.774
Oregano essential oil 0.2%	0	0.067	0.067	13.692	5.176	18.868	13.482	5.163	18.645
	0	0.25	0.25	13.757	5.318	19.075	13.546	5.140	18.687
Thyme essential oil 0.2%	0	0.245	0.245	13.348	5.015	18.362	13.120	4.834	17.954
	0	0.119	0.119	13.115	4.929	18.043	12.842	4.807	17.649
Std. (Tetracycline/cylcoheximide)	0	0.161	0.161	12.893	4.839	17.732	12.691	4.744	17.435
	0	0.01	0.01	13.068	4.850	17.919	12.892	4.768	17.659
No antimicrobial	0	0.191	0.191	13.240	5.201	18.441	7.035	1.087	8.122
	0	0.033	0.033	12.630	4.975	17.605	6.368	0.837	7.205

5.4. Conclusions

Volatile plant oils from thyme and oregano can act as antimicrobial agents in enzymatic saccharifications. Very low concentrations (0.2%) are required to inhibit microbial growth. At industrial scale, these recoverable volatile antibiotics can lower operating costs and eliminate toxic antibiotics. In the case of essential oils, the parent plant could be directly added in the biomass feed in the required quantity such that the final concentration of volatile oils in the slurry is 0.2%. After the volatile oils are recovered in the sugar concentration step, even smaller make-up quantity would be needed in continuous operation.

6. CONTINUUM PARTICLE DISTRIBUTION MODEL

6.1. Introduction

This chapter will briefly describe the Continuum Particle Distribution Model (CPDM) developed by Loescher [36] and will serve as a step-by-step guide to use CPDM to predict the performance of a four stage countercurrent fermentation.

Countercurrent fermentations are very time-consuming and take months to reach a steady-state acid concentration. Furthermore, thousands of man-hours are needed to investigate the performance of a countercurrent fermentation train with one VSLR and LRT. From a set of batch data, CPDM can simulate the performance of complex systems like countercurrent fermentations with reasonable accuracy (within 10%) [24, 38, 46, 59, 60].

A continuum particle is defined as one gram of solids in the initial unreacted state. For this study, a continuum particle is more precisely defined as one gram of non-acid volatile solids (NAVS), because the initial acid present is not a reactant.

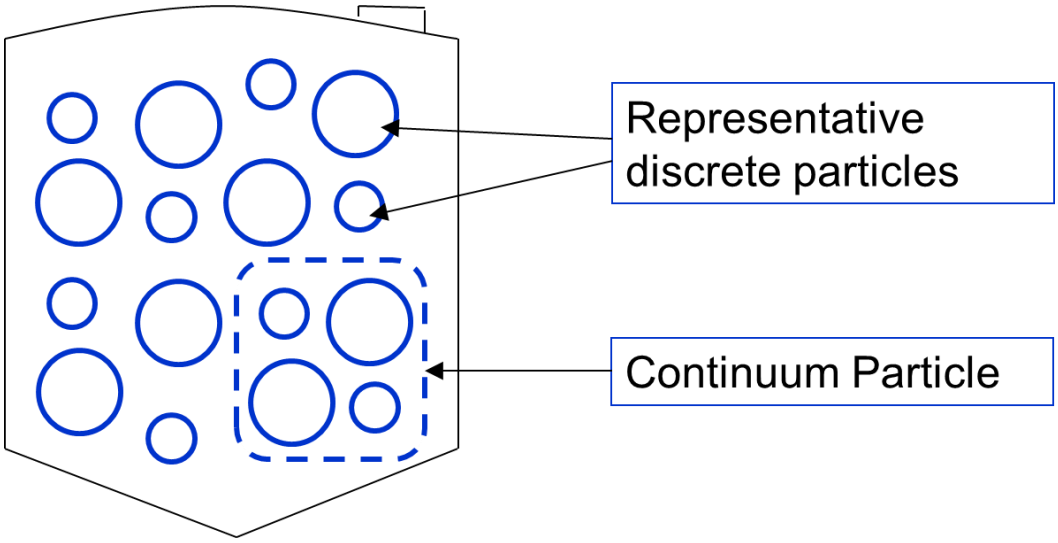

Figure 6.1 Illustration of a "continuum particle."

Other kinetic models, such as the Residence Time Distribution (RTD), use time-parameterized distribution functions. In contrast, CPDM uses a conversion-parameterized distribution function of these continuum particles. The overall rate equation is the product of the specific rate and the conversion-parameterized distribution function.

The number of particles with conversion between x_1 and x_2 is given by:

$$n_{x_1,x_2} = \int_{x_1}^{x_2} \hat{n}(x)dx$$

The initial particle concentration is the sum of all reacting particles and is given by:

$$n_0 = \int_0^1 \hat{n}(x)dx$$

The overall reaction rate (r) with the specific rate ($\hat{r}$) is given by:

$$r = \int_0^1 \hat{r}(x,A)\hat{n}(x)dx$$

6.2. Simulating a four stage countercurrent fermentation

6.2.1. Adaptation

Before performing batch fermentations, the first step is to adapt the mixed microbial culture. It is very important to perform a batch fermentation for about 3−4 weeks with the exact same conditions (such as substrate, nutrients, pH, and temperature) that will be used in the CPDM batch fermentations and subsequently in the countercurrent fermentation. Inoculum from this batch fermentation should be used as "adapted inoculum" to inoculate the CPDM batch fermentations with different substrate loadings.

6.2.2. Batch fermentations with five different substrate loadings

Batch fermentations consists of five different substrate loadings: 20, 40, 70, 100, and 100+ g dry substrate/L liquid. The 100+ fermentor had the same substrate loading as the 100 g/L, but an additional 20 g carboxylic acids/L (16 g/L acetic acid, 1 g/L propionic acid, and 3 g/L butyric acid) were added to capture any inhibitory effects of initially present product. The carboxylic acid concentration is monitored every day for 4

weeks. The carboxylic acid concentration is converted into acetic acid equivalents (Aceq).

6.2.3. Fit rate equations to batch data

$$\text{Aceq} = a + \frac{bt}{1+ct}$$

For each substrate loading, Equation 4 is fit to the Aceq (*t*) data individually (20, 40, 70, 100, and 100+) and parameters *a*, *b*, and *c* are determined by least-square regression in Microsoft Excel. To determine *a*, *b*, and *c*, the residual defined by the following equation is minimized.

$$\text{Residual} = \sum (\text{Aceq}_{\text{exp.}} - \text{Aceq}_{\text{calc.}})^2$$

The reaction rate for that substrate loading (Equation 6) is then calculated by differentiating Equation 4. The reaction rate is divided by the initial substrate loading to get the specific reaction rate (Equation 7).

$$\text{rate} = r = \frac{d(\text{Aceq})}{dt} = \frac{b}{(1+ct)^2}$$

$$\text{specific rate} = \hat{r} = \frac{r}{S_0}$$

$$\text{conversion} = x(t) = \frac{\text{Aceq}(t) - \text{Aceq}(t=0)}{S_0 \sigma}$$

where σ is the selectivity (g Aceq produced/g VS digested).

The biomass conversion (x) is calculated for each substrate loading using Equation 8. Selectivity is assumed constant for a particular substrate and should be determined from a countercurrent experiment.

Once we have the specific rate and conversion for each substrate loading, the next step is to fit the governing rate equation (Equation 9) to all the substrate loadings using least square regression in Microsoft Excel.

$$\hat{r}_{pred} = \frac{e(1-x)^f}{1 + g(\phi \cdot Aceq)^h}$$

where x = conversion

e, f, g, and h = empirical constants

ϕ = the ratio of total grams of carboxylic acid to total grams of Aceq

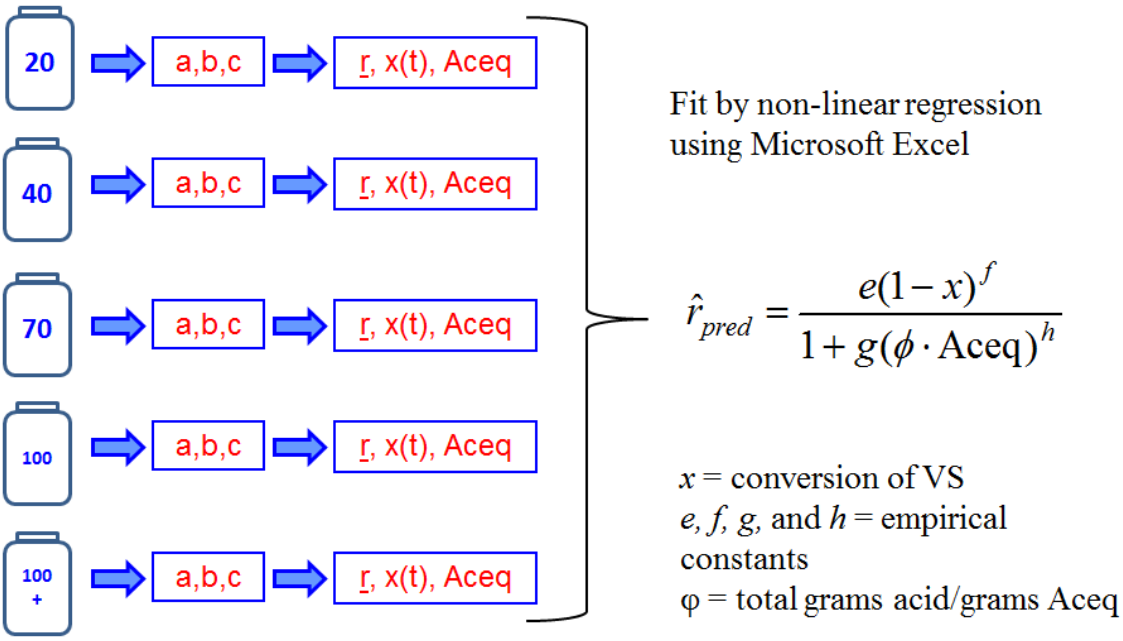

Figure 6.2 Governing CPDM rate equation from batch data.

The residual minimized (Equation 10) to determine *e*, *f*, *g*, and *h* is the summation of difference between specific rate (Equation 7) and the predicted specific rate using the governing equation (Equation 9) of all substrate loadings at once.

$$\text{Residual} = \sum (\hat{r} - \hat{r}_{pred})^2$$

Once the parameters *e*, *f*, *g*, and *h* are determined from batch fermentation data, the MATLAB code is run for the four-stage countercurrent fermentation (Appendix P). The other system specific parameters that will be needed are total volume of the liquid in

fermenters, holdup (ratio of liquids to solids in the wet cake), and moisture (in the solid feed).

7. CONCLUSIONS AND FUTURE WORK

7.1. Conclusions

The main goal of this research was to investigate the efficacy shock pretreatment and to improve the performance of the mixed-culture fermentations by altering the buffer and headspace gas composition.

To this end, adding shock pretreatment has proved beneficial in enzymatic saccharification, mixed-culture batch fermentations, mixed-culture countercurrent fermentations, and in CPDM predictions with corn stover.

Enzymatic saccharification of shock pretreated corn stover showed a modest benefit in terms of overall digestibility (glucan + xylan). At an enzyme loading (CTec2) of 48 mg protein/g glucan, SLP + shock pretreated corn stover had an overall digestibility of 81.9% as compared to 80.7% in SLP pretreated corn stover, whereas OLP + shock pretreated corn stover had an overall digestibility of 82.9% as compared to 80.7% in OLP corn stover. In terms of absolute percentage increase (2.2%), the benefit in digestibility seems modest; however, to achieve a given conversion, the enzyme dosage is reduced substantially. This is important to industry because enzyme costs contribute between \$0.68−1.47/gal of ethanol, depending on the scale of operation for ethanol produced via sugar platform [56].

In the carboxylate platform (mixed-culture fermentations), shock pretreatment exhibited a far greater benefit both in terms of total carboxylic acid concentration and

biomass conversions. This might result because a mixed-culture of microorganisms can use all biomass components (e.g., cellulose, hemicellulose, and proteins), whereas enzymes can only utilize cellulose. In batch fermentations at 100 g/L substrate concentration with $MgCO_3$ buffer, adding shock pretreatment increased the total carboxylic acid concentration to 34.5 g/L, a 13.5 % increase over lime-only pretreatment (30.4 g/L). The conversion (g VS digested/g VS fed) increased to 0.58 from 0.54 for lime-only pretreatment.

In the countercurrent fermentation experiment (VSLR of 1.875 g/(L·day) and LRT of 16 days) which lasted for 112 days, OLP + shock corn stover had a steady-state carboxylic acid concentration of 16.3 g/L, 14.8% higher than OLP corn stover (14.2 g/L). The experimental results matched closely with the CPDM model predictions for acid concentrations with an error of 3.38% for OLP corn stover train and 4.05% for OLP + shock corn stover train. CPDM predicts that at high VSLR rate of 12 g/(L·day) and LRT 30 days, adding shock pretreatment to lime pretreatment (SLP) increased the total carboxylic acid yields by 28.5%.

Short residence times (30 to 90 s) and small vessels ensure that shock pretreatment is economically feasible. Total cost of shock pretreatment is estimated to be $5/tonne [22], which is very small compared to conventional chemical pretreatments (~$45/tonne) [43].

The effect of magnesium carbonate as a buffer for mixed-culture fermentations in the MixAlco™ process was also evaluated. $MgCO_3$ buffer controlled the pH near

neutrality. Compared to traditional $CaCO_3$ buffer, $MgCO_3$ increased the carboxylic acid production, yield, conversion, and selectivity of mixed-culture fermentations. In batch fermentations with 100 g/L substrate, $MgCO_3$ increased carboxylic acid production (2.7 times for SLP and 2.6 times for SLP + shock corn stover). This suggests that magnesium carbonate should replace calcium carbonate as the buffer in mixed-culture fermentations.

The headspace gas fermentations study shows that using H_2:CO_2 (1:1) at 2.05×10^5 Pa (abs) in the fermenter headspace can increase total acetic equivalents produced by up to 37%. Using CO_2 at 2.05×10^5 Pa (abs) in the fermenter headspace reduced the total acids by 4%, but shifted the acid spectrum toward higher-molecular-weight acids. Because CO_2 is easily available in a plant, it can be used for this purpose without significant additional costs.

The effect of hydrogen and carbon dioxide compositions in the headspace of the mixed-acid fermentations is also studied for its impact on the total carboxylic acid production, conversions, and yields.

7.2. Future work

In this study, shock pretreatment with the shotgun shell at 2-L scale showed a great benefit in mixed-culture fermentations as well as enzymatic saccharification. However, at an industrial scale, shock pretreatment would be performed via gaseous detonation such as hydrogen and oxygen. Mixed-culture fermentation studies need to be performed on biomass shock pretreated with these techniques. Also, shock pretreatment needs to be studied on other lignocellulosic substrates to see if it has a similar effect.

Lignocellulosic biomass with lower lignin content might show a greater benefit from shock pretreatment. In that case, it may be possible to avoid expensive chemical pretreatments altogether.

Headspace gas fermentations with H_2:CO_2 (1:1) and pure CO_2 show great promise by improving the yields and product spectrum of carboxylic acids produced in mixed-culture fermentations, but detailed economic analysis needs to done. Also, mixed-culture fermentations with syngas (H_2:CO) needs to be included in this study.

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APPENDIX A

OXIDATIVE LIME PRETREATMENT

1. Introduction

The purpose of this procedure is to describe the oxidative lime pretreatment (OLP) for lignocellulosic biomass. Lime pretreatment exposes a mixture of lignocellulosic biomass, calcium hydroxide, and water to different conditions of temperature and pressure for a desired reaction time. Oxidative lime treatment refers to the addition of an oxygen source, which further improves performance (Kim & Holtzapple, 2005). Lime pretreatment has proven to selectively reduce the lignin content of lignocellulosic biomass and remove acetyl groups, while maintaining high carbohydrate yields (Sierra et al., 2009).

2. Procedure

OLP in 8-L Parr reactor

1. Fill reactor with 310 g biomass, 155 g calcium hydroxide, and 4,650 mL DI H_2O.
2. Mix reactor contents with a flat spatula until uniformly wet.
3. Using two half-round plates and ten bolts, close and secure reactor.
4. Connect reactor to O_2 line, Magdrive supply and return water lines, and internal coil supply and return cooling water lines. Place thermocouple in thermowell.

5. Turn on reactor controller display.
6. Supply at least 0.5 L/min water through Magdrive supply line.
7. Turn on stirring, set to 20% maximum velocity.
8. Set temperature controller to desired set point; turn on heating.
9. Once reactor reaches set point temperature, open O_2 cylinder and set pressure regulator to 100 psi. This is the initiation of the reaction time. See Table 1 for suggested reaction times.
10. When the reaction time has elapsed, close the O_2 cylinder, turn off heating power, and begin cooling water flow through the reactor coil.
11. Once reactor has cooled, slowly open gas relief valve to depressurize the reactor. Turn off Magdrive supply water and stirring.
12. Remove half-round plates and disconnect reactor from O_2 line, water, and cooling water lines.
13. Quantitatively transfer biomass from the internal coil. Using a two-person crew, empty reactor contents into two or more gallon-sized buckets. To effectively remove all biomass, rinse reactor with DI H_2O.
14. Follow the post-pretreatment conditioning procedure below.

Table A1: Summary of suggested biomass treatment conditions

Biomass	Lignin (%)	Lime Loading (g $Ca(OH)_2$/ g biomass)	Time	Temp (^oC)	O_2 Pressure (bar)
Pine[a]	34.1	not reported	2 h	140	20.7
Poplar[b]	29.3	0.23	2 h	160	13.8
Sugarcane bagasse[c]	23.7	not reported	2 h	130	6.9
Sorghum[a]	22.0	not reported	2 h	180	6.9
Switchgrass[d]	21.4	0.3	4 h	120	6.9
Corn stover[a]	20.9	not reported	4 h	110	6.9
Corn stover[e]	20.9	0.07	4 week	55	0.21

a (Sierra, 2010); b (Sierra et al., 2009); c (Meysing, 2011); d (Falls et al., 2011); e (Kim & Holtzapple, 2005)

Post-Pretreatment Conditioning

1. Using vacuum filtration or 140-mesh sieve, remove water and soluble components from biomass.
2. Place biomass in one or more 4-L plastic buckets. To each bucket, add 4-L DI H_2O, and stir.
3. Slowly add 5-N hydrochloric acid to each bucket until pH reaches 4. Monitor pH closely, allowing ~30 min for equilibration after acid addition.
4. Using a 140-mesh sieve, remove water from biomass.
5. To each bucket, add 4-L DI H_2O and stir for a minimum of 5 min.
6. Repeat Steps 4–5 an additional two times.
7. Using a 140-mesh sieve, remove water from biomass. NOTE: Pressing biomass with a spatula to push excess water through the screen facilitates drying.

8. Place biomass in a labeled container to air dry. Biomass should be at a depth no greater than 2 cm; stir at least once every 24 h to ensure proper drying.

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APPENDIX B

SUBMERGED LIME PRETREATMENT

This procedure describes the submerged lime pretreatment (SLP) in detail. Approximately 2.5 kg dry weight of biomass was mixed with weighed calcium hydroxide (0.1 g $Ca(OH)_2$/g dry biomass) and placed in a cylindrical jacketed steel vessel (volume = 65.3 L). The vessel was then filled with distilled water until it reaches a concentration ~ 0.05 kg dry biomass/kg water. A 6-inch free-board was left to avoid spills. A heat exchanger circulated hot water through the jacket and maintained the biomass treatment system at a constant temperature of ~50 °C (Figure B1). Air was scrubbed through a lime slurry container and then bubbled through the pile via an air scrubber in the bottom of the vessel.

1. Mix the raw biomass (e.g., 2.5 kg) with excess lime (0.1g $Ca(OH)_2$/g dry biomass). Mix well to ensure a complete contact between the lime and the biomass.
2. Fill the steel vessel with the lime/biomass mixture. Add distilled water to the vessel until it reaches a concentration ~ 0.05 kg dry biomass/kg water.
3. Fill the heat exchanger with water and start the circulation pump.
4. Set the temperature controller to 49 °C.
5. Adjust the air valve connected to the diffusers until the air gently bubbles up through the mixture.
6. Add more water to the heat exchanger every day so it does not evaporate dry.
7. Add more water to the vessel and keep the 6 inch free-board.

8. Check the system daily for leaks and monitor the circulation pump to ensure it retains prime.

9. Monitor the pH of the lime slurry to ensure basic conditions are maintained (e.g., desired pH > 9).

10. Maintain conditions for 24–28 days. At the end of the time period, turn off the temperature controller, the circulation pump and the air valve.

11. Add HCl, 5 N (~1.2 L) to reach pH ~ 4–5.

12. Remove the biomass slurry from the vessel and allow to cool to room temperature.

13. Centrifuge the biomass slurry and dispose the liquid.

14. Add distilled water to the biomass to be washed and repeat Step 13 three times.

15. Spread the mixture onto aluminum foil and allow to air dry (5–7 days). Store the dried biomass in a labeled container.

16. Clean the interior of the steel vessel and flush with distilled water.

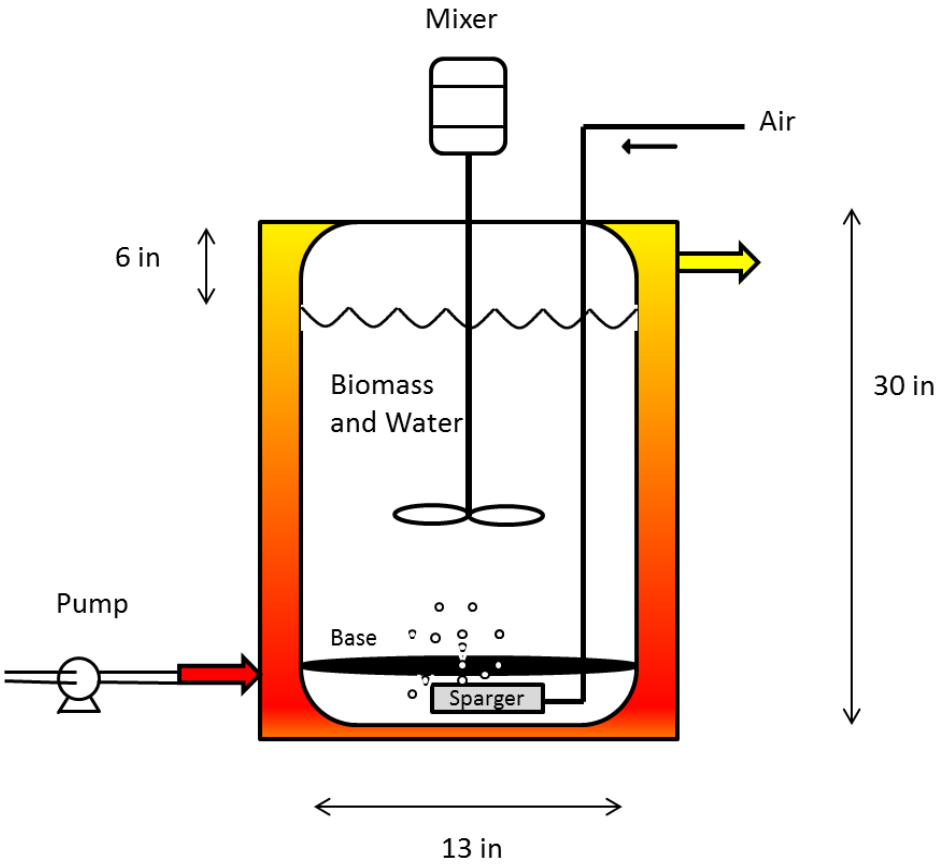


Figure B.1. Schematic process flow diagram of pretreatment apparatus.

APPENDIX C

SHOCK PRETREATMENT

1. Introduction

This procedure describes a mechanical pretreatment process, which uses a shockwave, or rapid pressurization, to render lignocellulosic biomass more amenable to biological and enzymatic digestion.

SCOPE:

- This procedure is valid when the shockwave is generated by means of a propellant cartridge containing a mixture of the following: high explosive solid primer, low explosive solid propellant, and lead or steel payload.
- Effectiveness has been demonstrated for sugarcane bagasse, corn stover, poplar wood, sorghum, and switchgrass.
- Shock pretreatment is most effective when couple with a chemical pretreatment, such as oxidative lime.

SAFETY PROCEDURES AND PRECAUTIONS

This process involves intentionally igniting an explosive charge inside of a closed and sealed vessel and is extremely dangerous! The following safety measures are mandatory:

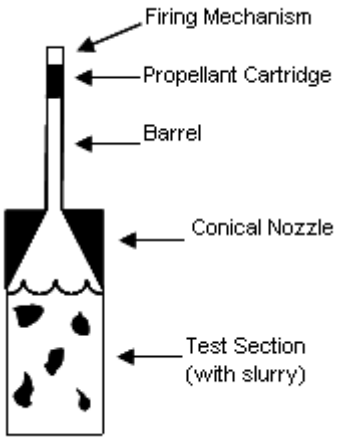
- There exist no relief device (such as a rupture disk or relief valve) capable of preventing a catastrophic vessel failure, thus *the shock tube must always be operated remotely*!
- A 'bunker' or protective blast shield must be used to contain any catastrophic pressure vessel failure and must be strong enough to contain the direct impact of any impending projectiles
- The propellant cartridge must be triggered by purely electrical or electro-mechanical means to facilitate automation and minimize human error.
- Only one person may handle explosive materials, *which must remain in their immediate control at all times*.
- Only one person may occupy the bunker while loading the propellant cartridge, all other personnel must be accounted for prior to loading and retreat to a safe location.
- The pilot plant gate must be closed to prevent any unauthorized entry during testing.
- In the event of a misfire, only one person is permitted to approach a 'hot' or 'live' cartridge to assess the cause of the ignition systems malfunction.
- The ignition system must be intentionally and manually disarmed/unplugged prior to loading the propellant cartridge into the shock tube, and immediately after ignition.
- Governing authorities (specifically TEES) must be aware of and have granted permission to control and possess firearm related 'contraband'.

- The propellant cartridges and any other flammable/reactive/or otherwise hazardous materials must be properly stored before and after use.
- Safety glasses MUST be worn at all times.
- All applicable chemical handling and laboratory safety procedures must be adhered.

TERMINOLOGY

Barrel – A piece of 1-in schedule 160 pipe used to ignite the pressure generated by the propellant and transmit it to the nozzle.

Biomass – Material that was, or is, a part of a living organism. For renewable energy applications, the definition is limited to those materials that are plant-derived, such as agricultural residues (e.g., wheat straw, corn stover), by-products of industrial processes (e.g., sawdust, sugar cane bagasse, pulp residues, distillers grains), or dedicated energy crops (e.g., switchgrass, sorghum, Miscanthus, short-rotation woody crops).



Firing Mechanism – An electronic AC current solenoid that pulls the spring loaded firing pin to ignite the primer (high explosive) upon receipt of the signal from the trigger in the control room.

Ignition System – The assembly that composes the firing mechanism, trigger button, and relay switch used to control the release of power to the AC solenoid on the firing mechanism and ultimately ignite the propellant cartridge.

Pretreated biomass – Biomass that has been mechanically, chemically, or thermally altered, possibly changing the structural composition.

Propellant Cartridge – A cartridge containing a mixture of the following: high explosive solid primer, low explosive solid propellant, and lead or steel payload.

Shock tube (ST) – An effective mechanical pretreatment method for lignocellulosic biomass that uses a shock wave to disrupt the lignocellulosic structure.

Slurry – Combined liquid and solid material resulting from biomass pretreatment.

Test Section – A piece of 4-in schedule 80 pipe which contains the slurry of biomass being used for the respective test.

Total solids ($\%TS_{105}$) – The amount of solids remaining in a sample after heating at 105 ºC to constant weight.

EQUIPMENT

- Personal Protective Equipment:
    - Safety glasses
    - Latex gloves
- Plastic bags
- Paper towels
- Nalgene bottles
- Marking pen
- Gaskets for 4-in pipe
- Electro-mechanical firing mechanism with trigger button
- 120-VAC power
- Air compressor
- Impact wrench
- Electric hoist in bunker
- Optional:
    - DAQ system
    - Pressure transducers

REAGENTS AND MATERIALS

- Prepared biomass samples
- Propellant cartridges
- Water (from local tap)

PRIOR TO ANALYSIS

- Moisture content should be less than or equal to 10%.
- Compute solids required given desired solids concentration and moisture content.
- Weigh biomass and store in a hermetically sealed bag.

2. Procedure

i. SAMPLE PREPARATION

1. Compute the moisture content of the biomass and preserve it in a sealed plastic bag prior to weighing the biomass for a particular run.
2. Using the moisture content, compute the amount of air-dried biomass required to meet the volume and solids concentration parameters for a specific run.
3. Weigh the calculated amount of biomass and store inside a freezer bag.
4. Measure, record and add enough water to thoroughly soak the biomass, without any excess.

ii. *SHOCK TREATMENT:* All personnel should have on safety glasses.

1. Gather all required material; verify that ancillary equipment is properly functioning prior to assembly. This should include the following:
    - Firing mechanism can actuate properly
    - Pressure transducers are installed and greased
    - DAQ system is ready to collect data
    - Electric hoist is functioning
    - Impact wrench is functioning
    - Bottom flange is tightened to the appropriate torque
    - Gaskets for the shock tube are identified
    - Biomass is weighed
    - Water hose has been connected
2. Rinse barrel and test sections to check for and eliminate exorbitant rust, residual particles, or other potential contaminant.
3. Measure the calculated amount of water into a graduated cylinder and mix with the dried biomass to reconstitute/rehydrate the mixture/slurry.
4. Dispense biomass slurry into test section. Pour remaining water into the test section and mix until the slurry is homogenized.
5. Measure the volume of the slurry to make sure the depth is at the fill line. If necessary, add additional water or remove some of the slurry to guarantee the volume is level with the fill line.

6. Place gasket on upper flange of test section and lower the barrel into place. Tighten the flange using the impact wrench.
7. DAQ System: Assign a filename and put DAQ system on standby.
8. Retract firing pin on the firing mechanism, insert hitch pin orthogonally through firing pin, and then insert steel rod into solenoid.
9. Remove all non-essential personnel from the area and verify they have retreated to the control room.
10. Verify that the ignition system is unplugged or 'disarmed' in order to prevent any accidental triggering.
11. Insert shotgun shell into barrel.
12. Thread firing mechanism on to barrel.
13. Double check that blast shields or bunker is in place.
14. Retreat to control room.
15. Arm the ignition system by plugging in the trigger, which connects to the firing mechanism.
16. DAQ System: Start data collection.
17. Push trigger button on the ignition system

ABORT PROCEDURE – If the shell does not ignite

- Wait 5 min before approaching the shock tube to verify the shell is a 'dud'
- Remove firing mechanism and shotgun shell

- Dispose of shotgun shell as a flammable/hazardous material
- Diagnose ignition problem, make appropriate modifications to prevent misfires

18. Unplug ignition system to ‘disarm’ system
19. DAQ System: Wait 5 seconds for data collection to cease, and 2 additional minutes for signal to be processed.
20. Approach shock tube, remove firing mechanism, verify that pressure has been relieved and remove shotgun shell.
21. Unflange shock tube.
22. Pour contents of test section into labeled nalgene bottles.
23. While pouring, remove and discard any visible remnants of the propellant cartridge such as the following:
    - Plastic wadding
    - Cork wadding
    - Steel BB’s or lead shot
24. Add wash water to rinse splattered biomass into test section, such that it may be poured out into any additional Nalgene bottles.
25. Once all biomass has been removed, add additional water to rinse any residual residue coating the interior of the shock tube.
26. Repeat above steps for any additional biomass requiring the shock treatment
27. Clean up and store all equipment.

iii. POST-SHOCK PROCEDURE:

1. Consolidate biomass into a single pre-weighed container. Mix slurry vigorously to homogenize.
2. Place an 80-mesh sieve over a pre-weighed plastic bucket to collect wash liquid. Slowly transfer the biomass slurry onto the sieve.
3. For a 5% solids run, wash the biomass repeatedly with small amounts of DI water until ~5 L of total liquid has been collected (included water in the slurry prior to adding DI water). Runs performed at 10 and 15% solids will require 10 and 15 L of total wash water, respectively.
4. Remove and discard any visible remnants of the propellant cartridge such as the following:
    - Plastic wadding
    - Cork wadding
    - Steel BB's or lead shot
5. Transfer the washed biomass from the top of the sieve to a pre-labeled aluminum tray, load the tray on a vented rack, and allow the biomass to air dry for ~48h.
6. Take six liquid samples (~40 mL) in 50-mL centrifuge tubes for further analysis. To achieve homogeneous liquid, a magnetic stirrer is used. Determine the moisture and ash content of wash liquid samples in triplicate.

7. Prior to harvesting the air dried biomass, determine the moisture content in triplicate. If the moisture content is above 10%, allow the biomass to dry for an additional day. If less than 10%, remove the dried biomass from the aluminum trays and store it in pre-labeled Ziplock bags. Record the total weight (biomass + bags). Determine the moisture and ash content for air-dried biomass.
8. Perform the mass balance for shock pretreatment.

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APPENDIX D

DETERMINATION OF STRUCTURAL CARBOHYDRATES AND LIGNIN IN BIOMASS

This procedure is adapted from NREL laboratory analytical procedure for the determination of structural carbohydrates and lignin in biomass (NREL/TP-510-42618).

1. Determine moisture content of biomass samples. Moisture content should be less than or equal to 0.10 (g water/g wet biomass).

2. Remove extractives prior to this analysis.

3. Prior to analysis, biomass should be ground to –20/+80 mesh size.

4. Place filtering crucibles (Coors, No. 60531) in muffle furnace set to 550°C for 4 h.

5. Remove filtering crucibles from the furnace and place immediately in a desiccator.

6. After cooling to room temperature, weigh crucible mass.

7. Repeat Steps 4–6 until mass difference is less than 0.001 g.

8. Performing triplicate analysis is strongly advised. For each sample, add 0.30 ± 0.01 g to a labeled test tube (16×100 mm or larger). Record sample weights as $w1$.

9. To each test tube, add 3 mL 72% sulfuric acid.

10. Place test tubes in a water bath set to 30°C for 60 min, stirring every 5–10 min with a glass stir rod.

11. Remove test tubes from water bath.

12. Using 84 mL DI H_2O, quantitatively transfer test tube contents to labeled glass autoclave bottles. Tighten a crimp cap on each bottle.

13. In a labeled glass autoclave bottle, prepare sugar recovery standard (SRS). Add amounts of each sugar being analyzed representative of the amounts present in the samples. To the bottle, add 84 mL DI H_2O and 3 mL sulfuric acid. Tighten crimp cap on bottle and shake contents. Immediately open bottle and transfer 15 mL into a labeled 50-mL conical vial. Replace crimp cap and perform Steps 14–18 on SRS as with other samples.

14. Autoclave samples at 121°C for 1 h.

15. Allow bottles to slowly cool to room temperature.

16. Using 1-L Erlenmeyer flasks, filtering crucibles, and crucible adapters, vacuum filter bottle contents.

17. Transfer 15 mL filtrate to a labeled conical vial.

18. Add calcium carbonate to neutralize 15 mL liquid.

19. Prior to carbohydrate analysis by HPLC, store vials in freezer.

20. Using 50 mL hot DI H_2O, transfer biomass residue from glass bottle to filtering crucible.

21. Place filtering crucibles in an oven set to 105°C for 24 h.

22. Remove crucibles from oven and immediately place them in a desiccator.

23. After cooling to room temperature, weigh and record mass as *w*2.

24. Repeat Steps 20–22 until mass difference is less than 0.001 g.

25. Place filtering crucibles in a muffle furnace set to 550°C for 4 h.

26. Remove crucibles from furnace and immediately place them in a desiccator.

27. After cooling to room temperature, weight and record mass as *w*3.

28. Thaw neutralized samples.

29. Vortex each sample for 5–10 s.

30. Place vials in centrifuge adapters.

31. Centrifuge at 4,000 rpm (3,297 × g) for 5 min.

32. Using a 1-mL syringe and 0.2-µm syringe filter, transfer liquid from vial to a labeled glass HPLC vial. Cap the vial.

33. Prepare calibration standards for applicable sugars. Common sugar calibrations are 0.5, 1, 2, 5, and 10 g/L.

34. Analyze calibration standards, two SRS samples, and biomass samples. Use an Aminex HPX-87P column, guard column, and refractive index detector. Use a degassed, 0.2-µm-filtered DI H_2O mobile phase, 20-µL injection volume, and 20-min run time.

Calculations

1. Acid insoluble lignin (AIL) is calculated by:

$$AIL = \frac{w2 - w3}{w1(1 - \mathrm{MC})}$$

where:

w1 = sample weight (g)

w2 = filtering crucible plus dry residue (g)

w3 = filtering crucible plus ash (g)

MC = sample moisture content

2. Mass fractions for each sugar (xi) are calculated by:

$$xi = \frac{\mathrm{RSRS}i \times Ci \times \mathrm{AHC}i \times 87\ \mathrm{mL}}{w1 \times (1 - \mathrm{MC}) \times 1000\ \mathrm{mL/L}}$$

where:

Ci = HPLC-determined sugar concentration for Sugar *i* (g/L)

AHC*i* = anhydro correction for component *i* (0.90 for glucan, 0.88 for xylan)

RSRS*i* = recovery of sugar recovery standard for Sugar *i*

*w*1 = sample weight (g)

3. Sugar recovery standard recovery for each sugar (RSRSi) calculated by:

$$\mathrm{RSRS}i = \frac{\mathrm{SRS2}i}{\mathrm{SRS1}i}$$

where:

SRS2*i* = final SRS concentration for Sugar *i* (g/L)

SRS1*i* = initial SRS concentration for Sugar *i* (g/L)

APPENDIX E

BATCH ENZYMATIC HYDROLYSIS PROCEDURE

This procedure is adapted from NREL laboratory analytical procedure "Enzymatic Saccharification of Lignocellulosic Biomass" (NREL/TP-510-42629).

Reagents and equipment needed:

1. Incubator capable of agitation at ~150 rpm
2. Glass culture tubes (20×150 mm) with screw-caps (VWR, part # 9825-20X)
3. Auto-pipettes (20–200-µL, 100–1000-µL, and 500–5000-µL)
4. Moisture content analyzer (Denver Instruments IR 120)
5. 50-mL polypropylene centrifuge tubes (Corning, model # 430828)
6. Analytic balance w/ 0.1 mg precision Teflon thread tape
7. Sealant tape (Fisher, cat # 11-865-28) 100-mL beakers or flasks (1 per sample set)
8. Substrate 0.1-M sodium citrate buffer
9. Dilute CTec2 enzyme
10. Deionized distilled water (DI H_2O)
11. Tetracycline, cycloheximide solutions

Determination of number of samples:

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 F

HPLC SAMPLE PREPARATION AND TESTING FOR SUGARS

This procedure describes the HPLC sample preparation and testing for sugars after enzymatic hydrolysis.

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 13,000 rpm for 10 min.

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 min per sample.

APPENDIX G

MIXED-CULTURE BATCH FERMENTATION PROCEDURE

Batch fermentations were performed in 1-L polypropylene plastic bottles with a rubber stopper capping inserted with a glass tube and two stainless steel pipes that aided mixing of contents of the fermentor. The fermentors were placed in a rotary incubator at 2 rpm, set at a temperature of 40˚C, and were monitored every 48 h.

Batch fermentation monitoring procedure

1. Remove the fermentors from the incubator and allow them to cool for 10 min at room temperature.
2. Puncture the fermentor septum with a needle and open the valve to release the gases in the fermentor headspace. Record the gas production. Take gas samples once every four days.
3. Remove the fermentor caps and using a nitrogen purge line, carefully remove the residual solids adhered to the stopper and metal bars. Measure and record the pH for each fermentor.
4. Use a regular solid centrifuge cap to seal the fermentors. Balance each pair of fermentors on the weighing machine. Pay attention to balance the centrifuge nitrogen.bottles before placing them in the centrifuge.
5. Centrifuge (4,000 rpm, 25 min) the fermentors to separate the solid and liquid fractions.

6. After centrifuging, carefully move the bottles to ensure that the solid and liquid do not remix.
7. Collect a 1-mL sample of the liquid fraction and store it in a 2-mL centrifuge tube.
8. Add $MgCO_3$ (not needed for $CaCO_3$ buffer) to the bottles and mix well. Keep adding $MgCO_3$ till the fermentor has reached a near neutral pH.
9. Add methanogen inhibitor, if there is a methane peak found in the gas sample.
10. Mix contents of all bottles thoroughly and purge each fermentor with N_2.
11. Replace fermentor caps and return to incubator.

APPENDIX H

CARBOXYLIC ACID ANALYSIS USING GC

For carboxylic acids analysis, at least 3 mL of liquid is sampled from the fermentor, placed in a 15-mL conical centrifuge tube, and stored in the freezer at –10 °C. When analyzed, the samples were defrosted and vortexed. If the acid concentration is high, it may require further dilution before using the method below.

GC LIQUID SAMPLE PREPARATION

1. Centrifuge the liquid sample for 5 min at 4000 rpm.
2. Pipette 0.5 mL of clear liquid broth into a 2.0-mL microcentrifuge tube.
3. Add 0.5 mL of internal standard 4-methyl-valeric acid (1.162 g/L internal standard, ISTD).
4. Add 0.5 mL of 3-M phosphoric acid to convert all salts to acid form.
5. Cap and vortex the tube.
6. Centrifuge the mixture in a microcentrifuge (8000 × g) for 10 min.
7. Remove the tube and decant the mixture into a glass GC vial and cap. The centrifuged sample in the vial is ready to be analyzed now.
8. If the prepared sample will not be analyzed immediately, it can be frozen. Before GC analysis, make sure to thaw and vortex the sample.

GC OPERATION

1. Before starting the GC, check the gas supply cylinders (compressed hydrogen, compressed helium and compressed air from Praxair Co., Bryan, TX) to insure at least 200 psig pressure in each gas cylinder. If there is not enough gas, switch cylinders. Make sure to place an order for new ones.
2. Check the solvent and waste bottles on the injection tower. Fill up solvent vials with methanol. Empty the waste vials in designated waste container.
3. Before starting the GC, replace the septum beneath the injection tower.
4. Up to 150 samples can be loaded in the autosampler tray in one analysis batch. Place the samples in the autosampler racks. Include a vial with the volatile acid standard.
5. Check the setting conditions in the method:

    a. Inlet Conditions:

    i. Temperature: 230 °C

    ii. Pressure: 15 psig

    iii. Flow rate: 185 mL/min

    b. Detector conditions:

    i. Temperature: 230 °C

    ii. Air flow rate: 400 mL/min

    iii. H2 flow rate: 40 mL/min

    iv. The (makeup) flow rate: 45 mL/min

    c. Oven conditions:

i. Initial temperature: 40 °C

ii. Initial hold time: 2 min

iii. Ramp rate: 20 °C/min

iv. Final temperature: 200 °C

v. Final hold time: 1 min

d. Total run time per vial: 20 min

6. Start the GC on the computer by selecting the method with the setting conditions mentioned above. Load the sample sequence.
7. For quality control, run the standard mix every 15–25 samples. At the end of the sequence table, set the GC into standby mode to save gas.

APPENDIX I

MOISTURE AND ASH CONTENT ANALYSIS

This procedure was modified from NREL Standard Procedures (2004). If volatile acids are present in sample, lime may be added to retain all acids for more thorough measurement of moisture content (Meysing, 2011). However, when lime is added, the ash content cannot be measured as directed below. In this case, a separate sample must be dried with no lime addition, and subsequently ashed.

1. Record the label and weight of a clean, dry crucible (*W*1).
2. Place a representative sample of the material (liquid or solid) into the crucible and record the weight (*W*2).
3. Dry the crucible at 105 °C for 1 day in the drying oven. In a desiccator, allow to cool to room temperature before weighing. Record the dry weight (*W*3).
4. Ash the crucible at 575 °C for at least 12 h. Remove and allow sample to cool to room temperature in a desiccator. Record the ash weight (*W*4).
5. The moisture content [61] of the sample is calculated as

$$\mathrm{MC} = \frac{W2-W3}{W2-W1}$$

6. The ash content (AC) of the sample is calculated as

$$\mathrm{AC} = \frac{W4-W1}{W4-W1}$$

APPENDIX J

DEOXYGENATED WATER PREPARATION

Deoxygenated water with cysteine hydrochloride and sodium sulfide was used as the liquid medium in all fermentation experiments.

1. Fill a large glass container (≥4 L) with distilled water. Place the container over a hot plate to boil.
2. Boil the distilled water for 10 min.
3. Seal the top of the container and cool to room temperature.
4. Add 0.275 g cysteine hydrochloride and 0.275 g sodium sulfide per liter of boiled water.
5. Stir the solution until both chemicals are completely dissolved and pour into storage tank.

APPENDIX K

COUNTERCURRENT FERMENTATION TRANSFER PROCEDURE

In countercurrent fermentations, liquid and solid flow in opposite directions. A typical countercurrent train has four fermentors. For a laboratory-scale countercurrent transfer, the transfer of liquid and solids is made every 2 or 3 days, operating in a semi-continuous manner. Countercurrent fermentations are initiated as batch fermentations. The experiments were performed in batch mode until the culture is established in the fermentor (1 to 2 weeks). After the culture developed, the countercurrent operation was started, and the liquid and solids were transferred using the method below. To minimize oxygen exposure in fermentations, solid caps were placed on the bottles at any time solid and liquid was not actively being moved and a nitrogen gas purge was utilized to remove all oxygen from fermentor headspace before the fermentors were returned to the incubator.

1. Remove the fermentors from the incubator and allow to cool for 10 min at room temperature.
2. Release and record the gas production.
3. Remove the fermentor caps and using a nitrogen purge line, remove the residual solids adhered to the stopper and metal bars.
4. Measure and record the pH for each fermentor.

5. Cap the fermentor with a regular solid centrifuge cap.

6. Balance each pair of fermentors using some additional weight supplements. Balance the centrifuge bottles before placing them in the centrifuge.

7. Centrifuge the fermentors to separate the solid and liquid. Centrifuge for 25 min at 4000 rpm and a brake level of 5.

8. After centrifuging, carefully move the bottles to ensure that the solid and liquid do not remix.

9. Place the liquid from Fermentor 1 (F1) into a previously weighed plastic graduated cylinder. Record the weight and volume of liquid.

10. Take a 3 mL liquid sample for carboxylic acids analysis. Decant the remaining liquid from F1 into a liquid collection bottle for further VS analysis. Store the sample and collection bottle in a freezer for future analysis.

11. Weigh the fermentor bottle with the remaining solids and compare against the goal weight. Remember that the regular centrifuge cap is not included in this weight. To achieve steady state, a constant wet cake weight must be maintained in each fermentor. If the fermentor weight (wet solids + centrifuge bottle) weighs more than the goal weight, remove the difference and the solids will be added to the next fermentor (F2 in Figure). To simplify the transfer calculation, the goal weight includes the desired wet cake plus the weight of fresh biomass to be added to F1.

12. Add fresh biomass to F1.

13. Pour the liquid from F2 into a preweighed graduated cylinder. Record the weight and volume.

14. Pour the liquid into F1.

15. Weigh F2. Remove the solids resulting of: Solid removed = (F2 wet solids + solids from F1) – the goal weight.

16. Add the solids from F1 to F2.

17. Repeat Steps 13−16 from Fermentors 3 and 4 (F3 and F4).

18. Add fresh liquid medium (Appendix C) to F4 according to the predetermined volume.

19. Place the solids removed from F4 in a solid collection bottle and store it in the freezer until the VS analysis is performed.

20. Add buffer, urea (if desired), and methane inhibitor to each fermentor.

21. Mix content well and measure and record the pH.

22. Purge each fermentor with nitrogen and replace fermentor caps.

23. Return fermentors to the incubator.

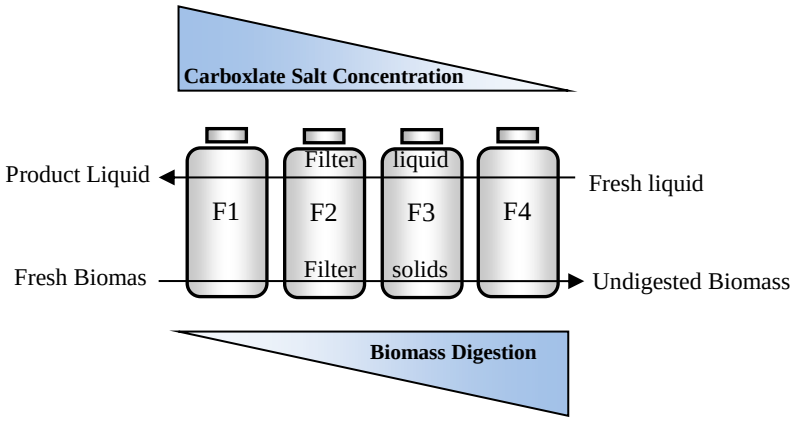
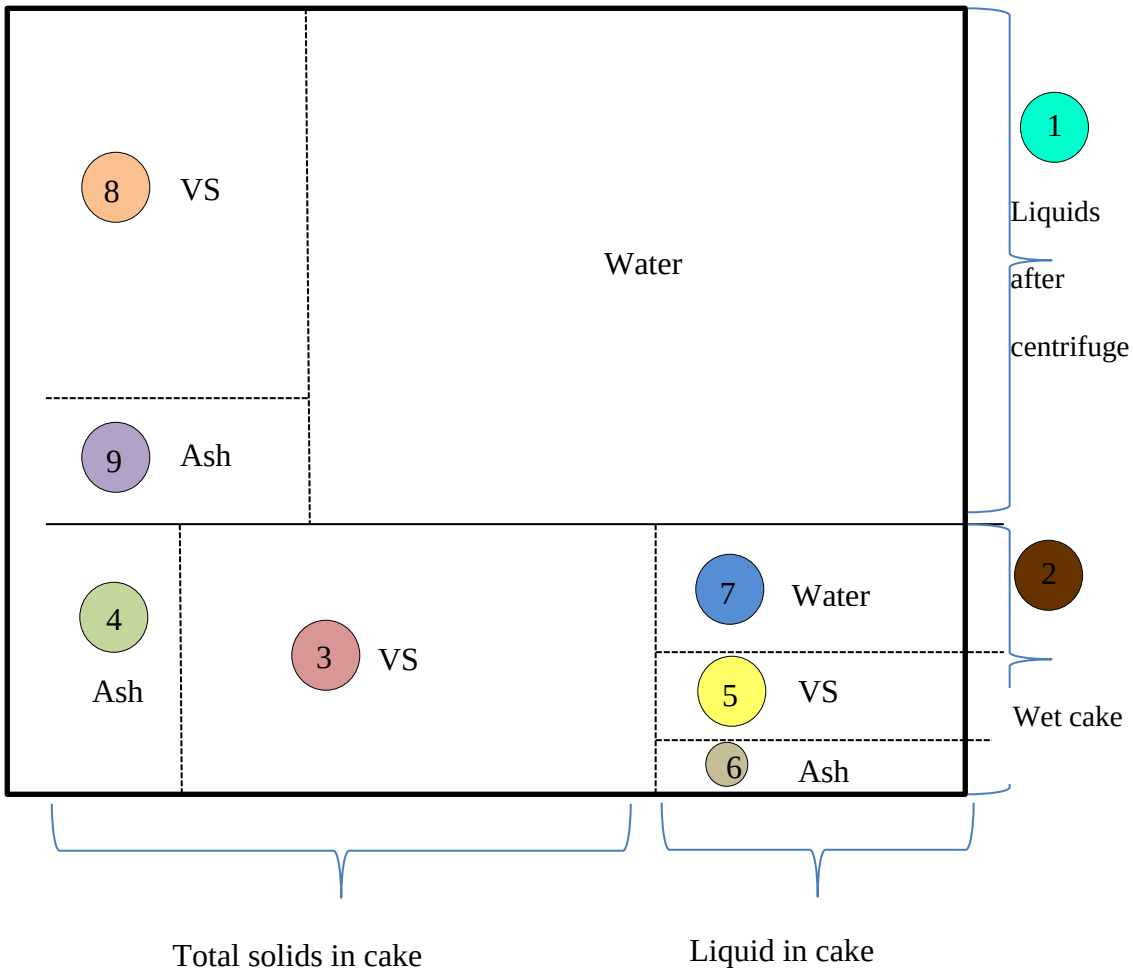


Figure K.1 Four-stage countercurrent fermentation

APPENDIX L

FERMENTATION PARAMETER CALCULATIONS



Calculated fractions:

1) $\text{Water}_{\text{liquid in cake}} = (\text{Wet cake} \times (1 - \text{TS}_{\text{wet cake}})$

2) $(\text{VS} + \text{Ash} + \text{Water})_{\text{liquid in cake}} = \frac{\text{Water}_{\text{liquid in cake}}}{(1 - \frac{\text{TS}_{\text{liquid}}}{100})}$

3) $(\text{VS} + \text{Ash})_{\text{wet cake}} = (\text{Cake} \times \text{TS}_{\text{cake separated}})$

4) $\text{Ash}_{\text{wet cake}} = \text{Wet cake} \times \frac{\%\ \text{TS}_{\text{cake}}}{100} \times \frac{\%\ \text{Ash}_{\text{cake}}}{100}$

5) $(\text{VS} + \text{Ash})_{\text{liquid in cake}} = (\text{VS} + \text{Ash} + \text{Water})_{\text{liquid in cake}} - \text{Water}_{\text{liquid in cake}}$

6) $(\text{VS} + \text{Ash})_{\text{dry cake solids}} = (\text{VS} + \text{Ash})_{\text{wet cake}} - (\text{VS} + \text{Ash})_{\text{liquid in cake}}$

7) $\text{Ash}_{\text{liquid in cake}} = (\text{VS} + \text{Ash} + \text{Water})_{\text{liquid in cake}} \times \frac{\%\ \text{Ash}_{\text{liquid}}}{100} \times \frac{\%\ \text{TS}_{\text{liquid}}}{100}$

8) $\text{VS}_{\text{liquid in cake}} = (\text{VS} + \text{Ash})_{\text{liquid in cake}} - \text{Ash}_{\text{liquid in cake}}$

9) $\text{Ash}_{\text{dry cake solids}} = \text{Ash}_{\text{wet cake}} - \text{Ash}_{\text{liquid in cake}}$

10) $\text{VS}_{\text{dry cake solids}} = (\text{VS} + \text{Ash})_{\text{dry cake solids}} - \text{Ash}_{\text{dry cake solids}}$

11) $(\text{VS} + \text{Ash})_{\text{separated liquid}} = \text{Liquid separated after centrifuge} \times \frac{\%\ \text{TS}_{\text{liquid}}}{100}$

12) $\text{Ash}_{\text{separated liquid}} = \text{Liquid separated after centrifuge} \times \frac{\%\ \text{TS}_{\text{liquid}}}{100} \times \frac{\%\text{Ash}_{\text{liquid}}}{100}$

13) $\text{VS}_{\text{separated liquid}} = (\text{VS} + \text{Ash})_{\text{separated liquid}} - \text{Ash}_{\text{separated liquid}}$

APPENDIX M

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 H2O.

2. Weigh 210 g of citric acid monohydrate and add to volumetric flask.

3. Once dissolved, weigh out approximately 35–40 g of NaOH 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 ± 0.05, 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 N

ANTIBIOTIC PREPARATION

Reagents and equipment needed:

Analytic balance w/ 0.1 mg precision

Weighing papers

Weighing spatula

Gloves (two 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 O

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 P

CPDM MATLAB PROGRAM

```
%MATLAB Code for CPDM Prediction
%This code is for a standard four-stage countercurrent fermentation
%Program predicts acid concentrations and conversion at varying VSLR and LRT.
%Department of Chemical Engineering, Texas A&M University, College St, TX
%CODE BY PRATIK 08/29/2014
clear all
close all
global so taus e1 f1 g1 h1
global holdup moist ratio stages loading tauloverall
global acid nnot factr1
global x_1 nhat_1 x_2 nhat_2 x_3 nhat_3 x_4 nhat_4

%Start Simulation
disp(['Program starts at: ', datestr(now)]);
tic;

VSLR_data=[4,6,8,10,12]';
LRT_data=[10,15,20,25,30]';
ACID = [];
CONVERSION = [];
VSLR_loop=12; %loop is for varying VSLR.
%To make map, set to lowest VSLR, otherwise, set to specific VSLR
while VSLR_loop<12.1 % if want loop, set to highest VSLR
   LRT_loop=31.1;   %loop is for varying LRT.
   %To make map, set to lowest LRT, otherwise set to specific LRT
   while LRT_loop<31.11 %if want loop, set to highest VSLR

      %%Basic parameters for Fermentation
      stages=4; %Fermentor stages
      so=0.77; %Aeq selectivity (gAEQ/g VS digested)
      %Please note that in older versions of the code (i.e. Loescher's)
      %this term referred to a VS selectivity of g VS/g total solids and
      %was carried over in the differiental equations in Ross and Fu.
      holdup =2.0; %ratio of liq to solid in wet cake (g liq/gVS cake)
      %Note: holdup is the liq in the solid cake NOT the lig of the
      %total slurry
      moist =.08; %ratio of liquid to solid in feed (g liq/gVS cake)
      SQ =1.0;
      ratio=0.76; %phi ratio of g total acid to g AEQ
      loading = VSLR_loop;
      tauloverall = LRT_loop;
      vol=[.48,.28,.28]'; %Liquid volime in each fermentor
      totvol=sum(vol);
      liquidfeed = totvol/tauloverall;
      nnotreal = [169,215,215,215]'; %VS concentration gVS/L (?in each fermentor?)
      solidfeed = loading*totvol; %Solid Feed (g dry weight)
```

```
Convrsn = [.1,.2,.3,.4]'; %Initial value for conversion
nnot = nnotreal./(1-Convrsn);
taus = nnot.*vol/solidfeed;
L =0.1*ones(stages+1,1); %L initial value for liquid flow rate in every reactor
taul = tauloverall/stages*ones(stages,1);

e1=0.017783; f1=3.3713656; g1=0.0004693; h1=-4.452693; %CPDM parameters
%acd=22.3; % acd need to trfer into the Function M file
rmodel = @(x1,acid) e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1);
syms x1 acid
drmodel_1 = diff(e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1),x1);
drmodel = @(x2,acid2) subs(drmodel_1,{x1,acid},{x2,acid2});

done = 0; %The index used to trace whether the condtion is satisfied
liqtoler = 0.01; %tolerance for Liquid flowrate 0.005
acidtoler = 0.1; %tolerance for acid concentration  0.02
nnottoler = 1; %tolerance for nnot

%Initial values for acid, acidold
%ans=ones(stages,1); % dont use ans it is a matlab variable.
acid=[30,20,15,5]';
acidold=ones(stages,1);
taulnew = 1000*ones(stages,1);  %column vector
nhatzero =100*ones(stages,1);  %CP concentration
creation = ones(stages,1);
destruction = ones(stages,1);
tauloverallnew = 20;

disp('Calculation is in progress.......');

while done < 0.50
   taulnew = 1000*ones(stages,1);  %Obtain Flowrate for each fermentor
   taulover_error = 0.001;
   while abs(tauloverall-tauloverallnew) > taulover_error
      liquidfeed = liquidfeed*(1+(tauloverallnew-tauloverall)/tauloverall*0.5);
      L(5) = liquidfeed;
      L(4) = L(5) + solidfeed/1000*holdup*(Convrsn(4)-Convrsn(3));
      L(3) = L(4) + solidfeed/1000*holdup*(Convrsn(3)-Convrsn(2));
      L(2) = L(3) + solidfeed/1000*holdup*(Convrsn(2)-Convrsn(1));
      L(1) = moist*solidfeed/1000 + L(2) - solidfeed/1000*holdup*(1.0-Convrsn(1));
      tauloverallnew = totvol/L(1);
   end

   taul = vol./L(1:stages);  %vol 4*1, L 5*1
   nnot = nnotreal./(1-Convrsn);
   taus = nnot.*vol/solidfeed;
   scale = ones(stages,1);

   disp([' nnot= ',num2str(nnot','%15.5f')]);

   %parameters for ODE45
```

```
        options = odeset('RelTol',1e-3,'AbsTol', 1e-3);
        x_low=0; x_high=0.99;

        %Reactor 1

        i=1;
        while abs(taulnew(i) - taul(i))> liqtoler  %liqtoler = 0.05
           nhat0 =nhatzero(i);
           [x,nhat]= ode15s(@Chan1,[x_low,x_high],nhat0,options);
           x_1=x;  nhat_1 = nhat;
           F_1 = @(x_1)interp1(x,nhat,x_1);
           factr1 = nnot(i)/quad(F_1,x_low,x_high);  %calculate factor
           F_11 = @(x_1) factr1*interp1(x,nhat,x_1).*rmodel(x_1,acid(i));
           robs = quad(F_11,x_low,x_high);
           F_12 = @(x_1) interp1(x,nhat,x_1).*x_1;
           Convrsn(i) = quad(F_12,x_low,x_high)/nnot(i)*factr1;
           taulnew(i) = (L(i)*acid(i) + solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
L(i+1)*acid(i+1))/(L(i)*robs);
           acid(i) = acid(i) + (taul(i)*robs-(L(i)*acid(i)+solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
L(i+1)*acid(i+1))/L(i))*.4;  %why 0.4 here?
        end
        disp(['  acid(',num2str(i),')=',num2str(acid(i),'%15.5f'),'
taulnew(',num2str(i),')=',num2str(taulnew(i),'%15.5f'),'  robs=', num2str( robs, '%15.5f')]);

        %Reactor 2

        i=2;
        nnottoler = nnot(i)/500;
        while abs(taulnew(i)-taul(i))>liqtoler;
           ndone = 0;
           while ndone <0.50
              nhat0=nhatzero(i);
              options = odeset('RelTol',1e-3,'AbsTol',1e-3);
              [x,nhat] = ode15s(@Chan2,[x_low,x_high],nhat0,options);
              x_2=x;  nhat_2=nhat;
              F_2 = @(x_2)interp1(x,nhat,x_2);
              nhattot=quad(F_2,x_low,x_high);
              disp(['  nhatzero= ',num2str(nhatzero(i), '%15.5f'),';  nhattot= ',num2str(nhattot, '%15.5f'),';
nnot(',num2str(i),')= ',num2str(nnot(i), '%15.5f')]);
              if abs(nhattot - nnot(i))<nnottoler;
                 ndone = 1;
              end
              if (nhatzero(i) + (nnot(i) - nhattot)*1.0)>0
                 nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.7;
              else
                 nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.2;
              end
           end

           F_22 = @(x_2)interp1(x,nhat,x_2).*x_2;
           Convrsn(i)= quad(F_22,x_low,x_high)/nnot(i);
           robs = solidfeed*so/vol(i)*(Convrsn(i)-Convrsn(i-1));
```

```
            taulnew(i) = (L(i)*acid(i) + solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
L(i+1)*acid(i+1))/(L(i)*robs);
            acid(i) = acid(i) + (taul(i)*robs-(L(i)*acid(i)+solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
L(i+1)*acid(i+1))/L(i))*.5;
            disp(['  taulnew(',num2str(i),')=',num2str(taulnew(i), '%15.5f'),'
taul(',num2str(i),')=',num2str(taul(i),'%15.5f'),]);
         end
         disp(['  acid(',num2str(i),')=',num2str(acid(i),'%15.5f'),'
taulnew(',num2str(i),')=',num2str(taulnew(i),'%15.5f'),'  robs=', num2str( robs, '%15.5f')]);

         %Reactor 3

         i=3;
         nnottoler = nnot(i)/500;
         while abs(taulnew(i)-taul(i))>liqtoler;
            ndone = 0;
            while ndone <0.50
               nhat0 =nhatzero(i);
               options = odeset('RelTol',1e-3,'AbsTol',1e-3);
               [x,nhat] = ode15s(@Chan3,[x_low,x_high],nhat0,options); %was chan3
               x_3=x;  nhat_3=nhat;
               F_3 = @(x_3)interp1(x,nhat,x_3);
               nhattot=quad(F_3,x_low,x_high);
               disp(['  nhatzero= ',num2str(nhatzero(i), '%15.5f'),';  nhattot= ',num2str(nhattot, '%15.5f'),';
nnot(',num2str(i),')= ',num2str(nnot(i), '%15.5f')]);
               if abs(nhattot - nnot(i))<nnottoler;
                  ndone = 1;
               end
               if (nhatzero(i) + (nnot(i) - nhattot)*1.0)>0
                  nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.7;
               else
                  nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.2;
               end
            end

            F_32 = @(x_3)interp1(x,nhat,x_3).*x_3;
            Convrsn(i)= quad(F_32,x_low,x_high)/nnot(i);
            robs = solidfeed*so/vol(i)*(Convrsn(i)-Convrsn(i-1));

            %taulnew(i) = (L(i)*acid(i) + solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-solidfeed/1000*(1-
Convrsn(i-1))*holdup*acid(i-1))/(L(i)*robs);
            %acid(i) = acid(i) + (taul(i)*robs-(L(i)*acid(i)+solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
solidfeed/1000*(1-Convrsn(i-1))*holdup*acid(i-1))/L(i))*0.5;
            taulnew(i) = (L(i)*acid(i) + solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
L(i+1)*acid(i+1))/(L(i)*robs);
            acid(i) = acid(i) + (taul(i)*robs-(L(i)*acid(i)+solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
L(i+1)*acid(i+1))/L(i))*.5;
            disp(['  taulnew(',num2str(i),')=',num2str(taulnew(i), '%15.5f'),'
taul(',num2str(i),')=',num2str(taul(i),'%15.5f'),]);
         end
```

```
        disp([' acid(',num2str(i),')=',num2str(acid(i),'%15.5f'),'
taulnew(',num2str(i),')=',num2str(taulnew(i),'%15.5f'),' robs=', num2str( robs, '%15.5f')]);


        %Reactor 4

        i=4;
        nnottoler = nnot(i)/500;
        while abs(taulnew(i)-taul(i))>liqtoler;
           ndone = 0;
           while ndone <0.50
              nhat0 =nhatzero(i);
              options = odeset('RelTol',1e-3,'AbsTol',1e-3);
              [x,nhat] = ode15s(@Chan4,[x_low,x_high],nhat0,options); %was chan4
              x_4=x;  nhat_4=nhat;
              F_4 = @(x_4)interp1(x,nhat,x_4);
              nhattot=quad(F_4,x_low,x_high);
              disp([' nhatzero= ',num2str(nhatzero(i), '%15.5f'),';  nhattot= ',num2str(nhattot, '%15.5f'),';
nnot(',num2str(i),')= ',num2str(nnot(i), '%15.5f')]);
              if abs(nhattot - nnot(i))<nnottoler;
                 ndone = 1;
              end
              if (nhatzero(i) + (nnot(i) - nhattot)*1.0)>0
                 nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.7; %25/nnot(i);
              else
                 nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.2;
              end
           end

           F_42 = @(x_4)interp1(x,nhat,x_4).*x_4;
           Convrsn(i)= quad(F_42,x_low,x_high)/nnot(i);
           robs = solidfeed*so/vol(i)*(Convrsn(i)-Convrsn(i-1));

           taulnew(i) = (L(i)*acid(i) + solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-solidfeed/1000*(1-
Convrsn(i-1))*holdup*acid(i-1))/(L(i)*robs);
           acid(i) = acid(i) + (taul(i)*robs-(L(i)*acid(i)+solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
solidfeed/1000*(1-Convrsn(i-1))*holdup*acid(i-1))/L(i))*0.5;
           disp([' taulnew(',num2str(i),')=',num2str(taulnew(i), '%15.5f'),'
taul(',num2str(i),')=',num2str(taul(i),'%15.5f'),]);
        end
        disp([' acid(',num2str(i),')=',num2str(acid(i),'%15.5f'),'
taulnew(',num2str(i),')=',num2str(taulnew(i),'%15.5f'),' robs=', num2str( robs, '%15.5f')]);
        disp([' Conversion in each stage (from nhat): ',num2str(Convrsn','%13.5f')]);

        if max(abs(acid-acidold))<acidtoler
           done=1;
        end
        acidold = acid;
     end
```

```
        %Output results section

        disp('Congratulations!  The simulation is successfully finished!')
        toc  %toc is used to check the whole time of the process

        for i3 = 1:(stages+1);
           disp(['  L(',int2str(i3),')= ',num2str(L(i3))]);
        end

        creation(1) = L(1)*acid(1) + solidfeed/1000*(1-Convrsn(1))*holdup*acid(2)-L(2)*acid(2);
        creation(2) = L(2)/acid(2) + solidfeed/1000*(1-Convrsn(2))*holdup*acid(3)-L(3)*acid(3)-
solidfeed/1000*(1-Convrsn(1))*holdup*acid(2);
        creation(3) = L(3)*acid(3) + solidfeed/1000*(1-Convrsn(3))*holdup*acid(4)-L(4)*acid(4)-
solidfeed/1000*(1-Convrsn(2))*holdup*acid(3);
        creation(4) = L(4)*acid(4) - solidfeed/1000*(1-Convrsn(3))*holdup*acid(4);

        %Calculation of Destruction

        destruction(1) = solidfeed/1000*(Convrsn(1)-0);
        for i3=2:stages;
           destruction(i3)=solidfeed/1000*(Convrsn(i3)-Convrsn(i3-1));
        end
        selectivi = creation./destruction;
        selec = L(1)*acid(1)/(solidfeed*Convrsn(4));

        %output the result and plot the result
        disp(['  Selectivity = ',num2str(selectivi','%15.5f')]);
        disp(['  Creation = ',num2str(creation','%15.5f')]);
        disp(['  Destruction = ',num2str(destruction','%15.5f')]);
        disp(['  selectivity = ',num2str(selec','%15.5f')]);
        disp(['  tauloverall = ',num2str(tauloverall,'%15.5f')]);
        disp(['  taus = ',num2str(sum(taus),'%15.5f')]);
        disp(['  acid levels = ',num2str(acid','%13.5f')]);

        disp(['  VSLR_LOOP = ',num2str(VSLR_loop),'  LRT_loop = ',num2str(LRT_loop)]);

        %Collect data for CPDM map
        ACID = [ACID;acid(1)];
        CONVERSION = [CONVERSION;Convrsn(4)];
        LRT_loop = LRT_loop + 5;
    end
    VSLR_loop = VSLR_loop + 2;
end

disp(['  acid levels = ',num2str(acid','%13.5f')]);
disp(['  convrsn levels = ',num2str(Convrsn','%13.5f')]);
 disp(['  VSLR = ',num2str(VSLR_data','%13.5f')]);
 disp(['  LRT = ',num2str(LRT_data','%13.5f')]);
 disp(['  Acid levels = ',num2str(ACID','%13.5f')]);
 disp(['  Conversions = ',num2str(CONVERSION','%13.5f')]);
```

```
 %%Section to draw CPDM "map"of product concentration and conversion
%tested 7/8/11
 %  mapdata = [VSLR,LRT,Conversion,acid];
mapdata = [opmap_table(:,1),opmap_table(:,2),CONVERSION,ACID];
VSLR_sorted=sortrows(mapdata,1); %sort
LRT_sorted=sortrows(mapdata,2); %sort
[map_num,map_1]=size(mapdata);

VSLR_sort=sort(mapdata(:,1));
uniqueM=[diff(VSLR_sort);1]>0;
VSLR_sort1=VSLR_sort(uniqueM);
VSLR_number=diff(find([1;uniqueM]));

LRT_sort=sort(mapdata(:,2));
uniqueM=[diff(LRT_sort);1]>0;
LRT_sort1=LRT_sort(uniqueM); %unique LRT
LRT_number=diff(find([1;uniqueM]));

temp1=zeros(length(VSLR_sort1)+1,1);
for j1=1:length(VSLR_sort1)
   temp1(j1+1)=temp1(j1)+VSLR_number(j1);
   mapdata_1=VSLR_sorted(temp1(j1)+1:temp1(j1+1),:);
   %for VSLR(j1)
   F=@(x)interp1(mapdata_1(:,3),mapdata_1(:,4),x,'spline');
   hold on;
   plot(mapdata_1(:,3),F(mapdata_1(:,3)),'k');
   if j1==1
      for j3=1:length(mapdata_1(:,3))
         text(mapdata_1(j3,3),mapdata_1(j3,4),[' ',num2str(mapdata_1(j3,2))], 'HorizontalAlignment', 'left');
      end
   end
end

temp1=zeros(length(LRT_sort1)+1,1);
for j1=1:length(LRT_sort1)
   temp1(j1+1)=temp1(j1)+LRT_number(j1);
   mapdata_2=LRT_sorted(temp1(j1)+1:temp1(j1+1),:);
   %for LRT(j1)
   F2=@(x)interp1(mapdata_2(:,3),mapdata_2(:,4),x,'spline');
   hold on;
   plot(mapdata_2(:,3),F(mapdata_2(:,3)),'k');
   if j1==1
      for j3=1:length(mapdata_2(:,3))
         text(mapdata_2(j3,3),mapdata_2(j3,4),[' ',num2str(mapdata_2(j3,2))], 'HorizontalAlignment',
'right');
      end
   end
end
hold off;
axis([0 1 0 60]);

%-----end of map ploting
```