EFFECTS OF PRODUCT REMOVAL USING ION-EXCHANGE RESIN AND SODIUM HYDROXIDE PRETREATMENT IN MIXED-CULTURE FERMENTATION

A Thesis

by

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ABSTRACT

In mixed-culture fermentations of lignocellulosic biomass, periodical product removal has proven to reduce product inhibition and thereby increase biomass digestion. Previously, ion-exchange resin (Amberlite IRA-67) was employed in countercurrent fermentation, and different amounts of resin loadings (10–40 g wet resin per 1.38 L total liquid volume) were studied. Using the pre-established culture (office paper and oven-dried chicken manure), this research used higher resin loadings (50, 60, and 80 g wet resin per 1.34 L total liquid volume). The 0.3-L resin column was sustained by CO_2 (gas flow rate: 1.5 L/min). Compared to 30 g resin loading, when higher loadings were introduced, biomass conversion dropped by 0.05–0.09 g $NAVS_{digested}$/g $NAVS_{feed}$. Furthermore, the yield plateaued at resin loadings greater than 40 g. Overall, the optimal resin loading for both biomass conversion and acid yield was 30–40 resin loading per 1.34 L total liquid volume, which corresponded to 21.74–29.20 g wet resin/L_{liq}.

As indicators of digestibility, cellulase (Ctec3) and hemicellulose (Htec3) were used to saccharify pretreated corn stover. Shock pretreatment subjects biomass to a high-pressure pulse to enhance enzyme accessibility to cellulose and hemicellulose. It was found that 5.52 bar (abs) initial H_2/O_2 filling pressure generated a sufficiently high shock pressure to produce good digestibility. Sodium hydroxide is a potent alkali for pretreatment. Shock treatment (5.52 bar (abs) filling pressure) followed by moderate

temperature (50°C) with 4 g OH^-/100 g dry biomass NaOH concentration for 1 h is the recommended treatment condition.

The continuum particle distribution model (CPDM) simulates four-stage countercurrent mixed-culture fermentation from data obtained from batch experiments. Using the recommended pretreatment conditions described above, three different extents of pretreated corn stover were studied: (1) shock only, (2) NaOH only, and (3) shock + NaOH. As the nutrient source, sewage sludge was used together with raw or pretreated corn stover. Compared with raw corn stover, the CPDM map showed improvements in conversion and product concentration for NaOH treatment alone and shock + NaOH treatments; however, shock only made the corn stover less digestible. With shock + NaOH-treated corn stover, the model predicts the total carboxylic acid concentration of 36.1 g/L and conversion of 0.432 g $NAVS_{digested}$/g $NAVS_{feed}$ at liquid retention time (LRT) of 35 day and volatile solid loading rate (VSLR) of 6 g/(L·day).

Dedicate to my beloved family: my grandparents, parents, and my sister, who constantly believed in me to search for my interests and to pursue my dream. Thank you for all sincere encouragements throughout my life, especially the time of my graduate school years.

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originate from the same concept: fermentation. I sincerely thank my two mentors who guided me to the field of Enology, and also Dr. Mark Holtzapple, who always provide me tremendous freedom to explore my interests beyond research.

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CHAPTER I

INTRODUCTION

Recently, there is growing interest in developing cost-effective renewable energy. Improvements in shale gas fracturing have plummeted energy prices, which caused the public to gradually withdraw support for biofuel manufacturing; however, the gasoline shortage in 1973 is considered a harbinger for the inevitable dwindling supplies of fossil fuels. Moreover, tense international relations, economic vicissitudes, and political mistakes may cause striking fluctuations in oil prices; therefore, alternative energy should be incorporated into future scenarios.

Switching from fossil fuels to biofuels will significantly contribute to the development of a sustainable society and help manage greenhouse gases. In a future carbon-constrained world, numerous changes will be necessary at all stages of energy production, distribution, and use. Through photosynthesis, biomass captures solar energy as fixed carbon. Because atmospheric carbon dioxide (CO_2) is utilized while plants grow, there is no net CO_2 increase by combusting biomass-derived fuels. This benign cycle allows fuel production to continue while maintaining a constant CO_2 concentration. The next generational change in the utilization of bioresources will integrate various plant resources, biomaterials, and bio-based energy (Figure 1-1).[1]

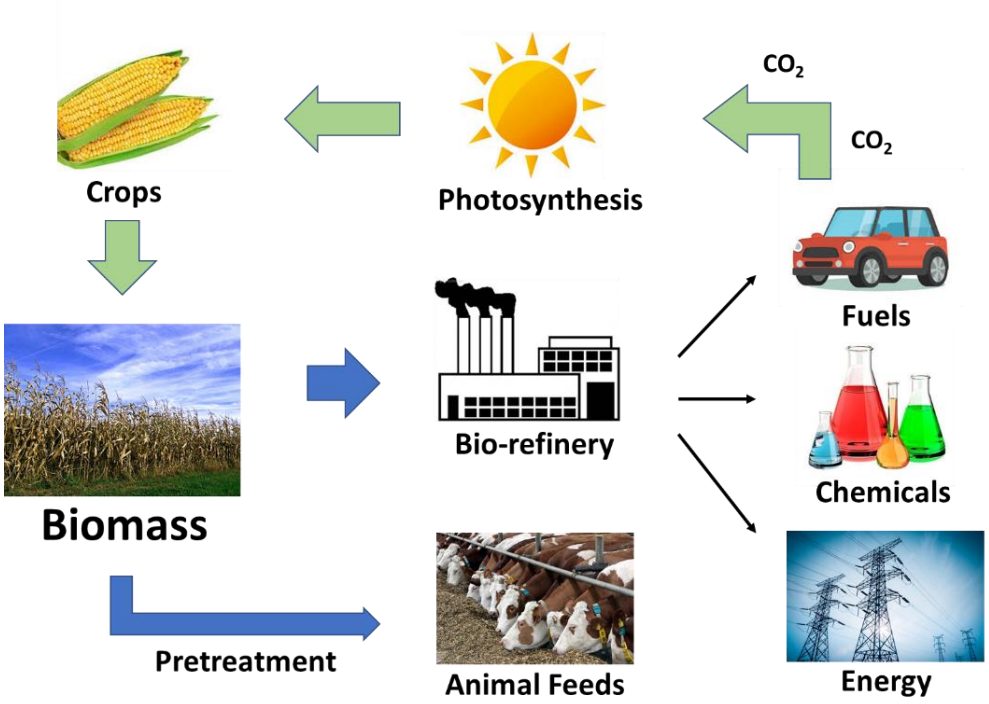


Figure 1-1 Sustainable biomass utilization cycle.

Nature has its own way to harness biomass. The ruminal fermentation is a very highly developed natural lignocellulose-degrading system. Consolidated bioprocessing (CBP) uses microorganisms that possess enzymes to hydrolyze pretreated biomass, and a fermentation pathway that converts the resulting hydrolytic products to ethanol and other compounds.[2] Mixed-culture ruminal fermentation is an example of CBP. Although the ruminal fermentation has its limitations, it is robust; engineering a bioreactor to mimic its function should be further investigated.[3-4]

To convert biomass to liquid fuels, the following platforms have been developed: thermochemical, sugar, and carboxylate. Among the three, the carboxylate platform has exhibited the highest product yields.[5] Developed by Holtzapple *et al.*, the

MixAlco® process is a robust example of the carboxylate platform, which employs mixed-culture acid-forming microorganisms to convert lignocellulose into carboxylate salts.[6-7] Lignocellulosic biomass typically undergoes pretreatment to obtain better utilization from the microorganisms, which usually leads to higher yields than from un-pretreated biomass.[8] The resulting carboxylate salts can be reacted to hydrocarbon fuels via a series of steps: ketonization, hydrogenation, and dehydration. Unlike the sugar platform, which demands a specific reaction environment, the essence of the carboxylate platform is that the fermentation is thermodynamically driven, and thus does not require sterile operating conditions, which makes it cost-effective and industrially favored.[9]

To substantiate the effectiveness and give insightful analysis of the MixAlco® process, research from various perspectives has been conducted. Pham provided techno-economic analysis of the process, which corroborates that MixAlco® is an economically viable way to convert biomass to fuels or chemicals.[9] Golub investigated the effects of different bioreactor configurations and countercurrent fermentation.[10] Roy analyzed carboxylic acid extraction from batch fermentations by ion-exchange resins.[11] Moreover, Bond proved that shock treatment, where a mixture of pressurized oxygen and hydrogen is detonated in a shock tube, improves the digestibility of corn stover efficiently and economically.[12]

Continuous removal of carboxylic acid products improves production yield, and pretreatment is considered the most expensive step in the biochemical conversion

of lignocellulose. This study provides an in-depth comprehensive investigation of two major aspects of biomass conversion: product removal via ion-exchange resin and pretreatment via shock and alkali. The results of this research may be used to ameliorate and reduce the cost of the MixAlco® process. The specific objectives of this study follow:

1. Examine the impacts of product removal on countercurrent fermentation performance, such as conversion, yield, and selectivity.
2. Observe total carboxylic acid concentration yields to determine if there is an optimal resin loading.
3. Search for optimal pretreatment conditions of corn stover.
4. Employ pretreated corn stover in mixed-culture fermentation to determine if the acid-forming potential increases in a manner similar to enzymatic saccharification.
5. Obtain a Continuum Particle Distribution Model (CPDM) for corn stover treated by shock and alkali.

CHAPTER II

MIXED-ACID COUNTERCURRENT FERMENTATION UNDER HIGH ION-EXCHANGE RESIN LOADINGS

2.1 Introduction

In the carboxylate platform, multi-staged countercurrent fermentation is commonly used, and has proven to enhance yield and conversion compared to a batch system.[13] As shown in Figure 2-1, fresh substrate and nutrient feeds enter at the opposite direction against the water feed. Depending on the research, the number of stages of countercurrent fermentation can be varied; from one to six stages have been studied by Golub.[14] More stages increase the acid concentration and selectivity, whereas fewer stages increase conversion. Four-stage countercurrent has been chosen in this experiment.

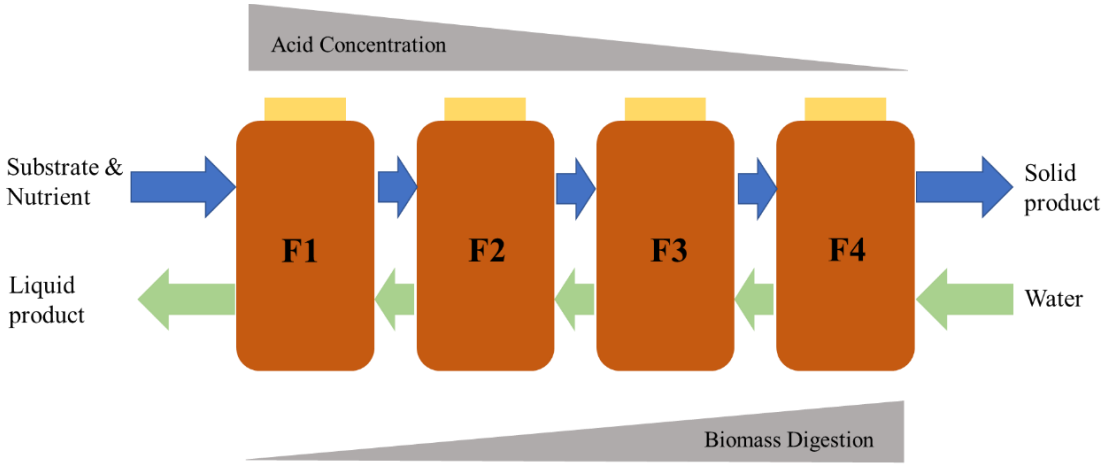


Figure 2-1 Schematic illustration of four-stage countercurrent fermentation.

Introducing ion-exchange resin into countercurrent fermentation improves product yield and substrate conversion. In this experiment, Amberlite IRA-67 weak-base ion-exchange resin has been selected. Detailed explanations regarding resin selection are explained by Roy. She found that Amberlite IRA-67 significantly enhanced batch fermentation.[11]

Wu combined countercurrent fermentation and ion-exchange resin adsorption.[15] The mixed-acid fermentation trains used commercial office paper energy source, oven-dried chicken manure nutrient, and urea nitrogen source. Iodoform was added as a methane inhibitor. In the study, each train had a total liquid volume of approximately 1.38 L. In 10-g increments, 10 to 40 g of Amberlite IRA-67 resin was used in four different fermentation trains. Approximately three months after commencing the fermentation, these trains reached steady state when carboxylate production became stable. Then, the ion-exchange resin was introduced to the countercurrent fermentation after maintaining two months of steady-acid production. Bubbling CO_2 enhanced fluidization of the resin and maintained a low pH in the extractor, which is required for effective acid adsorption.[15]

Ion-exchange resins can be incorporated with countercurrent fermentation. The increasing resin loadings employed by Wu reduced product inhibition and linearly improved fermentation performance: conversion increased by 128% to 0.409 g $NAVS_{digested}$/g $NAVS_{feed}$ and yield increased by 107% to 0.236 g carboxylic acid produced/g $NAVS_{feed}$. Compared to the non-resin group, incorporating the ion-

exchange resin improved both conversion and yield. In each train, the selectivity was fairly constant (approximately 0.65 g total carboxylic acid produced/g $NAVS_{digested}$).[15]

Countercurrent fermentation uses mixed-cultures of microorganisms to produce short- and medium-chain carboxylic acids. This chapter aims to extend past results by examining higher resin loadings, and to investigate the optimized outcomes into mixed-acid fermentation.

This chapter is a collaborative effort with Opeyemi Olodeke, a PhD candidate in the Department of Chemical Engineering, Texas A&M University.

2.2 Materials

2.2.1 Substrate

In this study, a mixture of unused white office paper, chick manure, and urea were used as the mixed-acid fermentation substrate.

The office paper (20 pounds, Caliber®) serves as energy source for the fermentation. Because office paper is vigorously pretreated during manufacturing, it requires no further pretreatment. Mechanical shredding using a paper shredder (AURORA® AS680S) was performed before use. The carbon-to-nitrogen ratio (C-N ratio) of office paper is reported as 519 g carbon/g nitrogen, where the carbon is 36.30% and the nitrogen is 0.07% by weight.[11]

$$\text{C-N ratio} = \frac{\text{total carbon mass}}{\text{total nitrogen mass}} \tag{2-1}$$

Fresh chicken manure was collected from the Department of Poultry Science at Texas A&M University (College Station, TX), and was homogenized and dried in the oven at 105℃ for 48 hours to maintain consistency and to avoid degradation throughout the experiment. Oven-dried chicken manure has C-N ratio of 12.8 g carbon/g nitrogen, where the carbon is 28.20% and the nitrogen is 2.20% by weight.[15]

Urea (>99.5%, Fisher Scientific) was added to the fermentors. The carbon and nitrogen contents of urea are 19.35% and 45.16% by weight, respectively. Its low C-N ratio makes it a desired supplemental source of nitrogen. In chicken manure/office paper fermentations, it is reported that the optimal C-N ratio range is 25–35 g carbon/g nitrogen.[16]

2.2.2 Fermentor

Figure 2-2 illustrates a schematic view of the fermentor. The fermentation was performed in 1-L polypropylene copolymer (PPCO) bottles (Nalgene®) capped by a rubber stopper with a glass tube, septum, and two 0.25-in. stainless steel bars in the middle. The metal bars facilitate better mixing while the fermentation is performed in the rolling incubator. The rubber septum allows convenient venting of gases.

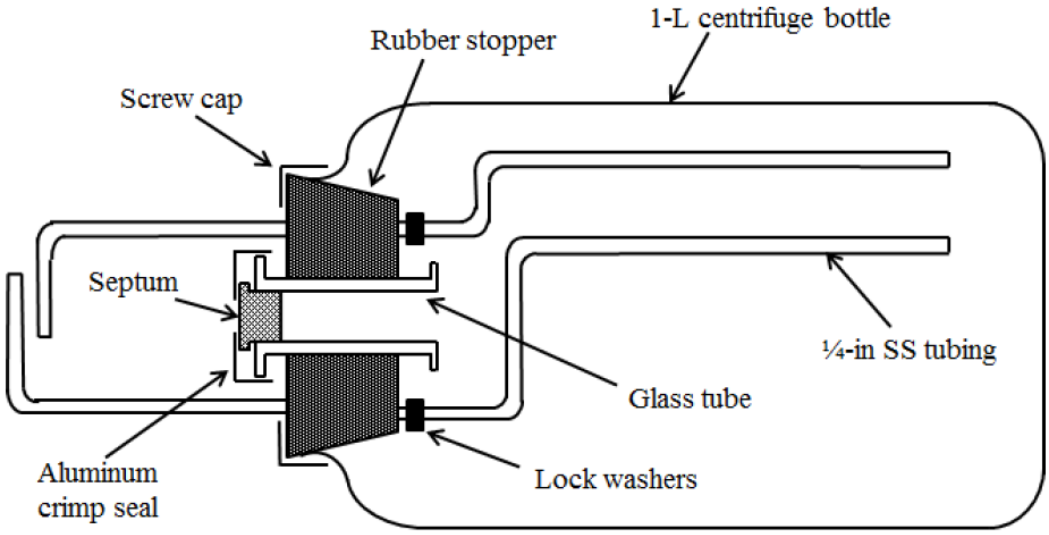


Figure 2-2 Mixed-acid fermentor.

2.2.3 Fermentation Media

For countercurrent fermentation, liquid waste was removed and fresh liquid was periodically replenished into the last fermentor of the fermentation train. Unlike batch fermentations, which typically use de-oxygenated water, de-ionized water was used as the liquid feed in these countercurrent experiments.

2.2.4 Inoculum

The original inoculum was a mixed-culture marine microorganisms from beach sediment in Galveston, Texas. The sediment was collected from the bottom of multiple half-meter-deep shoreline pits. Samples were immediately stored in airtight plastic bottles filled with de-oxygenated water, capped, and frozen at –20°C until use. Before inoculation, samples were thawed, shaken vigorously, and settled by gravity for at least 3 h. The supernatant was used as the inoculum, which occupied 12.5 vol% of the initial fermentation working volume.

To ensure the marine microorganisms can function under the newly introduced environment, inoculum adaptation was required before the experiment began. Alternatively, liquid product from the previous office paper/chicken manure using the same substrate concentration could also be used as inoculum.

2.2.5 Methanogen Inhibition

Iodoform (CHI_3) was used to inhibit methane production, and thus ensure production of carboxylic acids.

Every 2 days, 120 μL iodoform solution (20 g CHI_3/L 200-proof ethanol) was added into each fermentor. Because iodoform is light, temperature, and air sensitive, the solution was stored in a foil-wrapped amber-colored glass bottle at 4℃.

2.2.6 Ion-exchange Resin

According to the study done by Roy, Amberlite® IRA-67 ion-exchange resin (Alfa Aesar, Product No. 42253) was selected.[11] Measured amounts of ion-exchange resin were loaded into a 0.3-L refillable cartridge (Max Water, Part No. 104050) (Figure 2-3).

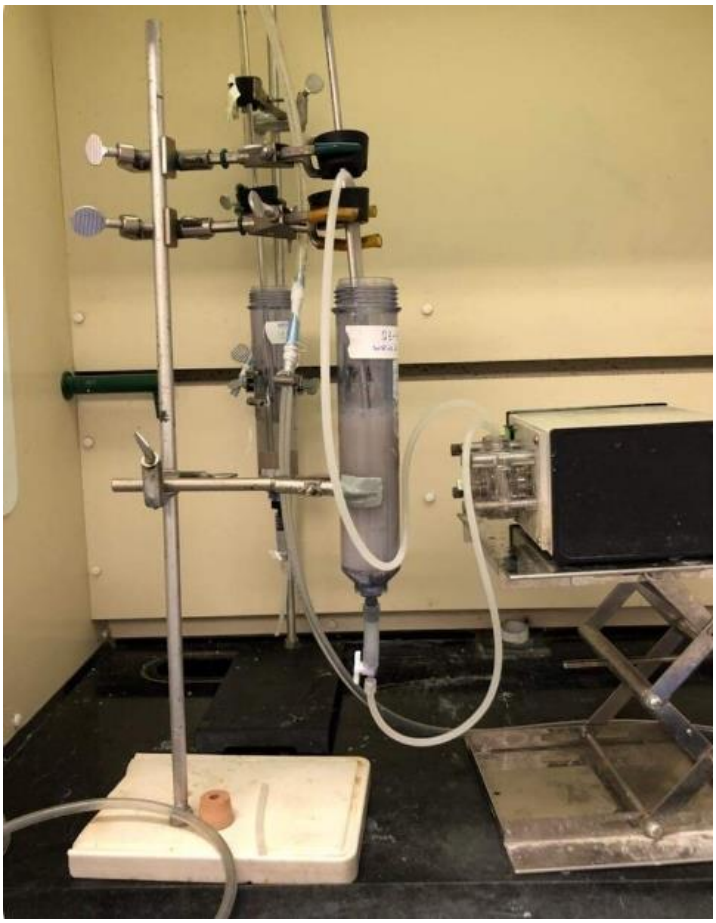


Figure 2-3 Resin adsorption apparatus.

2.2.7 Incubator

This study employed a standing incubator cabinet (Wheaton Modular Cell Production Roller Apparatus) (Figure 2-4). The fermentors were kept in the incubator and rotated horizontally at 2 rpm while maintaining a temperature of 40℃.

Figure 2-4 Thermostatic incubator equipped with horizontal roller.

2.2.8 Experiment Set-up

This experiment was the continuation of the previous study done by Wu, who used 10 to 40 g of resin per 1.38 L of total fermentor working volume.[15] The new study used the identical substrate concentration. The established culture from the previous fermentation was used in the new countercurrent fermentation. The only difference was increased resin loading. In this experiment, 50, 60, and 80 g of Amberlite IRA-67 resin loadings were employed, and they were continued from the culture that used 20, 30, and 40 g resin, respectively. Each specific resin loading was added into individual cartridges (Figure 2-3).

However, because the fermentation had hibernated for a few weeks after the termination of the previous experiment, the countercurrent fermentation was started without involving resin adsorption for one week. After that, the study began when the ion-exchange resin was introduced into the system.

As shown in Figure 2-5, the adsorption column (R) with a known amount of resin was incorporated with countercurrent fermentation (F) with an established culture. The fermentors were ordered respectively as F4, F3, F2, and F1, which are the four different fermentors using the same train. R1, R2, and R3 stand for the three adsorption steps, which were all operated in the same column. A portion of centrifuged fermentation broth was passed through the adsorption column. For example, liquid from F4 passed through R1, F3 passed through R2, and F2 passed through R3. The liquid from each fermentation stage was passed through the same resin, and the resin was not regenerated until the third use. The resin adsorption time in each step was set at 10 min.

As shown in the inset of Figure 2-5, the CO_2-sustained ion-exchange resin adsorption apparatus has a distributor situated in the middle of the cartridge, and its gas outlet is located at the lower part of the distributor. This arrangement facilitates fluidization of the system. The cartridge is connected with a peristaltic pump to circulate the fluid.

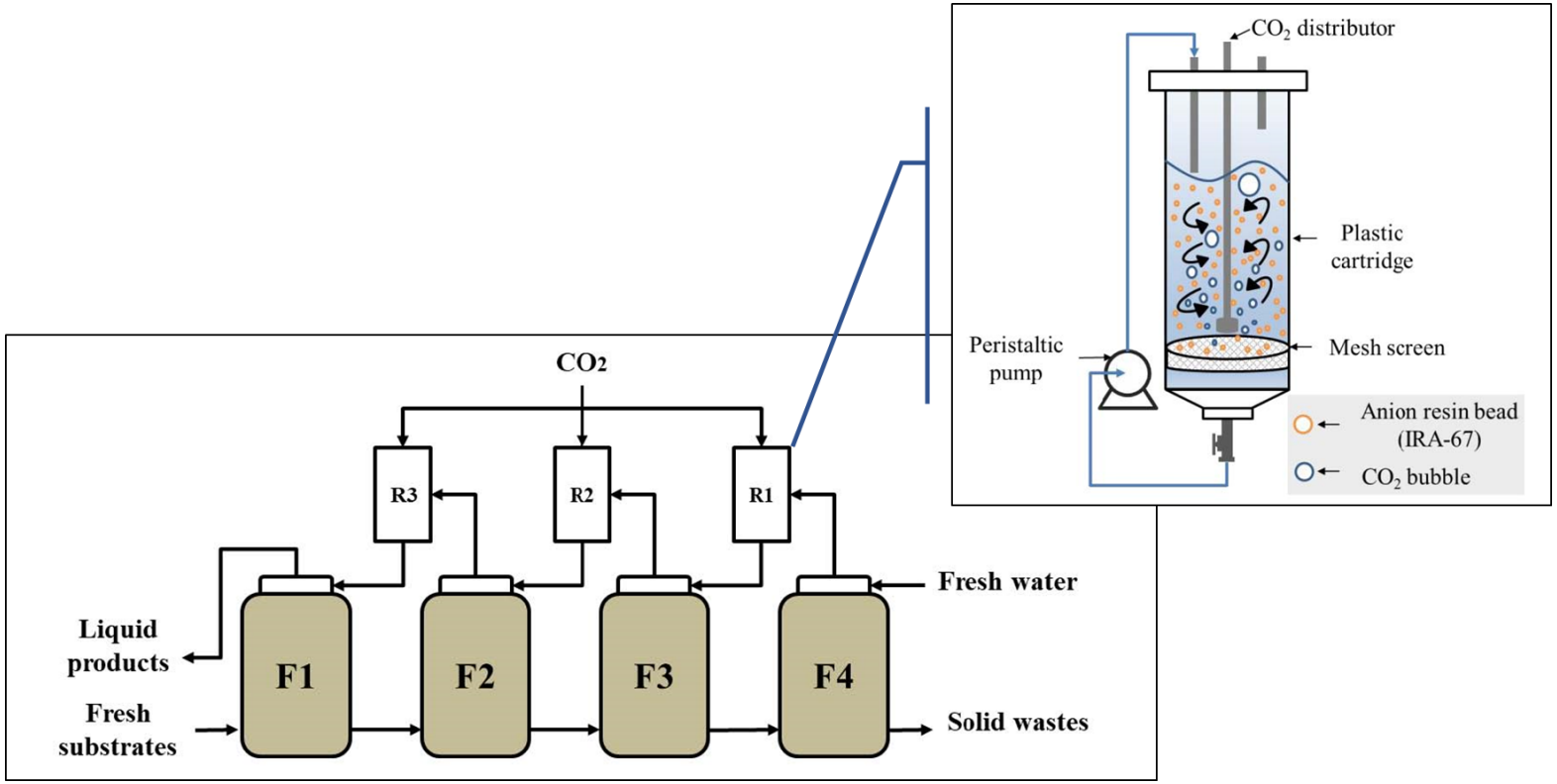


Figure 2-5 Schematic diagram for CO_2-sustained adsorption using ion-exchange resin in countercurrent mixed-acid fermentation. (Adapted from Wu.[15])

2.3 Methods and Calculation

2.3.1 Countercurrent Mixed-acid Fermentation

Three continuous four-stage trains were operated using the countercurrent fermentation apparatus shown in Figure 2-1. Because this experiment continued from an established paper-chicken manure environment, all three trains could bypass the batch fermentation and go directly to countercurrent fermentation.

To perform the experiment, solid and liquid transfers occurred every other day. To maintain the working volume in the fermentor, the mass of total solid and liquid were regulated to a specific set-point (Eq. 2-2). The residence time of the fermentation is based on the frequency of transfer, namely 48 h.

Prior to transfer, fermentors were cooled to room temperature, and the gas volume in each bottle was measured (Paragraph 2.3.5). Each vented fermentor was centrifuged (Beckman Coulter J-6B) at 4000 rpm for 10 min to achieve phase separation, forming liquid and solid cake (approximately 70–80% moisture content). The separated supernatant was sampled and decanted into a clean beaker. The weights of supernatant and retained solid cake were measured to determine the solid/liquid removal by the following equation:

Mass removal = Initial mass

+ mass transferred from other fermentor (or fresh feeding)

– mass set point (2-2)

As shown in Figure 2-1, in each train, liquid was transferred from the more-digested fermentor to the less-digested one; the liquid in F(n) was added into F(n – 1), where n = 2, 3, or 4. Solids were transferred from the less-digested to the more-digested fermentor; the solids in F(k) were added into F(k + 1), where k = 1, 2, or 3. The liquid removed from the least-digested fermentor (F1) is the liquid product, and the solids removed from the most-digested fermentor (F4) is the solid product. Set amounts of fresh substrate (office paper and chicken manure) were added into F1. De-ionized water and urea were added into F4 at specific amounts. A 1.5-mL pre-adsorption liquid sample of each fermentor was taken before the transfer occurred.

Once transfer was completed, post-transfer pH was measured to serve as an indication of buffer addition. Buffer was added into the liquid phase to maintain the pH at a near-neutral range (6.5–7.5), which is a preferred environment for the mixed culture. Buffer addition was required during the first week of "de-hibernation." After the ion-exchange resin was introduced into the experiment, seldom was sodium bicarbonate buffer added. Thereafter, 120 μL of methanogen inhibitor was added into each fermentor. To ensure an anaerobic environment, nitrogen was sparged into the fermentors to purge out oxygen introduced during handling. Lastly, the fermentors were capped with rubber stoppers immediately after purging, then placed back into the incubator.

2.3.2 Moisture and Ash Content

Moisture content (MC) is defined as the fraction of liquid evaporated from the wet sample after one-day heating in the 105°C oven. Volatile solids (VS) are defined as the mass loss from dry solid materials after one-day heating in the 550°C furnace. Ash is defined as the mass of residuals after 24-h combustion in the same furnace.[17]

Ash content (AC) was calculated on a dry basis. Samples were kept in the ceramic crucible and carried in a desiccator to minimize external factors (e.g., moisture adsorption from atmosphere) that could impact the weight. Moreover, to accurately measure volatile acids produced, additional calcium hydroxide ($Ca(OH)_2$) was added prior to weighing the liquid sample. Before drying in the oven, 33 mg $Ca(OH)_2$/(g liquid sample) was added to ensure all volatile acids were converted to their deprotonated form, and hence were non-volatile. Samples were dried in a 105°C oven for 24 h, and were subsequently heated in a 550°C furnace for the other 24 h. Appendix A shows the detailed procedures for this experiment.

2.3.3 Regenerate Ion-exchange resin

To regenerate the Amberlite IRA-67 resin, 1.0-N sodium hydroxide (NaOH) solution was used and passed through the resin column. As described by the IRA-67 manual, one bed volume (BV) is defined as 1 m^3 solution per m^3 resin. The saturated resin was rinsed with 8 BV NaOH solution for 30 minutes under 1.34 mL/s circulation rate.[18] After regeneration, 1.5-mL liquid sample was taken from the effluent. Appendix B shows the detailed procedures for this experiment.

2.3.4 Carboxylic Acids Adsorption

Using the previously described countercurrent fermentation, the ion-exchange resin adsorbed carboxylate anions from the fermentation broth. During each transfer, liquid passed through the CO_2-sustained resin column (R) (Figure 2-3). Each fermentation train employed a specific resin loading (Table 2-1). The presence of carbon dioxide served as buffer during adsorption. The fermentation pH was 6.5–7.5. In the 0.3-L resin column, the CO_2 flowrate was set to 1.5 L/min, which fully fluidized it. The operation time of resin adsorption was 10 min. Then, the circulation created by the peristaltic pump was terminated and the bottom pipeline was replaced with a tube connected to a graduated cylinder to collect the post-adsorption fermentation broth. Without regeneration, the acid-loaded resin was passed with liquid from the subsequent fermentor. This operation was done every 48 hours. A 1.5-mL liquid sample from the resin column drain was taken after each adsorption. Table 2-2 provides operating parameters for the experiment.

Table 2-1 Wet resin loading and normalized resin loading

Train	**Previous experiment**[15]				**T1**	**T2**	**T3**
Wet resin loading (g)	10	20	30	40	50	60	80
Normalized resin loading (g wet resin/L_{liq})	7.20	14.28	21.74	29.20	37.32	44.78	59.70

(Note: For T1 to T3, the total liquid volume (L_{liq}) = 1.34 L, and L_{liq} = 1.38 L for 10–40 g wet resin loadings.)

Table 2-2 Operating parameters for CO_2-sustained adsorption using ion-exchange resin on countercurrent fermentation

	Train	**T1**	**T2**	**T3**
Controlled parameters	Transfer frequency, *T* (h)	48	48	48
	Office paper (g/*T*)	10.2	10.2	10.2
	Oven-dried chicken manure (g/*T*)	2.4	2.4	2.4
	Urea (g/*T*)	0.2	0.2	0.2
	Fresh water feed rate (mL/*T*)	120	120	120
	Solid set-point in each fermentor (g)	200 (250 for F4)	200 (250 for F4)	200 (250 for F4)
	Liquid set-point in each fermentor (g)	200	200	200
	Inhibitor (μL)*	120	120	120
	Resin loading (g)	50	60	80
	Carbon dioxide flow rate during adsorption (L/min)	1.5	1.5	1.5
	Adsorption time (min)	10	10	10
	Regeneration time (min)	35	35	35

*The concentration of the methanogen inhibitor is 20 g CHI_3/L 200-proof ethanol.

2.3.5 Biogas measurement

The amount of gas produced by the fermentation is a factor that indicates fermentation activity and steady acid production. Because of mechanical limitations of the fermentors and the continuous biogas formation, it is crucial that the fermentors be vented periodically. At a two-day basis, biogas was vented by inserting a needle through the fermentor rubber septum. The amount of biogas was measured by connecting the needle to an inverted glass graduated cylinder, which contains 300 g $CaCl_2$/L solution (Figure 2-6). These measures prevent carbon dioxide adsorption and microbial growth. A vacuum pump lifted the water level prior to adding the gas.

Figure 2-6 Biogas measuring apparatus.

2.3.6 Carboxylic Acids Determination

To determine the carboxylic acid concentrations, samples from the countercurrent fermentation were analyzed by a gas chromatograph (GC, Agilent 6890 series) equipped with an automatic liquid sampler (Agilent 7683 series).

The frozen samples were thawed, vortexed to ensure uniform liquid concentration, and centrifuged to remove solids (Beckman Coulter Microfuge® 16) at a revolution speed of 13,300 rpm for 10 min. Because the products of the mixed-acid fermentation are carboxylic salts, they are acidified using phosphoric acid to ensure the carboxylic acids are volatile for GC analysis.

Supernatant (0.5 mL) from each sample was mixed with 0.5 mL of 3-M phosphoric acid (H_3PO_4) and 0.5 mL of internal standard solution (isocaproic acid, 1.16 g/L) to form the intermediate. The intermediate solution was centrifuged (13,300 rpm, 10 min) once again, then transferred into a glass vial for further GC analysis.

The GC system employs a 30-meter-long fused-silica capillary column (J&W Scientific, Model # 123-3232). The column head pressure was maintained at 2 atm abs. After sample injection, the GC temperature raised from 40 to 200°C at 20°C/min, and was subsequently held at 200°C for 2 min. The total run time for each sample was 11 min. Helium was used as the carrier gas. An external standard carboxylic acids mixture, which was injected before sample injection, was prepared for calibration (Table 2-3). Specific procedures for measuring carboxylic acid concentrations are described in Appendix C.

Table 2-3 Carboxylic acid concentration in external standard solution

Acid	Concentration (g/L)
Acetic Acid	4.000
Propionic Acid	3.030
Isobutyric Acid	1.002
Butyric Acid	1.999
Isovaleric Acid	0.807
Valeric Acid	1.570
Isocaproic Acid	1.160
Caproic Acid	0.812
Heptanoic Acid	0.399
Octanoic Acid	0.169

2.3.7 Fermentation Performance

Non-acid volatile solids (NAVS) aims to measure the mass of digestible solids, which is defined as the mass difference of volatile solids (VS) and carboxylic acids in the biomass.

$$\text{NAVS} = (\text{g total wet biomass})(1 - \text{MC})(1 - \text{AC}) - (\text{g carboxylic acids in wet biomass}) \quad (2\text{-}3)$$

where MC is the fraction of moisture of wet biomass and AC is the fraction of ash in dry biomass.

The volatile solid loading rate (VSLR) and liquid residence time (LRT) were regulated by controlling the transfer frequency (*T*), feed rates of NAVS and fresh water, and the amounts of centrifuged solid cake and centrifuged liquid retained in each fermentor. Table 2-4 shows the normalized parameters for the mean of steady-state values.

$$\text{VSLR} = \frac{\text{NAVS}_{\text{feed}}(\text{g})}{\text{Total liquid volume in all fermenters (L)} \cdot T(\text{day})} \quad (2\text{-}4)$$

$$\text{LRT} = \frac{\text{Total liquid volume in all fermenters (L)}}{\text{Flowrate out of fermentation train } (\frac{\text{L}}{\text{day}})} \quad (2\text{-}5)$$

Table 2-4 Normalized parameters for CO_2-sustained adsorption using ion-exchange resin on countercurrent fermentation

	Train	T1	T2	T3
Normalized parameters	Volatile solid loading rate, VSLR (g NAVS/(L_{liq}·d))	3.62 ± 0.03	3.64 ± 0.02	3.61 ± 0.04
	Liquid residence time, LRT (d)	33.9 ± 3.51	30.5 ± 1.29	31.8 ± 1.16
	Total liquid volume, TLV (L)	1.34 ± 0.01	1.33 ± 0.01	1.34 ± 0.01
	Total NAVS (g)	188.1 ± 14.7	203.3 ± 5.4	187.8 ± 8.0
	Volatile solid concentration (g NAVS/L_{liq})	140.5 ± 12.1	152.5 ± 4.8	139.8 ± 6.6
	Carbon-nitrogen ratio in feed, C-N ratio (g carbon/g nitrogen)	30.31 ± 0.00	30.31 ± 0.00	30.31 ± 0.00
	Urea addition rate (g urea/(L_{liq}·d))	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00

(Note: The errors are derived from 95% confidence intervals.)

Fermentation performance – conversion, yield, selectivity, and productivity – were analyzed during the steady-state period. The average rates of NAVS, products, and wastes were calculated using the Slope Method.[19] In this method, the accumulation of each component is plotted with respect to time, and the slope is the average rate. The performance parameters can be interpreted by the average rate using the following definitions:

$$\text{Conversion } (x) \equiv \frac{\text{rate } [\text{NADS}_{\text{feed}}(\text{g/d}) - \text{NADS}_{\text{exit}}(\text{g/d})]}{\text{rate } [\text{NAVS}_{\text{feed}}(\text{g/d})]}$$

$$= \frac{\text{rate } [(\text{NAVS}_{\text{feed}}(\text{g/d}) + \text{Ash}(\text{g/d})) - (\text{NAVS}_{\text{exit}}(\text{g/d}) + \text{Ash}(\text{g/d}))]}{\text{rate } [\text{NAVS}_{\text{feed}}(\text{g/d})]}$$

$$= \frac{\text{rate } [\text{NAVS}_{\text{digested}}(\text{g/d})]}{\text{rate } [\text{NAVS}_{\text{feed}}(\text{g/d})]} \quad (2\text{-}6)$$

$$\text{Yield } (Y) \equiv \frac{\text{rate } [\text{Total acid produced } (\text{g/d})]}{\text{rate } [\text{NAVS}_{\text{feed}}(\text{g/d})]} \quad (2\text{-}7)$$

$$\text{Selectivity } (s) \equiv \frac{\text{rate } [\text{Total acid produced } (\text{g/d})]}{\text{rate } [\text{NAVS}_{\text{digested}}(\text{g/d})]} \quad (2\text{-}8)$$

$$\text{Productivity } (P) \equiv \frac{\text{Total acid produced (g)}}{\text{Total liquid combined in all fermenters (L)} \cdot \text{Time (day)}} \quad (2\text{-}9)$$

where the term NADS (non-acid dry solid) is defined as the combination of NAVS and ash in biomass. The $\text{NADS}_{\text{feed}}$ and $\text{NADS}_{\text{exit}}$ are the NADS fed into and removed from the fermentation train, respectively. Throughout the fermentation, NAVS were gradually digested and transformed into carboxylic acid products. $\text{NAVS}_{\text{feed}}$ and

$NAVS_{exit}$ are the NAVS fed into and removed from the fermentation train, respectively. "Total acid produced" represents the amount of carboxylic acid produced each day. Ash is assumed to stay constant (Figure 2-7).

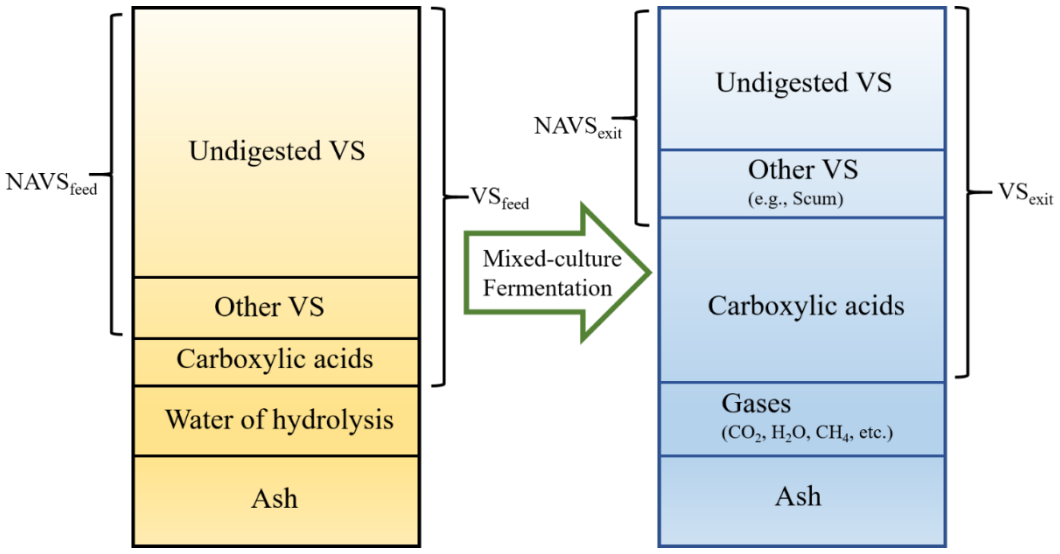


Figure 2-7 Schematic diagram on biomass conversion.

The product consists of short (C2) to long (C8) chains of carboxylic acids, where acetic acid, propionic acid, and butyric acid are the major products. Other carboxylic acids are less concentrated. Acetic acid equivalents (Aceq) are used to express the mixture with various carboxylic acids concentration as a single acid concentration. It equates the reducing potential of a carboxylic acid mixture to an energy-equivalent mass of acetic acid.[20]

$$\text{Aceq}\left(\frac{\text{mol}}{\text{L}}\right) = 1.00 \times [\text{acetic}]\left(\frac{\text{mol}}{\text{L}}\right) + 1.75 \times [\text{propionic}]\left(\frac{\text{mol}}{\text{L}}\right) + 2.50 \times [\text{butyric}]\left(\frac{\text{mol}}{\text{L}}\right) + 3.25 \times [\text{valeric}]\left(\frac{\text{mol}}{\text{L}}\right) + 4.00 \times [\text{caporic}]\left(\frac{\text{mol}}{\text{L}}\right) + 4.75 \times [\text{heptanoic}]\left(\frac{\text{mol}}{\text{L}}\right) + 5.5 \times [\text{octanoic}]\left(\frac{\text{mol}}{\text{L}}\right) \quad (2\text{-}10)$$

$$\text{Aceq}\left(\frac{\text{g}}{\text{L}}\right) = 60.05\left(\frac{\text{g}}{\text{mol}}\right) \times \text{Aceq}\left(\frac{\text{mol}}{\text{L}}\right) \quad (2\text{-}11)$$

Therefore, the yield can be expressed in terms of Aceq:

$$\text{Yield}\left(Y_{\text{Aceq}}\right) = \frac{\text{rate [Total Aceq produced (g/d)]}}{\text{rate [NAVS}_{\text{feed}}\text{ (g/d)]}} \quad (2\text{-}12)$$

2.4 Results and Discussion

2.4.1 Product Concentration

For each sample taken from the fermentors during the countercurrent fermentation, GC analysis determined the carboxylic acids composition and concentration. Trains 1, 2, and 3 (T1, T2, and T3) represent 50, 60, and 80 g resin, respectively. They were the continuation of previous experiment using 20, 30, and 40 g loading in the 1.38 L of total fermentor working volume.

As shown in Figures 2-8 to 2-10, Trains 1 and 2 fermented for approximately 150 days, whereas Train 3 was only 60 days. For Trains 1 and 2, the entire experiment had three parts: (1) previous resin loading, (2) current resin loading in Spring 2018, and (3) current resin loading in Summer 2018. In contrast, Train 3 had only two parts: (1) previous resin loading, and (2) current resin loading in Summer 2018. The acid profiles of Trains 1 and 2 show the total acid concentration reached steady state in the Summer 2018 section. The fluctuation of product concentration is relatively moderate compared to the Spring 2018 section. For Train 3, regardless of the loading amount, its acid profile was steady since the introduction of ion-exchange resin. Overall, the total carboxylic acid concentration in the liquid was relatively consistent from 13 to 15 g/L (Table 2-5).

Table 2-5 Fermentation performance for all trains

	Train	**T1**	**T2**	**T3**
Performance parameters	Resin (g)	50	60	80
	Total acids concentration (g/L)	14.95 ± 0.42	13.72 ± 0.41	13.08 ± 0.25
	Aceq (g/L)	19.41 ± 0.56	18.02 ± 0.60	17.78 ± 0.46
	Aceq/Acid Ratio	1.30 ± 0.01	1.31 ± 0.01	1.36 ± 0.02
	Conversion (g $NAVS_{digested}$/g $NAVS_{feed}$)	0.317	0.315	0.353
	Yield (g total carboxylic acid produced/g $NAVS_{feed}$)	0.215	0.229	0.211
	Selectivity (g carboxylic acid produced/g $NAVS_{digested}$)	0.679	0.726	0.600
	Productivity (g total carboxylic acid produced/(L_{liq} · day))	0.774	0.832	0.762

(Note: The errors are derived from 95% confidence intervals.)

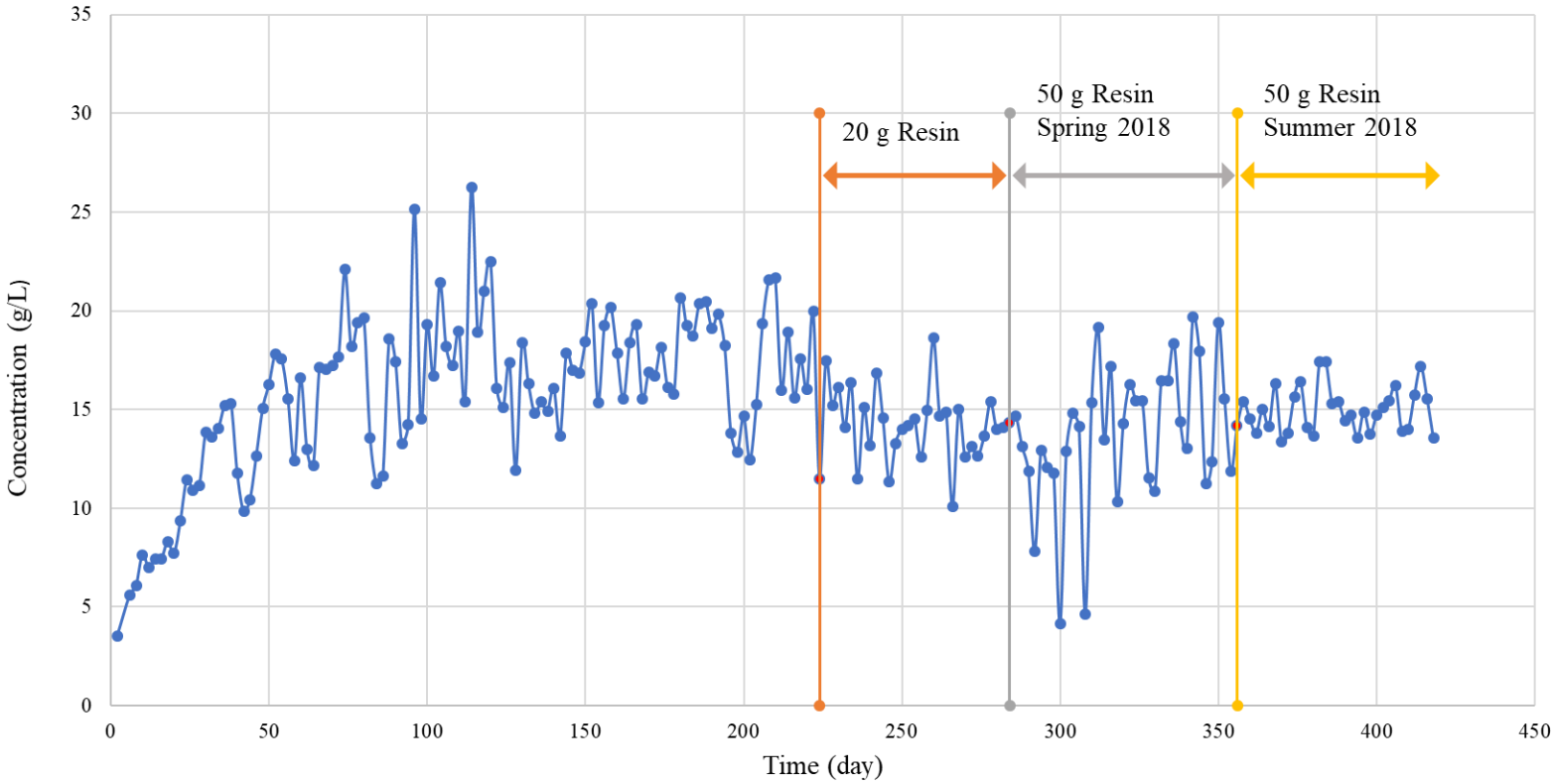


Figure 2-8 Total acid concentration profile of Train 1 exiting F1. (This research based on yellow arrow sector.)

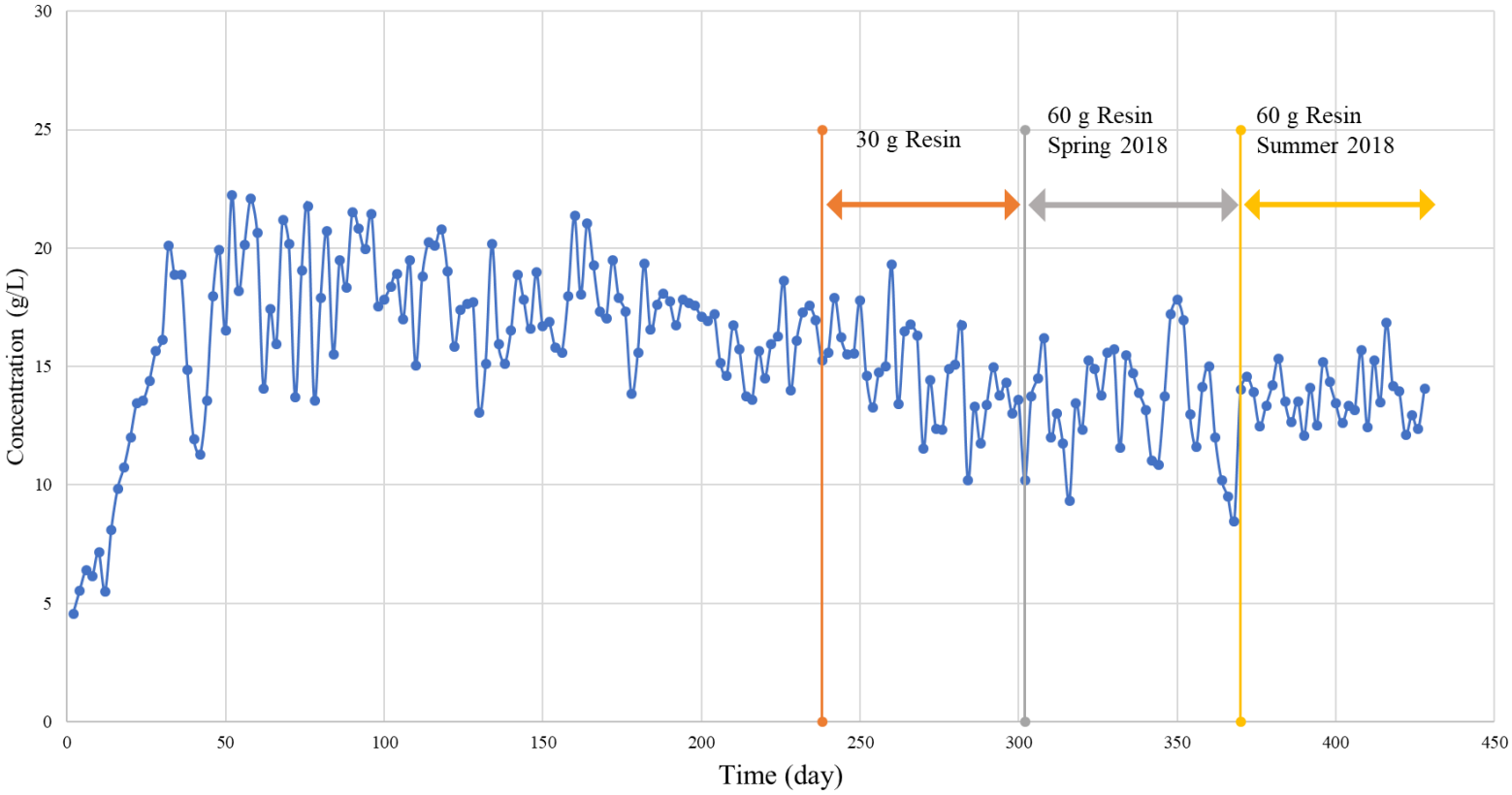


Figure 2-9 Total acid concentration profile of Train 2 exiting F1. (This research based on yellow arrow sector.)

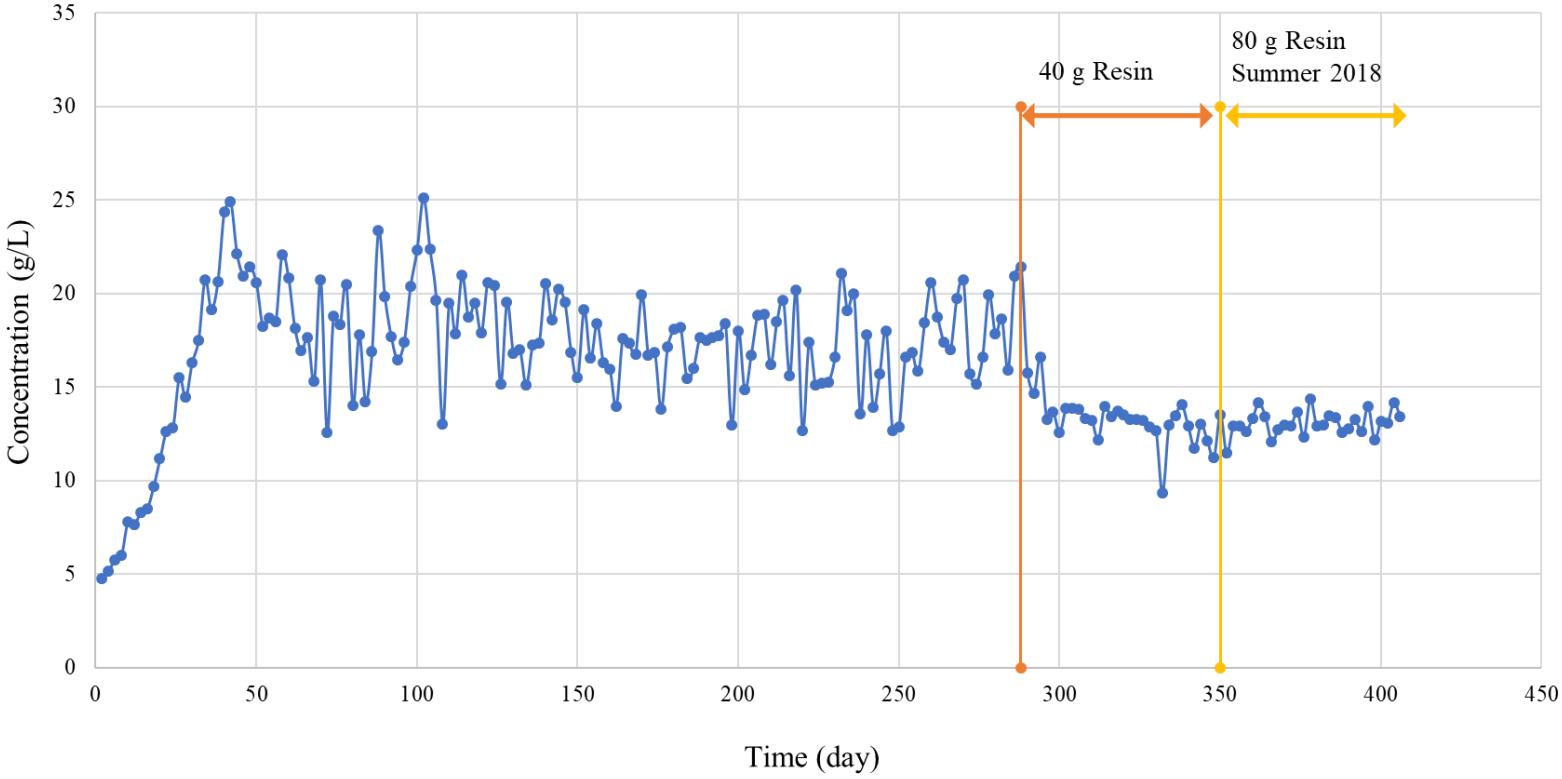


Figure 2-10 Total acid concentration profile of Train 3 exiting F1.

Furthermore, regarding the total acid concentration in each fermentor (F1–F4) of each train (T1–T3), the least-digested bottle (F1) produces the greatest concentration of carboxylic acids. When more biomass digests, less carboxylic acids form. For Trains 1 and 2, the acid concentrations in F2, F3, and F4 reached steady state during the experiment, and they have less fluctuation and a narrower confidence interval than F1. (See Figures 2-11 and 2-12, and Table 2-6.)

For Train 3, the acid concentrations in F2, F3, and F4 decreased until Day 20. Afterward, the concentrations increased and reached steady state for approximately one month. Because the greater resin loading had a greater acid adsorption potential, the concentrations initially declined because of the sudden change in environment. Only the liquid from F2, F3, and F4 passed through the ion-exchange resin; thus, the liquid product concentration varied less (Table 2-6). As the fermentation continued, the system gradually adapted to the new "rapid-acid-draining" environment. The acid concentration returned to where it started, and maintained steady. This demonstrates the outstanding endurance and environmental adaptability of the mixed-culture system (Figure 2-13).

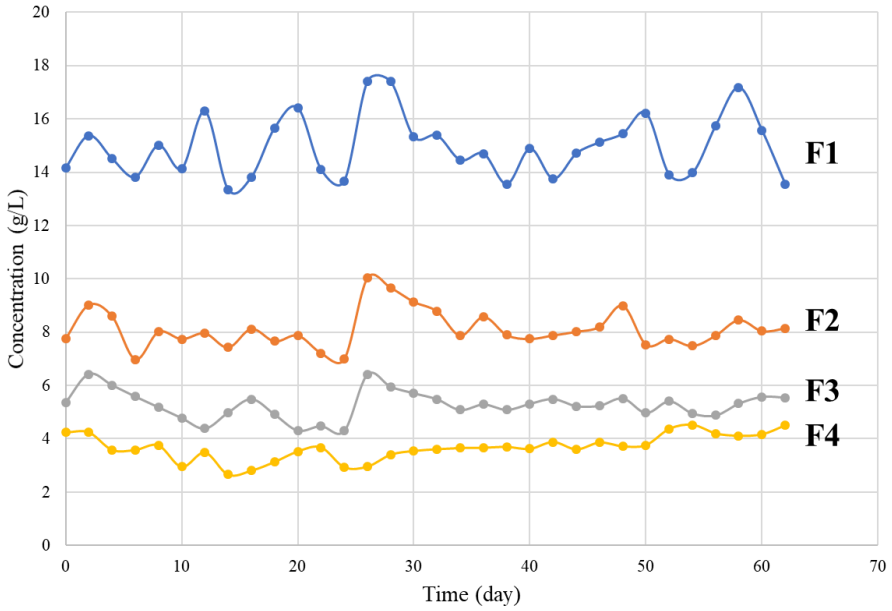


Figure 2-11 Total acid concentration during steady-state peroid in fermentors in Train 1.

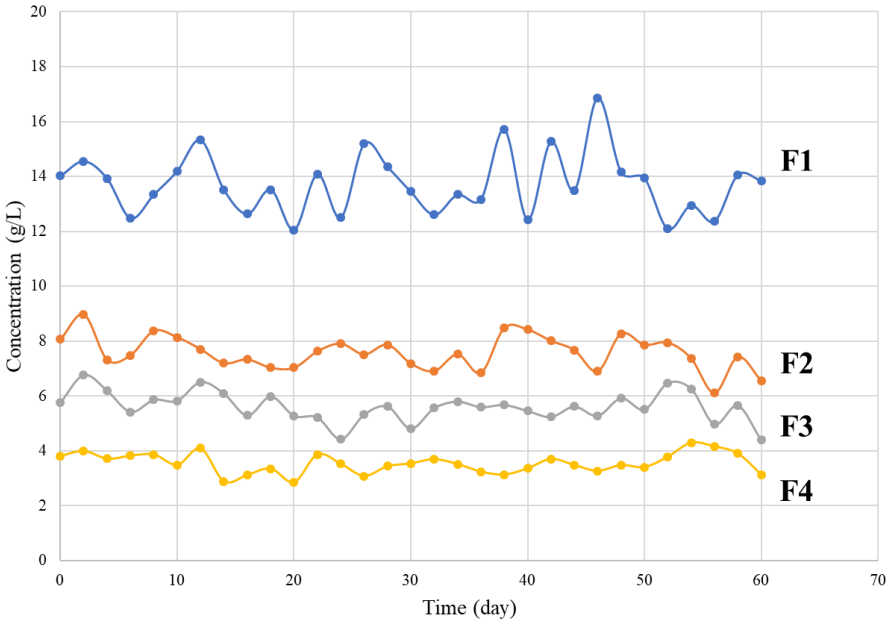


Figure 2-12 Total acid concentration during steady-state peroid in fermentors in Train 2.

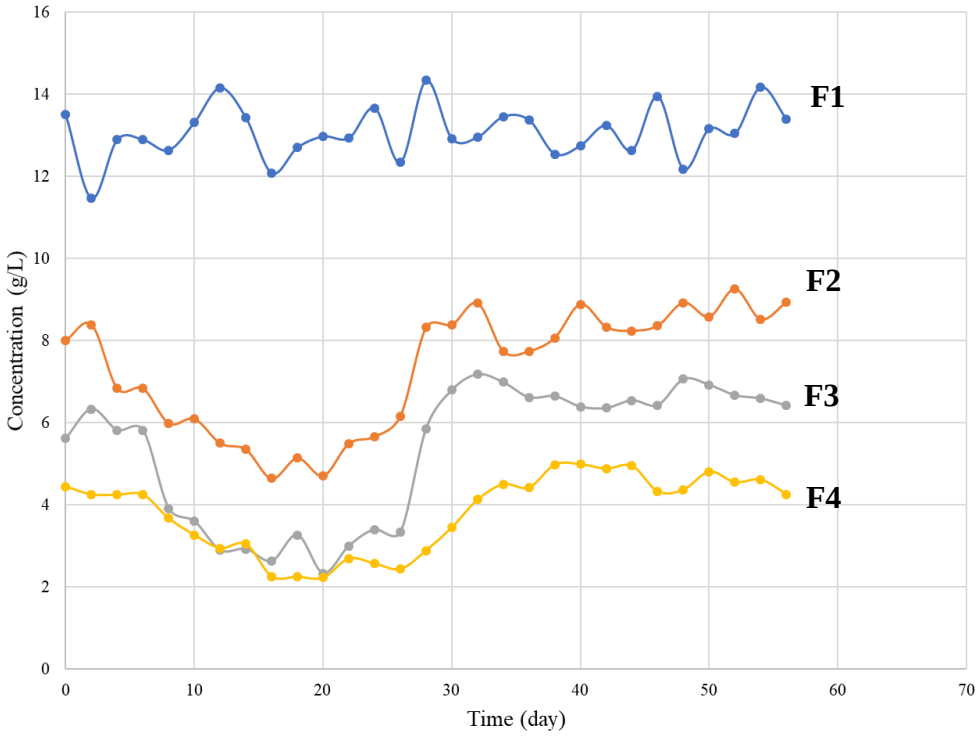


Figure 2-13 Total acid concentration during steady-state peroid in fermentors in Train 3.

Table 2-6 Total acid concentration during steady-state peroid in each train each fermentor

	T1	**T2**	**T3**
F1	14.95 ± 0.42	13.72 ± 0.42	13.08 ± 0.25
F2	8.11 ± 0.26	7.58 ± 0.23	7.32 ± 0.56
F3	5.27 ± 0.19	5.60 ± 0.20	5.33 ± 0.64
F4	3.67 ± 0.17	3.55 ± 0.14	3.82 ± 0.36

(Note: The errors are derived from 95% confidence intervals)

2.4.2 Fermentation Performance

Fermentation performance was determined by the Slope Method, which reduces the error significantly because it calculates parameters based on long-term trends instead of averaging instantaneous rates.[19] Figure 2-14 illustrates the aggregated amount of $NAVS_{feed}$, total acid, and $NAVS_{exit}$ during the steady-state period in all trains. The slopes of the linear-regression models were applied to calculate the fermentation conversion (Eq. 2-6), yield (Eq. 2-7), and selectivity (Eq. 2-8). The values are recorded in Table 2-5.

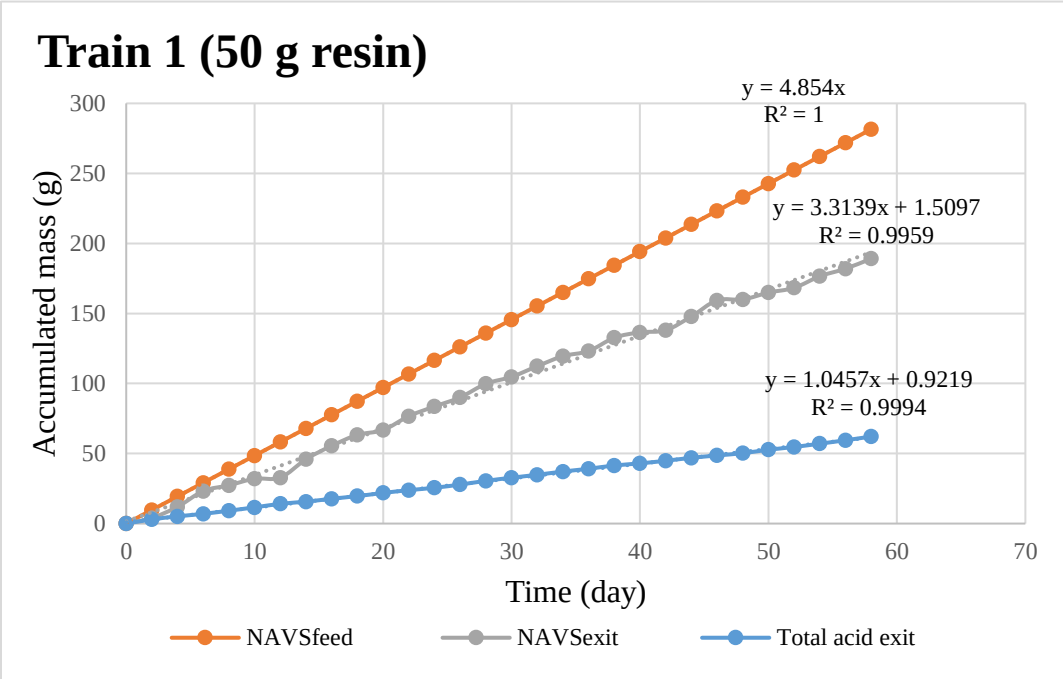
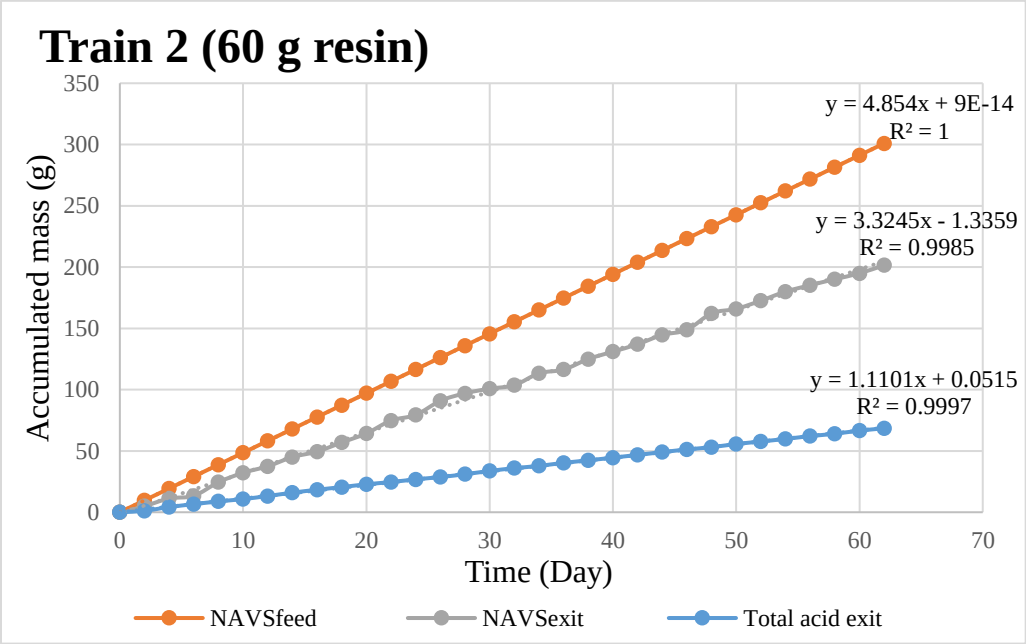


Figure 2-14 Accumulations of the $NAVS_{feed}$, $NAVS_{exit}$, and total acid exit for countercurrent fermentation trains during the steady-state peroid.



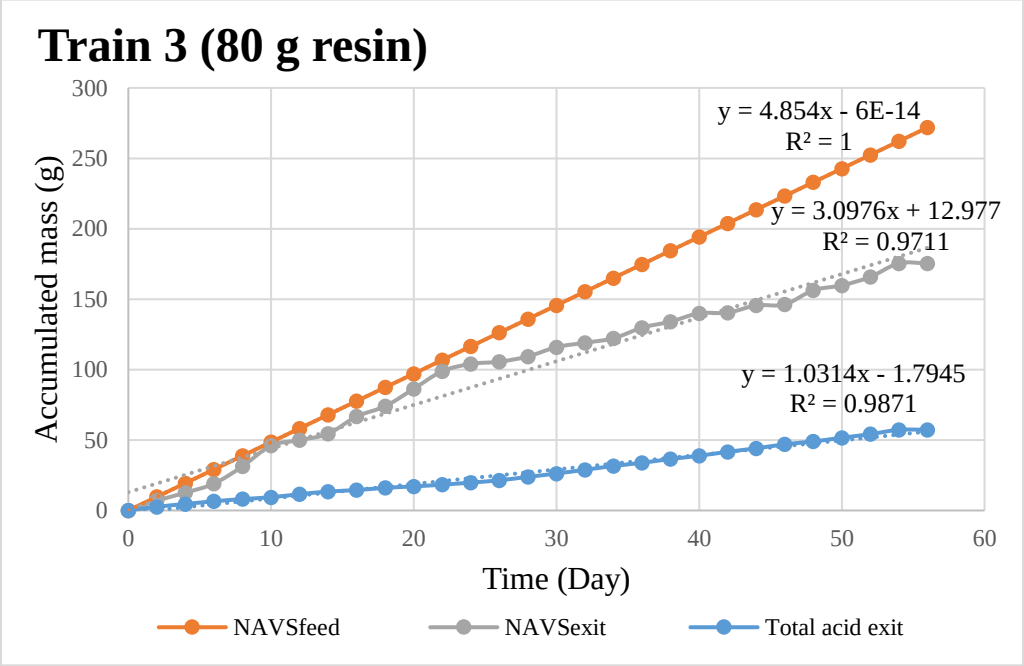


Figure 2-14 continued.

In all countercurrent fermentations that employed resin adsorption, the conversion and yield are all better than the control groups, which did not incorporate ion-exchange resin. Both performance parameters did not linearly increase. Yield reached a plateau (Figure 2-15) and conversion reached a peak (Figure 2-16). Selectivity fluctuated near 0.65 g total carboxylic acid produced/g $NAVS_{digested}$ without obvious trends (Figure 2-17).

The results suggest the optimal amount of resin loading is about 40 g for the 1.38 L of fermentor working volume. Train 2 (60 g resin) exhibits similar yield as 40 g resin; however, using less resin is more economically viable.

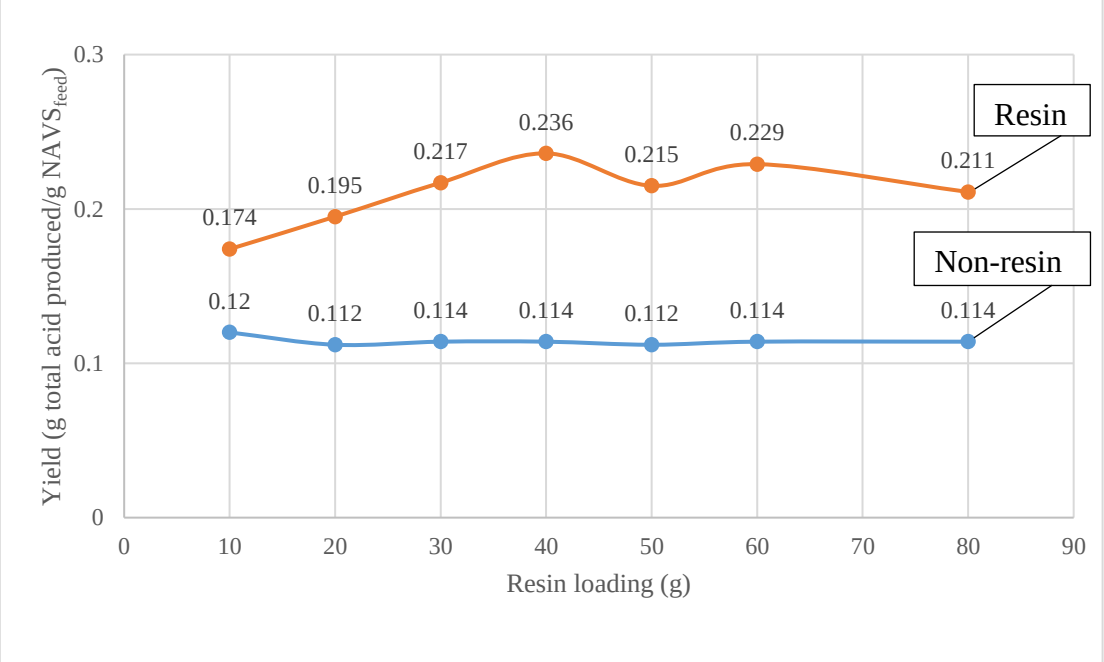


Figure 2-15 Yield versus resin loading during steady-state peroid. (Note: L_{liq} = 1.38 L for 10 to 40 g; 1.34 L for 50 to 80 g.)

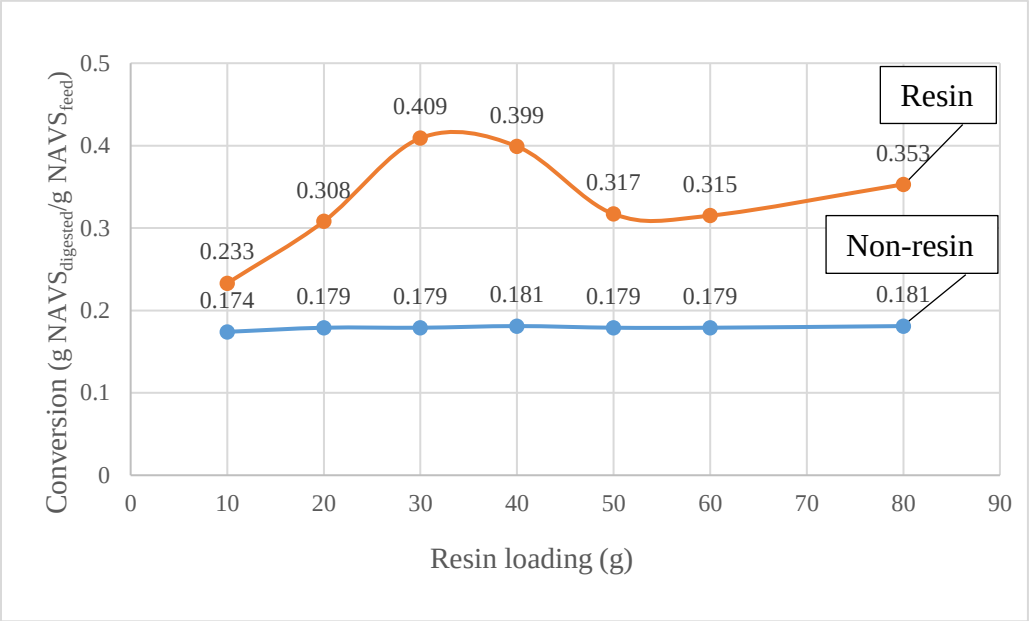


Figure 2-16 Conversion with resin loading during steady-state peroid. (Note: L_{liq} = 1.38 L for 10 to 40 g; 1.34 L for 50 to 80 g.)

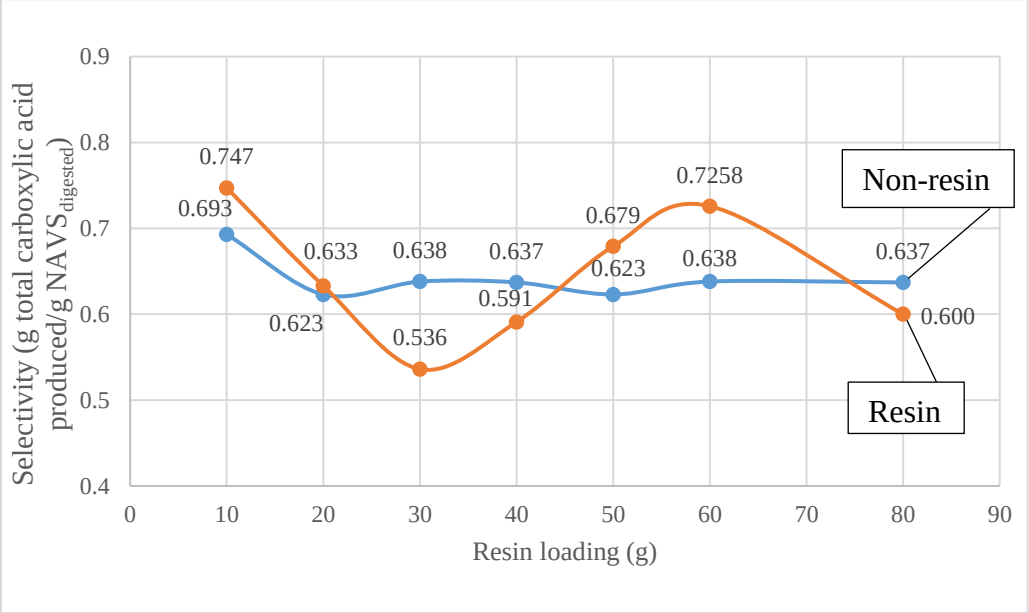


Figure 2-17 Selectivity with resin loading during steady-state peroid. (Note: L_{liq} = 1.38 L for 10 to 40 g; 1.34 L for 50 to 80 g.)

For industrial applications, the results are meaningful when they are expressed in a normalized manner (Figure 2-18). The values of normalized resin loading are shown in Table 2-1.

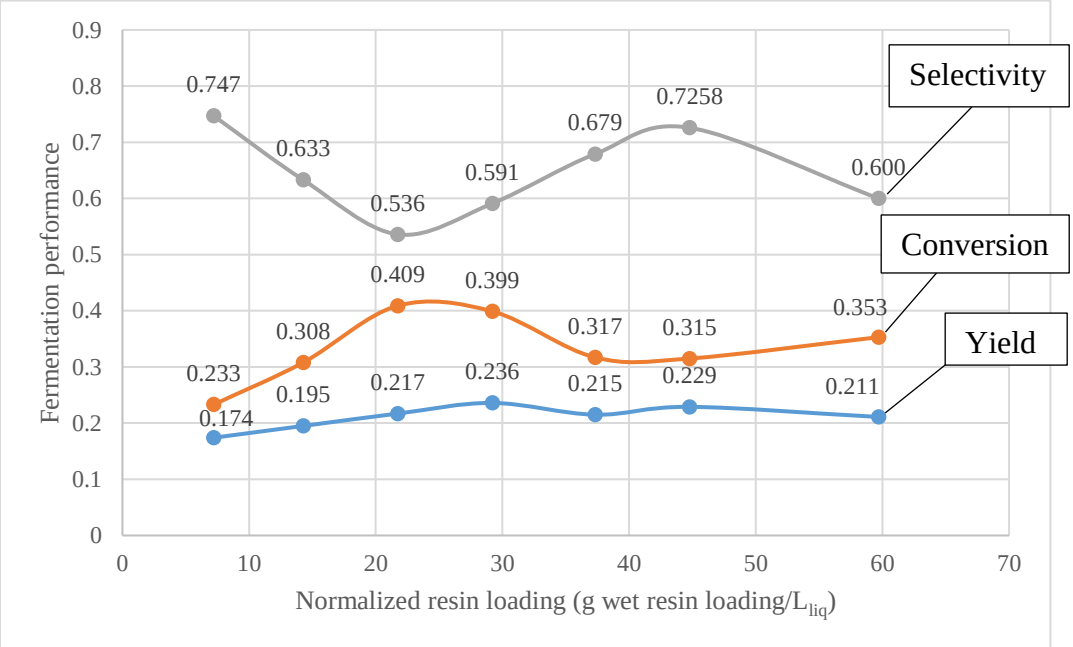


Figure 2-18 Fermentation performance with normalized wet resin loading.

2.4.3 Resin Adsorption Performance

IRA 67 ion-exchange resin was regenerated using 1-N NaOH solution. The regeneration trend was similar to the total acid adsorption. Most of the adsorbed acids on the resin could be successfully recovered (Figure 2-19).

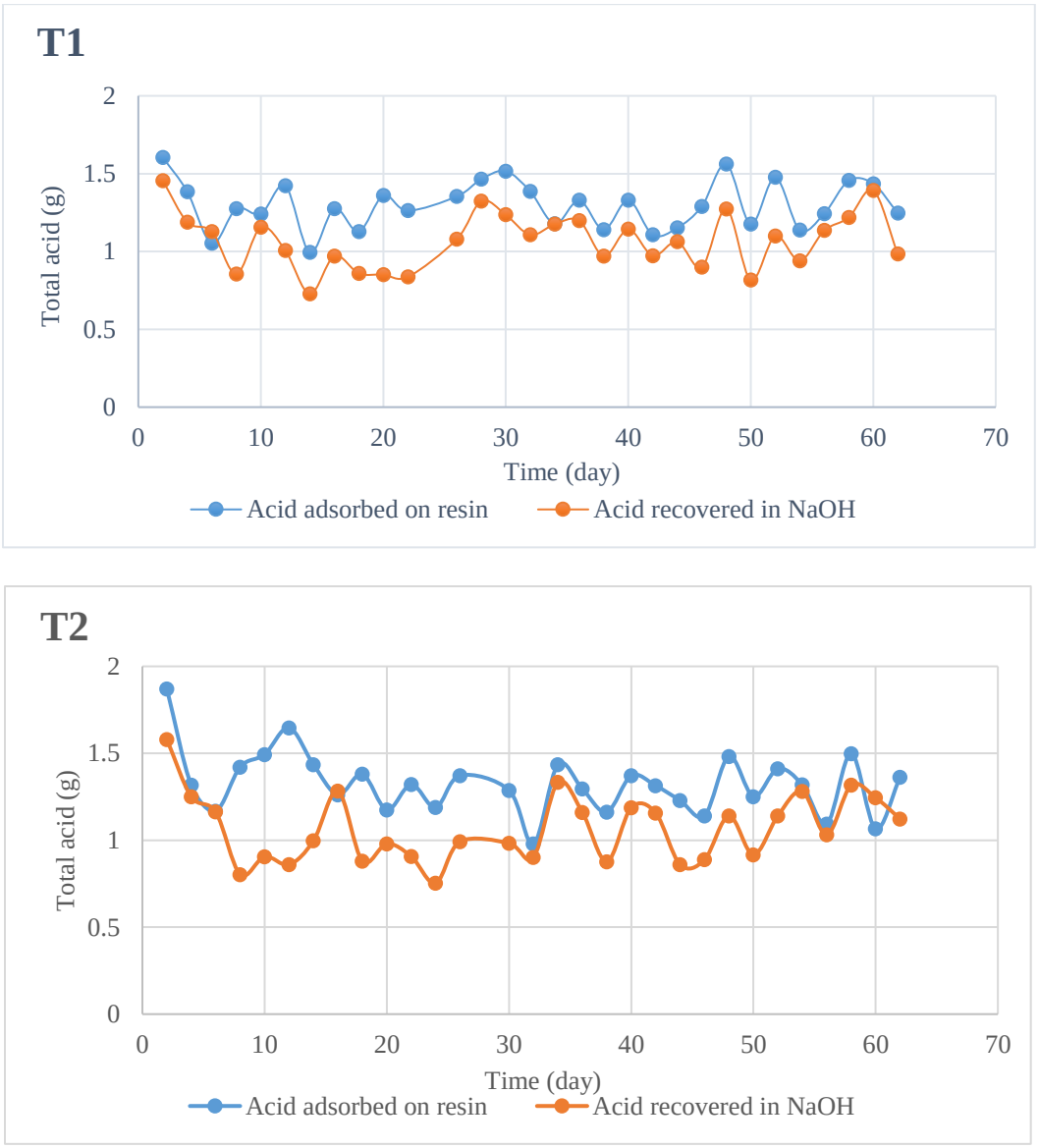


Figure 2-19 Acid adsorption and recovery.

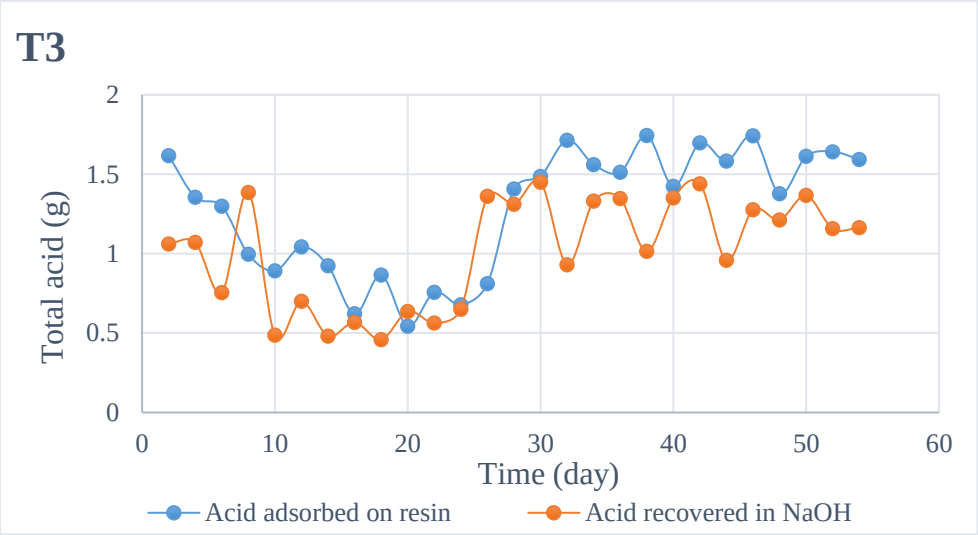


Figure 2-19 continued.

Figures 2-20 and 2-21 show the total carboxylic acid and Aceq concentrations, respectively. The figures include the results from the previous study (10–40 g resin loadings).[15] For total acid and Aceq concentrations, the 50 g resin loading had slightly higher values than the 60 and 80 g resin loadings, and only the 50 g resin loading exhibited better total acid and Aceq concentrations than its previous experiment (20 g). Among all trains, both total acid and Aceq concentrations were similar.

Figure 2-22 shows the total acid production and the proportion distributed between liquid and resin. After introducing ion-exchange resin, the acid in the liquid product slightly decreased; instead, additional acid production was adsorbed by resin. Compared to the control group, total acid production significantly increased and reached a peak at 40 g resin loading. The average amount of acid adsorbed by resin in 40 g loading is 0.685 g/d, and the total production is 1.155 g/d.

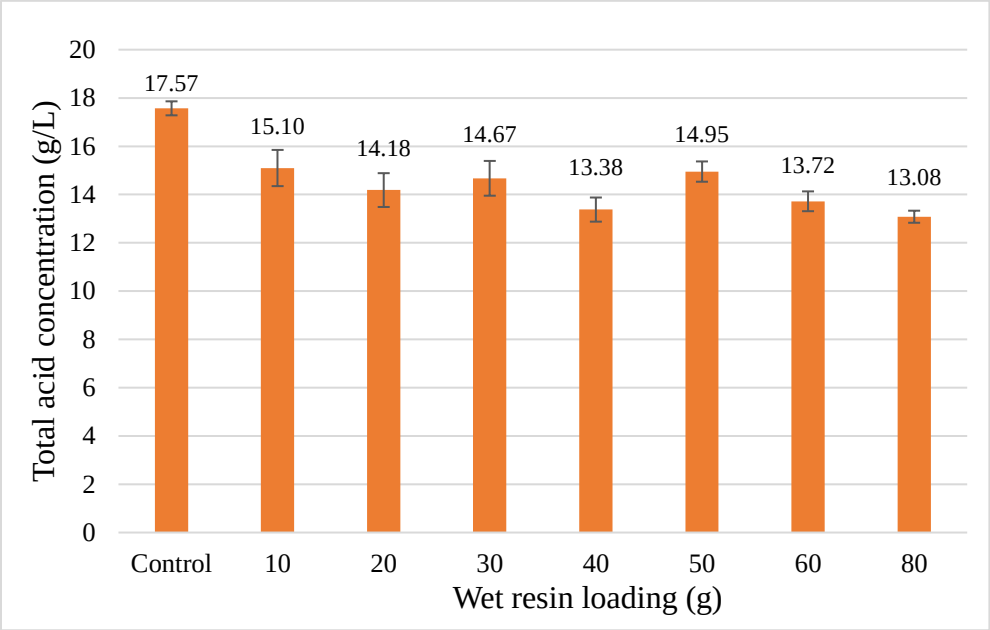


Figure 2-20 Total carboxylic acid concentration in liquid product of 10–80 g resin loadings in a total liquid volume of 1.38 L in 10–40 g, and 1.34 L in 50, 60, and 80 g. (Note: The errors are derived from 95% confidence intervals)

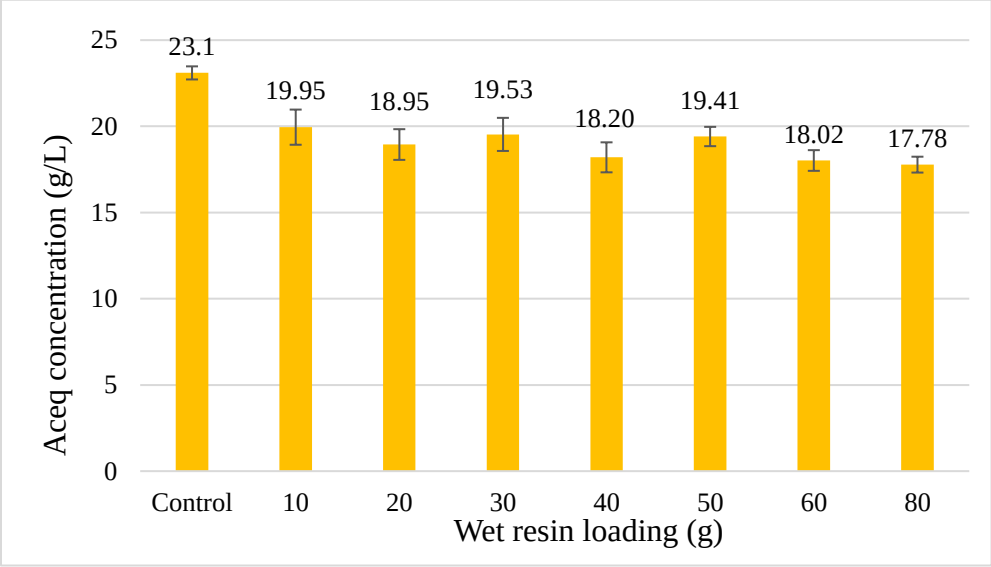


Figure 2-21 Total Aceq concentration in liquid product of 10–80 g resin loadings in a total liquid volume of 1.38 L in 10–40 g, and 1.34 L in 50, 60, and 80 g. (Note: The errors are derived from 95% confidence intervals.)

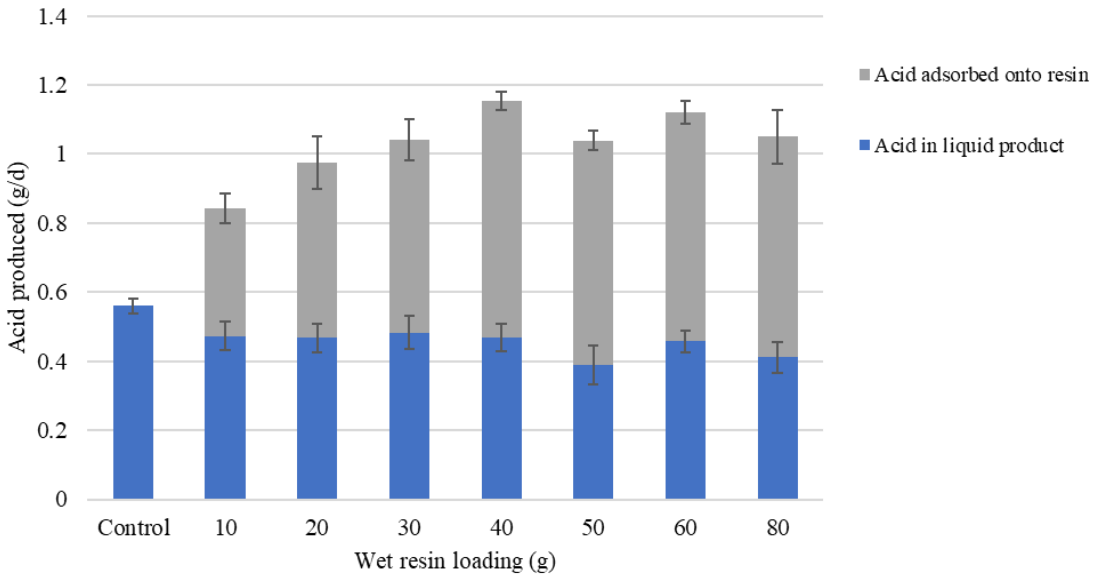


Figure 2-22 Total acid production contributed by liquid product and resin adsorption of 10–80 g resin loadings in a total liquid volume of 1.38 L in 10–40 g, and 1.34 L in 50, 60, and 80 g. (Note: The errors are derived from 95% confidence intervals.)

Figures 2-23, 2-24, and 2-25 show individual carboxylic acid spectra in the liquid product, adsorbed resin bed, and overall system (i.e., liquid product and resin adsorption), respectively. For liquid product, higher resin loadings did not significantly change the carboxylic acid composition profiles in the liquid product. The primary components were acetic acid, propionic acid, and butyric acids. The acetic acid fraction slightly decreased and propionic acid fraction slightly increased as the resins were introduced. Moreover, the fractions of longer-chain carboxylic acids, such as butyric acid and valeric acid, were slightly increased in 80 g resin loading.

The regeneration process captured most of the carboxylic acid adsorbed by the ion-exchange resin. The acid composition profile shown in Figure 2-24 shows the total acids that adsorbed during the three adsorptions between F2, F3, and F4. At resin

loadings higher than 30 g, the resin adsorbed more propionic acid than acetic acid. Compared to the 10 and 20 g resin loadings, the mass fractions of acetic and propionic acids are similar. The greatest mass fraction of propionic acid occurred in 50 and 60 g resin loadings.

Figure 2-25 shows the overall carboxylic acid spectrum, which consists of the acid in both the liquid product and acid adsorbed by resin. Compared to the 50 and 60 g loadings, 80 g loading has slightly higher fractions of longer-chain acids such as butyric and valeric acids and slightly lower fractions of shorter-chain acid such as acetic and propionic acids. However, similar profiles can be found in the 40 g resin loading. Additionally, 40 g has a higher fraction of propionic acid and a lower fraction of acetic acid compared to 80 g loading. The only resin loading that has higher propionic acid mass fraction than acetic acid is 30 g.

Compared to the control group, incorporating ion-exchange resin improves the formation of propionic acid. This may result because the system tried to compensate for the higher portion of propionic acid being removed from the liquid by the resin. Although the control group has the highest butyric acid fraction, the mass fraction of valeric acid improves on all other loadings with resin involved. Roy achieved similar results to this experiment, which substantiates her findings that ion-exchange resin improves the production of longer chain acid when using office paper as substrate.[11]

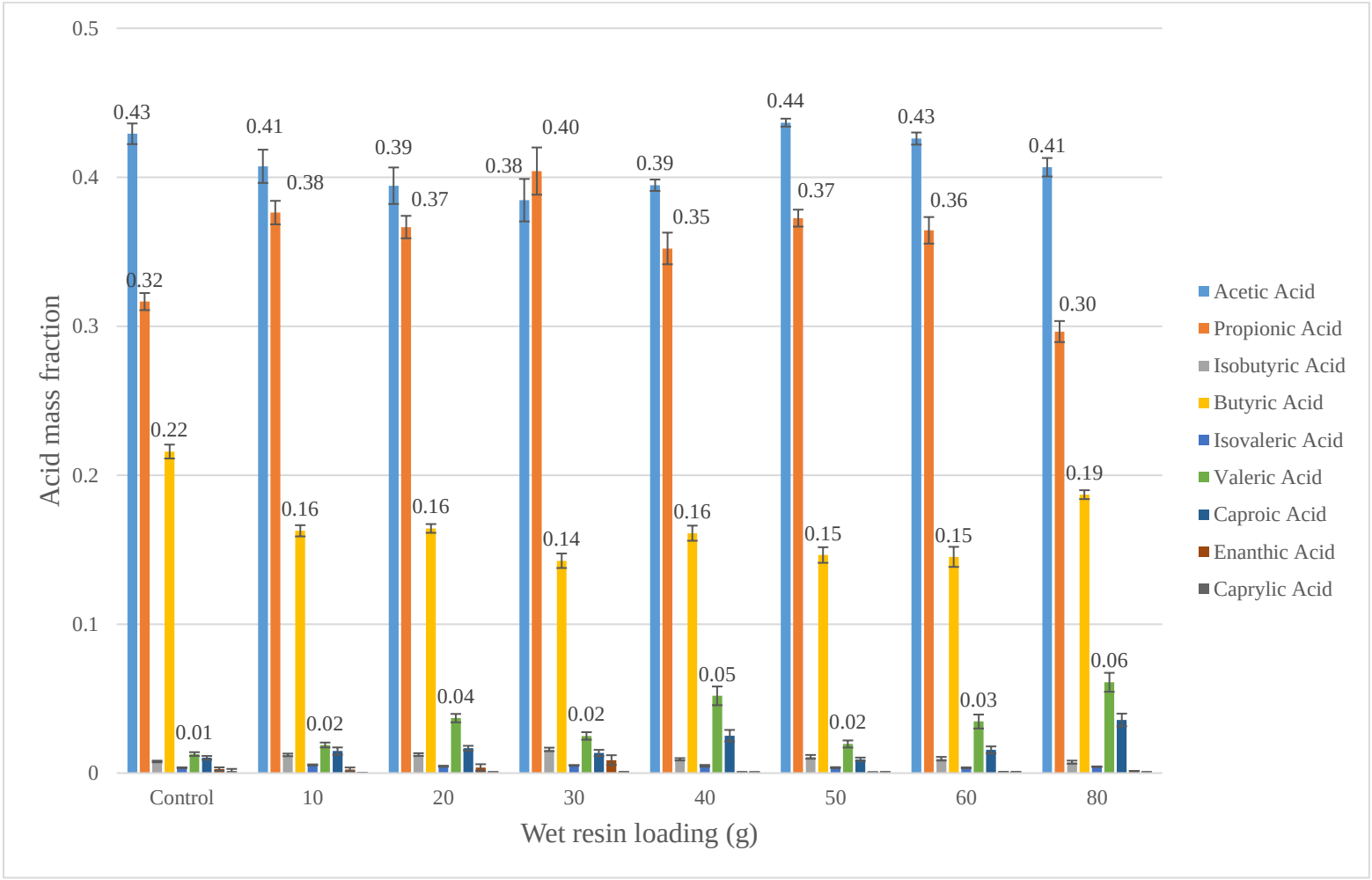


Figure 2-23 Carboxylic acid composition profiles in liquid product of 10–80 g resin loadings. (Note: The errors are derived from 95% confidence intervals.)

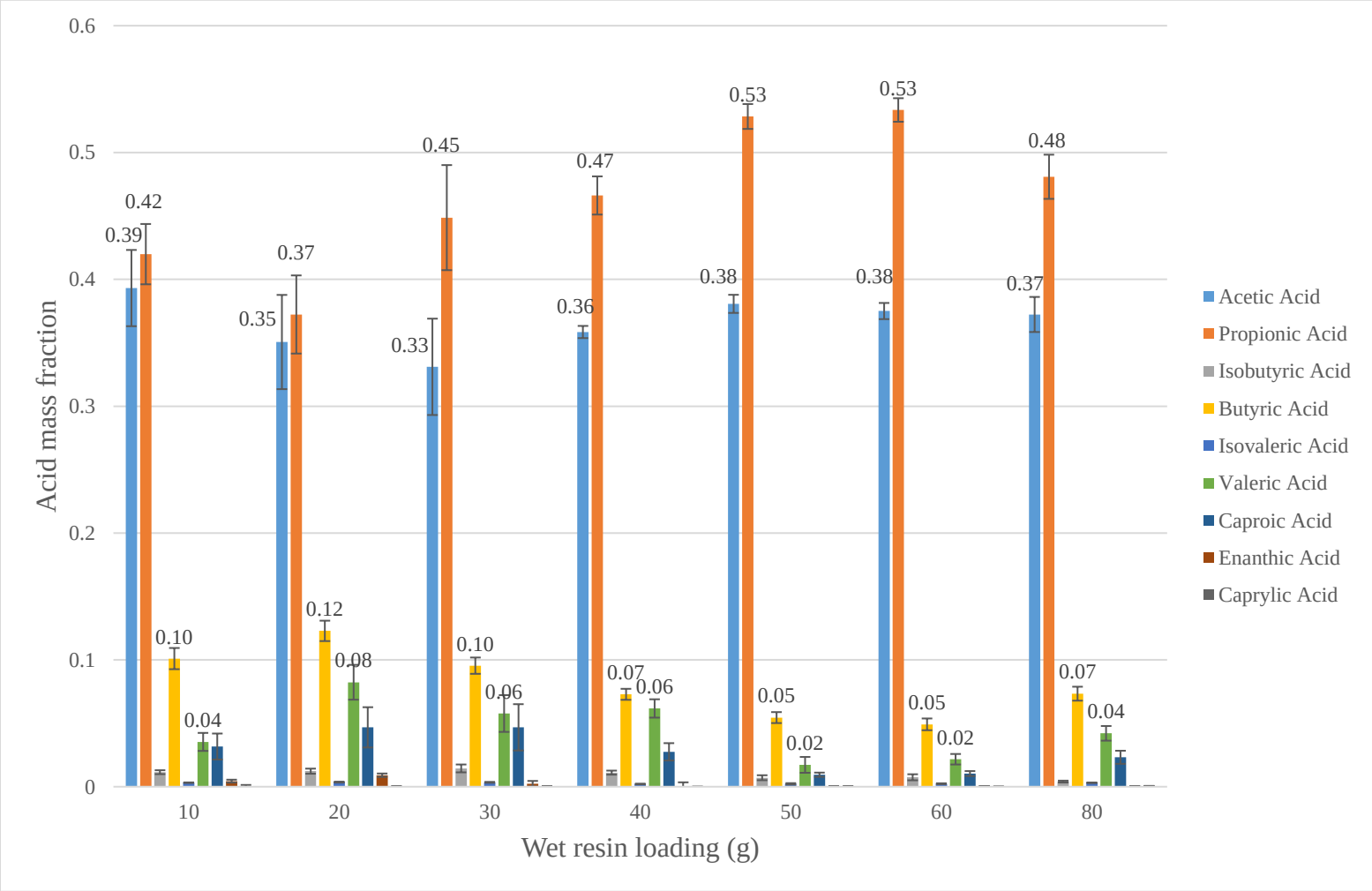


Figure 2-24 Carboxylic acid composition profiles in NaOH solution used in resin regeneration of 10–80 g resin loadings. (Note: The errors are derived from 95% confidence intervals.)

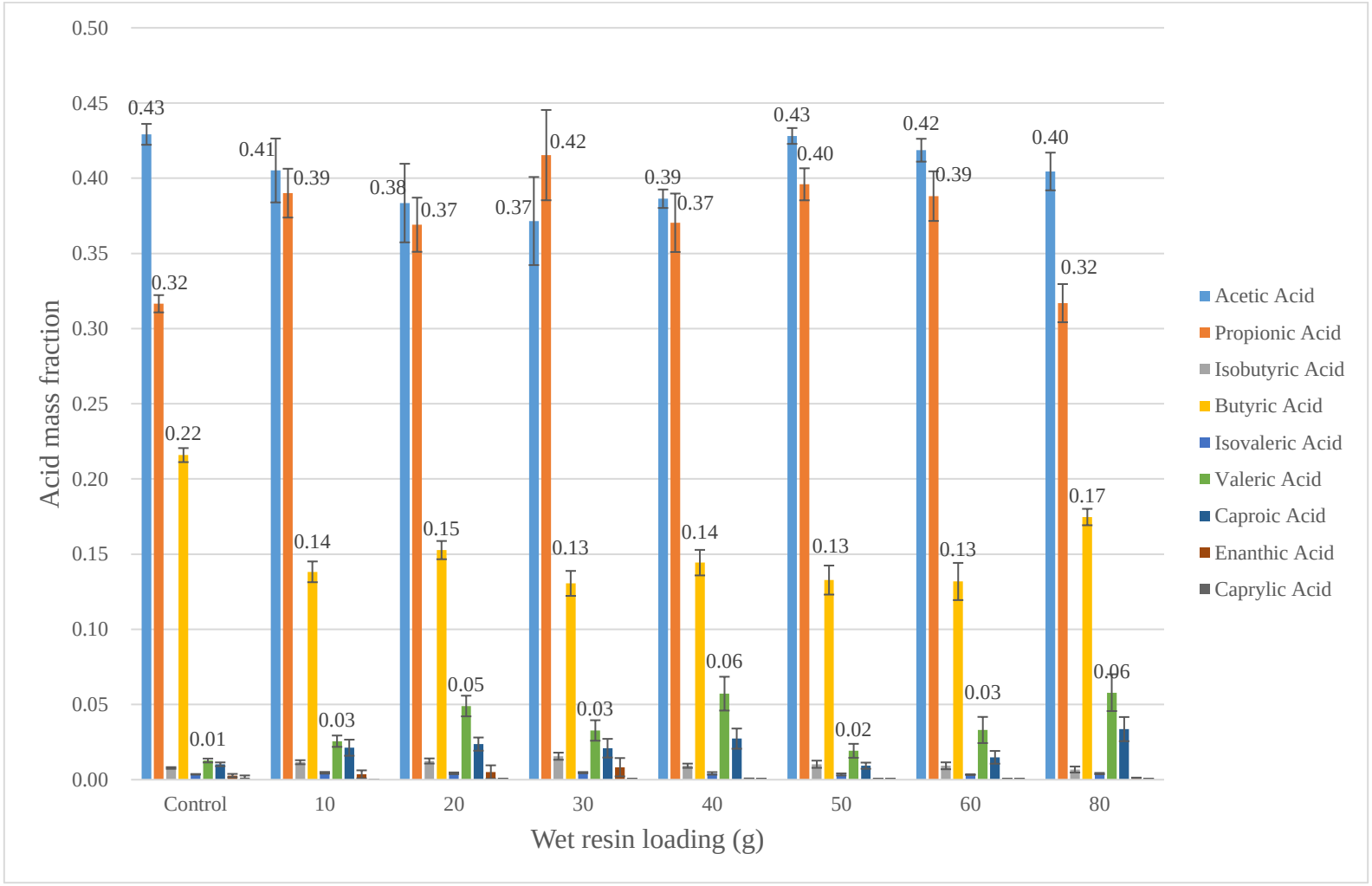


Figure 2-25 Carboxylic acid composition profiles for both liquid and resin using 10–80 g resin loadings. (Note: The errors are derived from 95% confidence intervals.)

The theoretical acid adsorption capacity for IRA-67 ion-exchange resin is determined based on the resin loading rate:

$$\text{Theoretical acid adsorption rate } \left(\frac{\text{mol}}{\text{d}}\right) = \frac{\text{Resin loading rate } \left(\frac{\text{g}}{\text{d}}\right) \cdot C_r \left(\frac{\text{eq}}{L}\right)}{\rho_{\text{r}} \left(\frac{\text{g}}{\text{L}}\right)} \cdot \frac{1 \text{ (mol)}}{1 \text{ (eq)}} \qquad (2\text{-}13)$$

According to the product data sheet for IRA-67, the total adsorption capacity (C_{r}) is 1.60 eq/L wet resin (free-base form) and the resin density (ρ_{r}) is 700 g/L wet resin. For carboxylic acids produced in mixed-acid fermentation, one mole of acid equals one equivalent (eq).

The average molecular weight ($M_{\text{w, avg}}$) is determined based on acid fractions (x_{C_i}) and molecular weights ($M_{\text{w,C}_\text{i}}$) of individual carboxylic acid recovered in the NaOH solution used in resin regeneration (Figure 2-24).

$$M_{\text{w,avg}} \left(\frac{\text{g}}{\text{mol}}\right) = \sum_{i=2}^{8} x_{C_i} \cdot M_{\text{w},C_i} \qquad (2\text{-}14)$$

The moles of acid adsorbed onto the resin is derived as follows:

$$\text{Actual acid adsorption rate } \left(\frac{\text{mol}}{\text{d}}\right) = \frac{\text{Rate of acid adsorbed onto resin } \left(\frac{\text{g}}{\text{d}}\right)}{M_{\text{w,avg}} \left(\frac{\text{g}}{\text{mol}}\right)} \qquad (2\text{-}15)$$

where the rate of acid adsorbed onto resin is determined by Figure 2-22.

The utilization of ion-exchange resin is the ratio of actual acid adsorption rate to the theoretical acid adsorption rate. In the calculation, the acid adsorption rate is shown on a daily basis, whereas the wet resin loading is based on each transfer (tr),

i.e., once every 2 days. The theoretical acid adsorption rate, which is calculated based on wet resin loading, must be adjusted to daily basis.

$$\text{Resin utilization} = \frac{\text{Actual acid adsorption rate (mol/d)}}{\text{Theoretical acid adsorption rate (mol/tr)}} \cdot \frac{2\ \text{(d)}}{1\ \text{(tr)}} \quad (2\text{-}16)$$

During the adsorption, approximately 10–45% resin capacity was used, which corresponds to 10–80 g wet resin loadings. The utilization ratio decreased as the resin loading increased (Figure 2-26).

Figure 2-26 The resin utilization.

Sample calculation (using 10 g wet resin loading as example):

1. $\text{Theoretical acid adsorption rate} = \frac{10\ \text{g}}{1\ \text{tr}} \cdot \frac{1.6\ \text{eq}}{\text{L}} \cdot \frac{\text{L}}{700\ \text{g resin}} \cdot \frac{1\ \text{mol}}{1\ \text{eq}}$
   $\cong 0.0229\ \left(\frac{\text{mol}}{\text{tr}}\right)$
2. $\text{Rate of acid adsorbed onto resin} = 0.3706\ \left(\frac{\text{g}}{\text{d}}\right)$
3. $M_{\text{w,avg}} = 72.44\ \left(\frac{\text{g}}{\text{mol}}\right)$
4. $\text{Actual acid adsorption rate} = \frac{0.3706\ \text{g}}{\text{d}} \cdot \frac{\text{mol}}{72.44\ \text{g}} \cong 0.0051\ \left(\frac{\text{mol}}{\text{d}}\right)$

5. Resin utilization = $\frac{0.0051\left(\frac{\text{mol}}{\text{d}}\right)}{0.0229\left(\frac{\text{mol}}{\text{tr}}\right)} \cdot \frac{2\text{ d}}{1\text{ tr}} = 0.447$

2.5 Conclusion

Using ion-exchange resins to adsorb acids in the countercurrent fermentations increased the yield and conversion, which is advantageous for the MixAlco® process. In contrast, selectivity was largely unaffected by using the resin. At high resin loadings, the carboxylic acid concentration dropped significantly initially. The acid concentration recovered showing the ability of mixed-culture microorganisms to adapt to diverse surroundings. From the standpoint of fermentation performance, the optimal resin loading for a four-stage countercurrent fermentation train is 30–40 g resin loadings per 1.38 L total liquid volume, which are 21.74–29.20 g wet resin/L_{liq} in this experiment. Higher loading does not appear to improve conversion, yield, and selectivity. Among all resin loadings, the carboxylic acid composition profiles are similar in the liquid product. Propionic acid had the greater tendency to be adsorbed by the resin. Among all other acids, its mass fraction increased when the resin loading was greater than the 30 g resin loading. Increasing resin loading above 30 g did not significantly change the acid profile. Among all trains, the highest resin loading (80 g) has the greatest chain-elongation effect. However, the profile is similar to 40 g resin loading, which required less resin and NaOH for regeneration, and therefore is more cost-effective.

CHAPTER III

ENZYMATIC HYDROLYSIS OF SHOCK- AND/OR ALKALI- TREATED CORN STOVER

3.1 Introduction

All lignocellulosic biomass, regardless of its origin, is comprised of different portions of cellulose, hemicellulose, and lignin. Together, they form a rigid structure. Because lignin and hemicellulose reduce accessibility of cellulase enzyme to cellulose, direct hydrolysis of lignocellulose biomass is almost impossible. Removing hemicellulose and lignin, or reducing cellulose crystallinity, increases the accessibility of cellulase (Figure 3-1).[21-22]

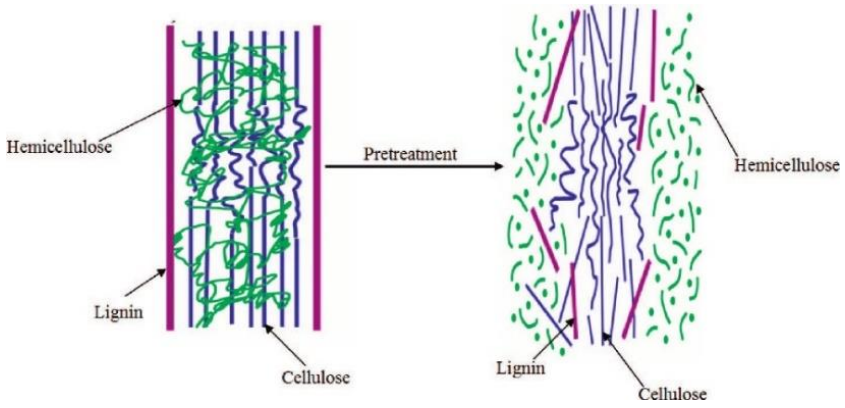


Figure 3-1 Schematic of the lignocellulosic biomass structure.[23]

Pretreatment processes improve the digestibility of cellulose before entering the fermentation. Numerous pretreatments have been proposed to improve hydrolysis, for example, mechanical pretreatment, acid or alkaline hydrolysis, biological

pretreatment, or explosion[23]. Developed by Holtzapple *et al.*, shock pretreatment is an economically favorable method that enhances enzymatic digestibility.[24]

Shock pretreatment enhances enzymatic digestibility of lime-pretreated biomass, and its apparatus and operating procedures have been elaborated and investigated by Bond.[12] Initially using solid explosives, the experiments were hindered because of contaminants. This problem was eliminated by using a gas explosion.[24] With this technique, it became crucial to examine its impact on real biomass. Further studies conducted by Kulozik focus on incorporating shock and caustic pretreatments using corn stover as the biomass source. The research investigates the enzymatic digestibility from various perspectives, including pre- and post-shock biomass, sodium-hydroxide-treated corn stover, pressurized oxygen, and temperature. This study shows that the dissolved corn stover should be collected, because it contributes significantly to digestibility (Figure 3-2).[25]

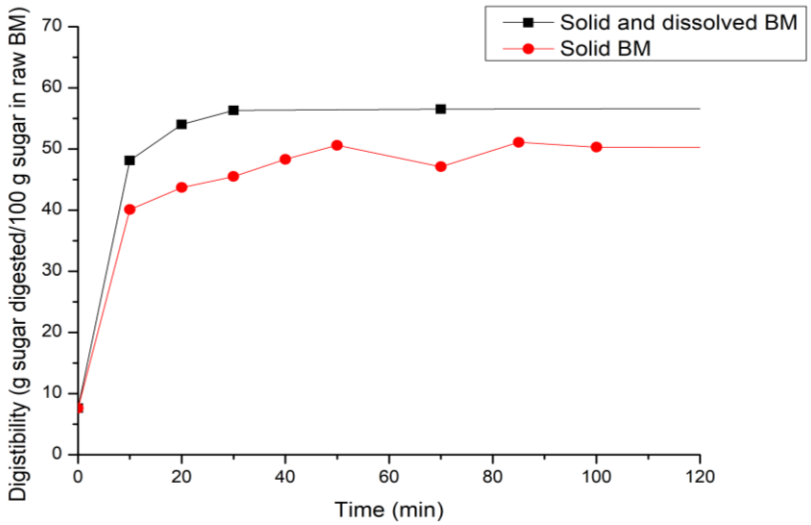


Figure 3-2 Digestibility on corn stover with and without dissolved biomass.[25] (Other conditions: 8 g OH^-/100 g dry BM in 100°C without shock treatment.)

Based on previous results, sodium hydroxide (NaOH) is a powerful alkali and increases digestibility significantly. Using high-temperature (100°C) treatment and high NaOH concentration (5–8 g OH^-/100 g dry BM) results in the highest enzymatic digestibility, but it also "conceals" the effect of shock pretreatment. The non-shock biomass results in even higher digestibility than shock-treated biomass. In contrast, biomass treated with moderate NaOH concentration (2–4 g OH^-/100 g dry BM) has good digestibility that can be further improved by applying shock treatment.[25] Sodium hydroxide pretreatment can be implemented in the MixAlco® process; however, sodium ions must be limited in ruminant animal feed because of health concerns. In this application, low-sodium pretreatments using lime can be used to upgrade ruminant animal feeds such as in dairy and beef cattle.[3]

This study aims to investigate conditions not previously explored with corn stover to determine the recommended pretreatment conditions. Hence, this chapter emphasizes the effect of different pretreatment conditions (e.g., initial shock-gun filling pressure, temperature, and hydroxide concentration) on enzymatic digestibility.

This chapter is a collaborative effort with Dr. Ju Huang, a visiting scholar at the Department of Chemical Engineering, Texas A&M University.

3.2 Materials

3.2.1 Biomass

This experiment uses unwashed, Champion-milled, Field corn stover harvested in 2012. To maintain constant moisture content, it is stored in a steel barrel.

3.2.2 Buffer

To reach optimum enzyme performance and maintain high enzyme activity, citrate buffer is used to adjust the pH of the biomass slurry. The desired pH range for each enzyme follows: [26-27]

- Cellulase CTec3: pH 4.75–5.25
- Hemicellulase HTec3: pH 4.80–5.20

Citric acid monohydrate (99.5%, ACROS Organics) and trisodium citrate dihydrate (99%, ACROS Organics) were added into deionized water to prepare 0.1-M citrate buffer. Detailed approaches are described in Appendix D.

3.2.3 Additives

A solution of tetracycline (crystalline powder, Alfa Aesar) and cycloheximide (95%, ACROS Organics) was prepared to prevent growth of microorganisms, which can consume the products. Tetracycline (a protein biosynthesis inhibitor) was dissolved in water/ethanol mixture (water: ethanol = 3:7 by volume) to form 10 g/L solution. Cycloheximide (an antibiotic) was dissolved into D.I. water to produce 10 g/L solution. The methods of preparation are described in Appendix E.

3.2.4 Enzyme

In previous research done by Liang, three types of enzymes – Novozymes Cellic® CTec2, CTec3, and HTec3 – were investigated. The results indicated that CTec3 performed better than CTec2.[28] CTec3 is the latest commercial enzyme for effectively hydrolyzing cellulose. It contains proficient cellulase components that are boosted by exclusive enzyme activities and a new array of hemicellulase activities.[26] HTec3 enzyme hydrolyzes insoluble and soluble hemicelluloses.[27] Because cellulose and hemicellulose contributed sugar production, two types of enzymes were introduced in this research: CTec3 and HTec3. The enzyme solutions involved in this experiment were diluted ten times compared to the stock solutions.

3.2.5 Alkali Source

In this research, sodium hydroxide (NaOH) was selected as the alkali source. Previously, lime was chosen because of its low operating cost and biological compatibility with ruminal animals; however, its poor water solubility is a significant drawback. Although it costs more, sodium hydroxide has great solubility in water and its better accessibility renders it a reasonable candidate for alkali treatment. NaOH solution (10 g/L) was prepared in this experiment.

3.2.6 Incubator

A temperature-controlled standing shaker was used to provide the optimum enzymatic saccharification environment. The shaker rotates horizontally at 120 rpm to ensure proper mixing. The desired temperature for each enzyme follows: [26-27]

- CTec3: 50–55℃
- HTec3: 40–45℃

3.3 Method and Calculation

3.3.1 Shock Pretreatment

Shock treatment is a new process that has relatively low operating cost (<$5/ton) compared to other existing conventional chemical pretreatment technologies (~$45/ton). Furthermore, shock treatment has the potential to be scaled up.[29]

High-pressure explosive gases were sealed in a shock tube together with biomass slurry. Shock pretreatment exerted its effect by detonation. The 2-L shock vessel included a conical section and run-up tube with biomass slurry (5% dry biomass by weight in water) in the bottom and was sealed with impact wrench. Figure 3-3 shows the shock tube and the conical vessel has the following dimensions:[30]

- Length (shock tube): 22 in.
- Length (conical vessel): 11 in.
- Length (run-up tube): 33 in.
- Outer diameter (d_1): 3.820 in.

- Inner diameter of wide end (d_2): 3.549 in.
- Inner diameter of run-up tube (d_3): 0.815 in.
- Divergence angle: 7.112° from the central axis

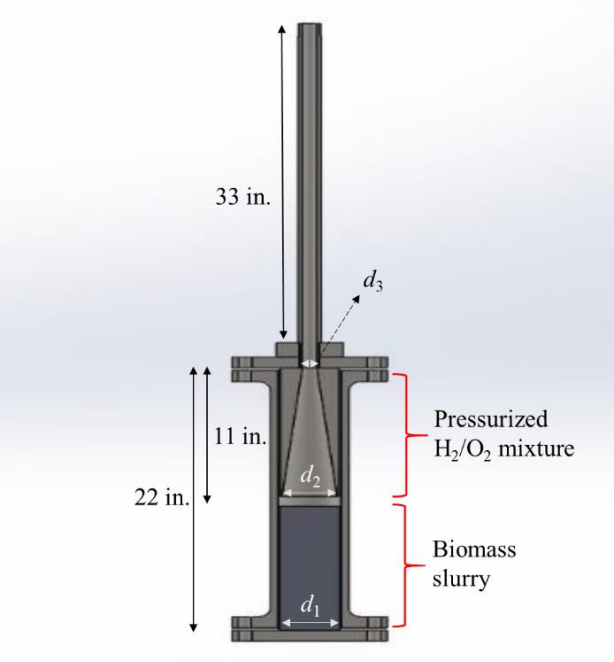


Figure 3-3 Cross-sectional view of 2-L shock tube and its dimensions.

Pressurized stoichiometric hydrogen and oxygen mixture was loaded into the tube with specified initial pressure (usually < 6.89 bar (abs)), which was low enough to prevent system failure. For gas filling, the vessel was purged with oxygen once, followed by adding hydrogen then oxygen.

The gas mixture was ignited by a glow plug. The resulting shock wave transferred into the aqueous slurry and mechanically disrupted the biomass structure, which increased microbial access to the biomass.[24]

In this experiment, the biomass source was corn stover. The shock-pretreated corn stover slurry was homogenized. Its moisture content was determined by heating

a portion of sample in the oven (105℃) for 4 hours. Detailed filling steps and operating procedures are described in Appendix F.

3.3.2 Alkali Pretreatment

NaOH-treated corn stover is more enzymatically digestible than lime-treated corn stover.[31] Moreover, shock treatment increases the digestibility of lime-treated corn stover.[12] In this set of experiments, shock treatment was combined with NaOH to investigate if it benefits enzymatic digestibility.

The amount of caustic used in the pretreatment was calculated by specifying the loading of hydroxide group (g OH^-/100 g dry BM).

$$W\left(\frac{\text{g caustic}}{100\text{ g dry BM}}\right)=\frac{[\text{OH}^-]\left(\frac{\text{g OH}^-}{100\text{ g dry BM}}\right)\cdot M_{caustic}\left(\frac{\text{g caustic}}{\text{mol}}\right)}{v_{caustic}\left(\frac{\text{mol OH}^-}{\text{mol caustic}}\right)\cdot M_{OH^-}\left(\frac{\text{g OH}^-}{\text{mol}}\right)} \tag{3-1}$$

$$V_{\text{caustic solution}}\left(\frac{\text{L}}{100\text{ g dry BM}}\right)=\frac{W\left(\frac{\text{g caustic}}{100\text{ g dry BM}}\right)}{c_{caustic}\left(\frac{\text{g caustic}}{\text{L}}\right)} \tag{3-2}$$

where $[\text{OH}^-]$ is the hydroxide loading, W is the NaOH loading required for the pretreatment, $M_{caustic}=40$ and $M_{OH^-}=17$ are molecular weights of NaOH and hydroxide group, respectively. $v_{caustic}$ is the number of dissociable hydroxide groups per mole of caustic (e.g., $v_{caustic}$ = 1 for NaOH). $c_{caustic}$ is the concentration of caustic solution ($c_{caustic}=10$ g NaOH/L in this research). $V_{\text{caustic solution}}$ is the

volume of NaOH solution per 100 g dry biomass required to provide desired amount of NaOH for pretreatment.

Measured amounts of caustic solution were mixed with the biomass under the condition of interest. Afterward, the caustic-loaded biomass was heated in the incubator. After the treatment was completed, the pretreatment reaction was terminated by soaking the reactor in a cold water bath, and waiting until it cooled down to room temperature. The pretreatment slurry was neutralized by adding 5-N hydrochloric acid (HCl) until the pH lowered to the enzyme-favored environment described at Paragraph 3.2.2.

3.3.3 Enzymatic Hydrolysis

Two enzyme solutions – Ctec3 and HTec3 – were utilized to conduct the enzymatic hydrolysis. The reaction was performed in 1-L HDPE bottles (Nalgene®, No. 2104-0032). Both cellulase (2 mg enzyme/g dry pretreated biomass) and hemicellulase (2 mg enzyme/g dry pretreated biomass) were added to the biomass sample. Then, cycloheximide (6 mL cycloheximide solution/L BM slurry) and tetracycline (8 mL tetracycline solution/L BM slurry) were added to prevent microbial growth. Citrate buffer solution was added to maintain pH at 4.8–5.0. Lastly, D.I. water was added to dilute the slurry to 5 wt% biomass. The mixture was kept in the incubator at 50℃ for 5 days. The reaction was terminated by placing the bottles in an 80℃ water bath for 10 min. The concentration of the major products – glucose and xylose – were determined by high-performance liquid chromatography (HPLC, Agilent 1260 Infinity)

using Aminex® HPX-87P column. Detailed procedures of sample preparation and HPLC operation are described in Appendix G.

3.3.4 Digestibility

The digestibility is defined as follows, and it is calculated based on the glucan and xylan in raw biomass:

$$\text{Digestibility} \equiv \frac{m_{\text{product}}}{m_{digestible\ BM}} \tag{3-3}$$

where m_{product} is the mass of produced glucose and xylose in the pretreated biomass slurry, and $m_{digestible\ BM}$ is the weight of equivalent glucose and xylose in raw biomass.

In this study, the enzymatic digestibility (D) is comprised of the digestibility from pretreated corn stover (D_{solid}) and pretreatment slurry (D_{slurry}):

$$D = D_{solid} + D_{slurry} = \frac{\left(m_{\text{product}}\right)_{\text{solid}} + \left(m_{\text{product}}\right)_{\text{slurry}}}{m_{digestible\ BM}} \tag{3-4}$$

The m_{product} can be determined by the HPLC and slurry volume V_{slurry} (L) can be measured:

$$m_{\text{product}} = \left(c_{\text{glucose}} + c_{\text{xylose}}\right) \cdot V_{\text{slurry}} \tag{3-5}$$

where c_{glucose} (g/L) and c_{xylose} (g/L) are glucose and xylose concentrations measured by HPLC.

During each treatment, biomass may be lost through the sieve and may dissolve into the liquid phase. Pretreatment yield (Y_{pre}) accounts for the mass loss from both shock (Y_{shock}) and alkali (Y_{alkali}) treatments.

$$Y_{pre} = Y_{shock} \cdot Y_{alkali} = \left(\frac{m_{\text{shocked}} + m_{MC,1}}{m^0_{\text{shock}}}\right) \cdot \left(\frac{m_{\text{chem}} + m_{MC,2}}{m^0_{\text{NaOH}}}\right) \quad (3\text{-}6)$$

where m^0_{shock} and m^0_{NaOH} are the dry weight of pre-shock biomass (100 g in this study) and biomass before alkali treatment (7 g in this study), respectively. Moreover, m_{shocked} is the dry weight of post-shock corn stover, where m^0_{NaOH} comes from a part of it. The dry weight of post-alkali-treated corn stover (m_{chem}) is used for subsequent enzymatic hydrolysis. $m_{MC,1}$ and $m_{MC,2}$ are the dry weight of the sample used for moisture content measurement after shock and alkali treatments (Figure 3-4).

Furthermore, when glucan and xylan are hydrolyzed into glucose and xylose, the mass increases from the water of hydrolysis. The correction factors ACC and ACX are 1.111 and 1.136, respectively. Bond determined the compositions of glucan and xylan in the corn stover used in this study.[12]

$$m_{digestible\ BM} = \left(x^0_{\text{glucan}} \cdot \text{ACC} + x^0_{\text{xylan}} \cdot \text{ACX}\right) \cdot \frac{m_{\text{chem}}}{Y_{pre}} \quad (3\text{-}7)$$

where x^0_{glucan} and x^0_{xylan} are the mass fractions of glucan and xylan in raw corn stover reported as 0.360 and 0.222, respectively.

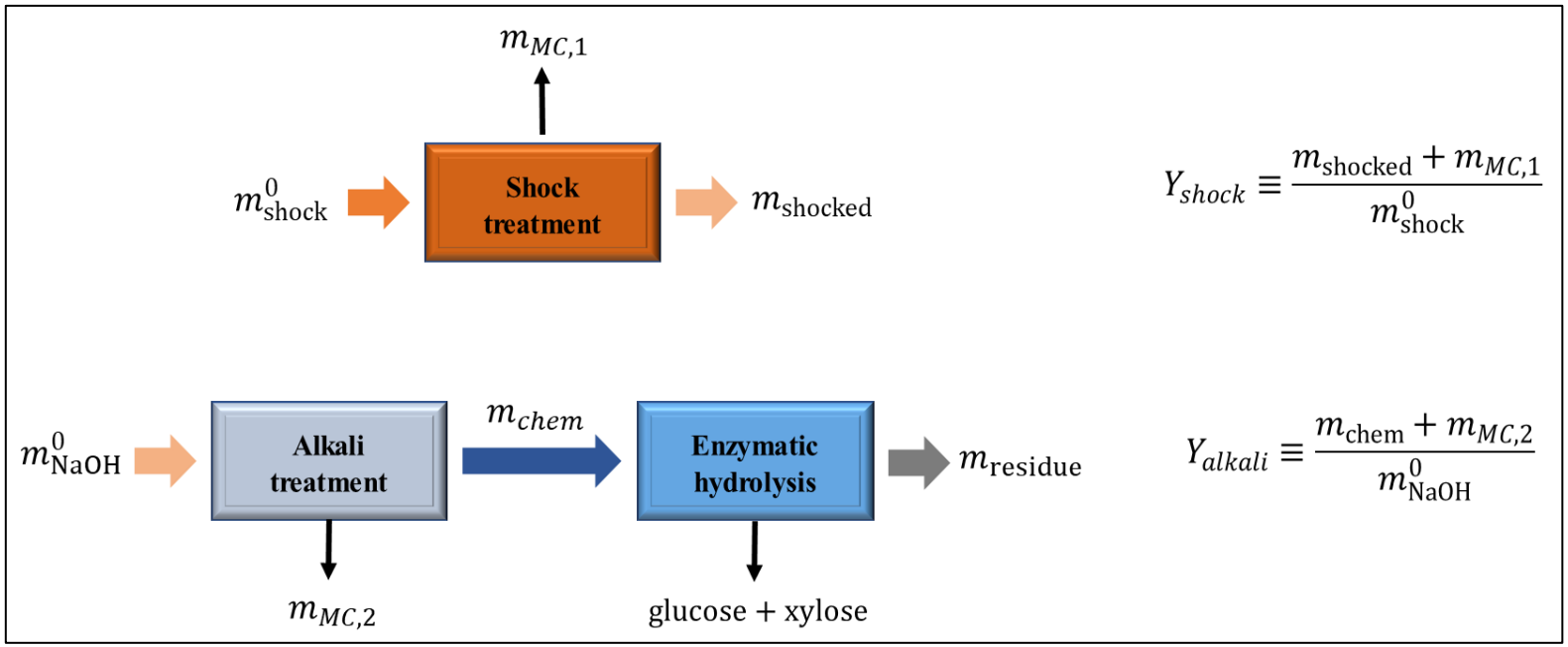


Figure 3-4 Schematic diagram of shock and alkali pretreatment and enzymatic hydrolysis.

3.4 Results and Discussion

In this study, the effects of different initial shock-gun filling pressure and pretreatment temperature on enzymatic digestibility were investigated to supplement the previous study.

The following points summarize previous corn stover pretreatment experiments conducted by Kulozik:[25]

1. Biomass dissolved in the NaOH pretreatment slurry must be included because it effectively increases enzymatic digestibility.
2. In NaOH pretreatments, pressurized oxygen has little benefit on enzymatic digestibility.
3. Shock pretreatment done prior to caustic pretreatment results in higher digestibility.
4. Shock treatment notably improves digestibility when biomass is treated under moderate temperature.
5. The optimal treatment time for NaOH treatment is 1 h.
6. Shock treatment alone cannot significantly improve enzymatic digestibility, so it must be incorporated with secondary treatment.

Increasing temperature increases digestibility; however, the linear increase plateaus at approximately 100℃, where the digestibility stops increasing (Figure 3-5) (Note: "Sugar" is the equivalent amount of monosaccharides produced from glucan and xylan.) At moderate temperatures (40–60℃), which is desired to lower energy costs, the digestibility ranges from 27.5 to 35.7 g sugar digested/100 g sugar in raw biomass. Other pretreatment conditions are listed in Table 3-1.

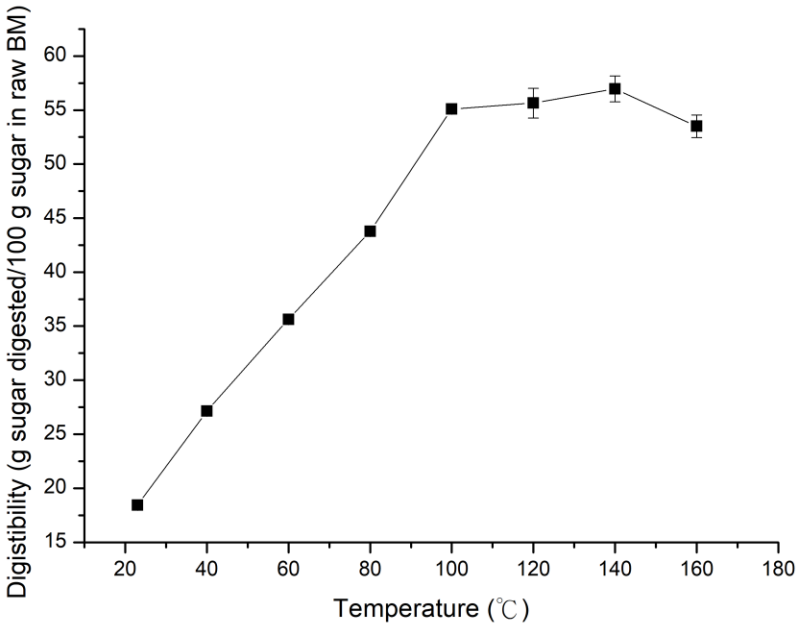


Figure 3-5 Effects of temperature of corn stover on digestibility.

Table 3-1 Additional pretreatment parameters for temperature effects

Parameters	Shock-gun initial filling pressure (bar (abs))	Pressurized oxygen	Duration (min)	Caustic concentration (g OH^-/100 g dry BM)	Caustic type
Value/ Response	5.52	N/A	60	4	NaOH

Previously, during shock pretreatment, 6.89 bar (abs) (100 psia) total shock-gun loading pressure of H_2/O_2 mixture was the default option. To see if lower pressures are effective, and hence reduce costs, experiments with initial pressure ranged between 3.45 to 6.89 bar (abs). As shown in Figure 3-6, the optimal initial loading pressure was around 5.52 bar (abs) (80 psia), because it had similar biomass digestibility as 6.89 bar (abs). Any pressures lower than 5.52 bar (abs) were less digestible. Other pretreatment conditions are listed on Table 3-2.

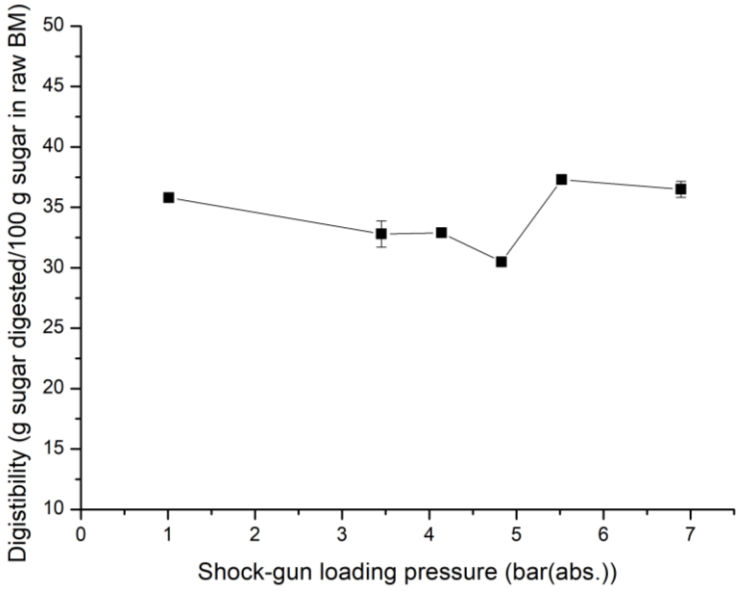


Figure 3-6 Effects of shock-gun initial filling pressure on digestibility.

Table 3-2 Additional pretreatment parameters for loading pressure effects

Parameters	Temperature (°C)	Pressurized oxygen	Duration (min)	Caustic concentration (g OH^-/100 g dry BM)	Caustic type
Value/ Response	50	N/A	60	4	NaOH

Because shock treatment is inexpensive (about $5/tonne), it might reduce the cost of conventional chemical methods by reducing the severity of the chemical pretreatment. Although higher temperatures significantly improve digestibility, shock treatment is less beneficial at high temperatures.[25] At moderate temperatures of 50°C, the digestibility of 29 g sugar digested/100 g sugar in raw biomass and was enhanced by shock treatment. At 50°C, metal reactors can be replaced by inexpensive plastic bottles (Nalgene® 1-L HDPE Bottle).

Next, the impact of hydroxide loading was examined. This set of experiment aims to determine the OH^- loading that provides the most economical way to pretreat biomass (Figure 3-7 and Table 3-3).

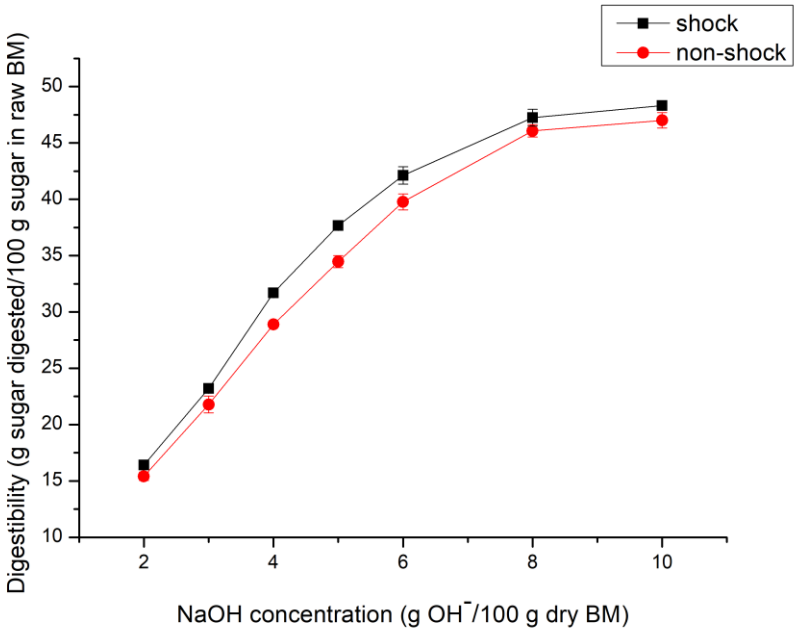


Figure 3-7 Enzymatic digestibility of shock and non-shock NaOH-treated corn stover.

Table 3-3 Additional pretreatment parameters for alkali concentration effects

Parameters	Shock-gun initial filling pressure (bar (abs))	Temperature (°C)	Pressurized oxygen	Duration (min)	Caustic type
Value/ Response	5.52	50	N/A	60	NaOH

Table 3-4 Mean digestibility of shock and non-shock NaOH-treated corn stover

OH^- concentration (g OH^-/100 g dry BM)		2	3	4	5	6	8	10
Digestibility (g sugar digested/100 g sugar in raw BM)	Shock	16.4	23.2	31.7	37.7	42.1	47.3	48.3
	Non-Shock	15.4	21.8	28.9	34.5	39.8	46.1	47.0
	Improvement	1.0	1.4	2.8	3.2	2.3	1.2	1.3

According to Figure 3-7 and Table 3-4, shock treatment modestly improved digestibility at all OH^- loadings at moderate pretreatment temperature (50°C). At 4–5 g OH^-/100 g dry BM loadings, shock treatment improves digestibility by more than 2.5 g sugar digested/100 g sugar in raw biomass; this improvement is better than other alkali loadings. At high alkali concentrations, shock treatment is unnecessary, whereas, at low alkali concentrations, shock enhances alkaline pretreatment, presumably by opening the biomass structure and improving diffusion.

For industrial applications, pretreatment must be economically feasible. Sodium hydroxide is a major expense, so it is worthwhile to investigate processing conditions that reduce alkali loading. Therefore, corn stover treated under 4 g OH^-/100

g dry BM incorporated with shock treatment (5.52 bar (abs) initial pressure) has been chosen as the biomass substrate for the next set of mixed-culture experiments.

3.5 Conclusion

These experiments supplement the previous study by investigating enzymatic digestibility by tuning the initial shock-gun pressures and varying temperature with alkali pretreatment. Increasing temperature improves digestibility below 100℃, but increases modestly above that. Furthermore, the highest digestibility was obtained at 6.53 bar (abs) initial gas filling pressure followed by 50℃ and 4 g OH^-/100 g dry BM treatments. Shock treatment had the best improvement at 4–5 g OH^-/100 g dry BM loading. The results suggest good performance when biomass is shocked with 5.52 bar (abs) initial filling pressure followed by 1-h treatment at 50℃, without pressurized oxygen, and with 4 g OH^-/100 g dry BM caustic loading. This biomass will be employed in mixed-culture fermentation. Because the goal of this study is to seek an economically favorable pretreatment method for the MixAlco® process, shock treatment and lower sodium hydroxide loading is preferred.

CHAPTER IV

ASSESSMENT OF SHOCK- AND/OR ALKALI-TREATED CORN STOVER ON MIXED-ACID FERMENTATION BY CPDM METHOD

4.1 Introduction

Continuum Particle Distribution Model (CPDM) is a technique developed by Loescher and Ross that has been extensively utilized to predict the performance of countercurrent mixed-acid fermentations.[32-33] A *continuum particle* is defined as a gram of solids in the initial unreacted state. In this study, it represents one gram of non-acid volatile solids (NAVS). Based on one set of batch fermentation data, this method can determine conversions and product concentrations of consolidated bioprocessing at a range of volatile solids loading rates (VSLR) and liquid residence times (LRT).

The CPDM method reduces labor by predicting the optimal countercurrent fermentation condition through mathematical modeling. Countercurrent fermentation is performed with only one set of VSLR and LRT and requires three to four months to reach steady state. To obtain data for a wide range of operating conditions would require many years of experiments, which is impractical. CPDM overcomes this problem by mathematically simulating the performance of multiple operating conditions.[34]

Previous chapters described several preliminary tests where various parameters on corn stover pretreatments were systematically varied to determine operating condition that maximize enzymatic digestibility.

From these enzymatic studies, the following operating conditions are recommended: initial pressure loading of 5.52 bar (abs) in the shock tube, following by 4 g OH^-/100 g dry BM NaOH treatment maintained at 50°C for 1 h. At moderate sodium hydroxide concentrations, shock treatment helps render pretreated-biomass more bio-available. Consequently, CPDM models of raw corn stover, shock-treated corn stover, NaOH-treated corn stover, and shock + NaOH-treated corn stover were conducted to investigate the potency of each pretreatment.

Furthermore, countercurrent fermentation of sewage sludge has been studied by Rughoonundun, which significantly improves carboxylic acid production.[35] The combination of sugarcane bagasse and sewage sludge resulted in 60.8 g/L in total acid concentration, which is the highest record among all other substrates used in countercurrent fermentation of the MixAlco® process.[36] In this chapter, the fermentation substrate is pretreated corn stover and air-dried sewage sludge.

This research is a collaborative effort with Opeyemi Olodeke, a PhD candidate in the Department of Chemical Engineering, Texas A&M University and Kejia Liu, a masters student in the Department of Chemical Engineering, Texas A&M University.

4.2 Materials

4.2.1 Substrate

4.2.1.1 Corn Stover

The corn stover used in this experiment comes from the same source described in Paragraph 3.2.1. The carbon-to-nitrogen ratio (C-N ratio) was measured by Soil, Water and Forage Testing Laboratory Texas A&M University (College Station, Texas). The test was based on a combustion method using an Elementar Variomax CN.[37] The C-N ratio was reported as 69.2 g carbon/g nitrogen (41.5 wt% total carbon and 0.6 wt% total nitrogen).

4.2.1.2 Sewage Sludge

According to the study by Rughoonundun, sewage sludge produced significant concentrations of carboxylic acids, two times more than using chicken manure.[35]

Sewage sludge was collected from the Carter Creek Wastewater Treatment Plant (College Station, Texas). The waste water was transferred into 1-L PCCO bottles and centrifuged at 4000 rpm for 10 min. Once the centrifuging process was completed, the supernatant was discarded immediately, leaving black sewage sediment at the bottom of the bottles. The process was repeated until all the wastewater was used. The supernatant was sterilized by mixing 1 part of 4% bleach (Cholorox® Regular Liquid Bleach) per hundred. The sewage sludge collected from the bottom sediment of the bottles was fan dried at room temperature for 48 hours (Figure 4-1). The moisture

content of the dried sewage sludge was measured from samples taken from at least five distinct sites of the metal tray.

Figure 4-1 Wet (upper) and air-dried (lower) sewage sludge.

4.2.2 Fermentation Media

De-oxygenated water (D.O. water) was used to ensure the system stays anerobic. Detailed procedures for D.O. water preparation are described in Appendix H.

4.2.3 Inoculum

The original inoculum was a mixed-culture of marine microorganisms collected from beach sediment in Galveston, TX. The sediment was collected from the bottom of multiple half-meter-deep shoreline pits. The following treatments are identical to those described in Paragraph 2.2.4.

4.2.4 Fermentor

Figure 2-2 shows a cross-sectional view of the fermentors. Fermentation was performed in 1-L PCCO bottles, which were capped by a rubber stopper with a glass tube, septum, and two 0.25-in. stainless steel bars in the middle.

4.2.5 Buffer

To maintain the pH in the near-neutral range (6.5–7.5), sodium bicarbonate ($Na(HCO_3)_2$, Fischer) was selected as fermentation buffer. As acids were produced, the carbonate and bicarbonate naturally adjust to the pH of interest (~7.0). Previously, magnesium carbonate ($MgCO_3$) was used for buffering; however, $MgCO_3$ is preferred for thermal conversion of the salts.[38] For extraction, which will be employed in future countercurrent experiments, sodium buffer is preferred because it forms less precipitate and avoids fouling in the acid-extraction apparatus.

4.2.6 Incubator

In this study, a standing incubator cabinet (Wheaton Modular Cell Production Roller Apparatus) was used. The fermentors were kept in the incubator and rotated horizontally at 2 rpm, and the temperature is controlled at 40°C.

4.3 Methods and Calculation

4.3.1 Carbon-to-Nitrogen Ratio

The carbon nitrogen ratio (C-N ratio) of the sewage sludge was measured by Soil, Water and Forage Testing Laboratory Texas A&M University (College Station, Texas). The test was based on a combustion method using an Elementar Variomax CN.[37] The carbon content was reported as 33.69 wt% and the nitrogen was 5.43 wt%. The C-N ratio was 6.20 g carbon/g nitrogen. To obtain higher production yields, Smith recommends the C-N ratio to be 13.0 to 25.0 g carbon/g nitrogen.[16] Therefore, the fermentation substrate used in this research combines 30 wt% sewage sludge and 70 wt% corn stover, which has a C-N ratio of 19.11 g carbon/g nitrogen.

4.3.2 Pretreatment

4.3.2.1 Shock Pretreatment

This shock process is described in Paragraph 3.3.1. The initial shock-gun loading pressure was set at 5.52 bar (abs) according to the results given in Chapter III.

4.3.2.2 Caustic Pretreatment

Sodium hydroxide (NaOH) was used as the caustic source and the procedures are described in Paragraph 3.3.2. Based on the results given by the previous chapter, shock pretreatment noticeably improves enzymatic digestibility at 4–5 g OH^-/100 g dry BM loading. Therefore, in this experiment, 4 g OH^-/100 g dry BM concentration was applied on both shock and non-shock corn stover.

According to Chapter III, alkali pretreatment dissolves some biomass into the liquid, so it is expected that including the slurry enhances the production of carboxylic acids. By directly inoculating the NaOH-treated corn stover, all dissolved biomass was included into the fermentor. More biomass dissolved into the fermentation broth at higher substrate concentrations.

Previously, the slurry was neutralized with 5-N HCl because the product (sodium chloride) did not impact HTec3 and CTec3 activities. However, high salinity may interfere with the mixed-microbial culture and undermine its acid-forming performance. Instead of using HCl, CO_2 was sparged into the pretreatment slurry through a gas distributor until it started to form large amounts of bubbles. The resulting sodium bicarbonate ($NaHCO_3$) served as naturally formed buffer. The final pH was controlled to 6.5–7.5, which is a favorable condition for further mixed-culture fermentation. Detailed caustic pretreatment procedures are described in Appendix I.

4.3.3 Microorganism Adaptation

To ensure mixed-microbial cultures function under the newly introduced environment, inoculum adaptation is crucial and was required prior to the CPDM batch experiment. Batch fermentation with the exact same conditions (such as substrate, nutrients, pH, buffer, and temperature) used in the CPDM experiment must be performed.

In this experiment, 30 wt% air-dried sewage sludge and 70 wt% corn stover were added into a 1-L PCCO fermentor described in Paragraph 2.2.2. The substrate

concentration was 50 g/L. D.O. water was added into the bottles containing marine soil from Galveston followed by the treatments described in Paragraph 2.2.4. Supernatant (50 mL) was collected and added into the fermentor. Buffer was added to adjust the pH to 6.5, 120 μL methanogen inhibitor was added to suppress methane formation, and D.O. water was added to reach the working volume of 400 mL. Adaptation is usually performed for approximately 2–3 weeks. Detailed procedures of inoculum adaptation are described in Appendix J.

4.3.4 Mixed-Culture Batch Fermentation

To initiate the mixed-culture fermentation, calculated amounts of substrate, nutrient, fermentation media (D.O. water), inoculum, buffer, and methanogen inhibitor were added into the fermentor to form the fermentation broth (Tables 4-2 and 4-4). The fermentor and the rubber stopper must be autoclaved before fermentation begins. The fermentation broth was purged with nitrogen and stored in the rolling incubator.

The transfer time for each fermentor is 2 days, although at the very end of the experiment, it could be lengthened to 5 days because less gas was produced. Initially, every 2 days, the fermentors were removed from the incubator, and the amount of biogas was measured and vented by the apparatus described at Paragraph 2.3.5. Two gas samples were randomly taken from two different fermentors and were analyzed for N_2, CO_2, CH_4, and H_2 using a gas chromatograph (Agilent 6890 series). The gas compositions indicated inhibitor efficacy and fermentation activity. Then, the fermentors were centrifuged at 4000 rpm for 10 min. The supernatant was removed

from the fermentor and transferred to a beaker, and 1-mL supernatant sample was collected for future carboxylic acids determination. The pH in each fermentor was measured. Additional buffer was added to adjust the pH if it was less than 6.5.

Lastly, 60 μL iodoform solution (20 g CHI_3/L 200-proof ethanol) was added into each fermentor followed by nitrogen purging. All fermentors were homogenized by vigorous shaking, then stored in the incubator.

4.3.5 Carboxylic Acids Determination

Liquid samples from each fermentor were measured by the gas chromatograph (Agilent 6890 series) with a flame ionization detector (FID) and autosampler (Agilent 7683 series). The methods for determining carboxylic acids concentration are described in Paragraph 2.3.6.

4.3.6 Continuum Particle Distribution Model (CPDM)

To construct the CPDM map, five different substrate loadings of the batch experiment must be conducted: 20, 40, 70, 100, and 100^+ g dry substrate/L liquid.[38] The 100^+ group has the same substrate concentration as 100 g/L, but with part of the fermentation media supplemented with 20 g/L mixed-acids to examine the inhibition effect of initially present product (Table 4-1). To increase the pH to neutrality prior to inoculation, buffer must be added to the 100^+ g/L fermentor. This measure prevented the mixed-microbial system from being overly acidic.

Table 4-1 List of carboxylic acids containing in the mixed-acids

Acid	Concentration (g/L)
Acetic acid	16
Propionic acid	1
Butyric acid	3

To increase accuracy, each substrate concentration was measured in triplicate. The biogas was vented and the volume was measured each time, and liquid sample was collected to monitor the carboxylic acids concentration throughout the experiment. The governing rate equation of CPDM modeling (Eq. 4-1) is derived from a set of batch fermentations with initial substrate concentrations. Once the specific rate and conversion are known, the governing equation of specific reaction rate, $\hat{r}(x, \mathrm{Aceq})$, can be fit by the least square method.[34]

$$\hat{r} = \frac{e(1-x)^f}{1 + g(\varphi \cdot \mathrm{Aceq})^h} \tag{4-1}$$

where x = conversion

e, f, g, and h are empirical constants

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

Aceq (g/L, acetic acid equivalence) is described in Eq. 2-10

To derive the final expression of CPDM (Eq. 4-1), batch fermentations were conducted to obtain empirical constants *e*, *f*, *g*, and *h*. Other parameters required to obtain the model were selectivity (σ), moisture content (MC), and ash content (AC) from feed substrates and ending solid cake.

Aceq(*t*) is the acetic acid equivalents at each instant during the entire batch fermentation, where the *t* is the time in days. Least square regression is applied to fit the Aceq(*t*) trend (Eq. 4-2), and the reaction rate (*r*) is its differentiation with respect to time (Eq. 4-3).

$$\mathrm{Aceq}(t) = a + \frac{bt}{1+ct} \tag{4-2}$$

$$r = \frac{d\big(\mathrm{Aceq}(t)\big)}{dt} = \frac{b}{(1+ct)^2} \tag{4-3}$$

The specific reaction rate ($\hat{r}$), which is the reaction rate per particle, is calculated by dividing the reaction rate (*r*) by initial substrate concentration (S_0) in each fermentor, where $S_0 = m_0/V$ (Eq. 4-4). In batch fermentation, m_0 is the mass of initial substrate (g VS) and *V* is the fermentor working volume (L).

$$\hat{r} = \frac{r}{S_0} \tag{4-4}$$

Biomass conversion (*x*) can be calculated through Eq. 4-5. This time-dependent parameter is used to determine the CPDM model.

$$x(t) = \frac{\mathrm{Aceq}(t) - \mathrm{Aceq}(0)}{S_0 \cdot \sigma} \tag{4-5}$$

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

For the CPDM model, selectivity (σ) is assumed constant throughout all substrate concentration and is calculated from the selectivity *s* (g acid produced/g VS digested), which is the ratio of acid produced per NAVS consumed. The average selectivity of CPDM batch fermentation were used, and the σ is calculated as follow:

$$\sigma = \frac{s}{\varphi} \tag{4-6}$$

Based on the resulting governing equations, MatLab code (see Appendix I and Appendix J from Fu[39]) was used to simulate four-stage countercurrent fermentation with different LRTs and VSLRs. The empirical constants *e, f, g,* and *h* were determined from the batch experiment data by using the least square method. Lastly, the resulting Aceq was converted back to total carboxylic acid using acid-to-Aceq ratio (φ).

This research is comprised with four different extents of biomass pretreatment according to the following codes:

1. RCS is the batch experiment that uses raw corn stover as fermentation substrate (Table 4-2).
2. SCS is the batch experiment that uses shocked corn stover as fermentation substrate (Table 4-2).
3. NCS is the batch experiment that uses NaOH-treated corn stover as fermentation substrate (Tables 4-3 and 4-4).
4. SNCS is the batch experiment that uses shock + NaOH-treated corn stover as fermentation as substrate (Tables 4-3 and 4-4).

Table 4-2 Initial loadings of each fermentor to start fermentation

	Label	Substrate Concentration (g/L)	Working volume (mL)	Inoculum (mL)	Dry corn stover (g)	Dry Sewage (g)	Carboxylic acids (g/L)	D.O. water (mL)
RCS	20-RCS	20	200	25	2.8	1.2	0	175
	40-RCS	40	200	25	5.6	2.4	0	175
	70-RCS	70	200	25	9.8	4.2	0	175
	100-RCS	100	200	25	14	6	0	175
	100^{+}-RCS	100	200	25	14	6	20	171.1
SCS	20-SCS	20	200	25	2.8	1.2	0	175
	40-SCS	40	200	25	5.6	2.4	0	175
	70-SCS	70	200	25	9.8	4.2	0	175
	100-SCS	100	200	25	14	6	0	175
	100^{+}-SCS	100	200	25	14	6	20	171.1

(Note: D.O. water stands for de-oxygenated water, and the densities of acetic acid, propionic acid, and butyric are 1.05, 0.99, and 0.96 g/cm^3. For the D.O. water required for 100^{+} group: $171.1 = 200 - 25 - 0.2 \cdot \left(\frac{16}{1.05} + \frac{1}{0.99} + \frac{3}{0.96}\right)$)

Table 4-3 NaOH pretreatment parameters on each substrate concentration

	Label	Substrate Concentration (g/L)	Dry corn stover (g)	Total reaction weight (g)	20 g/L NaOH (mL)	D.I. water (mL)
NCS	20-NCS	20	2.8	28	13.2	11.8
	40-NCS	40	5.6	56	26.4	23.6
	70-NCS	70	9.8	98	46.1	41.4
	100-NCS	100	14	140	65.9	59.1
	100^{+}-NCS	100	14	140	65.9	59.1
SNCS	20-SNCS	20	2.8	28	13.2	11.7
	40-SNCS	40	5.6	56	26.4	23.4
	70-SNCS	70	9.8	98	46.1	41.0
	100-SNCS	100	14	140	65.9	58.6
	100^{+}-SNCS	100	14	140	65.9	58.6

(Note: D.I. water stands for de-ionized water, and the density of 20 g/L NaOH solution is approximate at 1 g/cm^3. The moisture content of corn stover is 0.067; shocked-corn stover is 0.099. The total reaction weight is the sum of biomass (wet), NaOH solution, and D.I. water)

Table 4-4 Initial loadings of each fermentor to start fermentation

	Label	Substrate Concentration (g/L)	Working volume (mL)	Inoculum (mL)	Dry Sewage (g)	Carboxylic acids (g/L)	D.O. water (mL)
NCS	20-NCS	20	200	25	1.2	0	147
	40-NCS	40	200	25	2.4	0	119
	70-NCS	70	200	25	4.2	0	77
	100-NCS	100	200	25	6	0	35
	100^{+}-NCS	100	200	25	6	20	31.1
SNCS	20-SNCS	20	200	25	1.2	0	147
	40-SNCS	40	200	25	2.4	0	119
	70-SNCS	70	200	25	4.2	0	77
	100-SNCS	100	200	25	6	0	35
	100^{+}-SNCS	100	200	25	6	20	31.1

(Note: D.O. water stands for de-oxygenated water, and the densities of acetic acid, propionic acid, and butyric are 1.05, 0.99, and 0.96 g/cm^3. For the D.O. water required for 100^{+} group: $31.1 = 200 - 25 - 140 - 0.2 \cdot \left(\frac{16}{1.05} + \frac{1}{0.99} + \frac{3}{0.96}\right)$)

4.4 Results and Discussion

4.4.1 Batch Fermentation

To construct the CPDM map, four sets of batch fermentation representing different extent of pretreatment were conducted. Periodically, liquid samples were taken from each bottle. Because all substrate concentrations were performed in triplicate, the reported values are average concentrations. The acid concentrations were subsequently used for obtaining the governing equation (Eq. 4-1).

Figures 4-2 to 4-5 illustrated the Aceq concentration for each substrate concentration in each batch fermentation. The concentration profile shows the accumulative amount of Aceq in fermentor. Generally, higher substrate concentrations produce greater Aceq. Compared to raw corn stover (RCS), shock-alone pretreated corn stover (SCS) had slightly less amount of Aceq. Previous study done by Falls *et al*. determined whether shock treatment had an effect on biomass crystallinity. For corn stover, shock treatment slightly increased the crystallinity index.[40] The newly formed crystalline structure reduces microbial access to cellulose.[22] This explains why SCS exhibited poorer acid yield than raw corn stover (RCS).

In contrast, corn stover that underwent sodium hydroxide pretreatment (4 g OH^-/100 g dry BM, 50°C, 1 h) (NCS) effectively enhanced acid production when it was applied to mixed-acid fermentation. Moreover, shock + NaOH-treated corn stover (SNCS) exhibited even higher acid concentration than NCS at 70 and 100 g/L. Sodium hydroxide treatment had been found to reduce crystallinity and degree of

polymerization in lignocellulose biomass.[41] Although shock treatment increased biomass crystallinity, it presumably disrupted lignin structure, increased the internal surface area accessible to microorganisms. These effects compensated the drawback of shock treatment (increased crystallinity), making it easier for NaOH to penetrate into the biomass. Consequently, shock treatment combined with NaOH pretreatment resulted in greater acid production than using NaOH alone.

At 100^{+} g/L, product inhibition effect was significant. SNCS only had slightly higher Aceq concentration than the other groups, and RCS and NCS shared similar Aceq concentration trend. SCS had Aceq concentration that was significantly lower than all other groups in the first 25 days (Figure 4-6).

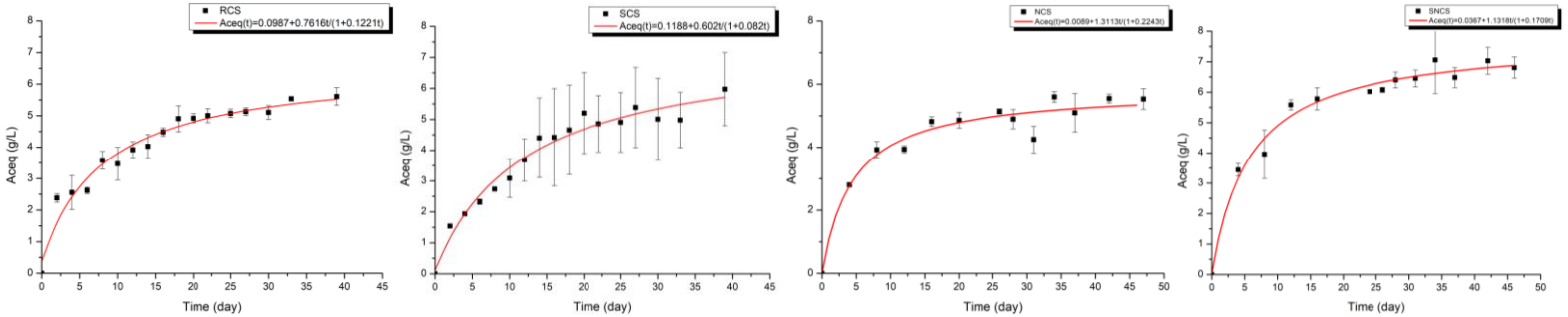


Figure 4-2 Aceq concentration profiles for each pretreatment condition based on 20 g/L substrate concentration. (Note: The errors are derived from 95% confidence intervals.)

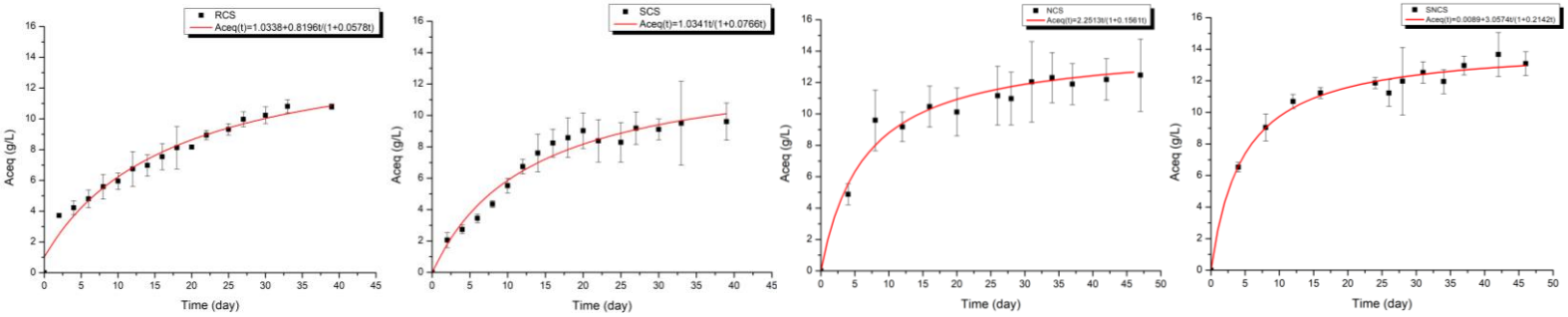


Figure 4-3 Aceq concentration profiles for each pretreatment condition based on 40 g/L substrate concentration. (Note: The errors are derived from 95% confidence intervals.)

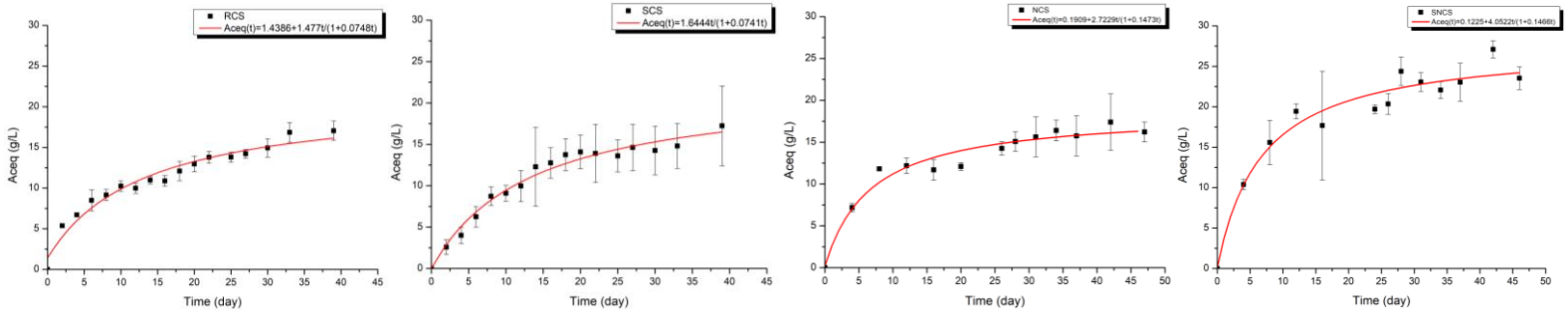


Figure 4-4 Aceq concentration profiles for each pretreatment condition based on 70 g/L substrate concentration. (Note: The errors are derived from 95% confidence intervals.)

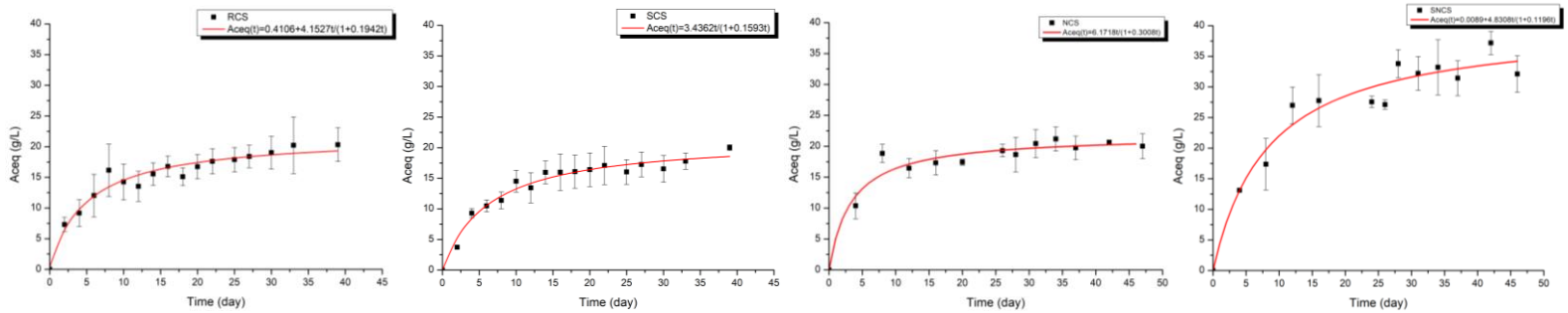


Figure 4-5 Aceq concentration profiles for each pretreatment condition based on 100 g/L substrate concentration. (Note: The errors are derived from 95% confidence intervals.)

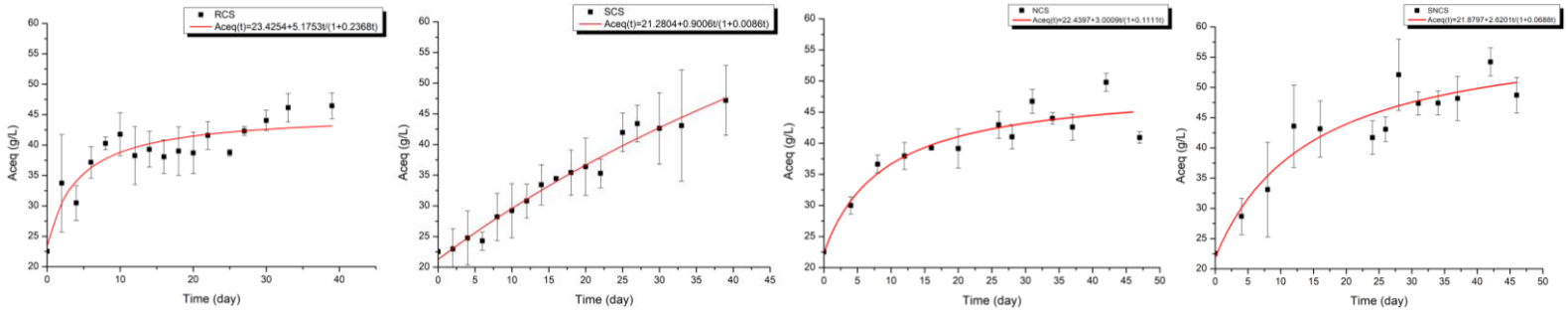


Figure 4-6 Aceq concentration profiles for each pretreatment condition based on 100^{+} g/L substrate concentration. (Note: The errors are derived from 95% confidence intervals.)

4.4.2 CPDM Predictions

Using the least square regression, the governing equation of CPDM model (Eq. 4-1) can be obtained by fitting the Aceq(t), conversion $x(t)$, and the acid-to-Aceq ratio (φ) in each substrate concentration. The specific rate ($\hat{r}$) models for RCS, SCS, NCS, and SNCS are as follow:

$$\hat{r}_{RCS} = \frac{0.041(1 - x)^{4.625}}{1 + 0.037(0.668 \cdot \text{Aceq})^{1.047}}$$

$$\hat{r}_{SCS} = \frac{0.033(1 - x)^{3.986}}{1 + 0.088(0.609 \cdot \text{Aceq})^{0.835}}$$

$$\hat{r}_{NCS} = \frac{0.065(1 - x)^{2.885}}{1 + 0.061(0.740 \cdot \text{Aceq})^{0.934}}$$

$$\hat{r}_{SNCS} = \frac{0.0701(1 - x)^{2.117}}{1 + 0.019(0.769 \cdot \text{Aceq})^{1.432}}$$

To run the MATLAB program, because the values of acid-to-Aceq ratio (φ) and selectivity (σ) were assumed constant, the averaged φ from all substrate concentrations and averaged σ from 100 g/L substrate concentration were used. The above rate equations were used to predict conversions and total acid concentrations for four-stage countercurrent fermentation with LRTs ranging from 5 to 35 days, and VSLRs ranging from 6 to 12 g/(L·day). This model was based on 70 wt% of pretreated (SCS, NCS, and SNCS) or raw corn stover (RCS) with 30 wt% air-dried sewage sludge. The biomass slurry which included dissolved biomass from NaOH treatment was fully utilized as fermentation media.

As shown in Figure 4-7, RCS had greatest predicted acid concentration and conversion at LRT of 35 day and VSLR of 6 g/(L·day), which was 27.5 g/L. At the same condition, the NaOH-treated corn stover (NCS) and shock + NaOH treated corn stover (SNCS) had even better carboxylic acid concentration, which were 33.5 and 36.1 g/L, respectively. But shocked-treated corn stover (SCS) had slightly poorer carboxylic acid concentration at 26.0 g/L. The NCS group reached its peak acid concentration (33.5 g/L) and had conversion of 0.396 g $NAVS_{digested}$/g $NAVS_{feed}$ at LRT of 35 day and VSLR of 6 g/(L·day). Compared to RCS, the CPDM map gradually shifted towards the upper right from the RCS to NCS, then from NCS to SNCS, whereas SCS slightly shifted lower left. This indicates reduced fermentation performance from shock treatment alone, the possible progression resulted from NaOH treatment, and advanced improvement by conducting shock treatment prior to NaOH treatment. The map showed the highest predicted acid concentration in the SNCS group when LRT is 35 day and VSLR is 6 g/(L·day). For industrial application, large LRT and small VSLR are preferred. At LRT of 35 day and VSLR of 6 g/(L·day), SNCS had improved acid concentration by 2.57 g/L and biomass conversion by 0.036 g $NAVS_{digested}$/g $NAVS_{feed}$ compared to NCS; 8.55 g/L improvement on acid concentration and 0.173 g $NAVS_{digested}$/g $NAVS_{feed}$ on biomass conversion compared to RCS.

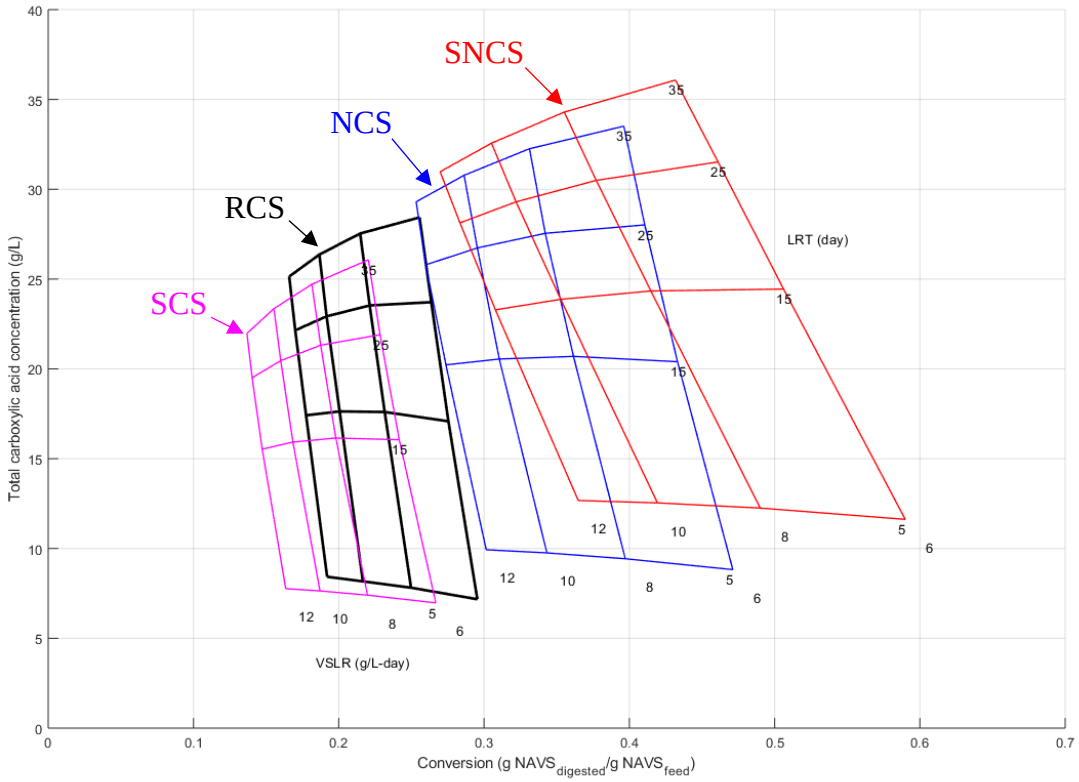


Figure 4-7 The CPDM maps for four-stage countercurrent fermentation using 70 wt% raw or pretreated corn stover and 30 wt% sewage sludge.

4.5 Conclusion

Shock pretreatment benefitted NaOH-treated corn stover under moderate hydroxide loading (4 g OH^-/100 g dry BM) and temperature (50℃) for 1 h, which matched the enzymatic hydrolysis results. However, because of possible increased crystallinity, the fermentation performance did not benefit from shock treatment alone. The acid concentration profiles indicated that SNCS had the greatest acid concentration compared to other groups. In the mixed-culture fermentation, microorganisms digested more shock + NaOH-treated corn stover than NaOH-treated corn stover and raw corn stover.

CPDM simulated the possible scenario when biomass was used in four-stage countercurrent fermentation. The CPDM map showed gradual improvement on conversion and final acid concentration when NaOH and both shock + NaOH pretreatments were applied. In the SNCS group, the total carboxylic acid yields and product concentrations were highest at LRT of 35 day and VSLR of 6 g/(L·day).

CHAPTER V

CONCLUSION AND FUTURE WORK

This study investigated two major steps regarding the MixAlco® process: fermentation and pretreatment.

Ion-exchange resin was a technique that successfully removed product and eliminated product inhibitory effect. Countercurrent fermentation benefitted from using ion-exchange resin. A previous study showed a linearly increasing trend on biomass conversion and yield. From the standpoint of fermentation performance, increasing resin loading did not promote production yield nor increase conversion. Biomass conversion had reached a peak at 30 g resin loading and decreased at loadings higher than 40 g. At 40 g resin loading, production yield reached the maximum at 0.236 g total carboxylic acid produced/g $NAVS_{feed}$, and it had the greatest total acid production from liquid product and resin adsorption in combine. Hence, the optimal resin loading is recommended at 30–40 g (21.74–29.20 g wet resin/L_{liq}). In the future, other techniques such as cells recycle[10], parallel nutrient addition[42], and using fresh nutrients can be incorporated with resin adsorption to investigate better fermentation performance.

As an example of real biomass feedstock, pretreated corn stover was enzymatically saccharified to calculate enzyme digestibility. Although higher temperature increased digestibility, moderate temperature (50°C) was preferred to reduce energy costs and accentuate the effect of shock treatment. Shock treatment

increased enzymatic digestibility using 5.52 bar (abs) initial filling pressure using 50℃ NaOH treatment temperature. Shock significantly improved digestibility using NaOH concentration at 4–5 g OH^-/100 g BM.

Different extents of pretreatment (RCS, SCS, NCS, and SNCS) were performed in this research. Among all substrate concentration (except 100^+ g/L), acid concentration ordered from low to high is SCS, RCS, NCS, then SNCS. CPDM map predicted possible results using different LRT and VSLR. The map indicated countercurrent fermentation could reach 36.1 g/L in acid concentration and 0.432 g $NAVS_{digested}$/g $NAVS_{feed}$ in conversion at LRT of 35 day and VSLR of 6 g/(L·day) using sewage and shock + NaOH-treated corn sotver.

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APPENDIX A

MOISTURE AND ASH CONTENT MEASUREMENTS

1. Record the weight of a clean, dried crucible (W_1).
2. Add approximately 0.1 g of $Ca(OH)_2$ if sample contains volatile acids. Record the weight (W_1'). (Only liquid sample requires additional lime.)
3. Weight approximately 3 g of sample into the crucible.
4. Record the weight with sample loaded (W_2).
5. 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).
6. Ash the crucible at 550 °C for at least 12 h. Remove and allow sample to cool to room temperature in a desiccator. Record the ash weight (W_4).
7. The moisture content (MC) of the sample is calculated as:

$$\text{MC (No lime added)} = \frac{W_2 - W_3}{W_2 - W_1}$$

$$\text{MC (Lime added)} = \frac{W_2 - W_3}{W_2 - W_1'}$$

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

$$\text{AC (No lime added)} = \frac{W_4 - W_1}{W_3 - W_1}$$

$$\text{AC (Lime added)} = \frac{W_4 - W_1}{W_3 - W_1'}$$

APPENDIX B

ION-EXCHANGE RESIN REGENERATION PROCEDURE

1. According to Paragraph 2.3.3, measure the desired amount of 1-N NaOH solution in an appropriate flask, and ensure the CO_2 flow is properly turned off.
2. Put both tubes from the resin column outlet and peristaltic pump inlet into a flask loaded with NaOH solution.
3. Switch on the lower valve of the resin column, and turn on the peristaltic pump to circulate NaOH solution for 35 minutes.
4. Once regeneration completed, remove the sodium hydroxide solution through draining and vacuum pumping. Weigh the NaOH recovery from the flask, and weigh the vacuum recovery.
5. Collect 1.5-mL sample from the NaOH recovery from the flask.
6. The resin in column is washed twice with 250 mL of D.I. water.
7. Store the resin column by adding 100 mL of D.I. water in it.

Figure B-1 Resin regeneration set up.

APPENDIX C

GAS CHROMATOGRAPH MANUAL

C-1. Sample Preparation

1. Centrifuge the liquid sample for 10 min at 13,300 rpm.
2. Prepare a 2-mL plastic microcentrifuge tube. Pipette 0.5 mL internal standard (4-methyl-valeric acid 1.162 g/L, ISTD) and 0.5 mL 3-M phosphoric acid into it.
3. Once centrifuging is completed, pipette 0.5-mL supernatant into its corresponding microcentrifuge tube.
4. Centrifuge the mixture (ISTD + 3-M H_3PO_4 + supernatant) for 10 min at 13,300 rpm for fully mixing.
5. Once centrifuging process complete, pipette 1.0 mL supernatant into a glass GC vial and cap it properly. The sample in the vial is ready to be analyzed.

C-2. Operating Procedure

1. Check the solvent and waste bottles on the injection tower. Dispose waste methanol and replenish the storage vial with new methanol. The methanol level must at least above the minimum amount.
2. Replace the septum beneath the injection tower with tweezers or with hand wearing a clean and new glove.

3. Check the gauge pressure of the gas cylinders. Replace it if needed. (Must turn off the machine while replacing.)
4. Purge the GC column with hydrogen flow (40 mL/min) for 15 min without heating.
5. Place the samples and external standards (ESTD) in the autosampler racks.
6. Check the setting conditions in the method.
7. Inlet Conditions:
    - Temperature: 230 °C
    - Pressure: 15 psig
    - Flow rate: 185 mL/min
8. Detector conditions:
    - Temperature: 230 °C
    - Air flow rate: 400 mL/min
    - H_2 flow rate: 40 mL/min
    - The (makeup) flow rate: 45 mL/min
9. Oven conditions:
    - Initial temperature: 40 °C
    - Initial hold time: 2 min
    - Ramp rate: 20 °C/min
    - Final temperature: 200 °C
    - Final hold time: 1 min

- Total run time per vial: 20 min

10. Start the GC on the computer program (on-line mode) by selecting the method with the conditions listed above.
11. Sequence → New sequence template → Save sequence template.
12. Update the sequence template.
13. Save the sequence template again.
14. To increase precision, the ESTD is calibrated in every 10 samples.
15. At the end of the sequence table, set the GC at STANDBY mode.
16. Run the sequence.

C-3. Data Retrieving

1. Turn on GC off-line mode once analysis completed.
2. Batch → Load batch → File of interested → Select all → Unclick the "STANDBY" sample → OK
3. Input the first guesses of the retention time corresponding to each acid. Make sure the retention time of each acid peaks are included in the range specified (blue background).
4. Calibrate the retention time by clicking the "scale sign".
5. Once calibration complete, click "START".
6. Batch → Option → change file name.
7. Batch → Output batch report.

APPENDIX D

CITRIC BUFFER PREPARATION

1. Use graduate cylinder to measure 1 L D.I. water.
2. Add 8.4 g citric acid monohydrate and 17.65 g trisodium citrate dihydrate. Stir until complete dissolved.
3. Measure the pH and make sure it is at the range of 4.78–4.82.
4. Store at 4℃.

APPENDIX E

ADDITIVES PREPARATION

1. Prepare the solution contains 70 vol% ethanol and 30 vol% water.
2. Weigh the chemical for 10 g/L solution.
3. Suspend tetracycline powder in measured amount of ethanol before adding water.
4. Do not store the solution, because tetracycline will precipitate over time, and it is hard to re-dissolve it. Weigh exact amount you need at a time.
5. Once dissolved, add measured amount of water.
6. Instead of using ethanol/water solution, cycloheximide use D.I. water as solvent. Measure exact amount of cycloheximide to prepare 10g/L solution.

APPENDIX F

SHOCK TREATMENT PROCEDURES

F-1 Pre-operational Check

1. Gather all required equipment for the experiment: biomass, sieve, impact wrench, 2-L graduated cylinder, 1-gal bucket, and 1-L HDPE Nalgene® sample bottle.
2. Upon arriving the pilot plant, empty the shock-gun, check the gas cylinders if it has leakage, connect the water hose, measure the biomass based on pre-measured moisture content, and check if the pressure transducer is properly connected and greased.
3. Turn on the water pipeline, oxygen and hydrogen gas cylinders, and air compressor.
4. Connect the impact wrench to the pressurized air pipeline.

F-2 Loading Shock-gun

1. Mix the weighed biomass with tap water to reach desired working volume. (In this study, it is 2 L. Desirably, add water to the biomass until 1.8 L, and use the remaining 0.2 L to wash the residual biomass on the wall.)
2. Pour the biomass slurry into the shock-gun reactor, and mix until homogenized.
3. Place gasket on upper flange of test section and lower the barrel on top.
4. Use impact wrench to tighten the flange.
5. Close the doors for both shock-gun reactor and control room.

F-3 Shock Treatment

1. Retreat all personnel to the control room.
2. Turn on the LabView control program (Manual Control).
3. Click “start” → click “upper exhaust” to close the exhaust → click “oxygen”
4. Fill up the shock-gun with 6.53 bar (abs) (100 psia) oxygen, and click “oxygen” again to stop the addition.
5. Click “upper exhaust” to open the exhaust to vent out the gas.
6. To ensure an oxygen-rich environment, repeat Steps 3 to Step 5 for 3 times to purge out the remaining nitrogen in the shock-gun.
7. Turn on “Main Control” file → click “start”.
8. Input file name and enter the required pressure of fuel (hydrogen) and oxygen prior to the program starts.
9. Press “start”, then press “fill sequence.”
10. Wait until the 20 min countdown stop.
11. Press the “easy” bottom to ignite the shock-gun.
12. From ignition to actual explosion may take approximately 20 seconds.
13. Once shock explosion completed, open the door and unlock the flange with impact wrench.

F-4 Product Collection

1. Turn on the water and prepare the 1-gal bucket to collect the pretreated biomass.

2. One person tilts the shock-gun and pours out the biomass slurry into the bucket, and the other person holds the bucket and washes out the remaining biomass.
3. Filter the pretreated biomass with 80-mesh sieve.
4. Wring out the excess water by hand, then store it in the sample bottle.

F-5 Cleaning Procedures

1. Turn off the water pipe and the power of air compressor.
2. Switch off the pressurized air and disconnect the impact wrench.
3. Switch off the hydrogen and oxygen cylinders.
4. Click the "Manual Control" → "start" → click "upper exhaust" → click "oxygen" to vent out the remaining oxygen in the pipeline.
5. Click "hydrogen" to vent out the remaining hydrogen in the pipeline.
6. Check the gauge to see if the pressure has lowered to atmosphere.
7. Shut down the computer and close the door of the control room.

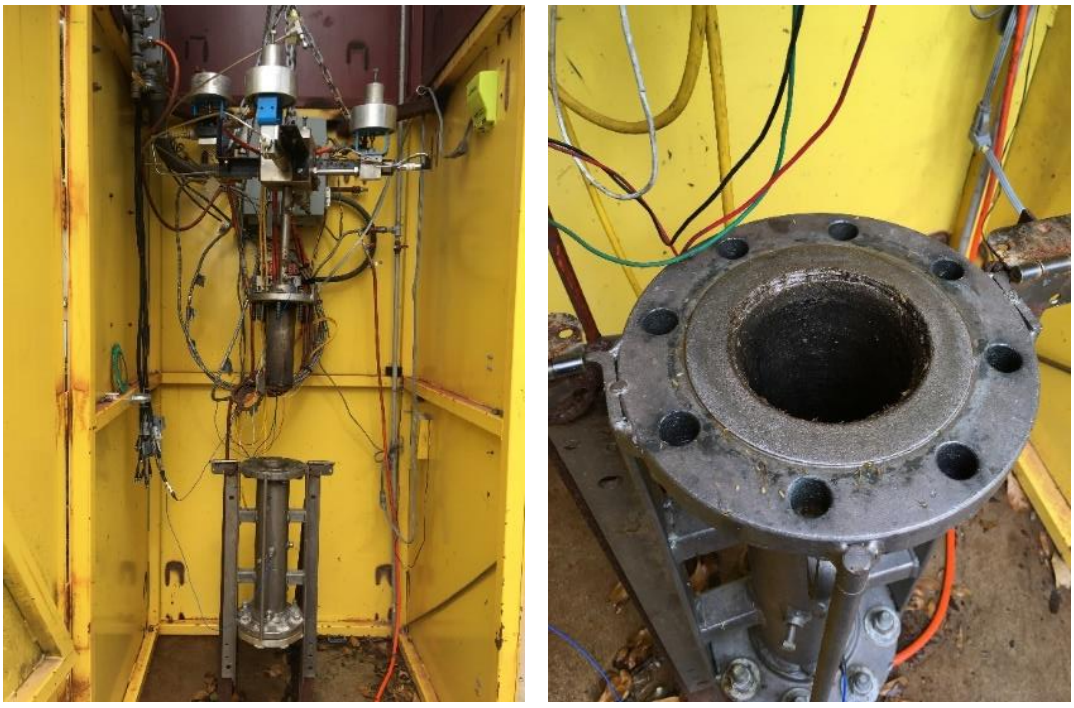

Figure F-1 Shock tube apparatus (left) and its biomass inlet (right).

APPENDIX G

HIGH PERFORMANCE LIQUID CHROMATOGRAPH MANUAL

G-1 Sample Preparation

1. Pipette 2 mL slurry into three 2-mL labeled sample vial, and store other two in freezer.
2. Make sure three sample vials are equally filled. Centrifuge them at 13,000 g for 10 min.
3. Use a 1-mL syringe to collect sample fluid.
4. Use a 0.21-μm filter to filter the fluid into a HPLC vial, there should be at least 0.5 mL in the vial.
5. Check if the fluid in the vial is clear. If not, dispose the filter and vial and return to Step 2
6. Cap the HPLC vial.
7. Label the glass vial and proceed to other samples (go back to Step 1).
8. Prepare a vial with 1 mL CVS (Control Verification Standard).
9. Prepare each one vial with 0.5 mL of 1, 2, 5, 10, and 25 g/L calibration solutions (adjust to your needs, they should cover the range of your concentration of interest.)

G-2 Preparation of HPLC

1. Turn on “HPLC online” program.

2. Backflush the needle and needle seat once in a month.
3. Open the purge valve (black nob on top left of HPLC).
4. Click on *Method* → *Edit entire Method* → click on *OK* twice → choose "Als" and continue with OK.
5. Find *Maximum Flow Gradient* in the "Advanced" section and set it to 1 mL/min^2.
6. Set *Flow* to 5 mL/min
7. Click *Apply* and close the window, this will purge the tubes.
8. Preset and turn on the temperature control of the column (85°C) and temperature control of the RID on (right click RID field and chose *Switch Heater On*, setting at 55°C.).
9. Repeat Step 4, and set *Maximum flow gradient* to 0 mL/min/min and *Flow* to 0 mL/min.
10. Close the purge valve, this will direct the flow through the column.
11. Repeat Step 4, and set *Maximum flow gradient* to 0.1 mL/min/min and *Flow* to 0.6 mL/min. The column pressure should be around 30 bar, but not above 40 bar.
12. Wait until RID turns green and RI signal (blue line) is constant. (This may take overnight.)

G-3 HPLC Operation and Data Collection

1. Put the samples, CVS and calibration solutions on the sample tray.
2. Choose *Sequence* → *Sequence table*
3. Name all vials in the table.

4. *Sequence* → *Sequence Parameters* → choose your directory.
5. Make sure there is enough HPLC grade water in the bottle (flow is 0.6 mL/min, HPLC stops if there is less than 0.35 L in the bottle).
6. Turn the UV-light on by right clicking the *DAD* field and choosing *Turn on UV*, wait for the *DAD* field and all other fields turn green.
7. Click *Sequence* → *Sequence table* → *Run sequence* this will start the analysis. The analyzing time for each sample is 25 min.
8. Once the analysis is completed, set the flow to zero, and turn off the UV and Column Heater.
9. Open *HPLC offline* program and click *Batch* → *Load Batch* and choose the result file.
10. Choose *Select all*, scroll to the bottom of the table and unclick *slowflow*.
11. Choose *Method* → *Load Method* Choose the SUGAR_INTEGRATION method.
12. Choose *Calibration* → *Calibration table*.
13. Choose your first calibration sample and click on C*alibration* → *Recalibrate* → R*eplace* → *level 1* → OK.
14. Repeat step 4 for all your standard samples (choose level according to the calibration table).
15. Check that all response factors are similar and the correlation is close to 1.
16. Click *Start*.
17. Click *Batch* → *Output batch report* to generate a pdf file of your results.

APPENDIX H

DE-OXYGENATED WATER PREPARATION

1. Fill a large glass flask (~4 L) with D.I. water. Place the flask over a hot plate until boiling.
2. Boil the water for 10 min.
3. Seal the top of the container with aluminum foil and cool down to room temperature.
4. Based on the remaining water volume, add 0.275 g/L cysteine hydrochloride and 0.275 g/L sodium sulfide into the boiled water.
5. Stir the solution until both chemicals are completely dissolved and pour into storage tank.

APPENDIX I

ALKALI TREATMENT PROCEDURES

1. Record the empty weight of petri dish.
2. Approximately 5 g of raw materials are weighted and placed on a petri dish, and transfer it in to the oven (105℃) for 4 h.
3. Pre-heat the shaker and water bath at 50℃.
4. Record the post oven weight, and calculate the moisture content (MC) of the sample.
5. Add pretreated biomass in dry weight basis into the reactor. The W_{Biomass} stands for the actual biomass weight used for experiment, and W_{dry} stands for biomass in dry weight basis.

$$W_{\text{biomass}}(\text{g BM}) = \frac{W_{\text{dry}}\ (\text{g BM})}{1 - \text{MC}}$$

6. Calculate $V_{\text{caustic solution}}$ according to Eqs. 3-1 and 3-2 based on the hydroxide concentration. In this study, $c_{caustic} = 20\ \text{g NaOH/L}$ solution was used.
7. Measure D.I. water until the slurry reaches 10 wt% total reaction weight. In this experiment, assuming the 20 g/L NaOH solution has the same density as water, so that $V_{\text{caustic solution}}$ can be approximated as its weight (W_{caustic}), and W_{water} is the weight of D.I. water:

$$10\ \text{wt\%} = \frac{W_{\text{biomass}}\ (\text{g BM})}{W_{\text{water}}\ (\text{g}) + W_{\text{caustic}}(\text{g}) + W_{\text{biomass}}(\text{g})}$$

8. Mix W_{water}, W_{caustic}, and W_{biomass} into a reactor (1-L PCCO Nalgene® bottle).
9. Put the reactors into 50℃ water for 10 min.
10. Transfer the reactors into the pre-heated shaker for 50 min.
11. The reactors were soaked into cold water bath for 5–10 min to terminate the reaction.
12. Connect the inlet of gas distributor with carbon dioxide (CO_2) pipeline, sealing the joint with Parafilm.
13. Insert the gas distributer into the pretreatment slurry in the reactor.
14. Turn on the CO_2 at the flow rate 0.5 L/min, then gradually increases to 1.0 L/min.
15. Turn off the CO_2 as soon as the bubble starts to form.
16. Test the pH.
17. Terminate the neutralization and collect the slurry back to the reactor if the pH is 6.5–7.0.
18. The pretreated biomass is ready for mixed-culture batch fermentation described in Paragraph 4.3.4.

APPENDIX J

INOCULUM ADAPTATION PROCEDURES

J-1 Initiation

1. Prepare enough amount of D.O. water.
2. Autoclave fermentor bottle and rubber stopper (with glass tube and septum).
3. Weigh 50 g/L dry solids of substrate into the autoclaved bottle. In this study, 400 mL is the working volume of the fermentor, thus 20 g dry solid is required.
4. Weigh 350 mL of D.O. water.
5. Weigh 50 mL of fresh Galveston inoculum. This corresponds to 12.5 vol% of the working volume.
6. Add all of abovementioned ingredients into the autoclaved fermentor.
7. Add 120 μL methane inhibitor solution into the fermentor.
8. Purge bottle with nitrogen, capped, and place in incubator.

J-2 Further Treatments

1. Every two days, remove the fermentor from the incubator.
2. Sample 30-mL gas with syringe, and analyze the gas composition by GC. Check if it contains methane, and CO_2 proportion.
3. Vent the rest of the gas by the apparatus described in Figure 2-5.
4. Centrifuge the fermentor bottle for 10 min at speed of 4000 rpm.
5. Decant the supernatant into a beaker.

6. Measure pH of the supernatant. Add appropriate amount of $NaHCO_3$ buffer to adjust the pH until 6.5–7.5.
7. Add 120 μL methane inhibitor solution if CH_4 is detected.
8. Purge bottle with nitrogen and place in incubator.
9. This routinely procedure must proceed for approximately 3 weeks.

J-3 Restart the Inoculum Adaptation

1. Centrifuge the fermentor bottle for 10 min with 4000 rpm.
2. Pour out the inoculum with established culture, storing in another autoclaved bottle.
3. Split the remaining solid cake into two fermentor bottles.
4. Redo the procedures described in Section J-1 Step 3 to Step 8.
5. Same procedures listed in Section J-2 must be done on both bottles.
6. This adaptation process usually takes shorter than the first one. The inoculum can be used for future experiment in 2 weeks.