MAXIMIZING MEDIUM-CHAIN CARBOXYLATES IN MIXED-CULTURE FERMENTATION

A Dissertation

by

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ABSTRACT

Global warming, steadily increasing energy demand, and limited fossil fuel reserves are growing concerns of modern society. In the past few decades, significant advances in renewable energy research have helped reduce dependence on conventional non-renewable energy sources. Biofuels are sustainable and can replace petroleum-based fuels. Biofuels can be produced through three different platforms: thermochemical, sugar, and carboxylate. Based on experimental results, this dissertation suggests process improvements in the carboxylate and sugar platform to make biofuels more economically attractive.

The carboxylate platform is a robust and scalable technology that produces fuels and chemicals from biomass. It employs methane-inhibited anaerobic fermentation to produce mainly short-chain fatty acids (SCFA, e.g., acetic, propanoic, butanoic, pentanoic). Medium-chain fatty acids (MCFA, e.g., hexanoic, heptanoic, octanoic acid) are more valuable than SCFAs. By feeding ethanol to the fermentor, MCFA formation is enhanced through chain elongation. To maximize MCFA production, alcohol concentrations and temperature were optimized in the mixed-culture fermentation. Chain elongation occurs at low temperatures (≤40 °C) and does not occur at 55 °C.

Using the sugar platform, enzymes are a major cost contributor in biofuel production. Conventionally, enzymatic saccharification is performed in batch. To more efficiently use enzymes, a new continuous countercurrent method is explored. Pseudo-continuous countercurrent saccharification was performed on lime-pretreated corn stover

at enzyme loadings of 1 mg CTec3/g dry biomass and (1 mg CTec3 + 1 mg HTec3)/g dry biomass and the results were compared with batch. To achieve the same glucan conversion as compared to batch, countercurrent saccharification reduced enzyme loading by 1.6 and 1.4 times at 1 mg protein/g biomass and 2 mg protein/g biomass, respectively.

In rapidly growing developing countries, waste disposal is a major challenge. To address this challenge, the MixAlco process was investigated as an alternative to create economic incentives for waste disposal. The MixAlco process is one example of carboxylate platform. This work focuses on fermenting municipal solid waste in batch fermentations. Using the Continuum Particle Distribution Model (CPDM), the performance of continuous countercurrent fermentation was predicted at different volatile solid loading rates (VSLR) and liquid residence times (LRT).

To my parents, brother, and sister who always believed

in me and encouraged me to pursue my dreams.

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NOMENCLATURE

SCFA	Short-chain fatty acids
MCFA	Medium-chain fatty acids
SLP	Submerged-lime pretreatment
CPDM	Continuum particle distribution model
NAVS	Non-acid volatile solids
DI water	Deionized water
Conc	Concentration
Temp	Temperature
Acetic acid	Ethanoic acid
Propionic acid	Propanoic acid
Butyric acid	Butanoic acid
Valeric acid	Pentanoic acid
Caproic acid	Hexanoic acid
Caprylic aid	Octanoic acid

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1. INTRODUCTION

Global population is expected to reach 9.7 billion by 2050 [1]. With modernization and improvements in living standards, energy consumption per capita is increasing rapidly. From 2012 to 2040, total world consumption of marketed energy will increase by 48% [2]. To meet this growing energy demand, energy production should increase at the same rate.

Today, fossil fuels provide most of the world's energy. Liquid fuels, natural gas, and coal account for 80% of total world energy consumption [2]. With extraction and burning of fossil fuels, underground carbon is released as carbon dioxide and accumulates in the atmosphere, which contributes to global warming. Global warming leads to increasing heat stress, severe weather conditions, melting of glaciers, and rising sea levels [3]. To avoid severe outcomes of global warming, global average temperatures rise should be limited to 2 °C relative to pre-industrial levels (about 1.1 °C above present levels) [4]. To meet increasing global energy demand while limiting global warming, renewable energy sources such as biofuels, solar, wind, etc., should be developed.

In the past few decades, advances in renewable energy research have developed efficient technologies to produce clean energy. Clean electricity can be produced by harvesting solar and wind energy and is gaining popularity; however, all liquid fuels consumed in the transportation and industrial sectors cannot be replaced with electricity. Transportation – including aviation, shipping, and heavy vehicles – require energy-dense fuels. Biofuels can replace conventional liquid fuels. To accommodate new technology,

replacing existing energy infrastructure is highly capital intensive. With minor modifications, biofuels can be produced and distributed using existing infrastructure.

For biofuels to compete economically with petroleum-derived fuels, it is important to have efficient technologies and inexpensive feedstocks. Biomass can be converted to fuels through three platforms: thermochemical, sugar, and carboxylate. The intermediates produced in the thermochemical, sugar, carboxylate platforms are syngas or bio-oil, sugars, and fatty acids, respectively. These intermediates are further converted to chemicals and fuels.

Lignocellulose is the least expensive and most abundant renewable material available on earth. It is comprised of about 35–50% cellulose, 15–25% hemicellulose, and 10–25% lignin. The remaining portion is made of minerals, free sugars, protein, etc. Cellulose is a polymer of glucose. Hemicellulose is a polymer of pentose sugars: arabinose, galactose, glucose, mannose, and xylose. Xylose is the most prevalent sugar in hemicellulose. Lignin acts as glue that binds cellulose and hemicellulose chains together. The recalcitrance of lignocellulosic biomass is overcome by pretreatment. Pretreatment removes lignin, thereby making binding sites in cellulose and hemicellulose more accessible. Most pretreatments use acid or base and/or high temperature. Pretreated biomass can be enzymatically converted to sugars or directly fermented to chemicals (e.g., carboxylic acids, alcohols, ketones, etc.).

The carboxylate platform is a robust and scalable technology that employs methane-inhibited anaerobic fermentation. The main advantages of the carboxylate platform include flexible feedstock, higher yields, scalability, and non-sterile operating

conditions. Pretreated biomass is anaerobically fermented using a mixed culture of microorganisms. The fermentation reactions occur in mainly three stages: hydrolysis, primary fermentation, and secondary fermentation. Hydrolysis breaks down polymers (e.g., cellulose) into monomers (e.g., glucose). The primary fermentation converts monomeric sugars into short-chain fatty acids (SCFA, e.g., acetic, propanoic, butanoic acid) and ethanol. During secondary fermentation, SCFAs and ethanol are converted to medium-chain fatty acids (MCFA, e.g., hexanoic, heptanoic, octanoic acid). In a typical anaerobic fermentation, carboxylic acids are further converted to methane by methanogens. In the carboxylate platform, methanogens are inhibited, which allows carboxylic acids to accumulate in the fermentation broth. The produced carboxylic acids can be converted to chemicals and fuels via three routes: Kolbe electrolysis, secondary alcohols, and primary alcohols [5]. The MixAlco process is one configuration of the carboxylate platform. One embodiment of the MixAlco process converts secondary alcohols to fuels.

In the MixAlco process (Figure 1-1), lime pretreatment removes lignin from lignocellulosic biomass. Pretreated biomass is anaerobically fermented to produce mixed fatty acids. To regulate pH, buffer is added to the fermentation, thus producing carboxylate salts. Methane formation is mitigated using methanogen inhibitors such as iodoform. The fermentation broth is dewatered to obtain dry carboxylate salts that are thermally converted to ketones. Ketones are hydrogenated to produce mixed secondary alcohols, which can be oligomerized using zeolite catalyst to produce hydrocarbons (gasoline or diesel).

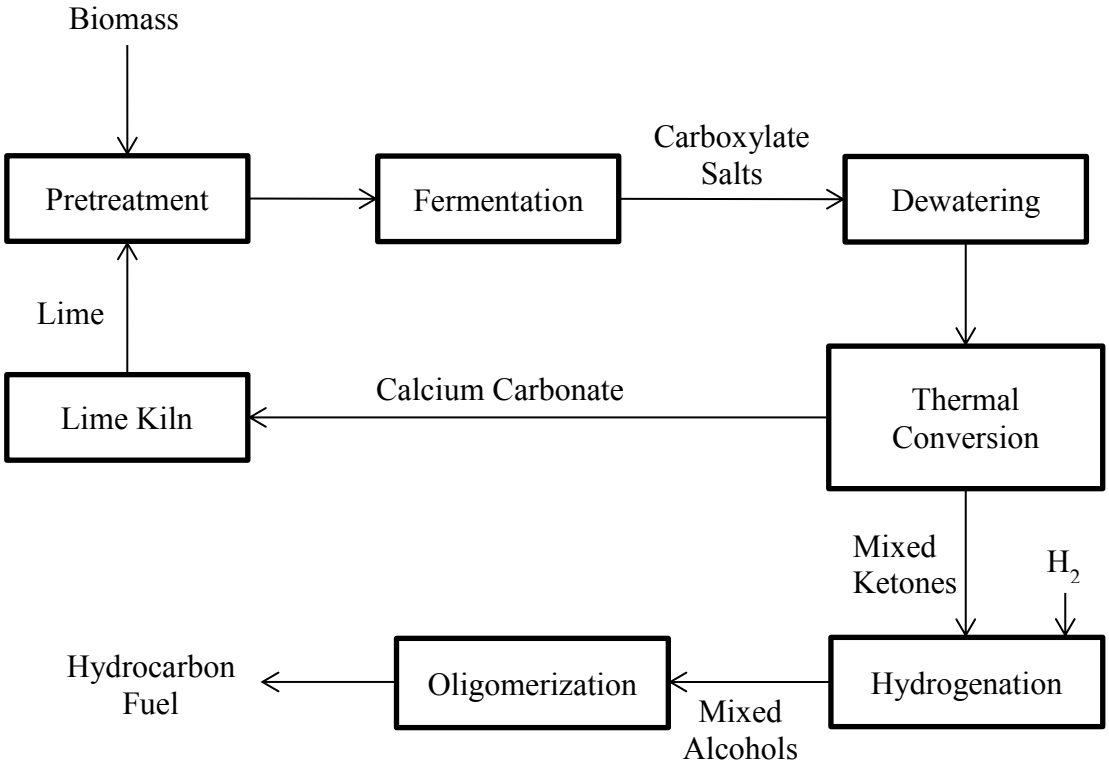

Figure 1-1. Schematic of the MixAlco process.

Bioethanol is a well-developed route of the sugar platform. First-generation bioethanol, which produces ethanol from starch and sugars, is a mature commercial technology. Because starch and sugar sources serve food markets, it is necessary to commercialize second-generation bioethanol, which produces ethanol from lignocellulose. Figure 1-2 shows process flow diagram of the sugar platform.

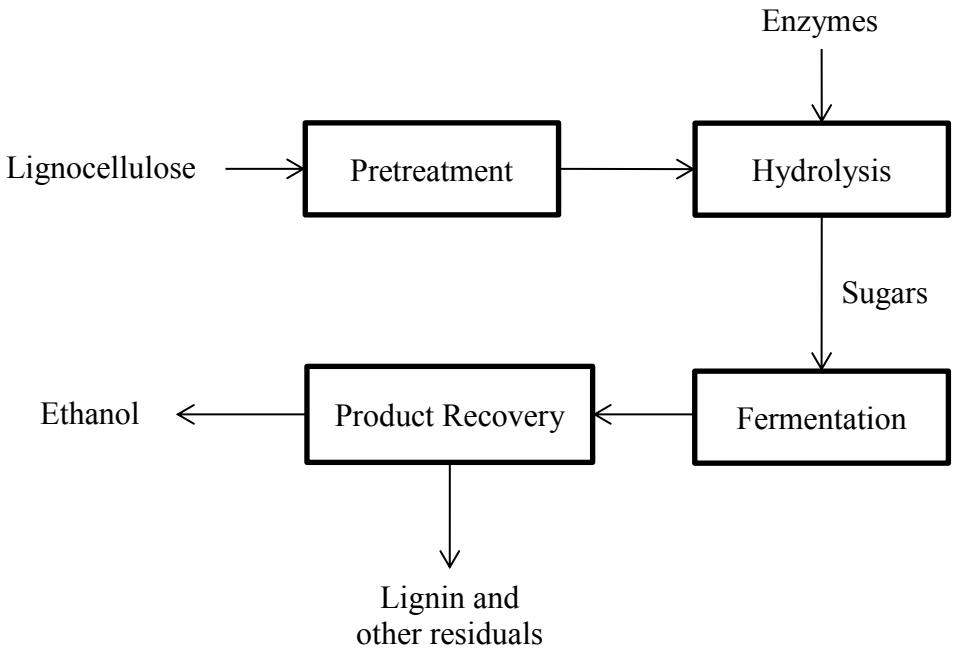

Figure 1-2. Simplified process flow diagram of the sugar platform.

The enzymatic hydrolysis of starch and lignocellulose is conceptually similar, but cellulose is far more resistant than starch. To hydrolyze lignocellulose, efficient mixtures of cellulase and hemicellulase enzymes are required. The sugars produced via enzymatic hydrolysis are fermented to ethanol. The fermentation broth is distilled to recover virtually pure ethanol in the overhead and solids and water in the bottoms. The solids are partially dried and used as boiler fuel. To commercialize cellulosic ethanol technology, major challenges are to reduce pretreatment and enzyme costs. To reduce enzyme costs, enzymes should be produced and recycled inexpensively, or their effectiveness should be enhanced.

To mitigate greenhouse gas emissions and control global warming, renewable energy sources are vital. Biofuels can replace liquid petroleum fuels. This work aims to modify existing biofuel production technologies to make them more economical and efficient. Recent advances in mixed-culture fermentations enable the production of medium-chain fatty acids from short-chain fatty acids and ethanol by chain elongation. To improve the productivity of medium-chain fatty acids, it is necessary to optimize the process parameters. In this dissertation, Sections 2 and 3 focus on chain elongation in mixed-culture fermentation. In the past, benefits of countercurrent saccharification have been explored for pure cellulosic substrates. Section 4 reports countercurrent saccharification of real substrate, i.e., lime-pretreated corn stover. Section 5 focuses on mixed-culture fermentation of municipal solid waste.

2. OPTIMUM ALCOHOL CONCENTRATION FOR CHAIN ELONGATION IN MIXED-CULTURE FERMENTATION[1]

2.1. Introduction

Traditionally, the carboxylate platform employs methane-inhibited anaerobic fermentation to produce mainly short-chain fatty acids (SCFAs) (acetic, propionic, butyric, and valeric acid). These SCFAs are valuable themselves; however, they can be converted into other valuable chemicals and fuels. The spectrum of the fatty acids produced via mixed-culture anaerobic fermentation can be altered by adding ethanol [6, 7]. In the presence of ethanol, microorganisms (e.g., *Clostridium kluyveri*) convert acetic acid and ethanol into butyric acid, which further combines with ethanol to form caproic acid [8]. This chain elongation allows the use of mixed-culture fermentation to produce medium-chain fatty acids (MCFAs) (caproic, heptanoic, and octanoic acid). Using the MixAlco process, this research focuses on the production of MCFAs by adding ethanol to mixed-culture fermentation of cellulosic biomass [9].

The MixAlco process is one example of the carboxylate platform, which produces fuels from waste biomass [10]. In one version of the MixAlco process, pretreated biomass is anaerobically fermented to produce carboxylate salts. The fermentation broth is dewatered to dry carboxylate salts, which are thermally decomposed to their corresponding ketones, as shown in Equation (2.1).

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[1]Reproduced in part with permissions from "Optimum alcohol concentration for chain elongation in mixed-culture fermentation of cellulosic substrate", Lonkar et al., 2016 (doi:10.1002/bit.26024). *Biotechnology and Bioengineering*, John Wiley and Sons. Copyright 2016 Wiley.

$$M^+(RCOO^-) + M^+(R'COO^-) \rightarrow RCOR' + M_2CO_3 \quad (2.1)$$

where R and R′ are alkyl groups and M^+ is a cation that depends upon the buffer used in the fermentation. The composition of ketones depends on the composition of fatty acids produced in the fermentation and can be predicted by using a random pairing model [11]. Ketones are hydrogenated to produce mixed alcohols, which can be dehydrated to hydrocarbons (e.g., gasoline or jet fuel). The hydrocarbon chain length for gasoline, jet fuel, and diesel ranges from C4–C12, C8–C16, and C8–C21, respectively. According to the U.S. Energy Information Administration [12], from 2014 to 2040 the demand for diesel is expected to increase by 21% whereas demand for gasoline will decrease by 24%.

Table 2-1 shows the typical acid distributions in mixed-culture fermentation under different conditions [5, 13] and Figure 2-1 shows their corresponding ketone distribution determined by the random pairing method. Thermophilic fermentations (55 °C) produce mainly acetic, propionic, and butyric acid [13] and results in ketones (and ultimately hydrocarbons) with carbon number in the range of C3 to C7 (mainly C3) which falls in the lower gasoline range. Mesophilic fermentations (≤40 °C) produce mainly short-chain acids but also small amounts of medium-chain fatty acids. The carbon number in corresponding ketones and hydrocarbons ranges from 3 to 11 with the majority of hydrocarbons between C3–C7 which falls in the gasoline range (Figure 2-1). With chain elongation in mesophilic fermentation, the fermentation acid distribution shifts towards medium-chain length (Table 2-1) and the corresponding ketones and hydrocarbons will be in the range of C7 to C11 (Figure 2-1), which falls in the lower jet

fuel and diesel range. Thus, the production of MCFAs in the MixAlco fermentation enables the production of jet and diesel fuels, which are more valuable than gasoline.

Table 2-1. Typical acid distributions in the mixed-culture fermentation under different conditions.

	40 °C (%wt)	**55 °C (%wt)**	**40 °C with ethanol addition[a] (%wt)**
Acetic acid	40	80	18
Propanoic acid	15	4	10
Butyric acid	20	15	13
Valeric acid	10	0.25	15
Caproic acid	11	0.25	42
Heptanoic acid	3	0.25	1
Octanoic acid	1	0.25	1
Total	**100**	**100**	**100**

[a]The acid distribution for fermentation at 40 °C with ethanol addition is obtained from the results discussed in this section. (The case of 10 g/L ethanol addition in Figure 2-5.)

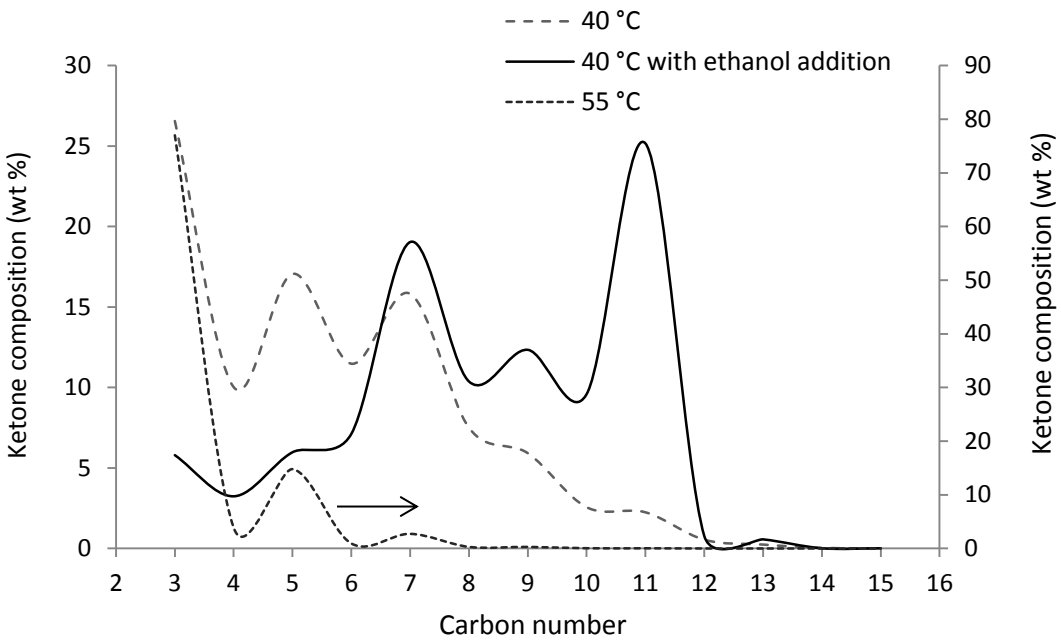


Figure 2-1. Predicted ketone distribution in the MixAlco process for acid spectrums produced at different conditions. (For each ketone, all isoforms were considered together.)

Compared to short-chain fatty acids, medium-chain fatty acids are more hydrophobic, which can be utilized to selectively separate the longer acids from fermentation broth. For example, selective extraction leaves behind SCFAs, which are necessary to produce MCFAs. High concentrations of carboxylic acids inhibit microorganisms during fermentation [14]; therefore, continuous extraction reduces inhibition.

Medium-chain fatty acids are used in the food, pharmaceuticals, and cosmetic industries [15, 16]. For example, ethyl caproate is used as a flavor [17, 18]. Methyl

caproate is used as a stabilizer and plasticizer for hand and face creams [18]. Caprylic acid is used to produce esters used in perfumery and in the manufacture of dyes [19].

Mixed-culture fermentation is very complex. Depending on fermentation conditions (temperature, pH, headspace gas composition, and substrate) different products are obtained. Agler et al. [20] summarized the reactions occurring in mixed-culture fermentation and their corresponding energy changes (ΔG). The biological pathways and the sequence of reactions occurring in mixed-culture fermentation are shown in Figure 2-2. At neutral pH and mesophilic temperature (40 °C), the primary reactions in mixed-culture fermentation convert cellulosic biomass into mainly SCFAs. Methane formation can be mitigated by adding methanogen inhibitors. In the presence of alcohols, secondary reactions convert SCFAs into MCFAs. Using microorganisms similar to *Clostridium kluyveri*, ethanol and acetate (C2) combine to form butyrate (C4), which again combines with ethanol to form caproate (C6). Similarly, by combining with ethanol, propionate (C3) forms valerate (C5) and ultimately heptanoate (C7). The reactions are represented as follows:

$$CH_3COOH + C_2H_5OH \rightarrow C_3H_7COOH + H_2O \quad (2.2)$$

$$C_2H_5COOH + C_2H_5OH \rightarrow C_4H_9COOH + H_2O \quad (2.3)$$

$$C_3H_7COOH + C_2H_5OH \rightarrow C_5H_{11}COOH + H_2O \quad (2.4)$$

$$C_4H_5COOH + C_2H_5OH \rightarrow C_6H_{13}COOH + H_2O \quad (2.5)$$

$$C_5H_{11}COOH + C_2H_5OH \rightarrow C_7H_{15}COOH + H_2O \quad (2.6)$$

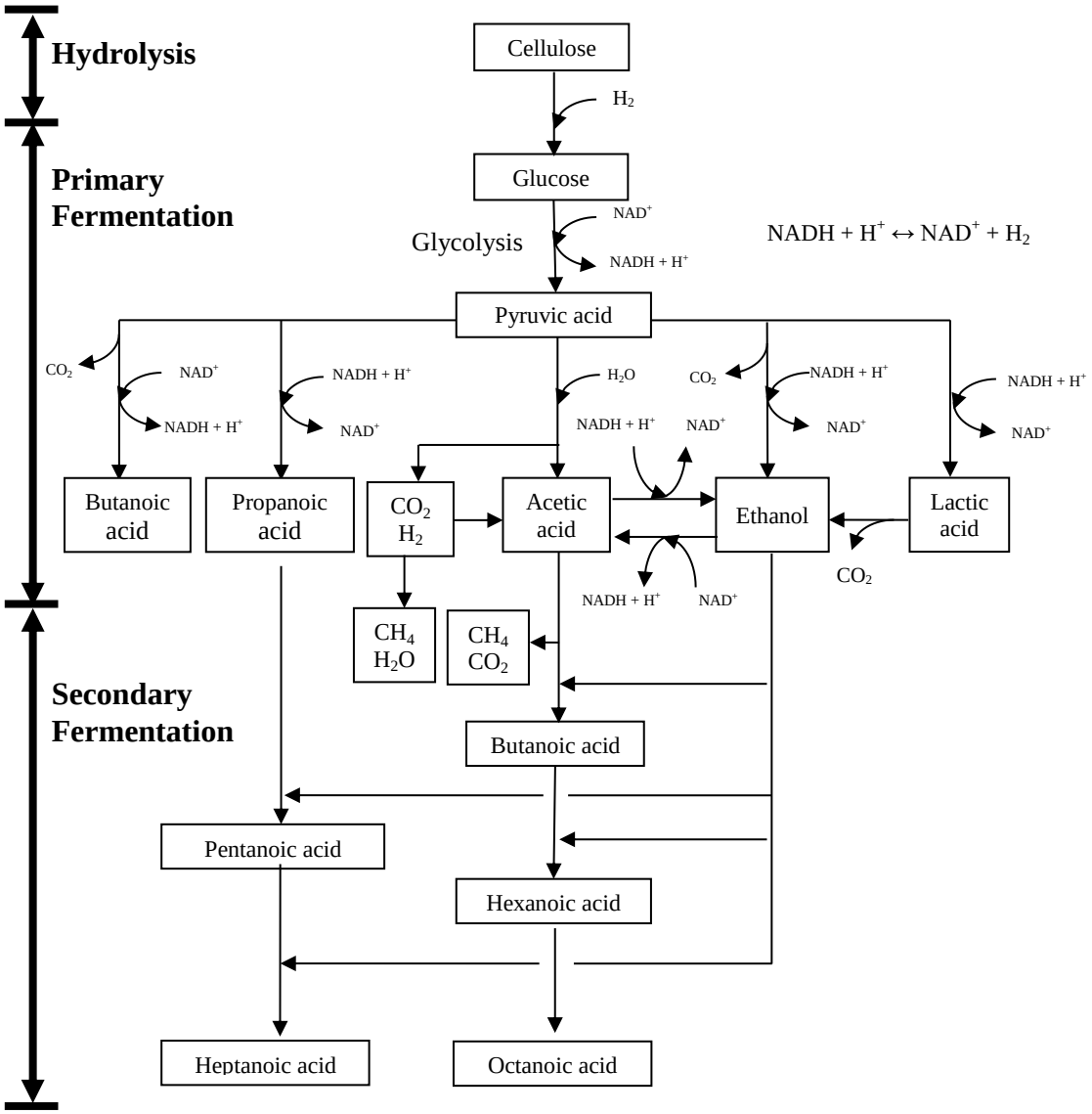


Figure 2-2. Biological pathways in the mixed culture anaerobic fermentation.

The first reports of caproic acid production in fermentation were published by Barker et al. [21]. They observed butyric and caproic acid in methane fermentation of ethanol. Kenealy et al. [22] used co-cultures of ruminal cellulolytic bacteria and *Clostridium kluyveri* to produce caproic acid from cellulose and ethanol. Recently, the unique metabolic features of *Clostridium kluyveri* were investigated by Seedorf et al. [8]. Grootscholten et al. [23, 24] studied the production of MCFAs via mixed-culture fermentation using acetate and ethanol as substrates. In anaerobic mixed-culture fermentation, chain elongation of SCFAs occurs via the reverse β-oxidation pathway [25]. Energy-rich, reduced compounds such as ethanol or lactate initiate chain elongation [25].

Previous literature studies have shown that adding ethanol to mixed-culture fermentation enhances chain elongation and produces more MCFAs. In the fermentation, very low concentrations of ethanol reduce the rates of chain elongation because substrate is not readily available. In contrast, very high concentrations of ethanol inhibit the acid-producing microorganisms resulting in low biomass conversions. Although ethanol is a substrate for chain elongating microorganisms, the optimal ethanol concentration is not reported in the literature. To improve yield, it is necessary to achieve high conversion and selectivity in the fermentation. To reduce downstream separation and processing costs, high acid concentrations are desired in fermentation broth. Considering all these factors, to commercialize the technology, it is important to determine the optimum ethanol concentrations that maximize MCFAs without adversely affecting conversions and selectivity.

Production of MCFAs from propionate and ethanol has been reported in the literature. Using mixed-culture fermentation, Grootscholten et al. [26] achieved a maximum heptanoate concentration of 3.2 g/L. They showed that different SCFAs combine with ethanol to form medium-chain carboxylates. Considering the versatility of mixed-culture fermentation, it is desirable to know the possibility of chain elongation of acids with alcohols other than ethanol. The objective of this research is to explore the optimum concentrations of ethanol and propanol to maximize the production of MCFAs in anaerobic mixed-culture fermentation. This study investigates MCFA production in batch fermentation at varying concentrations of ethanol and propanol. The conversion and selectivity were calculated to evaluate the performance of batch fermentation.

2.2. Materials and methods

2.2.1. Fermentor configuration

The fermentations were performed in 1-L polypropylene plastic bottles capped by a rubber stopper inserted with a glass tube (Figure 2-3). The glass tube was sealed using a rubber septum, which allowed gas sampling and venting. The ¼-inch stainless steel tubing inserted in the rubber stopper enhanced mixing of the slurry.

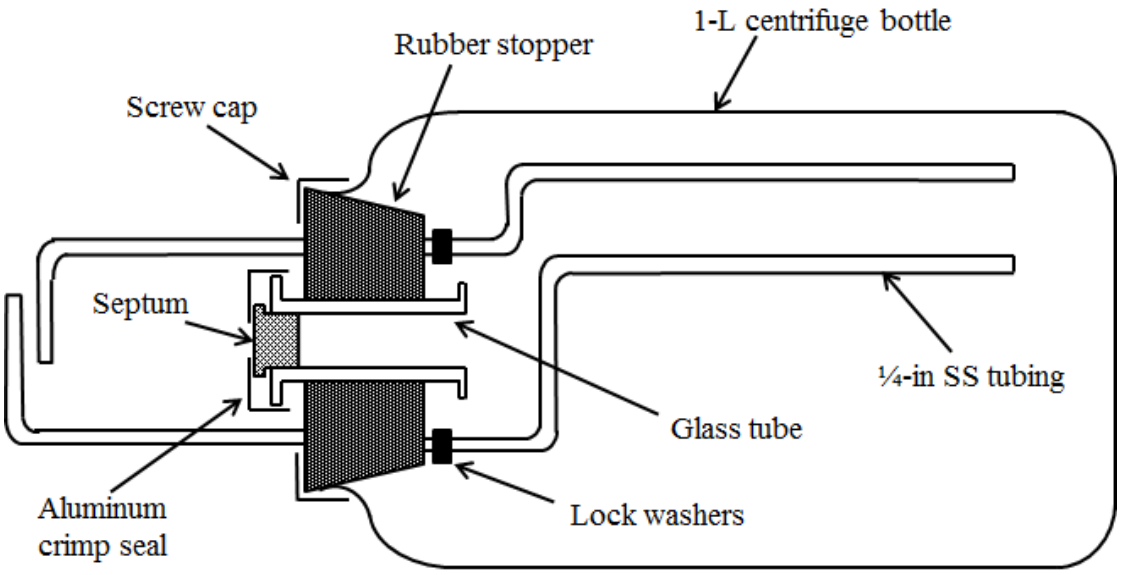


Figure 2-3. Schematic of fermentor.

2.2.2. Substrate

The substrate was shredded office copier paper (GP standard multipurpose paper). The nutrient source was dry chicken manure obtained from Feather Crest Farms, Inc. (Bryan, TX). Office paper and chicken manure were added in the ratio of 4:1 on a dry weight basis. Urea was added to adjust the carbon-to-nitrogen ratio in a range of 25–30 (w/w). Marine inoculum collected from beach sediment of Galveston Island (Galveston, TX) was used as the inoculum source for the mixed culture of microorganisms. The collected inoculum was kept in airtight plastic bottles and was stored at –10 °C until use. Before inoculation, the inoculum was thawed, shaken vigorously, allowed to settle by gravity, and then added to the fermentor. The inoculum

was 12.5% by volume of the total liquid present in the fermentor. The deoxygenated water added to the fermentor was prepared by boiling deionized water to liberate the dissolved gases and then letting it cool down in a covered vessel. Sodium sulfide (0.275 g/L) and L-cysteine hydrochloride (0.275 g/L) were added to further reduce the oxygen content of deoxygenated water. Ethanol (190 proof, USP, Koptec) and 1-propanol (Certified, Fisher Chemical) were added to corresponding fermentors to achieve desired initial concentrations.

2.2.3. Methane inhibition

To inhibit methanogens, 90 μL of iodoform solution (20 g CHI_3/ L acetone) was added to the fermentor every two days for the first two weeks of fermentation. Because ethanol concentration is one of the parameters under investigation, acetone was used to prepare iodoform solution rather than ethanol. Iodoform is sensitive to light, air, and temperature, so the glass bottle containing the solution was wrapped in aluminum foil and stored in the refrigerator at 4 °C [27].

2.2.4. Analytical techniques

2.2.4.1. Carboxylic acid and alcohol analysis

Dissolved carboxylic acids and alcohols were quantified using an Agilent 7890A gas chromatograph and Agilent DB-FFAP column (30m × 0.320mm). The column temperature limits were from 40 °C to 250 °C. Helium was used as the carrier gas. The frozen samples were thawed, vortexed, and centrifuged at 13,000 rpm for 10 min. Then,

the sample (0.5 mL) was mixed with 3-M phosphoric acid and an internal standard (4-methyl-valeric acid) to analyze the acid and alcohol concentration. The concentrations of carboxylic acids reported, include both the *n*- and iso- forms of acids.

2.2.4.2. pH measurement

The pH of the slurry in fermentors was measured using an Oakton (WD-35614) pH meter. The pH meter was calibrated every time before use. The pH in the fermentors dropped to around 6 when acids were produced. Magnesium carbonate buffer was added in steps to adjust the pH, i.e., it was added in steps of 0.1 g and fermentation slurry was stirred and pH was measured again. The same procedure was repeated until the pH reached 6.8 to 7. When the acid production rate was high, 0.2 to 0.8 g of buffer was added depending on pH adjustment.

2.2.4.3. Moisture and ash content

The volatile solids in the biomass sample are calculated using moisture and ash contents of the sample, which were measured using standard NREL analytical procedures [28]. At the time of termination of fermentations, the slurry was centrifuged to separate solids and liquids. Representative samples were taken from centrifuged solids for moisture and ash measurements.

2.2.5. Batch fermentation

The fermentors were maintained at a constant temperature of 40 °C in the incubator. The incubator is a roller apparatus with a rotational speed of 2 rpm maintained

at constant temperature. The fermentors were monitored every other day to vent the gases produced, take samples, and adjust the pH. The pH was adjusted to 6.8–7 using magnesium carbonate as buffer. At the end of monitoring, nitrogen gas was purged in the fermentors to maintain anaerobic conditions. The liquid samples collected from the fermentors were kept in the freezer at –10 °C until further analysis. The experiment was run in duplicate.

The performance of batch fermentation was evaluated using conversion and selectivity parameters, which are defined as follows:

$$\text{Overall conversion} = \frac{\text{NAVS digested (g)} + \text{Ethanol consumed (g)}}{\text{Total NAVS fed (g)} + \text{Ethanol fed (g)}} \tag{2.7}$$

$$\text{Overall Selectivity} = \frac{\text{Total acids produced (g)}}{\text{NAVS digested (g)} + \text{Ethanol consumed (g)}} \tag{2.8}$$

$$\text{MCFA Selectivity} = \frac{\text{MCFA produced (g)}}{\text{NAVS digested (g)} + \text{Ethanol consumed (g)}} \tag{2.9}$$

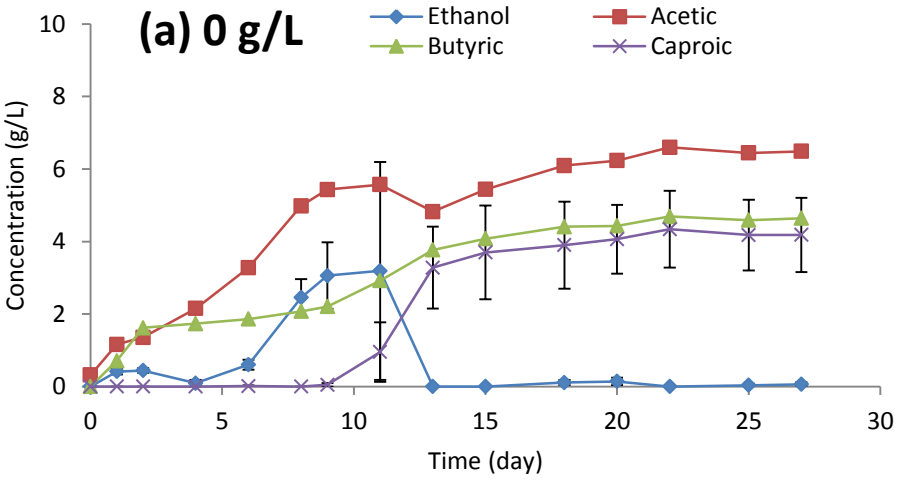
where NAVS is defined as non-acid volatile solids [29]. As the name indicates, NAVS are volatile solids excluding the fatty acids present in the sample.

2.3. Results and discussion

2.3.1. Addition of ethanol to mixed-culture fermentation

Batch fermentation of shredded office copier paper and dry chicken manure was run for a period of 27 days. In all fermentors, the solids concentration was 10% (100 g/L) and the total liquid volume in each fermentor was 400 mL. Ethanol was added initially to the fermentors along with the substrate. The concentration of initially added ethanol was 0, 5, 10, 20, and 40 g/L. The control is the fermentor without any added ethanol (0 g/L).

All fermentors produced MCFAs along with the SCFAs, but the compositions were different. Caproic acid was the major product in the fermentors that were initially fed ethanol. Ethanol consumption started after the first 5–6 days of fermentation (Figure 2-4). The concentrations of caproic acid and ethanol showed a consistent trend in all fermentors; the increase in caproic acid is accompanied by a corresponding decrease in ethanol. This crossover of ethanol and caproic acid concentrations indicates ethanol is consumed to produce MCFAs. Whenever ethanol concentration was constant during the fermentation or all the ethanol was consumed, caproic acid production stopped. The fermentors with higher concentrations of initially added ethanol (>10 g/L) consumed only some of the available ethanol.



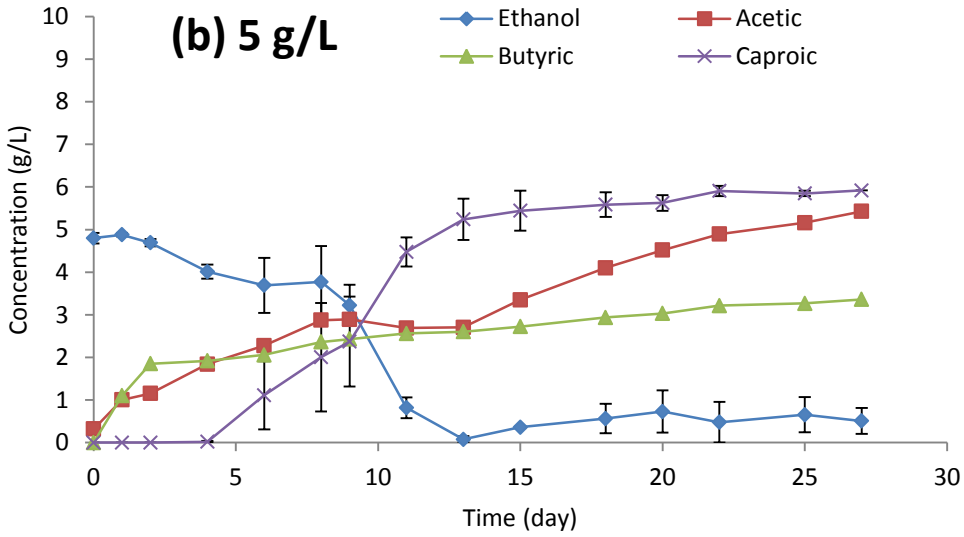
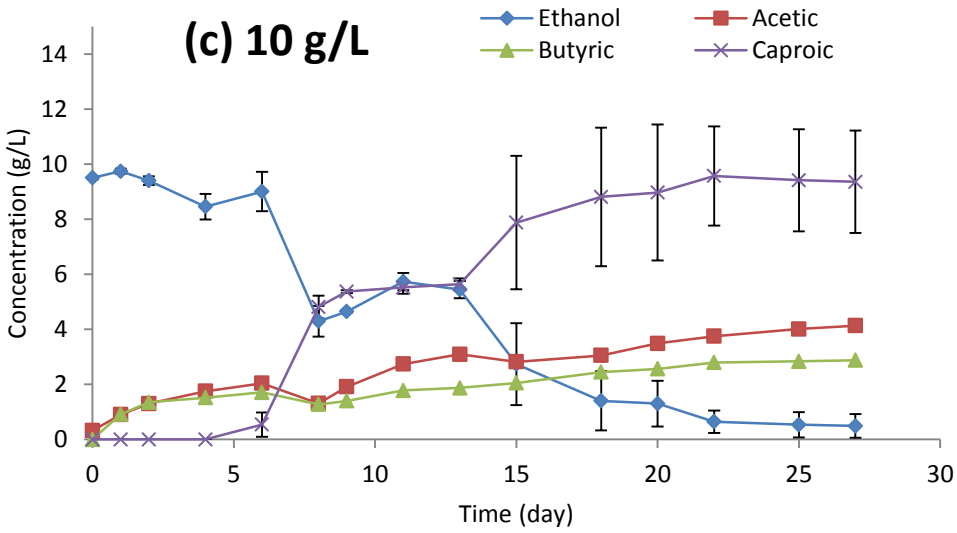


Figure 2-4. Concentration profiles (ethanol (♦), acetic acid (■), butyric acid (▲), caproic acid (×)) for fermentors with varying initial ethanol concentration (a) 0 g/L, (b) 5 g/L, (c) 10 g/L, (d) 15 g/L, (e) 20 g/L, (f) 40 g/L. (All values are average of two samples and error bars indicate the range. Error bars are shown only for caproic acid and ethanol to avoid overlapping.)



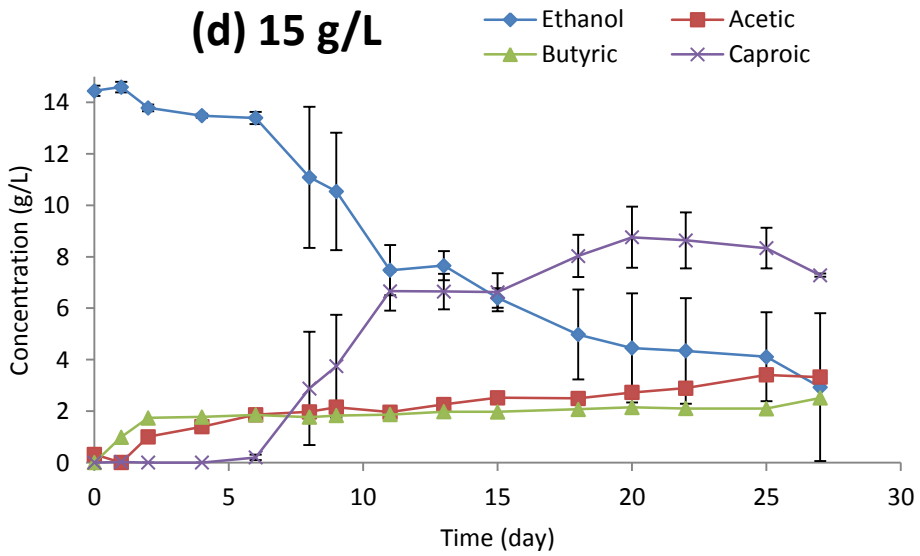
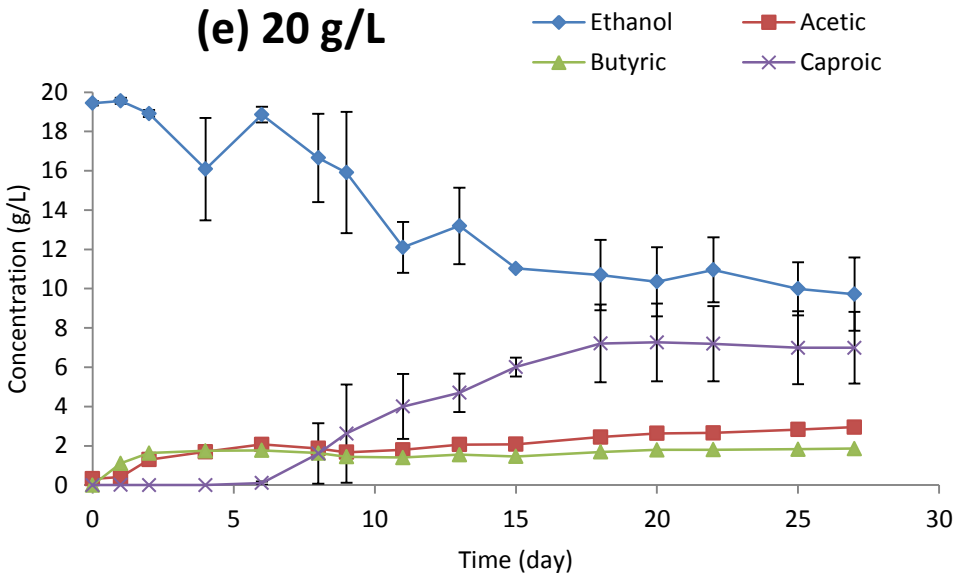


Figure 2-4. Continued.



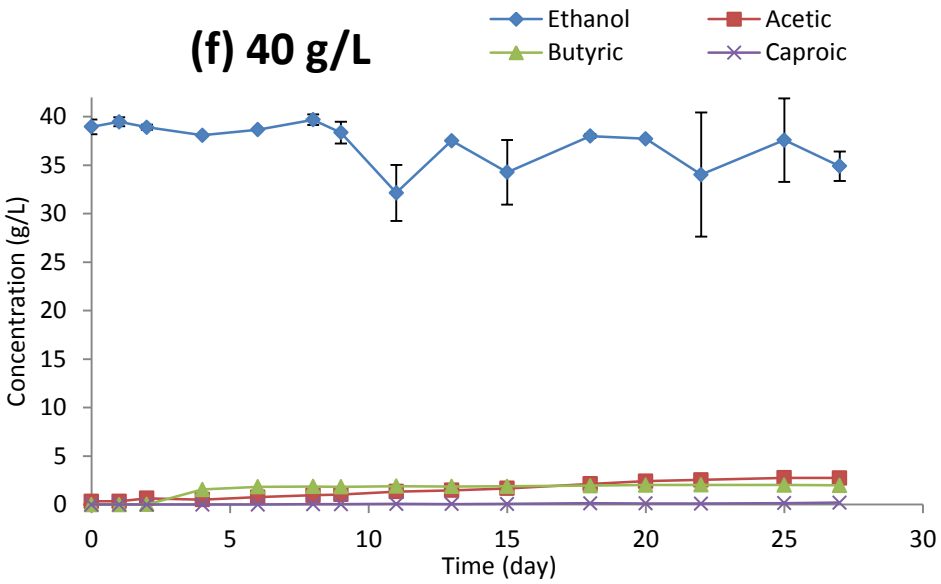


Figure 2-4. Continued.

The control fermentor also showed caproic acid formation up to 4 g/L. The ethanol concentration in the control fermentor started increasing after the first 5–6 days. Some microorganisms can convert acetic acid into ethanol if high hydrogen partial pressures are present in the headspace [20]. While converting biomass into carboxylic acids, anaerobic fermentations produce hydrogen and carbon dioxide as byproducts [30]. When methanogens are inhibited, the hydrogen partial pressure increases in the fermentor if gases are not vented continuously. Under elevated hydrogen pressures, acetic acid is converted into ethanol by the following reaction [20]

$$CH_3COO^- + H^+ + 2H_2 \rightarrow C_2H_5OH + H_2O \quad \Delta G = -7.22 \text{ kJ/mol at } 37\ ^\circ\text{C} \quad (2.10)$$

Ethanol formed in the control fermentor by the above-mentioned mechanism is consumed by other microorganisms that are responsible for chain-elongation, which forms butyric acid and ultimately caproic acid. Thus, the hydrogen generated *in-situ* during the formation of carboxylates from pyruvate can be utilized to elongate acids in the ways mentioned above.

As the length of carbon chain increases in the acid molecule, its water solubility decreases. In water, the solubility of pure valeric acid and caproic acid is 49.7 g/L and 10.8 g/L, respectively. Because the fermentations are performed near neutral pH, the solubility of acids increases by forming salts with the buffers. Buffers with monovalent cations (e.g., Na^+) are more effective than divalent cations because divalent cations tend to form scum with carboxylate ions at higher concentrations. The dissociated fatty acids

are less inhibitory to microorganisms than undissociated acids [31]. The pKa of short-chain and medium-chain fatty acids is 4.7–4.8; thus, at pH 7, more than 99% of fatty acids are dissociated. At neutral pH, the increase in solubility and the decrease in inhibition enhance production of medium-chain fatty acids. In industrial fermentors, the pH can be maintained near neutrality by adding buffer using automatic pH control.

The caproic acid concentration increased with increasing amounts of initially added ethanol from 0 to 10 g/L and decreased thereafter (15 g/L and above) (Figure 2-5). The maximum caproic acid concentration (up to 10 g/L) was observed in the fermentor that was initially fed with 10 g/L of ethanol (Figure 2-5). The same fermentor had a caproic acid composition as high as 40%. At ethanol concentration of 40 g/L, no production of caproic acid occurred even after 27 days, indicating the microorganisms responsible for chain elongation were inactive at such high ethanol concentrations. Clearly, the ethanol concentration is an important variable. Very low ethanol concentrations are unable to produce significant amounts of MCFAs because of substrate unavailability and very high ethanol concentrations (>40 g/L) inhibit the chain elongating microorganisms. Other than concentration of medium-chain fatty acids, conversion, selectivity, and total acid concentration are also equally important parameters to decide optimum ethanol concentrations.

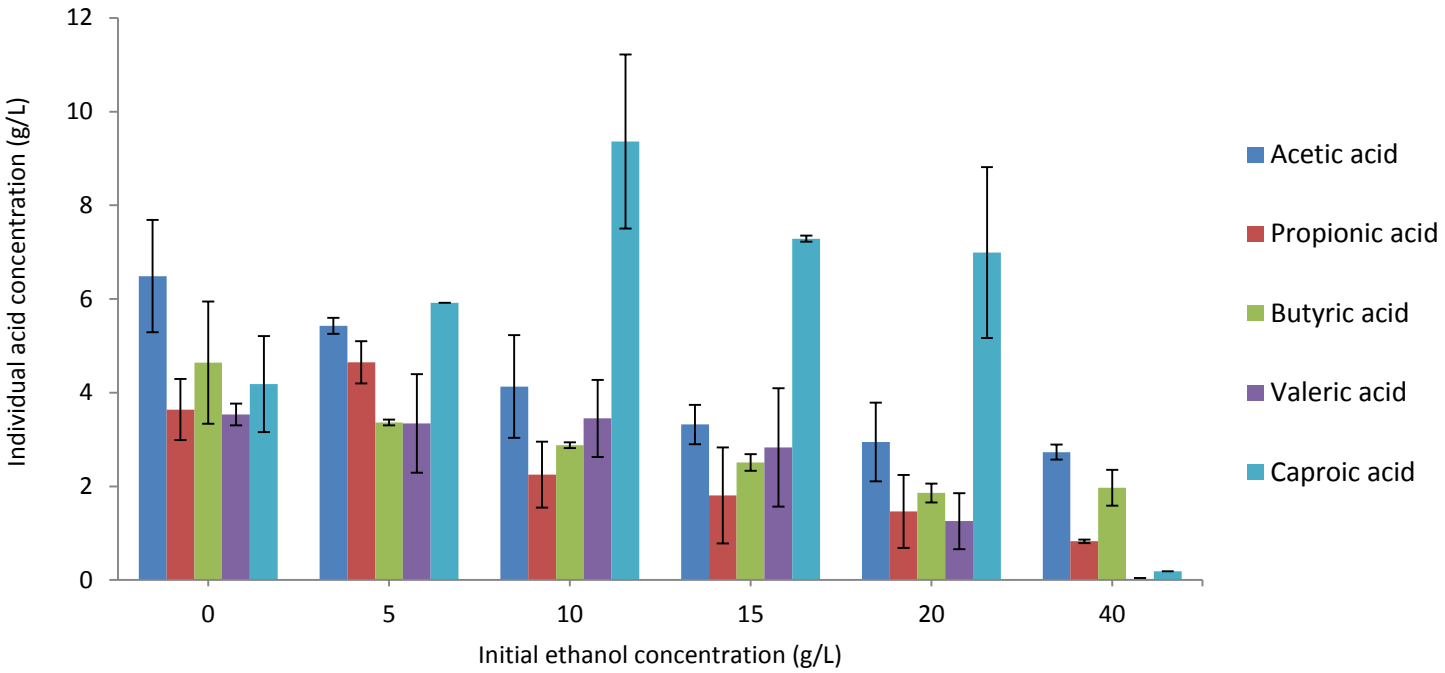


Figure 2-5. Concentration of individual acids achieved in the fermentors with different ethanol concentrations. (Values are average of two samples and error bars indicate the range.)

The fermentations were terminated when nearly no additional fatty acids were produced. The overall conversion is calculated using both biomass and ethanol consumed during fermentation. The control fermentor achieved maximum overall conversion of around 53% (Figure 2-6). The overall conversion decreases with increased ethanol concentration because of inhibition. To achieve high conversion (>40%), low ethanol concentrations (≤10 g/L) are preferred.

Overall selectivity is calculated using the total acids produced in the fermentation whereas MCFA selectivity considers only medium-chain fatty acids (caproic, heptanoic, and caprylic acid). Overall selectivity is in the range of 50 to 55% at low ethanol concentrations (0–10 g/L) and decreases at high ethanol concentrations (above 10 g/L) (Figure 2-6). High overall selectivity (>50%) is achieved at low initial ethanol concentration (<15 g/L). To produce medium-chain fatty acids, MCFA selectivity is of more interest. MCFA selectivity increases as the ethanol concentration increases from 0 to 10 g/L. The fermentors fed with 10–20 g/L of ethanol, showed MCFA selectivity of approximately 20–25% with maximum value at 10 g/L (23%) (Figure 2-6). At 40 g/L, the MCFA selectivity is almost zero.

The fermentors fed with 0–10 g/L ethanol produced around 20–25 g/L total acids (Figure 2-6). Above 10 g/L, the total acid concentration decreased with increasing ethanol concentration. The fermentor fed with 40 g/L ethanol produced only 6 g/L total acids with negligible amounts of MCFA.

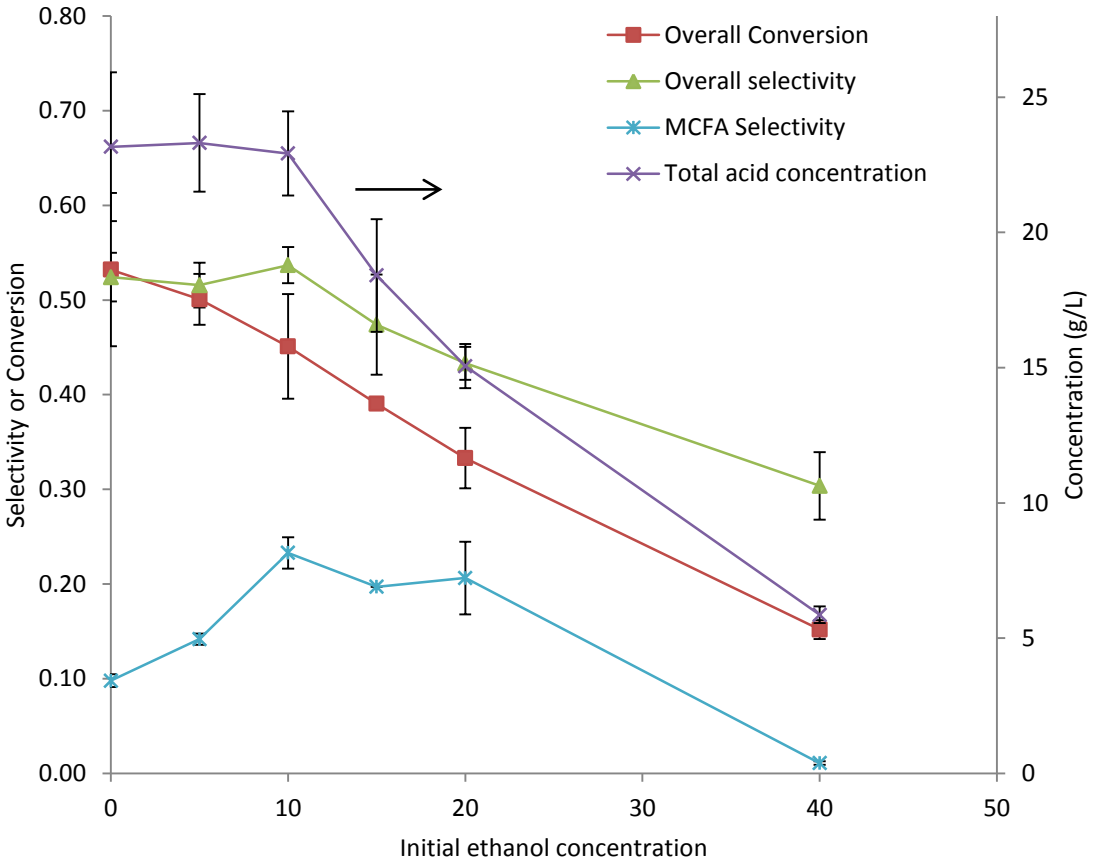


Figure 2-6. Effect of initial ethanol concentration on overall conversion (■), overall selectivity (▲), MCFA selectivity (∗), and total acid concentration (×). (Values are average of two samples and error bars indicate the range.)

Considering all the above parameters, fermentors fed with 5–10 g/L ethanol showed high total acid concentration (>20 g/L), high selectivity (>50%), and high conversion (>40%). The MCFA concentration was maximum between 10–20 g/L. Considering the cost of biomass (approximately $60/ton) and ethanol (approximately $500/ton), it would be more economical to use more biomass and less ethanol. Based on the results discussed above, the preferred ethanol concentration is 5–10 g/L.

Conversions can be improved by operating the fermentations in continuous countercurrent mode and MCFA selectivity can be improved by continuously extracting MCFAs from the fermentation broth. Selective extraction of MCFA will leave behind SCFAs, which are necessary to produce MCFA.

2.3.2. Addition of 1-propanol to mixed-culture fermentation

The experimental design was similar to the batch fermentation described in Section 2.3.1, except 1-propanol replaced ethanol. The initial propanol concentrations were 0, 5, 10, 15, 20, 45 g/L. The fermentor with initial propanol concentration of 0 g/L is the control fermentor. According to Equation 2.1, to produce ketones (and hydrocarbons) in jet and diesel fuel range using the MixAlco process, valeric or longer acids should be formed in the fermentation. It is expected that propanol will combine with acetic acid (which is the major product among all the fatty acids produced) to form valeric acid and ultimately caprylic acid by chain elongation. Also, part of the propanol may react with propionic acid to form caproic acid.

In the fermentors fed with 5 and 10 g/L of propanol, consumption of propanol was accompanied by the formation of valeric acid along with consumption of acetic acid (Figure 2-7 b, c). The concentration profiles show chain elongation of acetic acid and propanol form valeric acid. Between Days 5 and 10, ethanol was also formed probably because of the reduction of acetic acid in presence of hydrogen (*in-situ* generation). The consumption of ethanol eventually produced caproic acid.

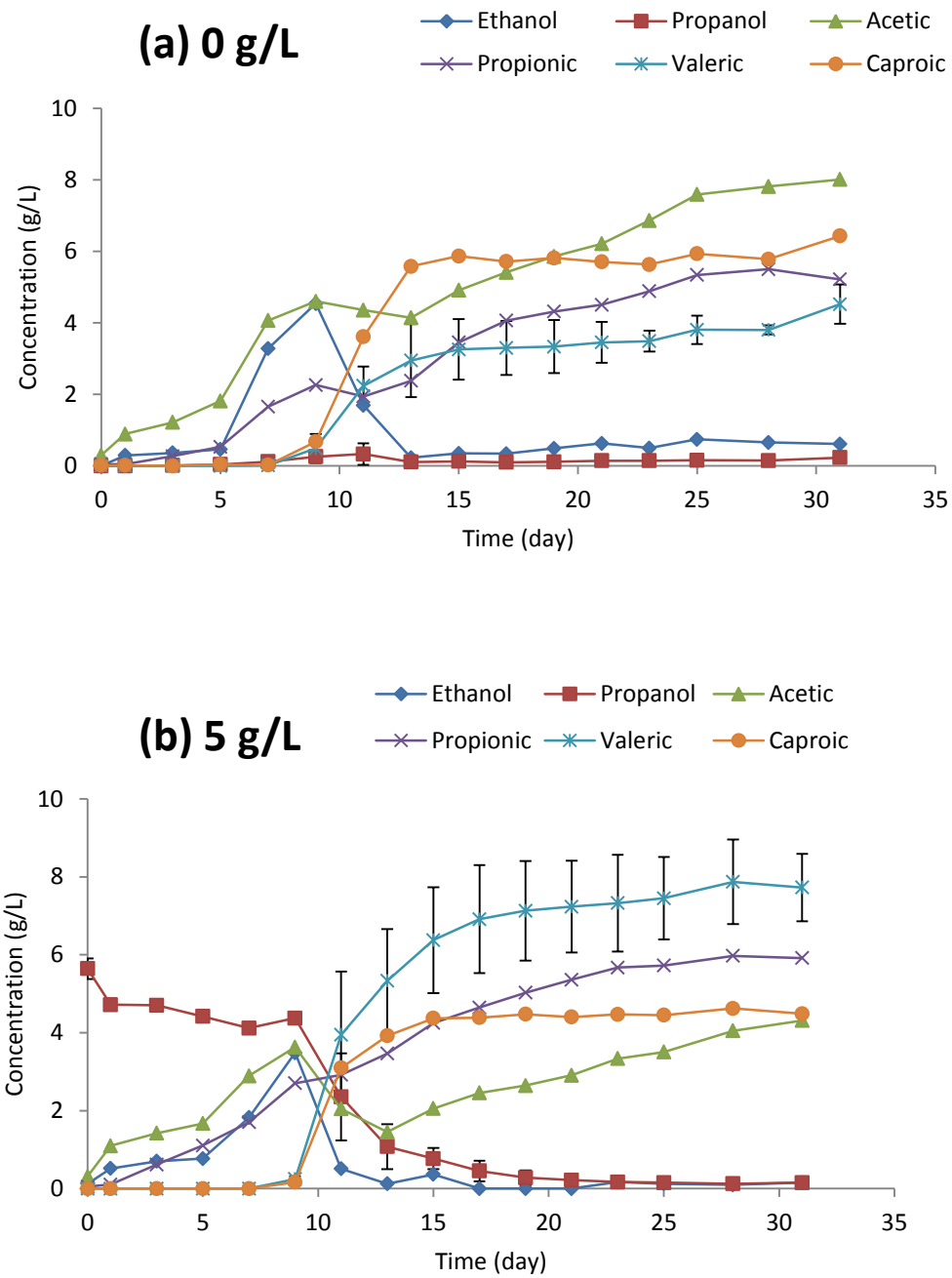
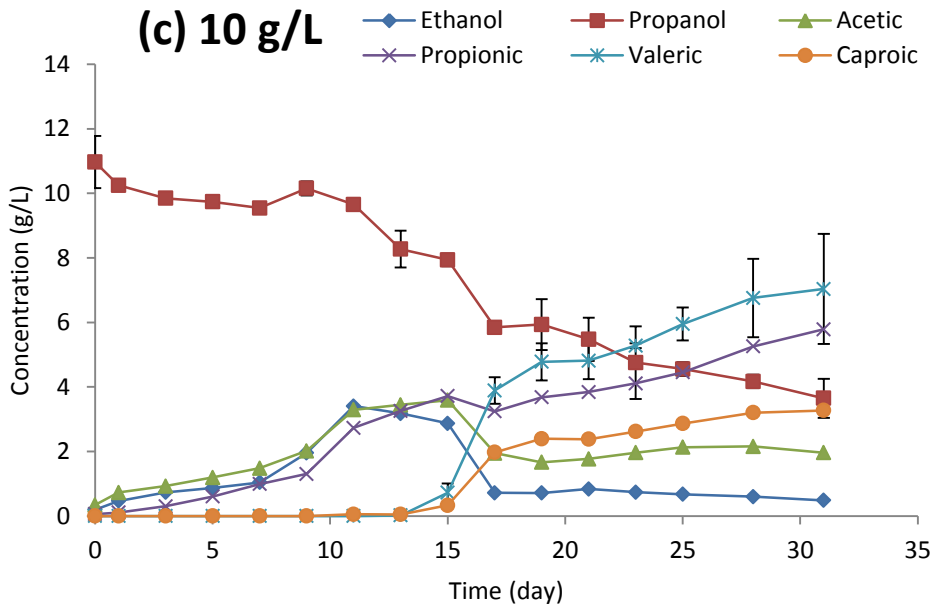


Figure 2-7. Concentration profiles for fermentors with varying initial propanol concentration (a) 0 g/L, (b) 5 g/L, (c) 10 g/L, (d) 15 g/L, (e) 20 g/L, (f) 45 g/L. (All values are average of two samples and error bars indicate the range. Error bars are shown only for valeric acid and propanol to avoid overlapping.)



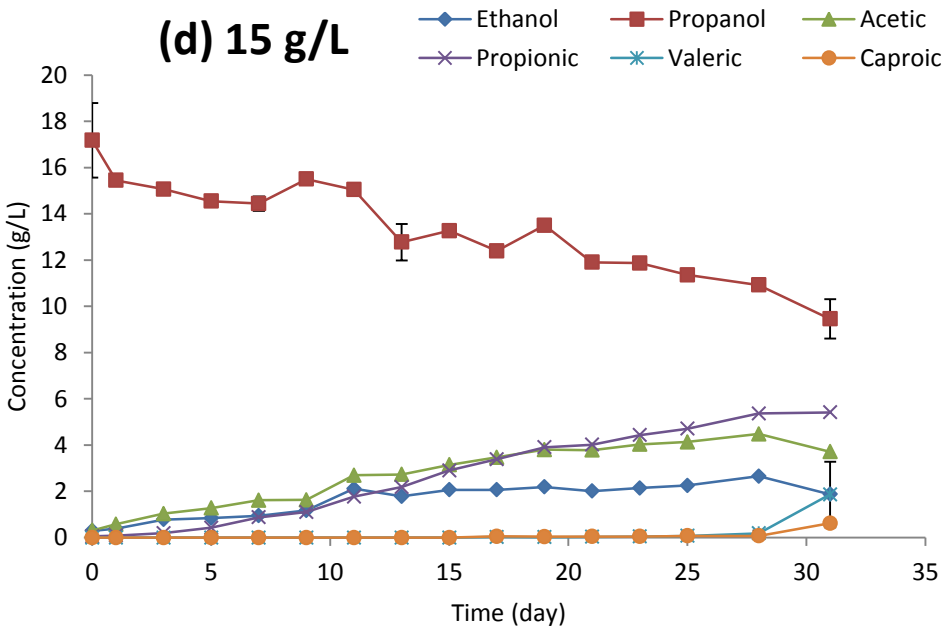
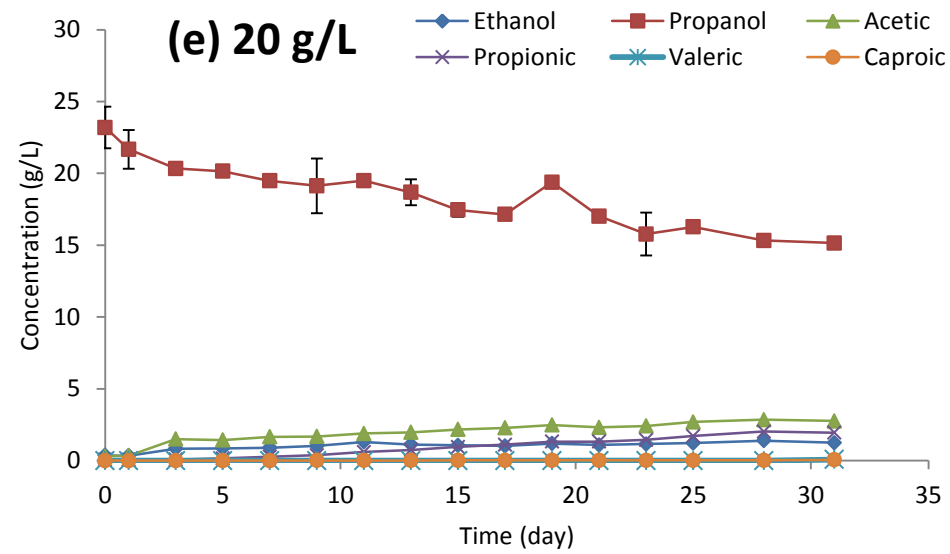


Figure 2-7. Continued.



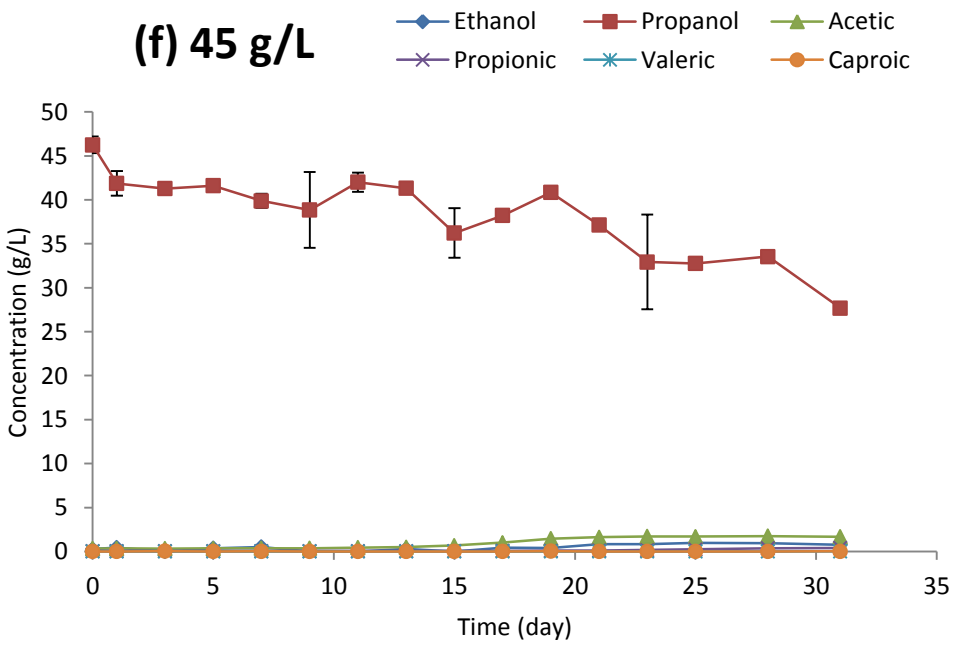


Figure 2-7. Continued.

Increased concentrations of valeric and caproic acid occurred only when propanol and ethanol were present in the fermentor. The heptanoic and caprylic acid concentration was less than 1 g/L in all the fermentors. At higher propanol concentrations (20 g/L and above), no chain elongation was observed, indicating inhibition of chain elongation at such high propanol concentrations. Propanol is more inhibitory than ethanol because microorganisms could produce MCFAs even at 20 g/L of ethanol concentration (Figure 2-5).

Some fermentors fed with propanol (5, 10, and 15 g/L) showed higher concentrations of propionic acid than acetic acid (Figure 2-8). Possibly propanol was converted into propionic acid by microorganisms, whereas acetic acid was utilized for chain elongation in the presence of propanol.

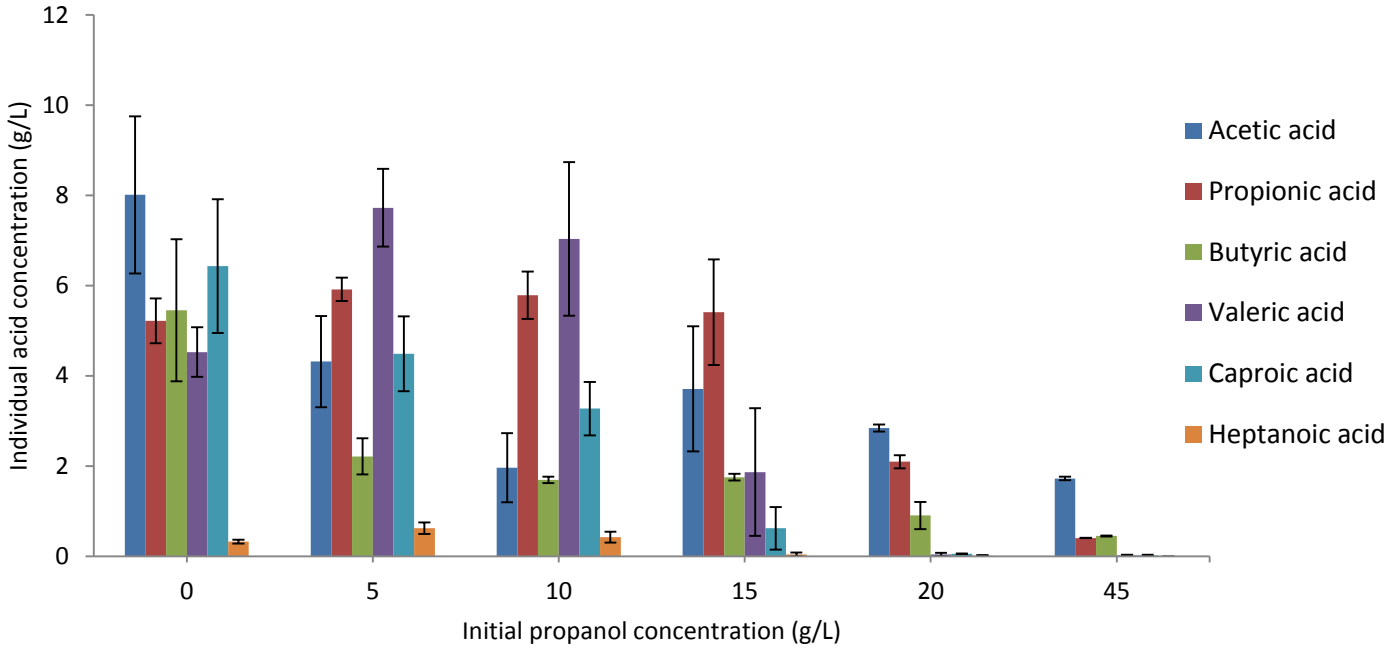


Figure 2-8. Concentration of individual acids achieved in the fermentors with different concentrations of propanol. (Values are average of two samples and error bars indicate the range.)

Because of propanol inhibition, conversion decreased with increasing propanol concentrations (Figure 2-9). The overall selectivity, MCFA selectivity, and total acid concentration also decreased with increasing propanol concentration (Figure 2-9). Propanol addition improves valeric acid production, but not MCFA. Based on the obtained results, it is preferred to use ethanol over propanol for chain elongation.

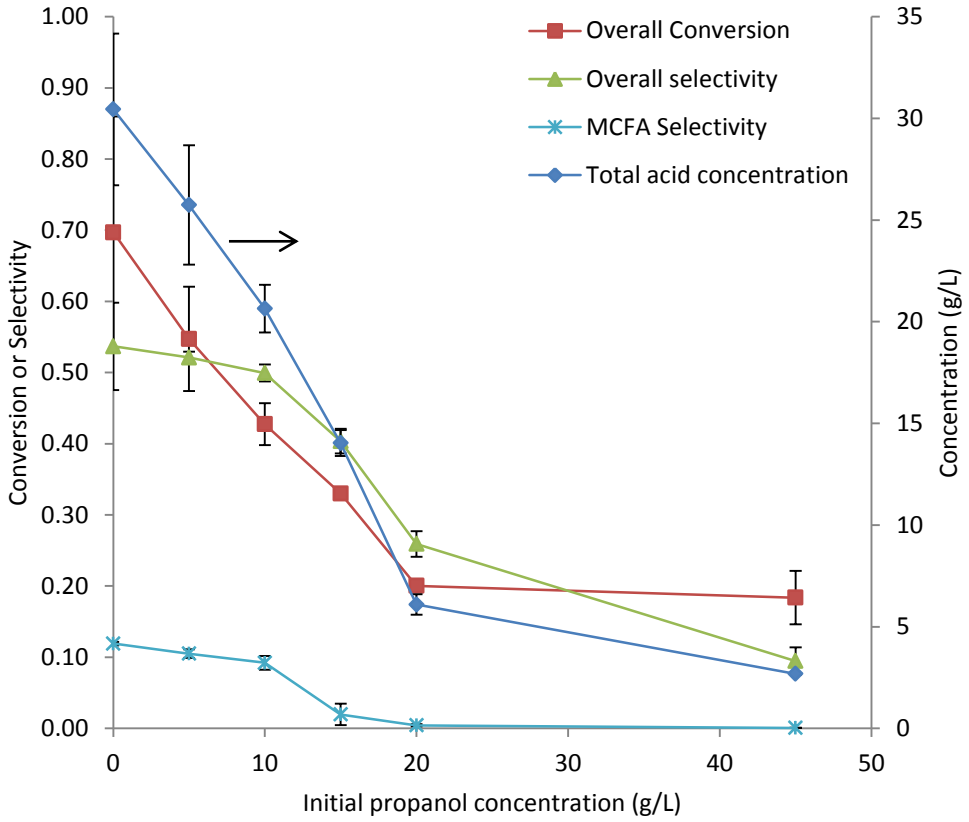


Figure 2-9. Effect of initial propanol concentration on overall conversion (■), overall selectivity (▲), MCFA selectivity (∗), and total acid concentration (♦). (Values are average of two samples and error bars indicate the range.)

2.4. Conclusions

In mixed-culture fermentation, production of medium-chain fatty acids is enhanced by adding ethanol. By feeding 10 g/L of ethanol (along with substrate), the fermentor produced 10 g/L of medium-chain fatty acids with 45% conversion, 53% selectivity, and 23 g/L total acid concentration. For chain elongation, the preferred concentration of ethanol is 5–10 g/L. Propanol also participates in the chain elongation mechanism. In the fermentors with initially added propanol (5–10 g/L), valeric acid was the major product. At very high concentrations of ethanol (>40 g/L) and propanol (>20 g/L), chain elongation does not occur. Ethanol is less expensive and less inhibitory, so it is preferred to use ethanol.

3. EFFECT OF TEMPERATURE ON CHAIN ELONGATION IN MIXED-CULTURE FERMENTATION

3.1. Introduction

In mixed-culture fermentation, chain elongation of short-chain fatty acids (SCFA) (acetic, propionic, butyric, valeric acid) with ethanol produces medium-chain fatty acids (MCFA) (caproic, heptanoic, and caprylic acid). Microorganisms (e.g., *Clostridium kluyveri*) combine acids and alcohols through a reversed β-oxidation pathway [25]. Addition of one ethanol molecule increases the acid chain length by two carbon atoms. The rate of chain elongation depends on both acid and alcohol concentration; high concentrations of each inhibit microorganisms and lower the fermentation rate.

In mixed-culture fermentation, various microbial activities are possible because of its complex nature. Adjusting process parameters (e.g., pH, temperature, substrate concentration, headspace gas composition) allows some selective control of fermentation. Previously, these parameters have been studied and optimized for carboxylic acid production from biomass [13, 32, 33]. More recently, emphasis has been placed on MCFA production using mixed culture; therefore, it is necessary to re-optimize those parameters to maximize MCFA formation.

High concentrations of caproic acid (up to 10–12 g/L) and heptanoic acid (up to 3 g/L) have been reported by Grootscholten et al. [24, 26]. Compared to acidic pH, chain elongation is favored at neutral pH [34]. At neutral pH, methanogens are active, but

methane formation can be mitigated by adding inhibitors such as iodoform [35]. The preferred ethanol concentration for chain elongation is 5–10 g/L [9]. At low ethanol concentrations (<5 g/L), the MCFA production rate is low whereas at high ethanol concentrations (>10 g/L), it decreases because of microbial inhibition. To optimize chain elongation, this research focuses on investigating temperature effects.

Chain elongation was compared at three different temperatures: room temperature (23–25 °C), 40 °C, and 55 °C. Control fermentations (without ethanol addition) were also studied. Ethanol and carboxylic acid concentrations were monitored. Based on the results, a process strategy is proposed to achieve high MCFA productivity.

3.2. Materials and methods

The experimental methods and analytical techniques were identical to the methods described in Section 2, except for fermentation temperature. The substrate was shredded office copier paper (GP standard multipurpose paper) and the nutrient source was dry chicken manure (4:1 ratio on a dry weight basis). The solids concentration was 10% (100 g/L) and total liquid in the fermentor was 400 mL. Marine inoculum was used as a source of microorganisms. Iodoform solution (20 g CHI_3/L acetone) was used to mitigate methane formation. Ethanol (190 proof, USP, Koptec) was added to the corresponding fermentors to achieve the desired initial concentrations. Magnesium carbonate was used as a buffer to adjust pH.

Dissolved carboxylic acids and alcohols were analyzed using an Agilent 7890A gas chromatograph. pH was measured using an Oakton pH meter. Moisture and ash

content of biomass were measured using standard NREL analytical procedures [36]. Overall conversion and selectivity were calculated using the following formulae:

$$\text{Overall conversion} = \frac{\text{NAVS digested (g)} + \text{Ethanol consumed (g)}}{\text{Total NAVS fed (g)} + \text{Ethanol fed (g)}} \tag{3.1}$$

$$\text{Overall Selectivity} = \frac{\text{Total acids produced (g)}}{\text{NAVS digested (g)} + \text{Ethanol consumed (g)}} \tag{3.2}$$

where NAVS is defined as non-acid volatile solids [29].

Fermentation temperature was maintained constant in the incubator, a roller apparatus with a rotational speed of 2 rpm. The fermentors were monitored every other day. The pH was adjusted to 6.8–7 using magnesium carbonate buffer.

3.3. Results and discussion

Microbial and enzyme activities depend on temperature and pH; therefore, these are natural parameters to adjust to maximize desired products. Compared to other parameters (e.g., microbial consortia, headspace gas composition), it is easier and less expensive to control fermentation temperature and pH. Gas solubility in water also depends on temperature. Hydrogen and carbon dioxide are the side products of primary fermentation reactions and are involved in secondary reactions. For example, hydrogen enhances chain elongation by reducing acetate to ethanol [37]. To study the temperature effect on acid production and chain elongation in mixed-culture fermentations, three

temperatures were investigated: room temperature (23–25 °C), 40 °C, and 55 °C with and without adding ethanol. Hereafter, room temperature is described as 25 °C. The experiments were performed in triplicate. Table 3-1 shows the experimental design.

3.3.1. Fermentation without added ethanol (Set A)

Batch fermentations of shredded office paper and chicken manure were performed at three different temperatures. The fermentations were run for 35–40 days. In Set A, no ethanol was added to the fermentors. The fermentors produced mainly short-chain fatty acids. At 25 and 40 °C, the final total acid concentration was 30 and 27 g/L, respectively. Small amounts (approximately 4 g/L) of MCFA were produced at low temperatures (≤40 °C). During the course of fermentation, ethanol was produced *in-situ* in small amounts, but was consumed later (Figure 3-1). Figure 3-2 shows composition of produced acids at different temperatures. Acetic acid was the major product followed by propionic and butyric acid.

At 55 °C, the final total acid concentration was lower (20 g/L) and the acid composition was significantly different as compared to 25 and 40 °C. Butyric and acetic acids were the major products at 55 °C. The concentrations of all other acids were less than 1 g/L. Ethanol was also produced (*in-situ* generation) and its concentration increased up to 4.6 g/L. At the end of the fermentation, acetic acid, butyric acid, and ethanol were the major products present. At thermophilic conditions (55 °C), the mixed culture shows high selectivity towards acetic and butyric acids. Forrest et al. [13] and Fu

et al. [38] have also reported similar trends in thermophilic fermentation of water hyacinth and sugarcane bagasse, respectively.

The conversions were similar at all tested temperatures (50–55 %) (Table 3-1). The fatty acid selectivity was similar at 25 and 40 °C (55–60%), but was low (38%) at 55 °C. Forrest et al. [13] also reported high biomass conversions and low fatty acid selectivity at 55 °C as compared to 40 °C. The formation of ethanol at 55 °C indicates that part of the digested biomass is utilized for ethanol production, which decreases fatty acid selectivity.

Table 3-1. Set of experiments and their performance parameters

Set	Ethanol conc (g/L)	Temp (°C)	Total acid concentration (g/L)	Overall conversion (g digested/g substrate fed)	Overall selectivity (g acids/g substrate digested)
Set A	0	25	30.16 ± 1.16	0.53 ± 0.02	0.60 ± 0.01
	0	40	27.57 ± 0.89	0.52 ± 0.01	0.58 ± 0.01
	0	55	20.33 ± 1.28	0.55 ± 0.03	0.38 ± 0.03
Set B	10	25	20.73 ± 1.27	NA[a]	NA
	10	40	26.11 ± 1.01	0.45 ± 0.01	0.55 ± 0.04
	10	55	17.99 ± 1.26	0.40 ± 0.02	0.40 ± 0.01

[a]The conversion and selectivity values are not available
Error is ±1 standard deviation

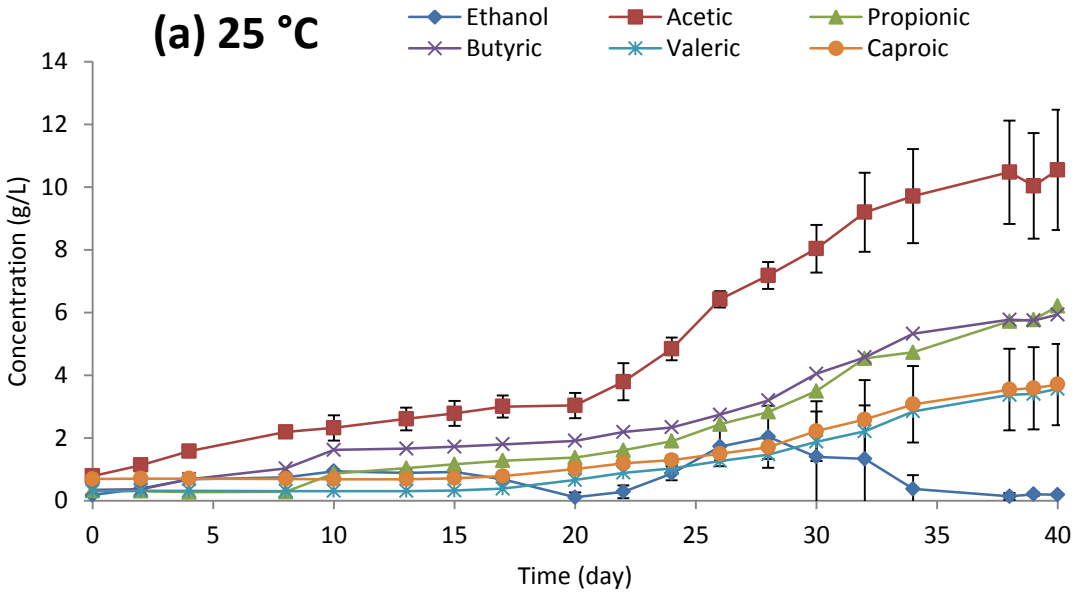
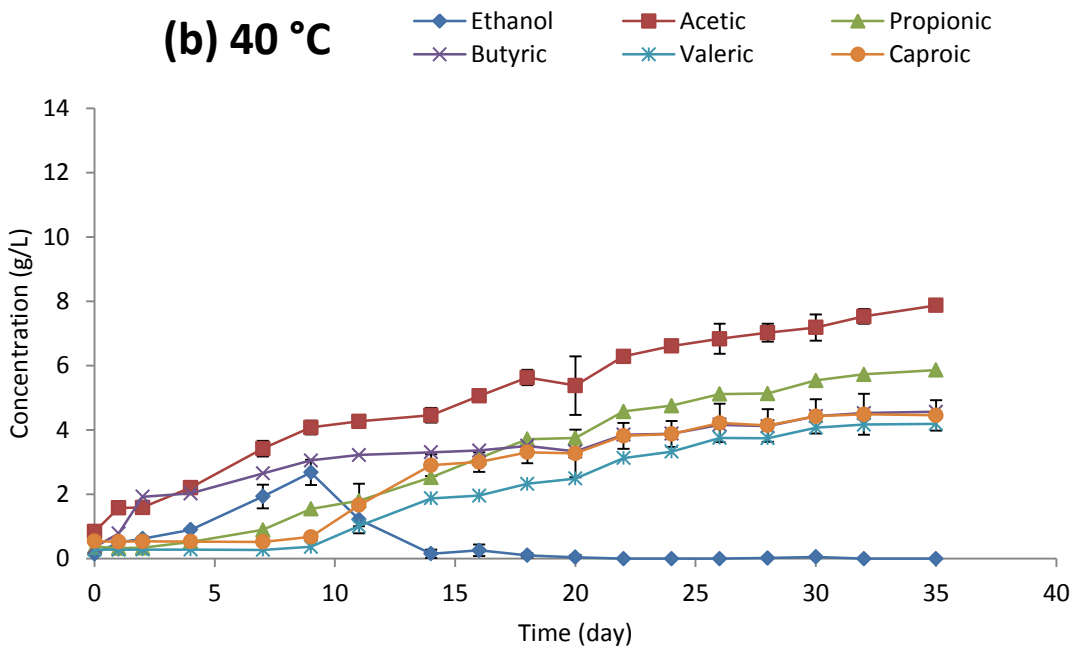


Figure 3-1. Effect of temperature on acid production in mixed-culture fermentation. Concentration profiles are shown for (a) 25 °C, (b) 40 °C (c) 55 °C. Values are average of three samples and error bars indicate ±1 standard deviation.



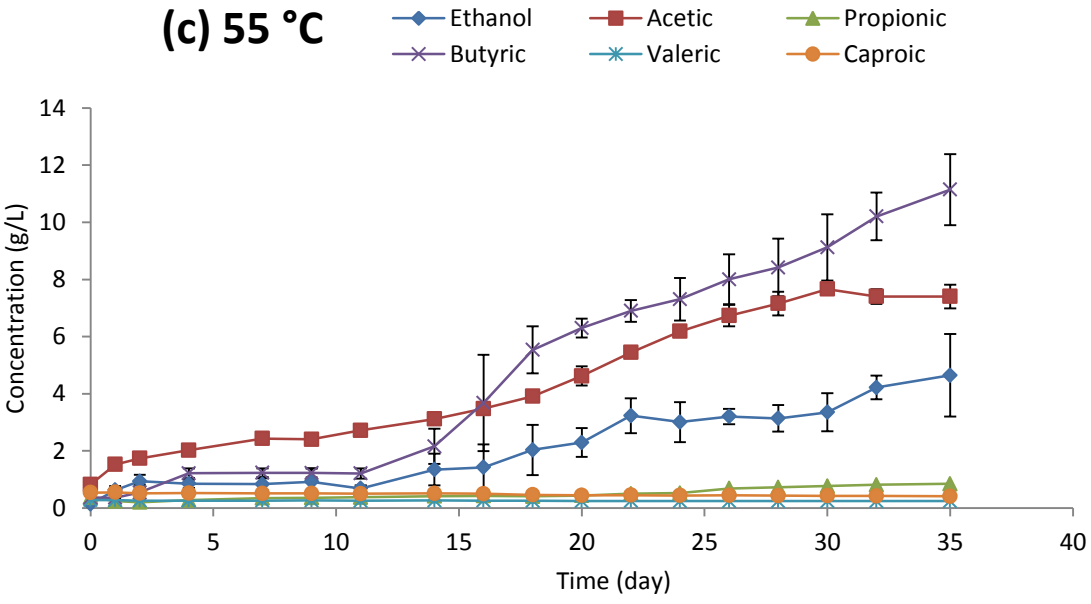


Figure 3-1. Continued.

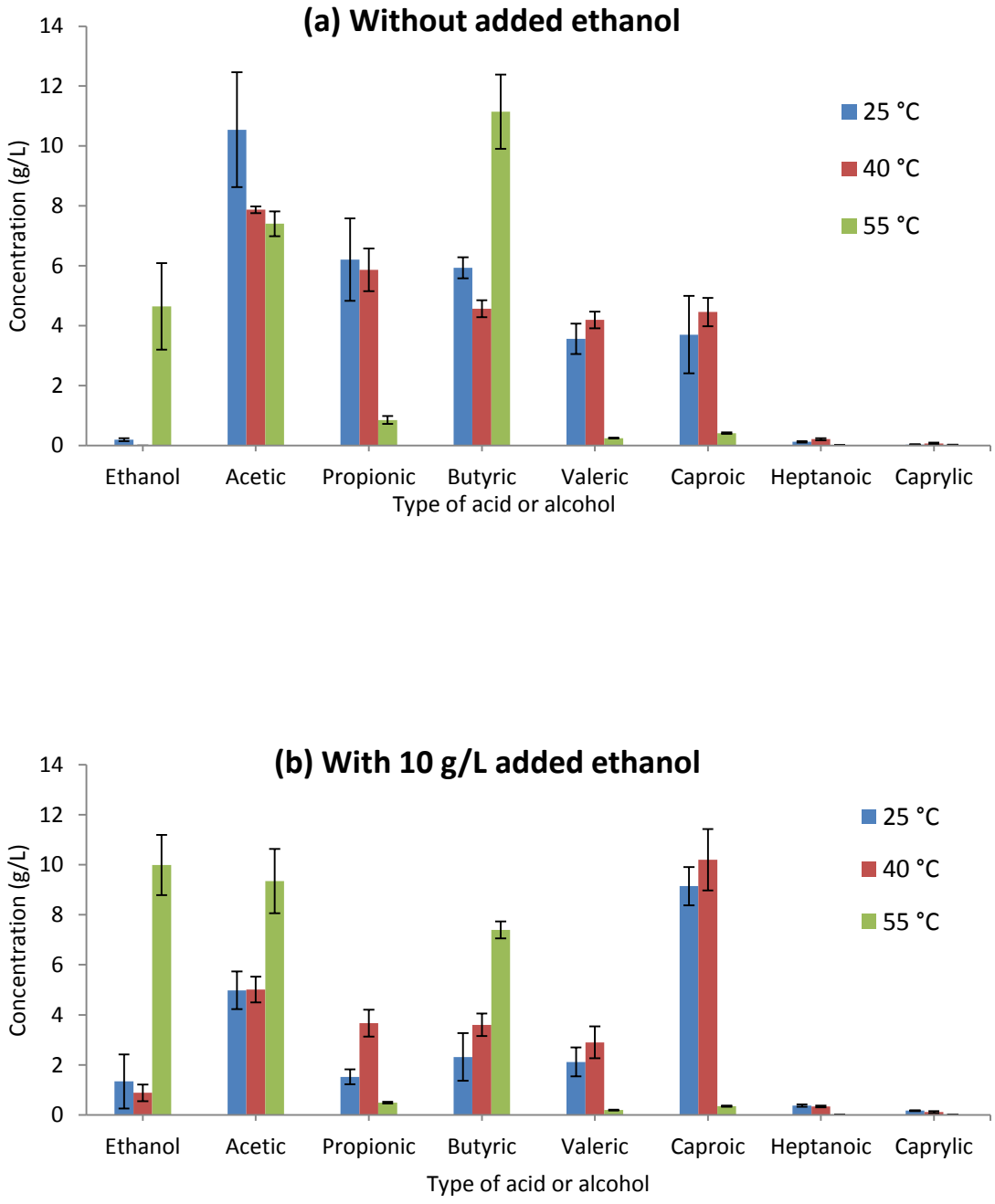


Figure 3-2. Distribution of acids at the end of the batch fermentation at different temperatures (a) without and (b) with added ethanol. (*Note:* 10 g/L ethanol was added initially to all fermentors in (b).) Values are average of three samples and error bars indicate ±1 standard deviation.

3.3.2. Fermentation with added ethanol (Set B)

The effect of temperature on chain elongation was investigated by adding ethanol to the fermentors at three different temperatures. It was speculated that ethanol and SCFAs would combine to form MCFAs. In all fermentors, the initial ethanol concentration was 10 g/L. All other fermentation conditions were similar to Set A (Section 2.3.1).

At low temperatures (25 and 40 °C), almost all the available ethanol was consumed (Figure 3-3 a, b). The decrease in ethanol concentration was accompanied by a corresponding increase in caproic acid concentration. The final total acid concentration was approximately 26 g/L at 40 °C, whereas it was 20 g/L at 25 °C (Table 3-1). Caproic acid was the major product followed by acetic acid. In both cases, the maximum caproic acid concentration was approximately 10 g/L. Low temperatures (40 °C and below) are favorable for chain elongation.

At 55 °C, the total acid concentration was 18 g/L. Ethanol was not consumed even after 30 days. Acetic acid and butyric acid were the major products, whereas other acids were negligibly produced (less than 1 g/L) (Figure 3-3). This was also observed in the fermentors without added ethanol. Clearly, at higher temperatures (55 °C), the chain elongating microorganisms (e.g., *Clostridium kluyveri*) are inactive and acetic and butyric acids are selectively produced. Agler et al. [39] also reported inhibition of chain-elongating microorganisms at thermophilic conditions (55 °C) and low pH (5.5).

At 40 and 55 °C, the overall conversion was around 40–45%. Overall selectivity was higher at 40 °C than 55 °C (Table 3-1) indicating higher MCFA productivity by utilizing ethanol at lower temperatures as compared to higher temperatures.

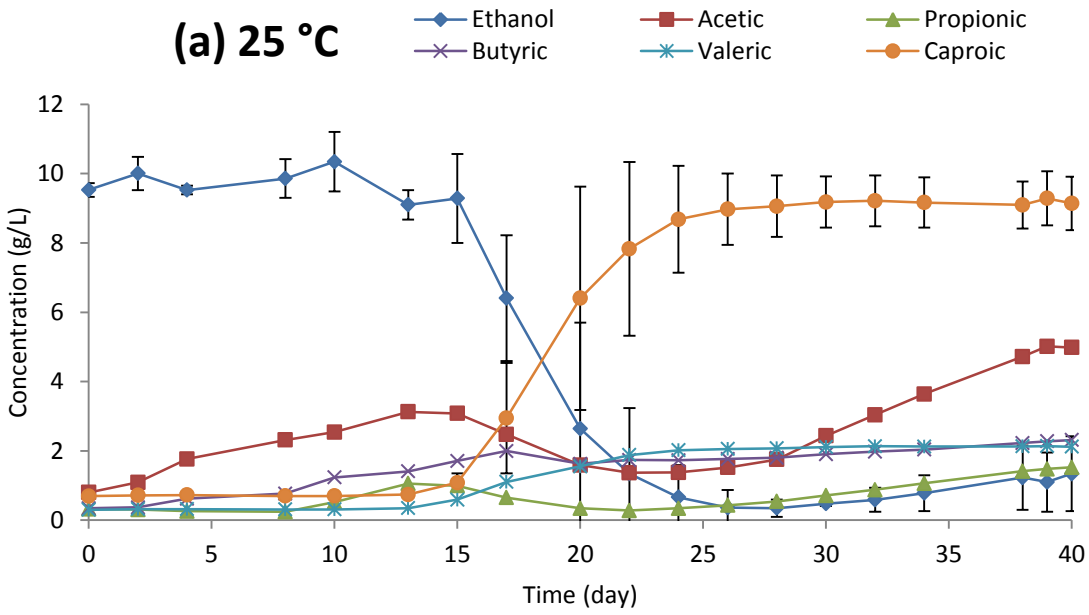
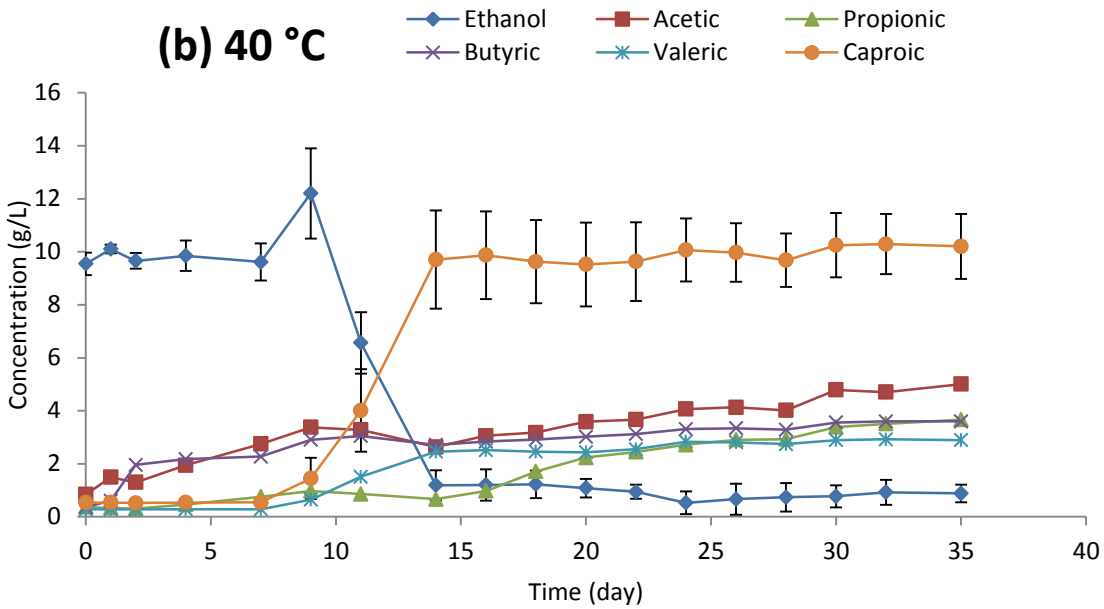


Figure 3-3. Effect of temperature on chain elongation in mixed-culture fermentation. Chain elongation occurs when ethanol is consumed to form longer chain acids. Concentration profiles are shown for (a) 25 °C, (b) 40 °C (c) 55 °C. Values are average of three samples and error bars indicate ±1 standard deviation.



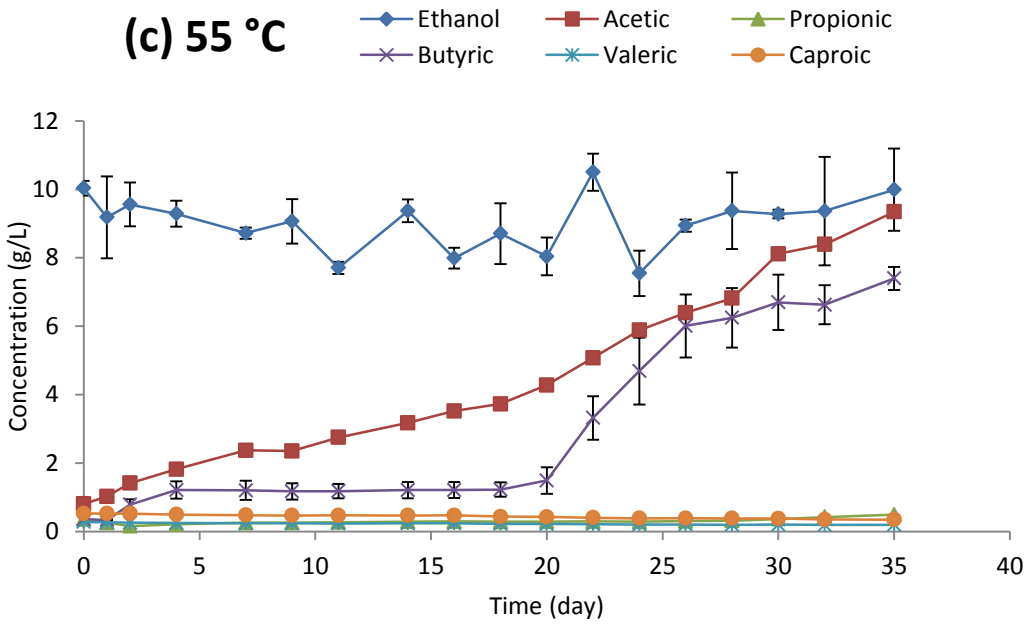


Figure 3-3. Continued.

3.3.3. Strategy to maximize MCFA production

MCFA production can be enhanced by continuously extracting acids from fermentation broth [40]. The separation of carboxylic acids becomes easier with higher carbon chain length. Based on results reported in the literature and obtained in this research, caproic acid is the longest fatty acid that can be produced in high concentrations (up to 10 g/L) in mixed-culture fermentation [9, 23, 34]. Caproic acid is produced by combining ethanol and butyric acid. Butyric acid is produced directly from biomass and also by combining acetic acid with ethanol. At high temperature, high acetic and butyric acid selectivity can be exploited to direct the process towards the production of caproic acid, which is easier to extract than valeric acid. Valeric acid formation can be reduced by avoiding propionic acid formation. At high temperatures, in addition to acids, ethanol is also produced from biomass (*in-situ* generation), which will reduce the external ethanol needed for chain elongation and therefore, will improve process economics. Also, the stoichiometric amount of ethanol needed to convert butyric acid to caproic acid is half that needed to convert acetic acid to caproic acid. At 55 °C, production of butyric acid directly from biomass (not by chain elongation of acetic acid and ethanol) reduces the amount of external ethanol needed.

To achieve high caproic acid production with minimal addition of external ethanol, acidification and chain elongation can be performed in separate fermentors. Acidification is performed at 55 °C where mainly acetic acid, butyric acid, and ethanol are selectively produced. The fermentation broth from the acidification fermentor is fed to the chain-elongation fermentor where external ethanol is added to convert acetic and

butyric acid to caproic acid (Figure 3-4). Chain elongation is performed at lower temperature (≤40 °C). The major product in the chain elongation fermentor is caproic acid, which can be selectively recovered by solvent extraction or ion exchange.

Separating acidification and chain elongation avoids the competition between acid-producing and chain-elongating microorganisms. Stable consortia of microorganisms with specific function (acid production or chain elongation) will dominate in each fermentor. To avoid the toxic effects of both high concentrations of MCFAs and ethanol on hydrolysis, Grootscholten et al. [41] also used separate fermentors for acidification and chain elongation. The temperatures of the acidification and chain-elongation fermentors were 35 and 30 °C, respectively. They observed higher MCFA production rates in two-stage system as compared to single-stage system.

Based on the results obtained in this study, Figure 3-4 shows the schematic of proposed two-stage system with acidification and chain elongation at different temperatures with extraction system coupled to chain-elongation reactor.

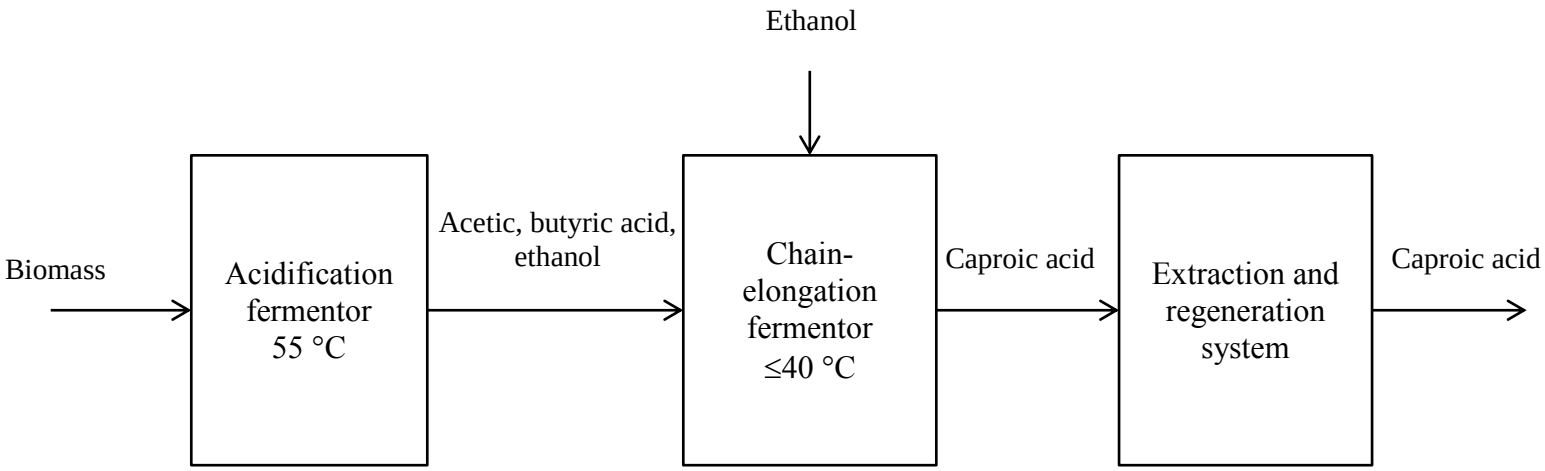


Figure 3-4. Schematic of two-stage system for MCFA production.

3.4. Conclusion

In mixed-culture fermentation, temperature affects product composition. At low temperatures (≤40 °C), both SCFAs and MCFAs are produced and acetic acid is the major product. At thermophilic conditions (55 °C), acetic acid, butyric acid, and ethanol are the major products. Chain elongation occurs at low temperatures (≤40 °C). Selective production of caproic acid can be achieved by performing acidification and chain elongation in separate fermentors. Acetic acid, butyric acid, and ethanol are selectively produced at 55 °C, which are necessary for caproic acid production by chain elongation. The fermentation broth from the acidification fermentor is fed to the chain-elongation fermentor. Chain elongation is performed at low temperatures (≤40 °C). At thermophilic conditions, inhibition of propionate-forming microorganisms drives the overall process to form caproic acid; therefore, by taking advantage of the temperature effect on mixed-culture fermentation, caproic acid can be selectively produced. In the acidification fermentor, ethanol formation reduces the amount of external ethanol needed for chain elongation making the process more economically attractive.

4. COUNTERCURRENT SACCHARIFICATION OF LIME-PRETREATED CORN STOVER

4.1. Introduction

The sugar platform is one of the well-studied routes for biofuel production from lignocellulosic biomass. In the sugar platform, pretreated biomass is enzymatically hydrolyzed to sugars. Enzymes are a major cost in a biomass-to-ethanol process. The contribution of enzyme cost to biofuels depends on various factors, such as type of feedstock, pretreatment effectiveness, enzyme loading, and biofuel yield. The production cost of cellulase is approximately $5–10/kg [42-44]. The challenge of enzyme cost can be addressed by using them more efficiently.

Conventionally, enzymatic saccharification is performed in batch with typical reaction times of 3–7 days. At the end of batch saccharification, enzyme activity remains, but leftover enzymes are usually discarded. To reduce the enzyme costs by reusing the leftover enzymes, recycle strategies have been studied in the past [45-47]. This study investigates the reduction in enzyme requirements for lime-pretreated corn stover by using countercurrent saccharification rather than batch.

For chemical processes, countercurrent systems are generally more efficient than batch and offer advantages such as more efficient utilization of substrates, continuous processing that avoids loading and unloading idle times, and less product inhibition. In a countercurrent saccharification system, the biomass and liquid flow in opposite directions. The fresh biomass encounters product liquid at one end and digested biomass

encounters fresh liquid at other end. The enzyme addition point can be selected strategically to maximize enzyme utilization. The enzymes present in product liquid are used by fresh active biomass at one end reducing product inhibition. At the other end, digested biomass is washed with fresh liquid to recover spent enzymes and product sugars, thus improving process efficiency. The liquid product can potentially reach high sugar concentrations because it last contacted fresh highly reactive biomass.

The benefits of countercurrent saccharification have been investigated in the past [48-50]. As compared to batch saccharification, Fox et al. [48] and Jeffries and Scharman [49] observed yield improvement by a factor of 1.27 and 1.39 in countercurrent saccharification, respectively. These studies used only three stages in the simulated countercurrent system and enzymes were added in the terminal stage. Zentay et al. [50] compared countercurrent and batch saccharification of pure cellulose substrates and reported significantly higher yields in countercurrent system than batch. An eight-stage countercurrent train with 5 mg protein/g biomass (Train 1) achieved 87.8% glucose conversion, whereas a train with 2 mg protein/g biomass (Train 2) achieved 56.1% glucose conversion [50]. Compared to 5-day batch saccharification, to achieve the same glucose conversion, enzyme requirements were reduced by factors of 16.8 and 8 for Trains 1 and 2, respectively. Also, the eight-bottle trains employed in this study had substantial remaining enzyme activity at both ends. Based on these lessons, a new set of experiments was designed to investigate countercurrent saccharification of real substrate (pretreated corn stover). To ensure complete utilization of enzyme, a sixteen-stage train was used in this research.

This section describes pseudo-continuous countercurrent saccharification of lime-pretreated corn stover. Instead of adding enzymes to a terminal stage, the enzymes were added to a fixed intermediate stage. This study uses Novozymes' newest commercial enzymes (CTec3 and HTec3) available in the market. Sugar yields were calculated at steady state, which was validated using the Slope Method [50]. The countercurrent saccharification sugar yield was compared with batch results. The sugar concentration distribution across the countercurrent train was analyzed to determine where the enzyme is active in the system.

4.2. Materials and methods

4.2.1. Submerged-lime-pretreatment (SLP)

Corn stover was pretreated using long-term submerged lime pretreatment [51]. The water and lime loadings were 10 kg water/kg dry biomass and 0.15 kg $Ca(OH)_2$/kg dry biomass, respectively. CO_2-free air was used for the pretreatment. The pretreatment time was 30 days and the temperature was maintained at 50 °C. The pretreated corn stover was washed with water, air dried at room temperature, and stored in Ziploc bags.

4.2.2. Compositional analysis of biomass

The composition of raw and pretreated biomass was determined using standard NREL procedure [28]. The biomass used for Sections 1 and 2 of the countercurrent saccharification contains 42.59% glucan and 19.79% xylan.

4.2.3. Saccharification

4.2.3.1. Substrate

Raw, lime-pretreated, and lime + shock treated corn stover was saccharified.

4.2.3.2. Citrate buffer

Optimal performance of cellulase CTec3, cellulase CTec2, and hemicellulase HTec3 occur at pH 4.75–5.25, pH 5.0–5.5, and pH 4.8–5.2, respectively [16-18]. Citrate buffer at 0.1-M concentration and pH of 4.8 was used to maintain relatively high enzyme activity. To prepare the buffer, citric acid monohydrate and trisodium citrate dihydrate were added to deionized (DI) water.

4.2.3.3. Antibiotics

To prevent growth of contaminating microorganisms that could consume produced sugars, an antibiotic cocktail was added to each bottle. The cocktail was composed of tetracycline and cycloheximide solutions. Tetracycline solution (10 g/L) was prepared in an aqueous solution of 70% ethanol. Cycloheximide solution (10 g/L)

was prepared in deionized water. To each batch saccharification vial, 40 μL of tetracycline and 30 μL of cycloheximide solution were added per 10 mL of solution.

4.2.3.4. Enzyme solutions

Three different Novozymes enzymes were used in this study: Cellic® CTec2, CTec3, and HTec3. CTec2 is a blend of aggressive cellulases with high levels of β-glucosidases and hemicellulases that degrade lignocellulose into sugars [52]. CTec3 is Novozymes' newest commercial enzyme product for effective hydrolysis of cellulose. It contains proficient cellulase components boosted by proprietary enzyme activities and a new array of hemicellulase activities [53]. HTec3 is the newest commercial enzyme product from Novozymes for effective hydrolysis of insoluble and soluble hemicelluloses [54].

4.2.3.5. Incubator

Optimal performance of CTec2, CTec3, and HTec3 occur at temperatures of 45–50°C, 50–55°C, and 40–45°C, respectively. In this study, a standing incubator cabinet was used. The incubator is a roller apparatus with a rotational speed of 2 rpm maintained at constant temperature (50°C).

4.2.4. Countercurrent saccharification

Countercurrent saccharification of lime-pretreated corn stover was performed using 16 1-L centrifuge bottles (Thermo Fisher Scientific, catalog# 05-562-25). All 16 bottles were started as batch saccharification with the same initial solid concentration

(100 g/L) and total volume of 250 mL. Tables 4-1 and 4-2 show the enzyme loadings and experimental details, respectively. Figure 4-1 shows the schematic of the experiment. In this section, the 16-bottle countercurrent system is often described as a countercurrent “train” and the monitoring procedure is referred as a “transfer.”

Table 4-1. Enzyme loadings used in countercurrent saccharification of lime pretreated corn stover.

Section	CTec3		HTec3		
	(mg protein/g dry biomass)	(mg protein/g glucan)	(mg protein/g dry biomass)	(mg protein/g glucan)	(mg protein/g xylan)
1	1	2.3	0	0	0
2	1	2.3	1	2.3	5.1

Table 4-2. Initial loading of countercurrent saccharification experiment in Bottles 1–16.

Citrate Buffer (mL)	125
Water (mL)	95.13
Substrate (g)	27.35
[1]Tetracycline (mL)	1
[2]Cycloheximide (mL)	0.75
[3]CTec3 1 mg protein/ g dry biomass (mL)	0.767
Total Volume (mL)	250

[1] Concentration = 10 g/L
[2] Concentration = 10 g/L
[3] Protein concentration = 32.6 g/L

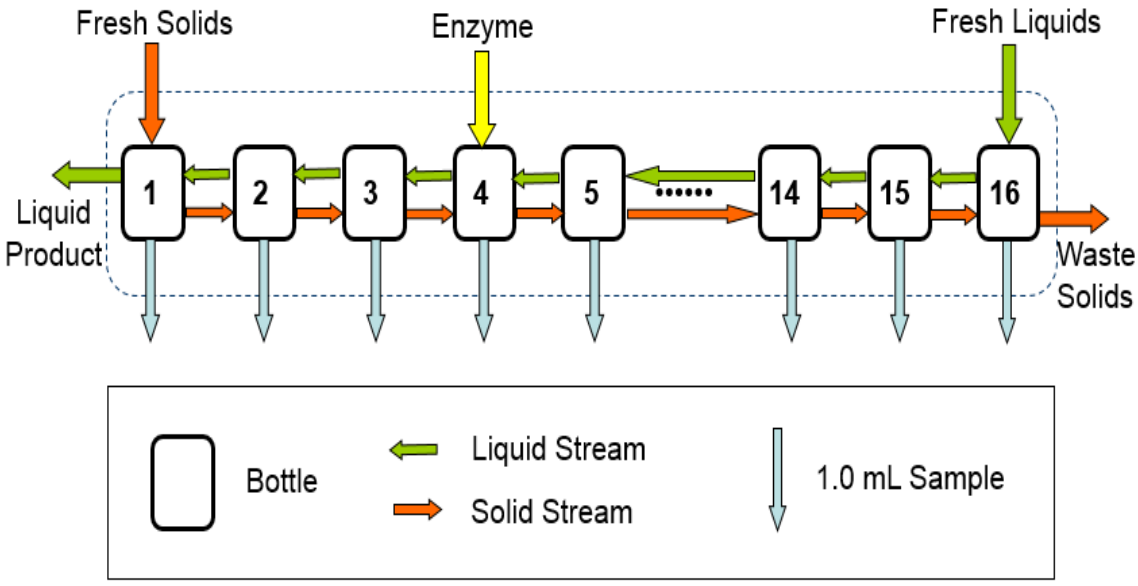


Figure 4-1. Schematic of countercurrent saccharification.

4.2.4.1. Monitoring of the countercurrent saccharification

The countercurrent train was monitored every other day (48 hours) to take samples and transfer solids and liquids. During every transfer, each bottle was centrifuged to achieve phase separation of liquid and solid wet cake (70–80% moisture content). For each bottle, the volume and mass of separated liquid and weight of wet cake were recorded. The pH of the liquid was measured to ensure it was compatible with the enzymes. Liquid samples (1 mL) were taken from every bottle and analyzed by HPLC to determine sugar concentrations. When the sugar concentrations from each bottle did not change significantly over a relatively long time (e.g., 20 days), the system was determined to reach steady state.

In Figure 4-1, Bottle 1 is the front end of the train and Bottle 16 is the back end. The liquid was transferred from "back" to "front" while calculated wet cake was moved in the opposite direction. At the end of each transfer, a target wet weight of 90 g was maintained in each bottle. All the free liquid was transferred from Bottle *N* to Bottle *N*–1. The amount of solid transferred between bottles is calculated as follows:

Wet cake transferred from Bottle *N* to Bottle *N*+1 (g) =

[Wet cake weight in Bottle *N* (g)] +

[Wet cake transferred from Bottle *N*–1 to Bottle *N* (g)] –

[target wet cake in Bottle *N* (g)] (4.1)

The solid concentration of the total system was about 100 g/L, similar to the batch hydrolysis. The total slurry volume in each bottle was about 180 mL. During each transfer procedure (every 48 h), 10 g dry biomass was added to Bottle 1 and 90 mL liquid consisting of 50 mL citrate buffer and 40 mL DI water was added to Bottle 16. Antibiotic cocktail (0.4 mL tetracycline and 0.3 mL cycloheximide) was also added to each bottle. The enzymes tend to attach to the solid substrate, thus they should be added closer to the fresh solids addition location [11]. The enzymes were not added to Bottle 1 because some enzymes remain in the liquid phase and are not adsorbed on the solid substrate [7]. To improve enzyme utilization, it is necessary that enzymes are used before leaving the system; therefore, enzymes were added to Bottle 4.

4.2.4.2. Calculation method

To calculate the glucose and xylose yields, the amount of sugars entering and exiting the countercurrent system must be determined. In every transfer, 10 g dry lime-pretreated corn stover was added to Bottle 1, i.e., 4.57 g equivalent glucose and 2.25 g equivalent xylose entered the system. The sugars exiting the system are the summation of sugars exiting from Bottles 1 and 16, and sugars in liquid samples collected from all 16 bottles. Glucose yield is calculated by using Equations 4.2 to 4.7. Xylose yield was calculated using a similar method.

$$\text{Yield}_{\text{glucose}} = \frac{\text{Mass}_{\text{glucose, out}}}{\text{Mass}_{\text{glucose, in}}} \times 100\% \quad (4.2)$$

$$\text{Mass}_{\text{glucose, out}} = \text{Mass}_{\text{glucose, Bottle 1}} + \text{Mass}_{\text{glucose, Bottle 16}} + \text{Mass}_{\text{glucose, samples}} \quad (4.3)$$

$$\text{Mass}_{\text{glucose, Bottle 1}} = \text{Vol}_{\text{liq, 1}} \times \text{Conc}_{\text{glucose, 1}} \quad (4.4)$$

$$\text{Mass}_{\text{glucose, Bottle 16}} = \text{Mass}_{\text{cake, 16}} \times \text{MC}_{16} \times \text{Conc}_{\text{glucose, 16}} \quad (4.5)$$

$$\text{Mass}_{\text{glucose, samples}} = \sum \left(\text{Conc}_{\text{glucose},i} \times \text{Vol}_{\text{samples},i}\right), i = 1 \text{ to } 16 \quad (4.6)$$

$$\text{Mass}_{\text{glucose, in}} = \text{Mass}_{\text{air-dry biomass}} \times (1 - \text{MC}_1) \times \text{Frac}_{\text{glucan}} \times f_{\text{glucose}} \quad (4.7)$$

where,

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

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

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

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

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

$Mass_{glucose,\ samples}$= summation of glucose in all liquid samples (g)

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

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

$Frac_{glucan}$ = fraction of glucan in pretreated corn stover

$Conc_{glucose,i}$ = the glucose concentration of Bottle i (i =1 to 16) (g/L)

$Vol_{liq,\ 1}$ = volume of liquid product exiting from Bottle 1 (L)

MC_1 = moisture content of substrate entering Bottle 1

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

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

$f_{glucose}$ = correction factor due to hydrolysis of glucan (1.111) (*Note*: For xylose, the appropriate correction factor is 1.136)

4.2.5. Slope Method for steady-state analysis

The Slope Method was used to analyze steady-state data. It uses regression to validate the steady state. Details of the method are explained in Zentay et al. [50]. Sugars entering with the enzyme cocktail are negligible and were neglected in the yield calculations.

4.3. Results and discussion

4.3.1. Batch saccharification

Figure 4-2 shows that with enzyme loadings of 1–25 mg protein/g dry biomass, the glucose yield of lime-pretreated corn stover is much higher than that of raw corn stover, which indicates lime pretreatment is very effective.

Novozymes reports that CTec3 has at least 1.5 times higher conversion efficiency than that of CTec2 [53]. At 5 mg protein/g dry biomass, CTec3 improved glucose yield by 16% and 14% for lime-pretreated and lime + shock treated corn stover, respectively (Figure 4-2). At high enzyme loadings, the difference of glucose yield between CTec3 and CTec2 becomes smaller (5–10%). For raw corn stover, when enzyme loading increases (>10 mg protein/g dry biomass), the difference of glucose yield between CTec3 and CTec2 remains unchanged. Based on these results, CTec3 was selected for countercurrent saccharification.

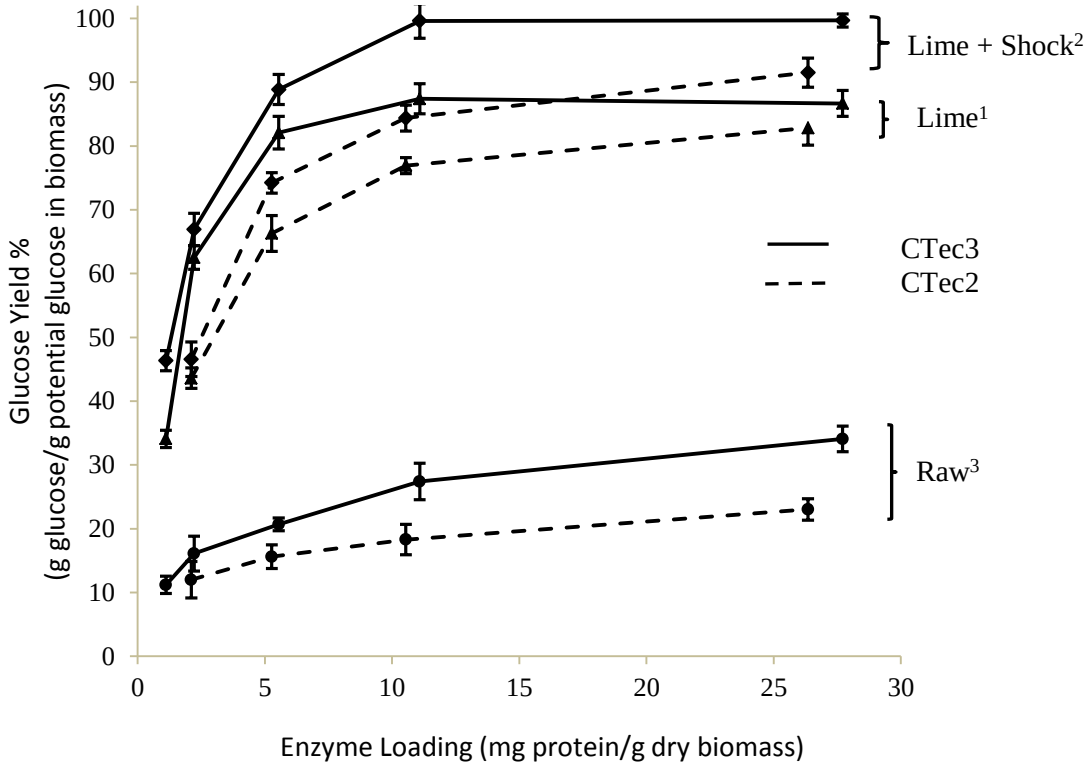


Figure 4-2*. Effect of pretreatment methods, enzyme type, and loadings on glucose yield. The results were obtained in batch saccharification. ([1]Lime represents substrate is lime-pretreated corn stover. [2]Lime + shock represents substrate is lime + shock treated corn stover. [3]Raw represents substrate is raw corn stover.) Error bars indicate ± 2 standard deviation.

*Reproduced with permissions from Chao Liang (Liang et al. [55])

4.3.2. Countercurrent saccharification

In the countercurrent saccharification of lime-pretreated corn stover, two different enzyme loadings were investigated: 1 mg CTec3/g dry biomass and (1 mg CTec3 + 1 mg HTec3)/g dry biomass. Once the experiment was started, the system took about 60 days to stabilize. Figure 4-3 shows glucose and xylose concentrations in the system for over 210 days. When the concentrations were constant for more than 20 days, steady state was validated using the Slope Method. The sugar concentration across the 16-bottle system varies with enzyme loading, which will be discussed later in this section.

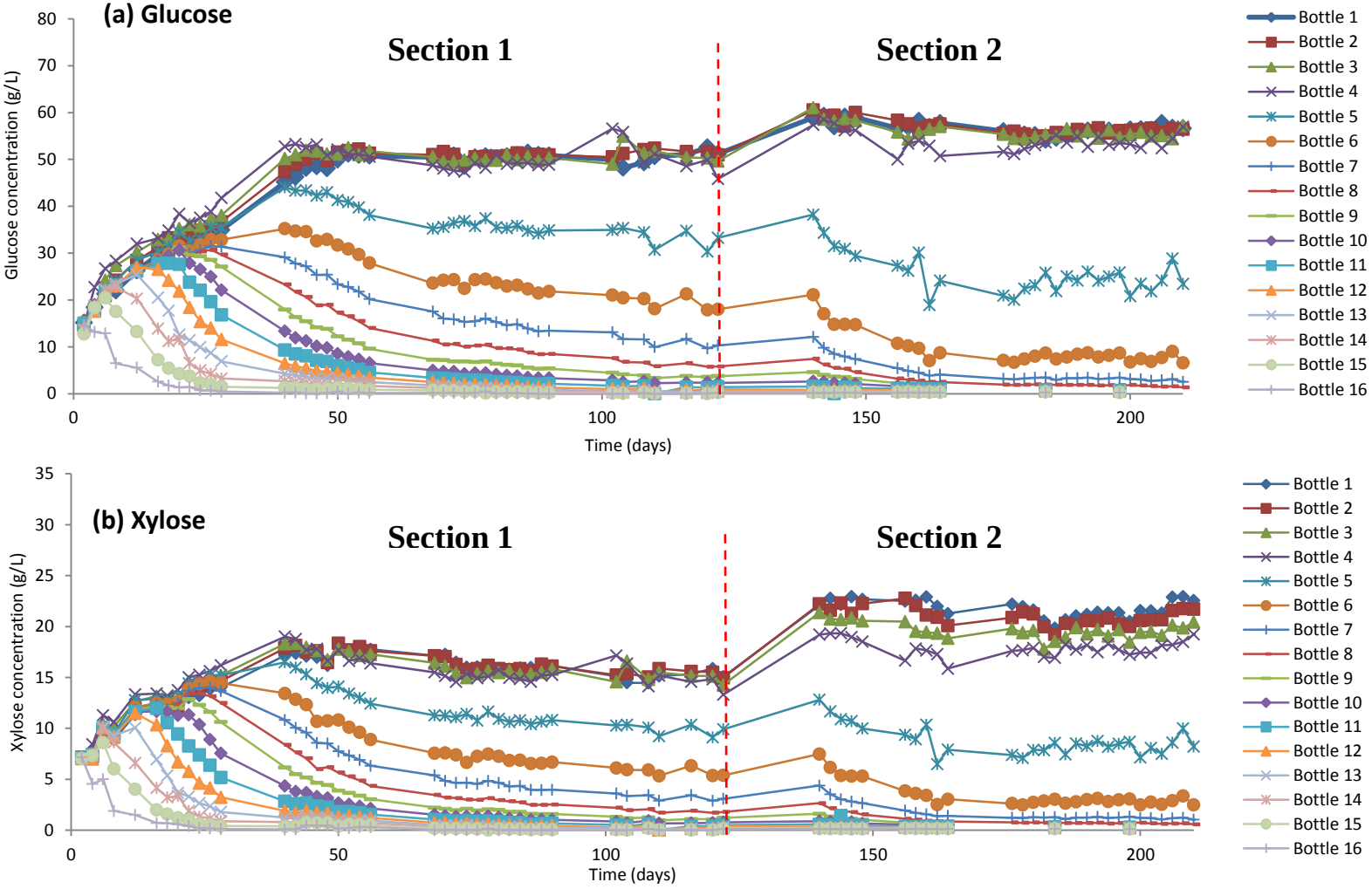


Figure 4-3. Glucose (a) and xylose (b) concentration as a function of bottle number and time from Day 0 to Day 210.

4.3.2.1. 1 mg CTec3/g dry biomass (Section 1)

Figure 4-4 shows glucose and xylose concentrations during the steady state (Days 68–90) at an enzyme loading of 1 mg CTec3/g dry biomass. The glucose and xylose concentrations in the liquid product leaving the system from Bottle 1 were approximately 50 g/L and 17 g/L, respectively. The steady-state data were analyzed using the Slope Method by plotting cumulative sugars exiting the system (Figure 4-5). Excellent fit of linear regression to the data (R^2~1) validates the steady state. After 22 days, the glucose, xylose, and total yields were 61%, 41%, and 55%, respectively. To achieve the same glucose yield, countercurrent saccharification required 1.6 times less enzyme as compared to batch (Table 4-3). In the case of xylose yield, countercurrent saccharification showed no benefit over batch. The loss in xylose yield can be attributed to less affinity of hemicellulases towards substrate, which is discussed in Section 4.3.2.3.

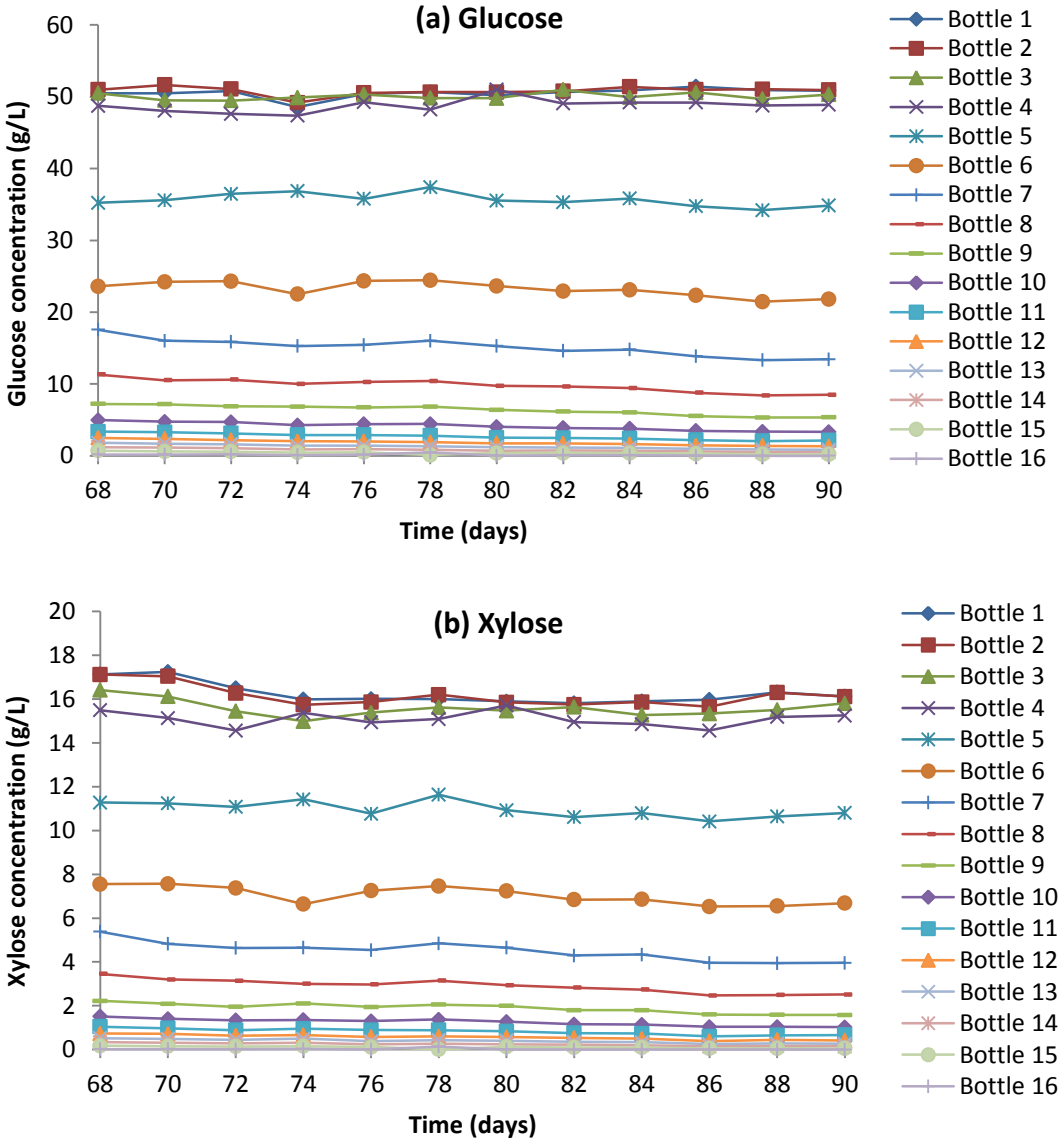


Figure 4-4. Section 1: Glucose (a) and xylose (b) concentration as a function of time and bottle number between Days 68 and 90.

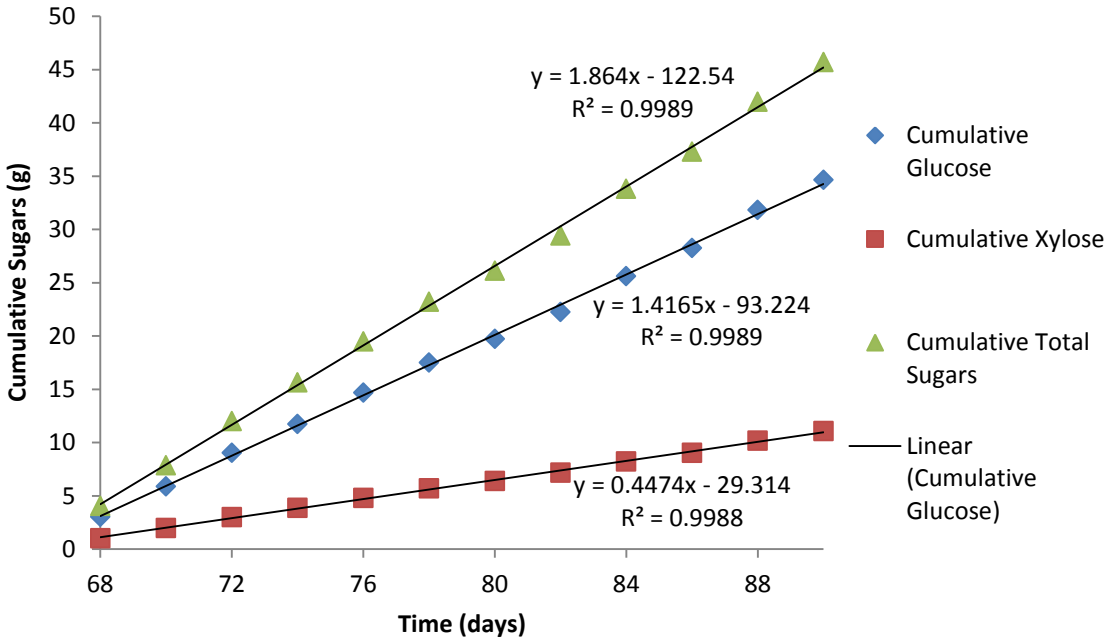


Figure 4-5. Sugars exiting the countercurrent system during Section 1. (Days 68 and 90)

Table 4-3. Comparison of enzyme requirements for batch and countercurrent saccharification.

	Section 1	Section 2
Glucose conversion (%)	61	67
Enzyme loading (mg protein/g biomass)		
Continuous countercurrent	1	2
Batch	1.6	2.8
Factor improvement	1.6	1.4
Xylose conversion (%)	41	53
Enzyme loading (mg protein/g glucan)		
Continuous countercurrent	1	2
Batch	0.7	1
Factor improvement	0.7	0.5

4.3.2.2. (1 mg CTec3 + 1 mg HTec3)/g dry biomass (Section 2)

Lime pretreatment retains most of the hemicellulose in raw biomass. CTec3 contains hemicellulases, but based on xylose yields reported in Section 4.3.2.1, these are not sufficient to digest hemicellulose. It was decided to supplement the system with HTec3, a hemicellulase that should boost xylose yields. Liang et al. [55] reported increase in xylose yield of lime + shock treated biomass with HTec3 addition. Figure 4-6 shows steady-state concentrations of glucose and xylose at (1 mg CTec3 + 1 mg HTec3)/g dry biomass (Days 176 – 210). The glucose and xylose concentrations in the product liquid leaving the system from Bottle 1 were approximately 55 g/L and 22 g/L, respectively. The Slope Method validated the steady state (Figure 4-7). After 34 days, glucose, xylose, and total sugar yields were 67%, 53%, and 62%, respectively. Adding HTec3 to the countercurrent saccharification also increased glucose yields along with increasing xylose yields. The digestion of hemicellulose enhances the hydrolysis of lignocellulose making cellulose more accessible to enzymes. To achieve a glucose yield of 67%, countercurrent saccharification required 1.4 times less enzyme than batch (Table 4-3).

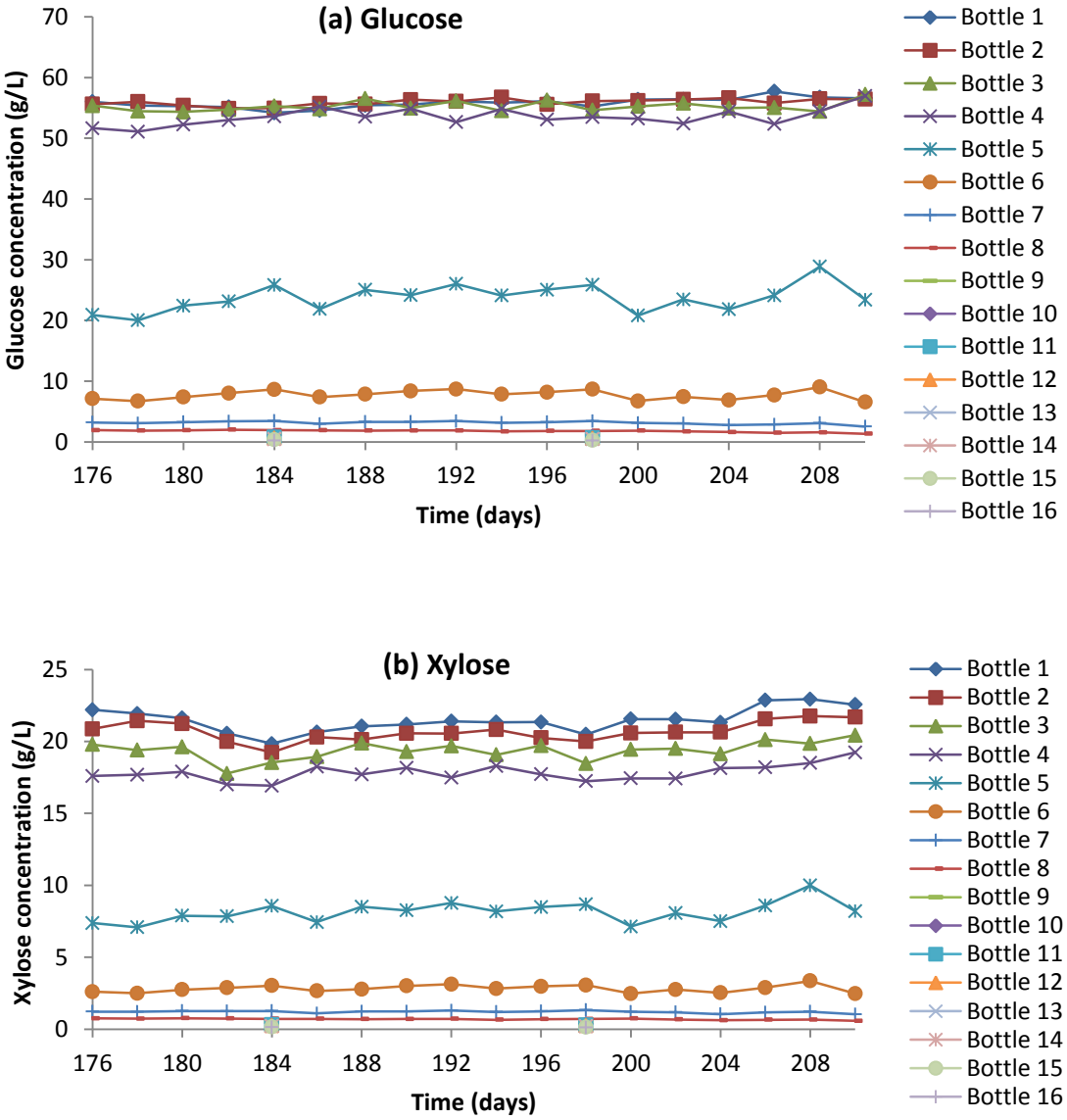


Figure 4-6. Section 2: Glucose (a) and xylose (b) concentration as a function of time and bottle number between Days 176 and 210. (Because the sugar concentrations in Bottle 9 to 16 were less than 1 g/L, they are shown only for Days 184 and 198).

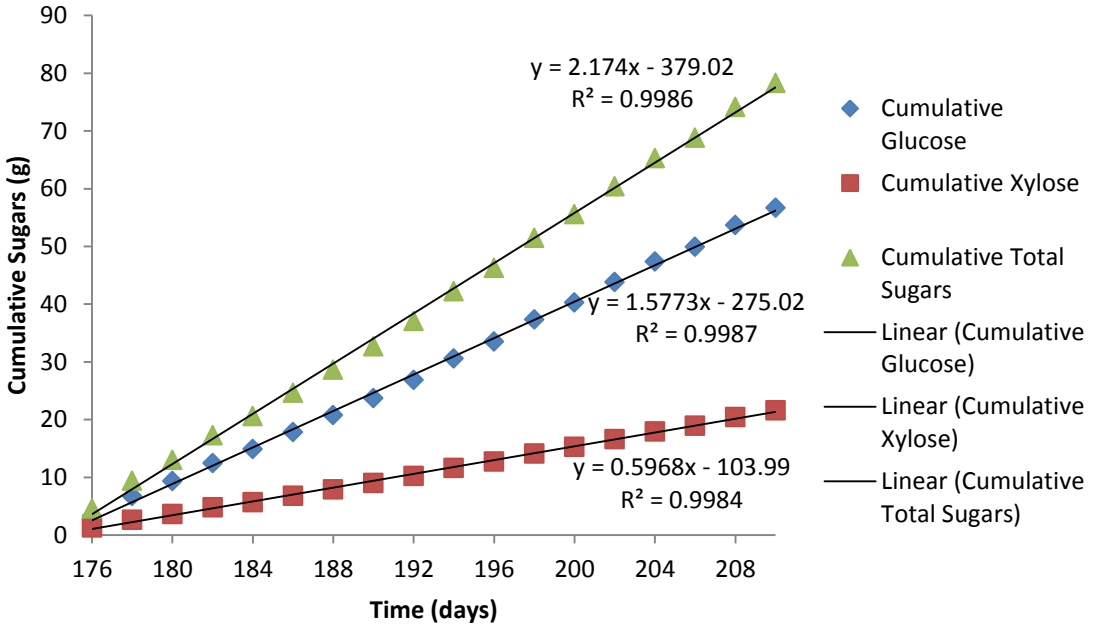


Figure 4-7. Sugars exiting the countercurrent system during Section 2. (Days 176 and 210)

4.3.2.3. Sugar concentration variation across the system

To utilize the enzymes more efficiently, they were added to Bottle 4 so that enzymes that distribute into the liquid phase (not adsorbed to substrate) are used when liquid flows from Bottles 4 to 1. At both enzyme loadings investigated, the glucose concentration was nearly the same in Bottles 1 to 4, which indicates that cellulases strongly bind to the substrate (Figure 4-8 a). In Section 1, the xylose concentration was almost the same in Bottles 1 to 4, whereas in Section 2, it increased from Bottles 4 to 1, which indicates that hemicellulases have less affinity for substrate compared to cellulases (Figure 4-8 b).

Another possible explanation is inhibition of enzymes by product sugars. The enzyme addition point is Bottle 4 where the glucose and xylose concentrations are approximately 40 g/L and 15 g/L, respectively. Based on the batch saccharification results discussed in Section 3.1.4, sugars bind to enzymes. Because the xylose concentration is low (~20 g/L) in Bottles 1 to 4, the hemicellulases are less inhibited and are active to digest hemicellulose. In contrast, because of high glucose concentration in Bottles 1 to 4 (~50 g/L), cellulase inhibition was higher, which affects cellulose digestion. This inhibition effect can be overcome by lowering the liquid residence time in the system. Liquid residence time (day) is defined as the volume of total liquid in the system (L) divided by the fresh liquid flow rate entering the system (L/day). Reducing the liquid residence time will decrease the product sugar concentration; thus, there is a trade-off between enzyme inhibition and the sugar concentration in the product.

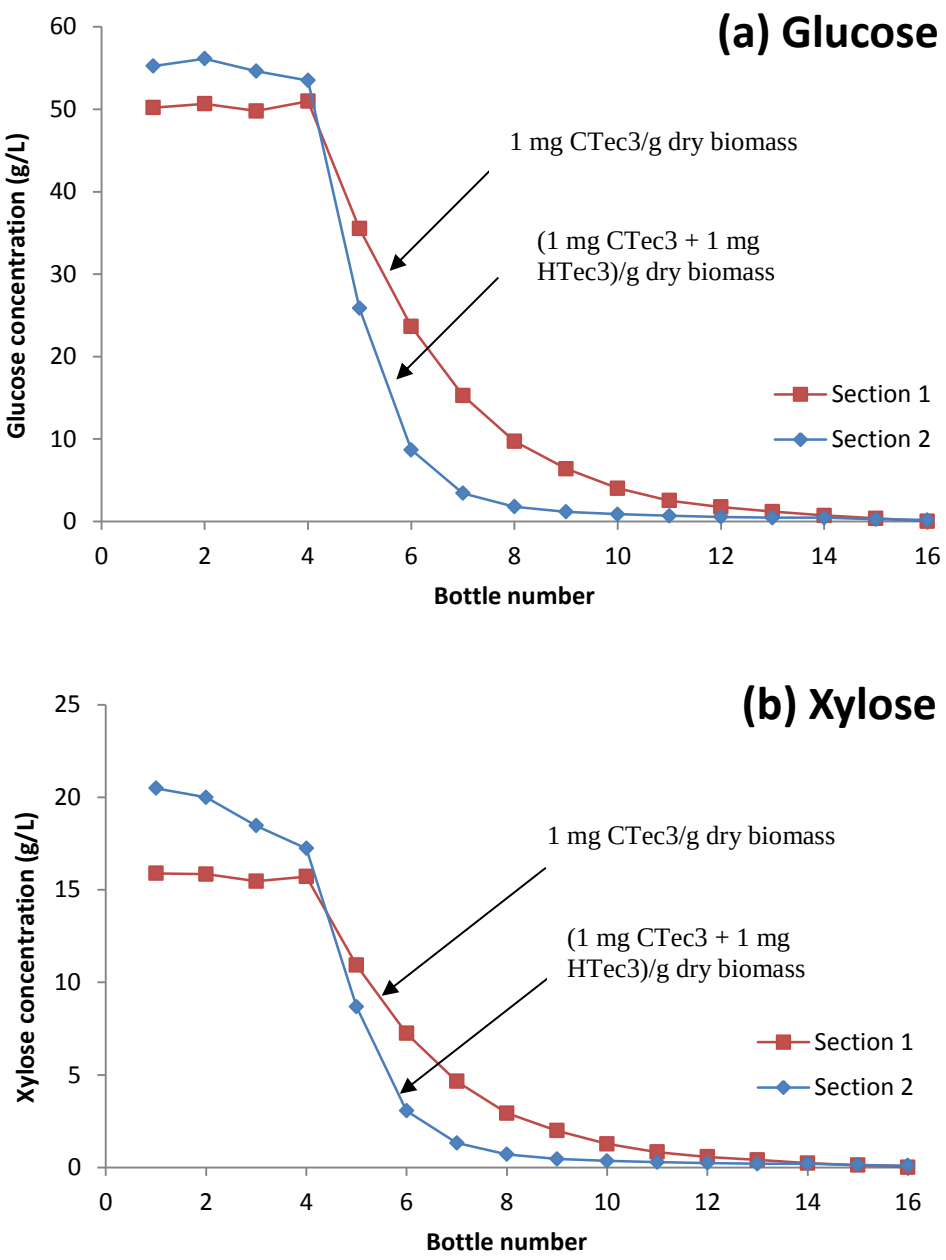


Figure 4-8. Variation of glucose (a) and xylose (b) concentration across the countercurrent train at different enzyme loadings.

In Section 1, more than 95% of the digestion occurred in only eight bottles (Bottles 4 to 12) (Figure 4-8). Enzymes were added to Bottle 4. Along the train, enzyme activity is inferred by the variation in sugar concentration. A large bottle-to-bottle change indicates large enzyme activity, and vice versa. The enzyme activity along the train was predicted based on variation in sugar concentration across the system. Sugar production up to Bottle 12 shows that enzymes are active for a longer period in the system. In the last four bottles (Bottles 13 to 16), the sugar concentration is less than 1 g/L. After increasing the enzyme loading for Section 2, achieving 95% of the digestion required even fewer bottles compared to Section 1. During Section 2, Bottles 9 to 16 had sugar concentration less than 1 g/L. Because of higher enzyme loading, most of the digestion occurs in Bottles 4 to 8 and the biomass remaining in the system thereafter is mainly lignin. The inactivity of enzyme in the later part of the train might be caused by irreversible binding of enzymes to lignin, which is reported to be a competitive cellulase adsorbent [56, 57]. Also, it has been suggested that residual lignin blocks the progress of cellulase down the cellulose chain [58, 59]. Kumar and Wyman [57] have reported an increase in cellulase effectiveness when lime-pretreated biomass was delignified. A pretreatment that can remove most of the lignin from biomass is desirable to remove unproductive binding and thereby increase the benefit of countercurrent saccharification.

4.4. Conclusion

Experimental analysis of countercurrent saccharification was performed on lime-pretreated corn stover at different enzyme loadings. The preferred enzyme mix included

both CTec3 and HTec3. Hemiellulase enzyme HTec3 was used to boost xylose yield. The Slope Method was used to validate steady state and to calculate sugar yields. At an enzyme loading of 1 mg CTec3/g dry biomass, glucose, xylose, and total sugar yield were 61%, 41%, and 55%, respectively. At enzyme loading of (1 mg CTec3 + 1 mg HTec3)/g dry biomass, the yields were 67%, 53%, and 62%, respectively. Adding hemicellulases not only increases xylose yield, but also improves glucose yield. Simultaneous conversion of cellulose and hemicellulose acts synergistically to increase the hydrolysis rate by "disentangling" the lignocellulosic substrate. Hemicellulases have less affinity for solid substrates than cellulases and therefore the addition point should be moved further downstream (towards the end where fresh liquid is added to the system).

Product sugars inhibit enzymes and reduce sugar yields. In countercurrent saccharification, there is a trade-off between reducing product inhibition and maximizing product sugar concentration. Countercurrent saccharification reduces enzyme requirements by efficiently using enzymes. In the case of lime-pretreated corn stover, to achieve glucan conversion of 61 and 67%, countercurrent system required 1.6 and 1.4 times less enzyme as compared to batch. In the case of α-cellulose, Zentay et al. [50] observed enzyme reduction of 8 and 16.8 times for countercurrent saccharification at enzyme loadings of 2 and 5 mg protein/g biomass, respectively. As compared to α-cellulose, lime-pretreated corn stover showed less reduction of enzyme required to reach a specified conversion. The major difference between the two substrates is the presence of lignin. Lime-pretreated corn stover contains about 8% lignin. Binding of enzymes to

lignin may render them inactive. The benefits of countercurrent saccharification may be increased by using pretreatments that more extensively remove lignin.

5. CREATING ECONOMIC INCENTIVES FOR WASTE DISPOSAL IN DEVELOPING COUNTRIES USING THE MIXALCO PROCESS

5.1. Introduction

In developing countries, high population density, rapid urbanization, and lack of infrastructure create challenges for managing wastes. Conventional disposal methods are expensive and unsustainable; thus, alternative solutions that provide incentives for waste collection and disposal are required [60].

In 2010, global municipal solid waste (MSW) generation was approximately 1.3 billion tonnes per year and by 2025 it is expected to increase to 2.2 billion tonnes per year [61]. Most low-income countries dispose of waste in open landfills, some of which openly burn wastes, thus releasing pollution that adversely impacts human health.

Alternatives to landfills include incineration, gasification, aerobic composting, and anaerobic digestion [61]. *Incineration* contributes to pollution and is expensive without energy recovery. After incineration, 20–30% of the original dry mass remains as ash, which requires further management. *Gasification* requires intensive pre-processing and is not suitable for wet waste. Depending on MSW composition, harmful compounds are released to the product syngas, which can cause environmental and operational problems. *Aerobic composting* has severe health risks through exposure to treated soil and dispersed dust (bioaerosols). Composting facilities require large space and

[1]Reproduced in part with permissions from "Creating economic incentives for waste disposal in developing countries using the MixAlco process", Lonkar et al., 2016 (doi: 10.1007/s12010-016-2213-6). *Applied Biochemistry and Biotechnology*, Springer. Copyright 2016 Springer.

marketing the product is challenging. *Anaerobic digestion* decomposes organic matter to generate biogas [62], and is commonly used in both developed and developing countries. The biogas is burned for home cooking or used in a diesel engine to make electricity. Although this approach works, biogas has some technical and economic challenges: (1) biogas is contaminated with carbon dioxide and toxic H_2S, which is expensive to remove; (2) biogas is difficult to transport; (3) methane has a low value; and (4) engines that generate electricity are expensive to purchase and operate.

The MixAlco process Figure 1-1 is similar to anaerobic digestion, except the methanogens are inhibited. The fermentation accumulates carboxylate salts, which can be chemically converted to fuels and chemicals [5, 10]. Lignocellulose requires pretreatment to remove lignin; however, in the case of municipal solid waste, paper and food scraps constitute major components of organic matter, and do not require pretreatment. In the fermentation, mixed consortia of microorganisms digest complex organic molecules into simple sugars, which are further fermented to short- and medium-chain fatty acids. The fermentation broth is dewatered to obtain dry carboxylate salts, which are heated to high temperatures (around 400 °C) to form ketones by decarboxylation. The ketones can be hydrogenated to produce mixed alcohols, which can be oligomerized using zeolite catalyst to produce hydrocarbons (gasoline and jet fuel). The estimated selling price ranges from $1.76 to $2.56 per gallon depending on the scale [63, 64].

The main advantages of the MixAlco process include flexible feedstock, high yields, scalability, and non-sterile operating conditions, which make it suitable for

disposing of MSW. The initial steps that produce ketones are very simple and appropriate for developing countries. The ketones are energy dense and can be shipped to an oil refinery where they are upgraded to gasoline and jet fuel.

In developing countries, urban areas commonly have shanty towns that suffer from inadequate infrastructure. Foul smelling and unhygienic waste disposal are common problems that can affect the whole community. For example, lack of sewer infrastructure leads to open defecation. Commonly, wastes are dumped into nearby water bodies. To improve living standards in shanty towns, proper facilities must be provided. Rather than install expensive sewer infrastructure, low-cost portable toilets can be used to collect the waste. Raw wastes from the portable toilets can be collected and transported to waste disposal facilities where they are upgraded to valuable products.

In developing countries, wastes are dominated by energy-rich materials such as agricultural residues (e.g., bagasse) and MSW (e.g., paper). To produce industrial chemicals and fuels from these wastes, the MixAlco fermentation requires a nutrient source, which can be supplied from animal manure or sewage sludge [38, 65, 66]. In this work, humanure (i.e., raw human feces and urine) is explored as a possible nutrient source using batch fermentations. The change in microbial community distribution with fermentation duration is studied. To study the product inhibition in fermentation, batch experiments spiked with carboxylic acids are performed. Using the experimental data, different model equations are compared to accurately predict the inhibition parameter. The Continuum Particle Distribution Model (CPDM) is used to predict the carboxylic

acid concentration and conversion of a continuous countercurrent fermentation of MSW and humanure.

5.2. Methods and materials

5.2.1. Substrate

In developing countries, MSW has a different composition than in the United States. Assuming that the waste will be sorted to recover valuable recyclables, a "synthetic" MSW was created by blending "organic matter" (i.e., foods scraps from Texas A&M University canteen) and paper (shredded white office paper) in a 3.6:1 ratio on a dry basis using compositions from the literature [61, 67, 68]. Food scraps were mainly composed of raw vegetables, fruits, banana peels, meat, rice, etc. Human feces and urine (humanure) were collected off-campus in 1-L polypropylene bottles and were autoclaved to make it sterile. The autoclaved bottles were brought to the campus and were pooled and mixed to create a uniform supply of human waste that was stored in the freezer (–10 °C) until further use.

Corn stover was provided by Texas A&M AgriLife Extension. It was air-dried at room temperature to moisture content of around 10% and stored in Ziploc bags to reduce contact with air. To remove lignin and improve the digestibility, corn stover was treated with submerged lime pretreatment (SLP) [69]. SLP was performed at 50 °C for 30 days with lime loading of 0.15 g $Ca(OH)_2$/g dry biomass in a 60-L jacketed vessel. Throughout the 30-day duration, the pH remained at 11.5. To provide oxygen, CO_2-free air was used. After pretreatment, biomass slurry was neutralized with 5-N HCl and then

washed thrice with distilled water. The washed biomass was air dried at room temperature and was stored in air-tight Ziploc bags to reduce contact with air.

5.2.2. Batch fermentation

Deoxygenated water was used as the medium. It was prepared by boiling deionized water to liberate dissolved gases and letting it cool in a covered vessel. Sodium sulfide (0.275 g/L) and L-cysteine hydrochloride (0.275 g/L) were added to further reduce the oxygen content of the deoxygenated water.

Fermentations were performed in 1-L polypropylene bottles capped by a rubber stopper (Figure 2-3). The fermentors were maintained at 40 °C in the incubator. The inoculum source was marine sediment collected from Galveston beach, Texas, USA. To accumulate carboxylic acids in anaerobic fermentation, it was necessary to inhibit methanogens. Iodoform has been shown to be an effective methanogen inhibitor in mixed-culture fermentation [35, 65]. Fu et al. [70] reported complete inhibition of methane formation at iodoform addition rate of 1.2 mg/(L·day). Based on these results, 90 μL iodoform solution (20 g CHI_3/L ethanol) was added to each fermenter every 2 days. Every 48 hours, the bottles were opened to vent accumulated gases, adjust pH, and to take 1-mL liquid samples. The liquid samples were stored at –10 °C until further analysis. At acidic pH, product inhibition is higher than at the neutral pH [10]. The pKa of short- and medium-chain fatty acids is 4.7–4.8 and at neutral pH, more than 99% of the acids are dissociated. Dissociated fatty acids are less inhibitory than undissociated acids. Therefore, the pH was adjusted to 6.8–7 using calcium or magnesium carbonate

buffer. When the acid production rate was high, 0.2–1 g of buffer was added depending on pH adjustment. The buffer information specific to the experiment is provided in figure and table captions. The experiments were run in duplicate.

5.2.3. Analytical methods

Dissolved carboxylic acids were analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (FID). The frozen samples were thawed, vortexed, and centrifuged at 13,000 rpm for 10 min. Then, the sample (0.5 mL) was mixed with 3-M phosphoric acid and an internal standard (4-methyl-valeric acid) to analyze the carboxylic acid concentration. The pH of the fermenter slurry was measured using an Oakton pH meter. The pH meter was calibrated every time before monitoring the fermentation.

Moisture content and ash content were measured using standard NREL methods [36]. Batch fermentation performance was analyzed in terms of conversion and selectivity, which are calculated using the following formulae:

$$\text{Conversion } (x) = \frac{\text{Volatile solids digested (g)}}{\text{Volatile solids fed (g)}} \tag{5.1}$$

$$\text{Selectivity} = \frac{\text{Total acids produced (g)}}{\text{Volatile solids digested (g)}} \tag{5.2}$$

5.2.4. DNA extraction

To determine the microbial community in the fermentation, slurry samples (approximately 0.5 g) were collected periodically from the fermentors. The samples were stored at –10 °C until DNA extraction. PowerFecal™ DNA isolation kit (Catalog No. 12830-50; MO BIO Laboratories Inc.) was used to extract DNA from the slurry samples per manufacturer's instructions. Before DNA extraction, samples were thawed and 0.25 g of sample and 750 µL of bead solution were added to the dry bead tube. Then, 60 µL solution 'C1' was added and the tube was vortexed briefly followed by 10 min incubation at 65°C. After incubation and bead beating for 10 min, the tubes were centrifuged and supernatant was collected. Supernatant (500 µL) and solution 'C2' (250 µL) were mixed and incubated in ice for 5 min. The samples were centrifuged again and 600 µL of supernatant was collected and mixed with solution 'C3'. After incubation on ice for 5 min, tubes were centrifuged and 600 µL of supernatant was mixed with 1200 µL of 'C4' solution. The supernatant was then applied onto the spin filter to bind DNA to the silica membrane of the filter. The spin filter was washed with solution 'C5' to remove impurities. The DNA was finally eluted out in 100 µL of solution 'C6'. The DNA samples were stored at –10 °C until further analysis. Purified DNA samples were submitted to MR DNA (www.mrdnalab.com, Shallowater, TX, USA) for sequencing.

5.2.5. DNA sequencing

The 16S rRNA gene V4 variable region PCR primers 515/806 [71] were used in a single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA)

under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, after which a final elongation step at 72°C for 5 min was performed. Sequencing was performed by MR DNA on an Ion Torrent PGM and sequence data were processed using a proprietary analysis pipeline (MR DNA). In summary, sequences were depleted of barcodes and primers, then sequences <150 bp were removed, and finally sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were de-noised, OTUs generated, and chimeras removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database [72].

5.2.6. Continuum Particle Distribution Model (CPDM)

CPDM has been used to quantify the kinetics of reactions occurring at the interface between solid and fluid phases. Using batch fermentation data, CPDM is a powerful tool to predict the product concentration and conversions of continuous countercurrent fermentation [73, 74]. Batch fermentations are performed using varying initial substrate concentrations. Some batch fermentations are spiked with product carboxylic acids to capture inhibition effects.

5.3. Results and discussion

5.3.1. Feasibility studies

To determine the feasibility of humanure as a nutrient source in the MixAlco process, batch fermentations of different substrates were performed. Table 5-1 shows the substrates used and the experiment details. To represent agricultural waste, raw and submerged-lime-pretreated (SLP) corn stover were used. Fermentations were terminated after carboxylic acid production stopped.

Table 5-1. Experiment design for feasibility studies (Buffer = calcium carbonate)

Biomass	Biomass:humanure ratio (on dry weight basis)	Total solids loading (g/L)
Office paper	80:20	100
Raw corn stover	80:20	100
SLP-treated corn stover	80:20	100
Synthetic MSW	90:10	100

In combination with humanure, all tested biomass feedstocks demonstrated good carboxylic acid production in a mixed-culture fermentation (Figure 5-1). Synthetic MSW had the highest carboxylic acid production rate and produced up to 40 g/L of acids in 30 days. Pretreated corn stover produced more carboxylic acids (37 g/L) than raw corn stover (29 g/L) and office paper (23 g/L). The results show that humanure can be used as a nutrient source for mixed-culture fermentation.

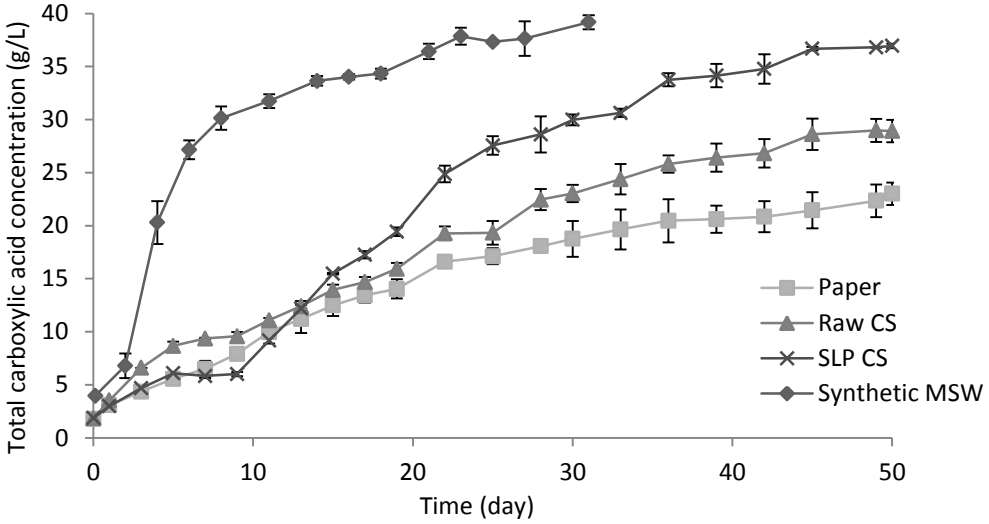


Figure 5-1. Carboxylic acid concentration profiles for various biomass materials combined with humanure. Error bars are the range of duplicate experiments. Buffer = calcium carbonate.

5.3.2. Analysis of microbial population in fermentation

Mixed cultures are extremely well suited to digest a variety of feedstocks under non-sterile conditions. The dominant microbial communities affect the performance and end-products of fermentation. Depending on substrate, temperature, pH, and headspace gas composition, particular microbial communities dominate the culture. In the MixAlco fermentation, the microbial population has previously been studied [75]. Under thermophilic conditions, *Thermoanaerobacterium*, Clostridia, and Bacilli dominated, whereas under mesophilic conditions, Clostridia, Bacteroidia, Proteobacteria, and Actinobacteria dominated [75]. To study the changes in microbial population in the

fermentation of office paper, raw corn stover, and lime-pretreated corn stover, slurry samples were collected periodically and analyzed. In all the tested substrates, Bacteroidia and Clostridia were the dominating communities (Figures 5-2, 5-3, and 5-4). These are common cellulose-degrading species. At mesophilic anaerobic conditions, many Clostridia species (e.g., *Clostridium acetobutylicum, C. cellulolyticum, C. cellulovorans, C. josui*) can produce complex cellulase systems (cellulosomes) that can digest cellulose [76]. Some Bacteroidetes are known to degrade hemicellulose-derived pentose sugars [77]. Raw and SLP corn stover have more hemicellulose than office paper [69]; therefore, when microbial cultures were stable (Day 50), corn stover showed more Bacteroidia composition (around 50%) than paper (around 30%). During the adaptation period, Bacilli and Gammaproteobacteria were observed in paper and lime-pretreated corn stover fermentation. Some Bacilli species (e.g., *Bacillus agaradhaerens, B. amyloliquefaciens, B. cellulyticus, B. circulans, B. pumilus,* and *B.* subtilis) have been reported to produce cellulose-degrading enzymes [78]. Gammaproteobacteria and Bacteroidetes are part of cellulose-degrading microbial communities present in the marine environment [79]. Marine inoculum was used for batch fermentations and the presence of these microbes can be attributed to their source and availability of cellulose as substrate. During the 50 days of batch fermentation, the microbial community did not change significantly.

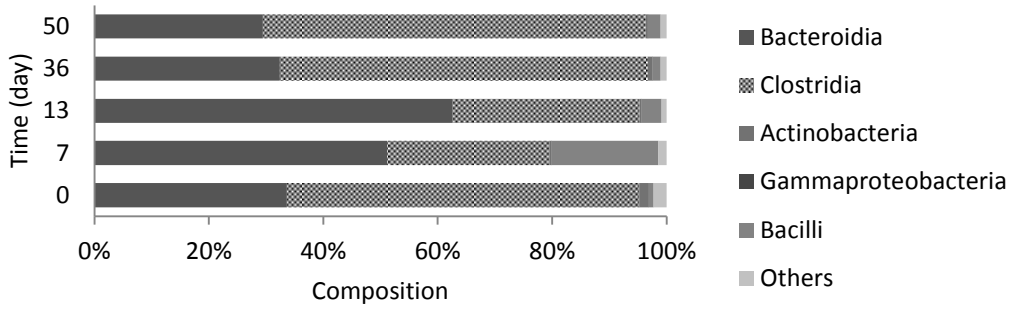


Figure 5-2. Variation in microbial population distribution in fermentation of paper and humanure. Buffer = calcium carbonate.

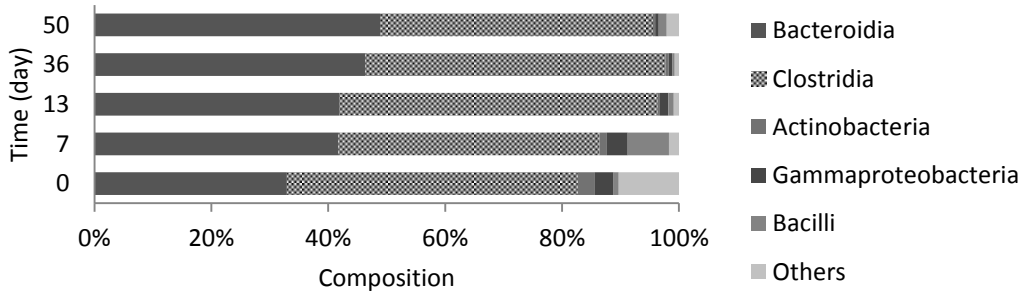


Figure 5-3. Variation in microbial population distribution in fermentation of raw corn stover and humanure. Buffer = calcium carbonate.

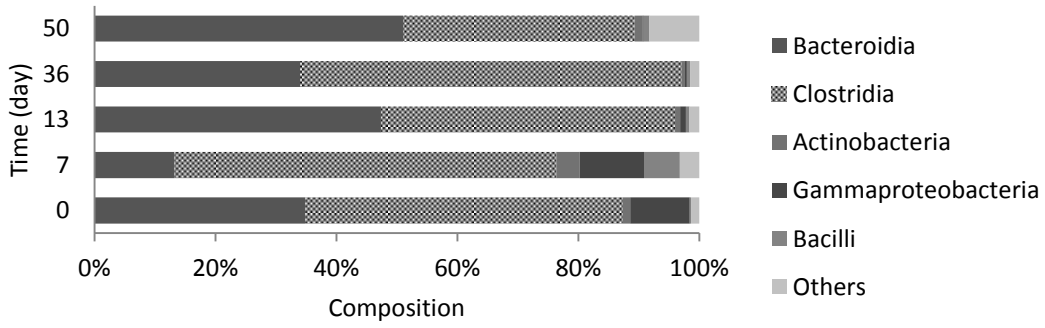


Figure 5-4. Variation in microbial population distribution in fermentation of SLP corn stover and humanure. Buffer = calcium carbonate.

5.3.3. Optimum ratio of nutrient-to-carbon source

For anaerobic fermentation, the carbon-to-nitrogen ratio is an important parameter and has been studied previously for different substrates [33, 80]. To use humanure as a nutrient source, it is necessary to re-optimize the nutrient-to-biomass ratio. Experiments were designed to determine the optimum humanure-to-biomass ratio for maximum carboxylic acid production. On a dry weight basis, humanure (10–60%) was mixed with office paper, the inoculum was adapted marine sediment, and calcium carbonate was the buffer. The total solid loading was 100 g/L in each fermentor. The fermentations were terminated after carboxylic acid production stopped.

The fermentors with nutrient:biomass ratios of 40:60 and 60:40 produced slightly more carboxylic acids (around 25 g/L) than those of 10:90 and 20:80 (around 21 g/L) (Figure 5-5). This is potentially important because it implies a wide range of feedstock ratios can be deployed with only a slight impact on performance. Considering the volumes of municipal solid waste and humanure waste produced in a typical developing country, it was decided to use 10% humanure in the subsequent fermentation studies.

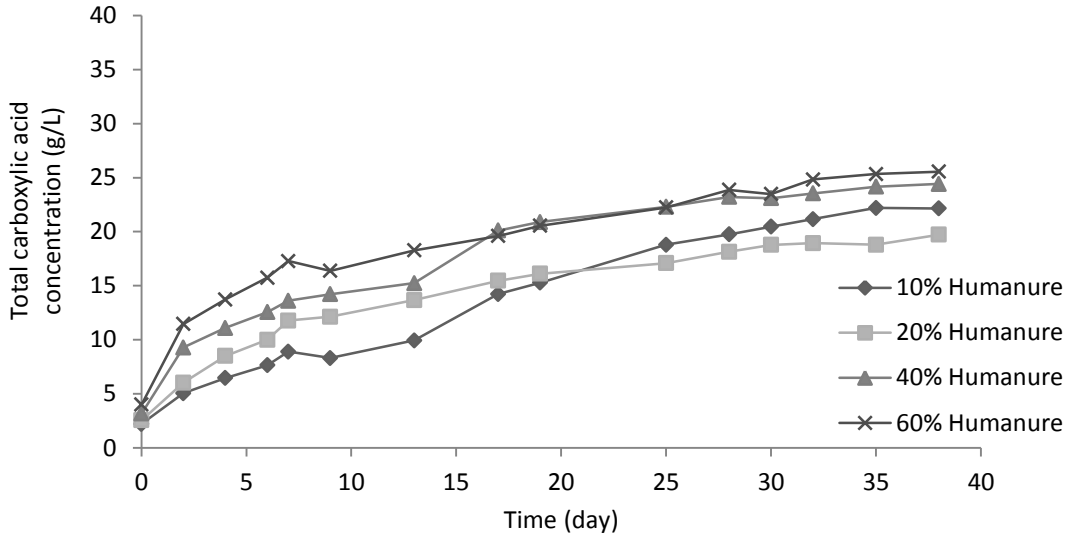


Figure 5-5. Carboxylic acid concentration profiles for varying ratios of humanure to carbon source on dry weight basis. Buffer = calcium carbonate.

5.3.4. Continuous fermentation performance prediction using CPDM

At commercial scale, continuous processes are preferred over batch to avoid loading and unloading idle times. Large-scale operations improve the economics of processes. Considering the volume of municipal solid wastes generated, a continuous process is envisioned for the production of fuels. In the MixAlco fermentation, product carboxylic acids inhibit the microorganisms, which can be mitigated by employing countercurrent operations.

To analyze the performance of countercurrent fermentations, steady-state data are used. Laboratory countercurrent fermentations are very time-and-resource-consuming and may take two to four months to reach steady state; thus, it may take years

to optimize fermentation for a single feedstock. To predict the performance of continuous fermentation using batch fermentation data, Loescher [74] developed CPDM. For different biomass fermentations, CPDM predicts product concentrations and conversions within 20% of the experimental results [51, 65, 70, 81, 82]. The details of CPDM have been described by Fu et al. [73].

Using empirical rate models developed from batch fermentation data, CPDM simulates four-stage countercurrent fermentation to estimate conversions and carboxylic acid concentrations at various volatile solids loading rates (VSLR) and liquid residence times (LRT), which are defined as follows:

$$\text{VSLR } (\text{g/(L} \cdot \text{d)}) = \frac{\text{Volatile solids fed to the system}}{\text{Total liquid in all fermentors} \times \text{Time}} \tag{5.3}$$

$$\text{LRT (d)} = \frac{\text{Total liquid in all fermentors}}{\text{Flow rate of liquid out of the fermentor train}} \tag{5.4}$$

Using the results at different VSLR and LRT, a CPDM map is obtained.

In batch experiments, the carboxylic acid concentration is represented as acetic acid equivalents, Aceq, using the following formulae:

$$\begin{aligned}\alpha\,(\text{mol/L}) = {} & 1.0 \times \text{acetic(mol/L)} + 1.75 \times \text{propionic(mol/L)} + 2.5 \times \text{butyric(mol/L)} + \\ & 3.25 \times \text{valeric(mol/L)} + 4.0 \times \text{caproic(mol/L)} + 4.75 \times \text{heptanoic(mol/L)}\end{aligned} \tag{5.5}$$

$$\text{Aceq (g/L)} = 60.05 \times \alpha \text{ (mol/L)} \tag{5.6}$$

The governing empirical rate equation (Equation 5.7) relates specific reaction rate ($\hat{r}$) with acetic acid equivalent concentration (Aceq) and conversion of VS (x).

$$\hat{r}_{pred} = \left(e(1-x)^{f}\right)i \tag{5.7}$$

where e and f are empirical constants and i is the inhibition parameter, which varies from 0 to 1 and is a function of product concentration expressed as Aceq. These constants are determined using batch fermentation data.

Batch fermentations of synthetic MSW (78.5% food scraps and 21.5% office paper) and humanure were performed at varying initial solid concentrations. The ratio of MSW to humanure was 9:1 on dry weight basis. To capture inhibition effects, additional batch fermentations were performed with initially spiked carboxylic acids. The composition of the spiked acid mixture was 47% acetic acid, 13% propionic acid, and 40% butyric acid, which is similar to a typical fermentation broth acid composition. All fermentations were performed in duplicate. Table 5-2 shows the design of the batch fermentation.

Table 5-2. Experiment design for CPDM batch fermentations (Buffer = magnesium carbonate)

Label	Solid loading (g/L)	Initial carboxylic acid spike concentration (g/L)	Duration of batch fermentation (days)
20	20	0	26
40	40	0	26
70	70	0	26
100	100	0	26
100+5	100	5	30
100+10	100	10	30
100+20	100	20	30
100+30	100	30	23
100+35	100	35	30
100+50	100	50	23
100+70	100	70	23
100+100	100	100	23

In food scraps, the availability of simple sugars and starches makes it easily digestible. Figures 5-6 and 5-7 show carboxylic acid production in CPDM batch fermentations. The fermenters loaded with 70 and 100 g/L of initial carboxylic acid concentration produced around 20 and 15 g/L of additional acids in 23 days, whereas, the fermenter loaded without any carboxylic acids produced around 40 g/L of carboxylic acids in the same time (Figure 5-7). At high initial carboxylic acid concentration (>50 g/L), acid production decreases because microbial activity is inhibited significantly. In

the MixAlco process, fermentation broth is dewatered to obtain dry carboxylate salts which are thermally converted to ketones. Thus, high carboxylic acid concentrations in the fermentation broth are desired to reduce dewatering costs.

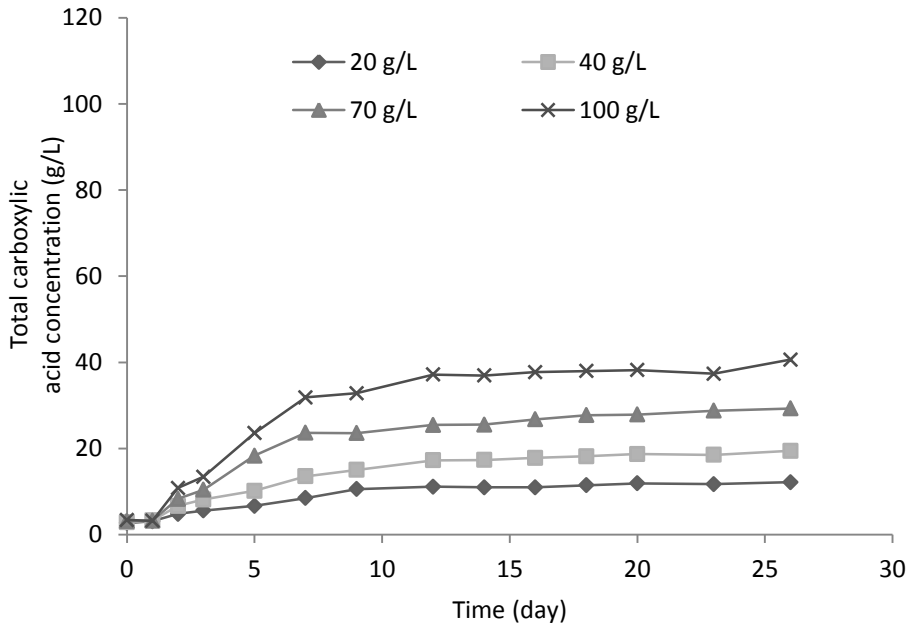


Figure 5-6. Carboxylic acid production in batch fermentation of MSW and humanure at varying initial solid concentration. Buffer = magnesium carbonate.

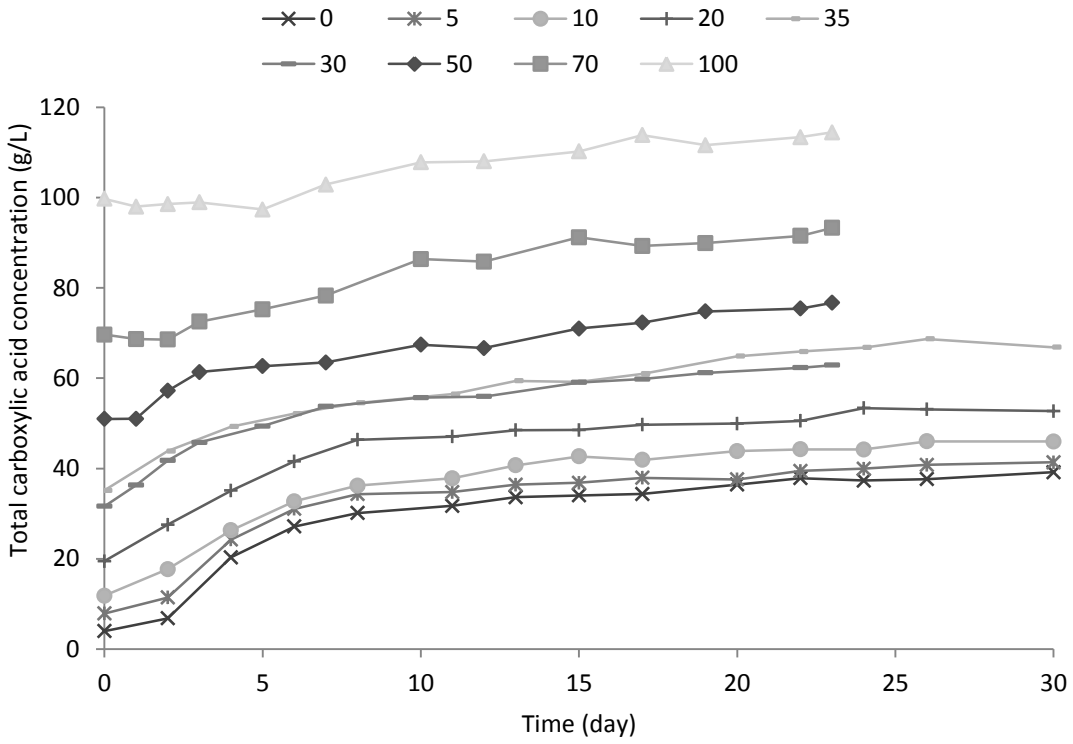


Figure 5-7. Carboxylic acid production in batch fermentation of MSW and humanure at varying initial carboxylic acid concentration. The initial solid concentration was 100 g/L. Buffer = magnesium carbonate.

5.3.5. Rate equation

High product carboxylic acid concentrations inhibit microorganisms. In the empirical rate equation (Equation 5.7), parameter *i* accounts for inhibition effects. For accurate predictions using CPDM, it is important to have a rate equation with an appropriate inhibition parameter *i*. To identify the best model that improves prediction

accuracy, batch fermentations with varying carboxylic acid loadings (Figure 5-7) were performed and the following model was fit to the acetic acid equivalent data.

$$\text{Aceq} = a + \frac{bt}{1+ct} \tag{5.8}$$

The reaction rate was calculated by taking the derivative of this equation, $d(\text{Aceq})/dt$. The initial reaction rate is determined at $t = 0$. The specific reaction rate $\hat{r}$ is calculated by dividing the reaction rate with initial substrate VS concentration. In Equation 5.7, the inhibition parameter i can take many forms, but is constrained by $i = 1$ at Aceq = 0, $i = 0$ at Aceq = ∞, and $0 \le i \le 1$. The selected empirical equations follow:

$$\hat{r} = e(1-x)^f \left(\frac{1}{1+g(\phi \cdot \text{Aceq})^h} \right) \tag{5.9}$$

$$\hat{r} = e(1-x)^f \left(\frac{1}{1+g(\phi \cdot \text{Aceq})} \right)^h \tag{5.10}$$

$$\hat{r} = e(1-x)^f \left(\frac{1}{1+m(\phi \cdot \text{Aceq}) + n(\phi \cdot \text{Aceq})^2} \right) \tag{5.11}$$

$$\hat{r} = e(1-x)^f \left(\frac{1}{1+m(\phi \cdot \text{Aceq}) + n(\phi \cdot \text{Aceq})^2} \right)^h \tag{5.12}$$

$$\hat{r} = e(1-x)^f \left(\frac{1}{1+g(\phi \cdot \text{Aceq})e^{h(\phi \cdot \text{Aceq})}} \right) \tag{5.13}$$

$$\hat{r} = e(1-x)^f \left(\frac{1}{1+g(\phi \cdot \text{Aceq})e^{\phi \cdot \text{Aceq}}} \right)^h \tag{5.14}$$

where the parameter ϕ represents the ratio of moles of carboxylic acids to moles of acetic acid equivalents and *e*, *f*, *g*, *h*, *m*, and *n* are empirical parameters. These rate equations were all evaluated at $x = 0$, such that $(1 - x)^f = 1$. Using the experimental data, initial specific reaction rate was plotted against initial carboxylic acid concentration (Figure 5-8). Using least-square regression, Equations 5.9–5.14 were fit to the data. Table 5-3 shows the corresponding empirical parameters and root mean square (RMS) values of the differences between experimental and calculated data.

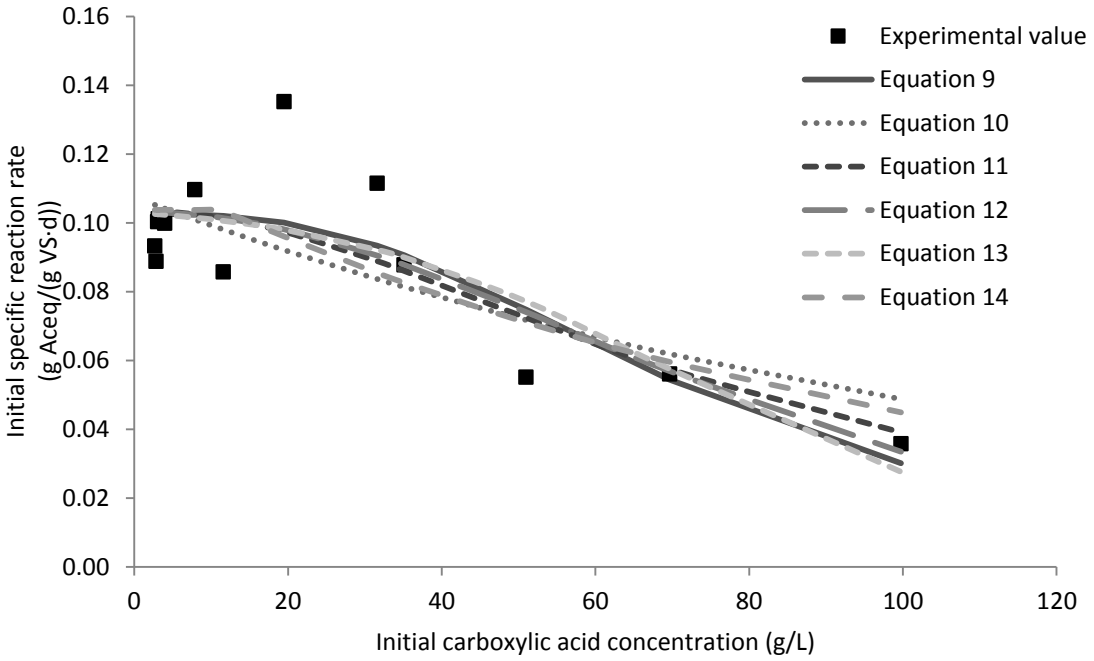


Figure 5-8. Comparison of model equations for product acid inhibition studies in mixed-culture fermentation.

Table 5-3. Empirical parameters obtained by regression

Parameters	*e*	*g*	*h*	*m*	*n*	RMS value
Equation 5.9	0.1027	6.8×10^{-6}	2.78	–	–	0.0141
Equation 5.10	0.1075	3.5×10^{-6}	2276.16	–	–	0.0172
Equation 5.11	0.1036	–	–	0	16.54×10^{-5}	0.0149
Equation 5.12	0.1032	–	3.39	0	3.96×10^{-5}	0.0147
Equation 5.13	0.1030	1480.9×10^{-6}	0.029	–	–	0.0149
Equation 5.14	0.1038	74.4×10^{-6}	0.009	–	–	0.0159

Based on RMS values in Table 5-3, Equation 5.9 fits best to the data as compared to other equations; therefore, Equation 5.9 was used to predict carboxylic acid concentrations and conversions using batch fermentation data. The governing rate equation is

$$\hat{r}_{pred} = 0.104(1-x)^{2.41}\left(\frac{1}{1+0.000376(\phi \cdot \text{Aceq})^{1.73}}\right) \tag{5.15}$$

where $\hat{r}_{pred}$ has units g Aceq/(g VS·d) and Aceq has units g/L.

Using Equation 5.15, a CPDM map is plotted (Figure 5-9) for a four-stage continuous countercurrent fermentation. It gives predicted carboxylic acid concentration as a function of conversion at different VSLR and LRT. High carboxylic acid

concentrations and high conversions can be achieved at low VSLR and high LRT. At VSLR = 10 g/(L·d), LRT = 25 d, and substrate concentration of 100 g/L liquid, the model predicts carboxylic acid concentration of 57 g/L and conversion of 53%. The conversion reduces significantly with increasing VSLR. For a specified VSLR, conversion remains almost the same at all LRTs, whereas carboxylic acid concentration increases with increasing LRT.

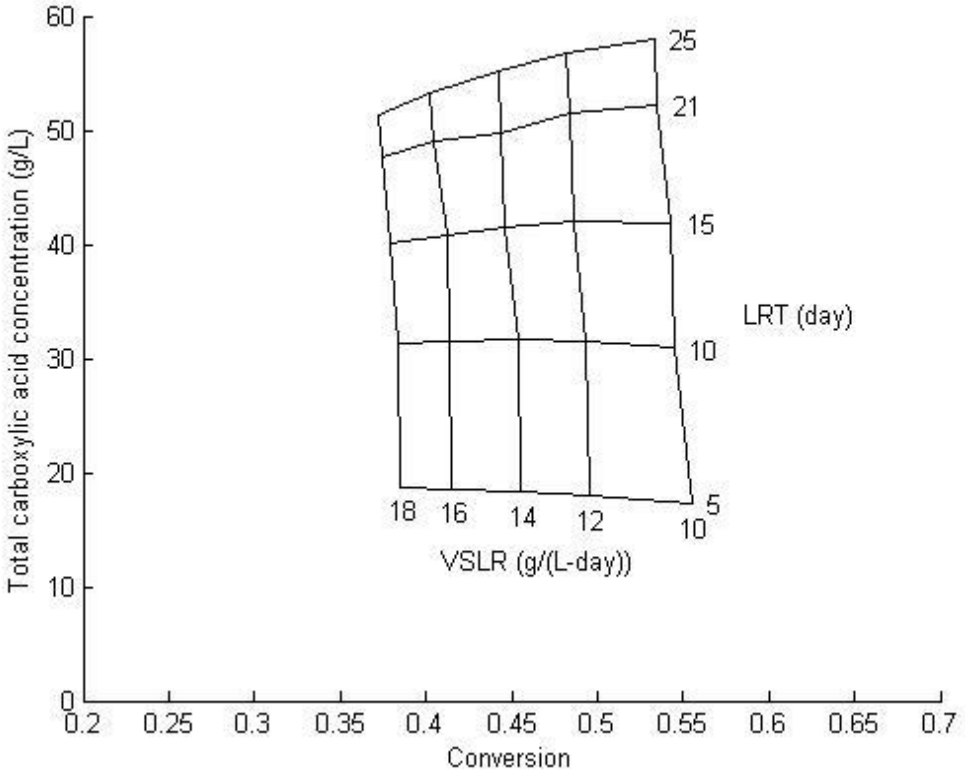


Figure 5-9. CPDM map of four-stage countercurrent fermentation of MSW and humanure. Substrate concentration = 100 g/L liquid.

5.3.6. Commercial vision

High conversions and high carboxylic acid concentrations make the MixAlco process an effective solution for municipal solid waste disposal. Small (50 tonnes per day) to medium-scale (500 tonnes per day) MixAlco plants can be built near every metropolitan area. Using existing collection and separation methods, the organic fraction of MSW and humanure can be transported to the nearby MixAlco plant. The produced ketones can be transported to nearby oil refineries where they are upgraded to gasoline and jet fuel and distributed using existing pipelines. The undigested residues of the MixAlco fermentations can be used as compost to upgrade soil quality in nearby agricultural land. This system will help create wealth from the waste collected in developing countries.

5.4. Conclusion

The MixAlco process allows agricultural waste, municipal solid waste, office waste, etc. to be converted to gasoline and jet fuel. Humanure can be used as a nutrient source in the MixAlco fermentations which creates an economic incentive for its collection in developing countries. At neutral pH and mesophilic conditions, Bacteroidia and Clostridia dominate the mixed-culture fermentations. Product inhibition in mixed-culture fermentation was studied and different empirical equations were compared to obtain the best governing rate equation. Using CPDM, carboxylic acid concentration and conversion of four-stage continuous countercurrent fermentation were predicted for municipal solid waste and humanure. At VSLR = 10 g/(L·d), LRT = 25 d, and substrate

concentration = 100 g/L, CPDM predicts carboxylic acid concentration of 57 g/L and conversion of 53%.

6. CONCLUSIONS AND FUTURE WORK

6.1. Conclusions

Medium-chain fatty acids (MCFA) can be produced in mixed-culture fermentation by adding ethanol to fermentors. Microorganisms (e.g., *Clostridium kluyveri*) elongate the fatty acid chain by combining them with ethanol. In mixed-culture fermentation of cellulosic substrate at neutral pH, the optimum ethanol concentration for chain elongation is 5–10 g/L. Caproic acid is the major product with maximum concentration of around 10 g/L. High ethanol concentrations (≥40 g/L) inhibit chain elongating microorganisms. In the presence of hydrogen partial pressure, *in-situ* generation of ethanol is beneficial for MCFA production in mixed-culture fermentation.

Propanol also participates in the chain elongation. In the fermentors fed with propanol (5–10 g/L), valeric acid is the major product. Propanol is more inhibitory than ethanol. Ethanol is less expensive and more readily available, so it is preferred to use ethanol for chain elongation.

Mixed-culture fermentation produces both short and medium-chain fatty acids at low temperature (≤40 °C) whereas only short-chain fatty acids are produced at high temperatures (≥55 °C). Chain elongation occurs at low temperature (≤40 °C) and does not occur at 55 °C and above. At 55 °C, the microorganisms selectively produce acetic acid, butyric acid, and ethanol. To maximize MCFA production, acid production and chain elongation can be performed in two stages maintained at different temperatures. Short-chain acids and ethanol can be produced under thermophilic conditions (55 °C),

which are then sent to chain-elongation fermentor operated at low temperature (≤40 °C). High selectivity towards acetic and butyric acids in the first stage directs the process towards high selectivity of caproic acid. The chain elongation process does not require pure ethanol; thus, any waste stream containing high concentration of ethanol can also be used.

Based on the results obtained in this study, the following recommendations are made to make the processes more economically attractive. If MCFA are desired products and the following are major and cheaper substrates:

1. Biomass: Two stage fermentation (acidification at 55 °C and chain elongation at ≤40 °C) can be employed to reduce requirements of external ethanol.
2. Ethanol: Directly added to fermentors for chain elongation. SCFA can be either produced from biomass or can be added externally.
3. Hydrogen: *In-situ* ethanol production can be enhanced by maintaining high partial pressure of hydrogen in fermentor headspace.
4. Acetic acid: Directly added to fermentor with ethanol stream. If both the streams are pure, monocultures can be employed to improve selectivity.

Countercurrent saccharification reduces enzyme requirements as compared to batch to achieve same conversions. In the case of lime-pretreated corn stover, to achieve glucan conversion of 61 and 67%, countercurrent system required 1.6 and 1.4 times less enzyme as compared to batch. At an enzyme loading of 1 mg CTec3/g dry biomass, glucose, xylose, and total sugar yield were 61%, 41%, and 55%, respectively. At enzyme

loading of (1 mg CTec3 + 1 mg HTec3)/g dry biomass, the yields were 67%, 53%, and 62%, respectively.

The MixAlco process can be used to create economic incentives for waste collection and disposal in developing countries. Humanure can be used as a nutrient source for the MixAlco fermentations. Using CPDM, carboxylic acid concentration and conversion of four-stage continuous countercurrent fermentation were predicted for municipal solid waste and humanure. At VSLR = 10 g/(L·d), LRT = 25 d, and substrate concentration = 100 g/L, CPDM predicts carboxylic acid concentration of 57 g/L and conversion of 53%. To predict product inhibition more accurately, different rate equations were compared. Based on the results obtained in this study, the equation that gives high prediction accuracy for inhibition is given by

$$\hat{r} = e(1-x)^{f}\left(\frac{1}{1+g(\phi \cdot \mathrm{Aceq})^{h}}\right) \tag{6.1}$$

The ketones produced from waste using the MixAlco process can be transported to the nearby existing refinery where it can be upgraded to hydrocarbon fuels (gasoline or diesel).

6.2. Future work

The ability to produce MCFA in mixed-culture fermentation allows modifications in the downstream processing. In the past, SCFAs have been extracted from fermentation broth using solvent extraction. Because of higher hydrophobicity of MCFA, they can be separated efficiently using solvent extraction or ion-exchange resins.

Commonly used extractants for recovery of carboxylic acids are long chain tertiary amines, quaternary amines, and phosphine oxides. Extraction can be performed at acidic or neutral pH and regeneration at basic pH. Figure 6-1 shows the simplified process.

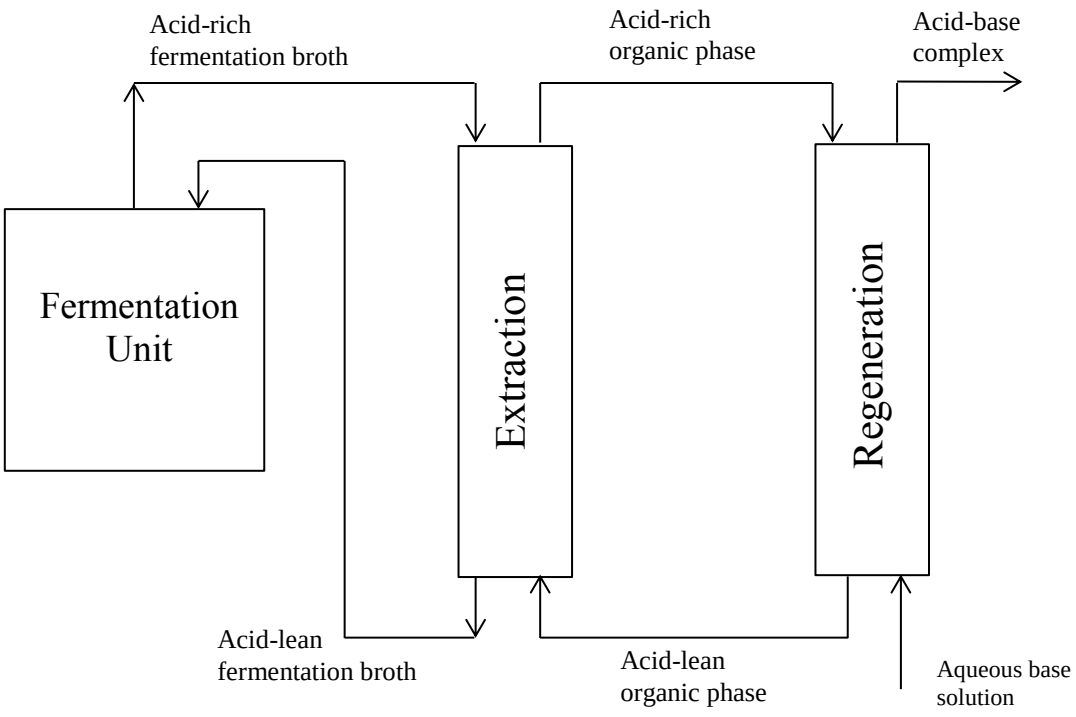


Figure 6-1. Schematic of extraction process.

Future research should investigate types of extractants and diluents for efficient separation. Optimum pH for regeneration should be investigated. Selective separation of MCFA will leave behind SCFA that are needed for chain elongation. Continuous

separation of acids will reduce the product inhibition in fermentation and improve the productivity.

Maximum concentration of caproic acid observed in this study and reported in literature is around 10–12 g/L. The concentrations of heptanoic and octanoic acids were less than 1 g/L. Low concentrations of higher acids (heptanoic and above) may be attributed to their low solubility in water or inability of microorganisms to elongate higher acids. It is speculated that because of their low solubility, higher acids may remain stuck to biomass in the fermentation. This can be investigated by washing the undigested biomass with water and analyzing its acid composition.

As compared to batch, countercurrent saccharification of lime-pretreated reduced enzyme requirements to achieve same glucan conversion. In a 16-bottle system, enzymes were added to Bottle 4. It was observed that hemicellulases have less affinity for substrate. In future, hemicellulase should be added further down in the system (Bottle 6–8 in a 16-bottle train) to improve xylose yield.

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APPENDIX A

DEOXYGENATED WATER PREPARATION

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

1. Fill a large glass container (≥4 L) with deionized water. Place the container over a hot plate to boil.
2. Heat the water until it starts boiling.
3. Seal the top of the container with aluminum foil and cool to room temperature.
4. Add 0.275 g cysteine hydrochloride and 0.275 g sodium sulfide per liter of boiled water.
5. Stir the solution until both chemicals are completely dissolved and pour into storage tank.
6. Seal the storage tank airtight

APPENDIX B

MIXED-CULTURE BATCH FERMENTATION PROCEDURE

Batch fermentations were performed in 1-L polypropylene plastic bottles (Thermo Fisher Scientific, Catalog# 05-562-25) with a rubber stopper capping inserted with a glass tube and two stainless steel pipes that aided mixing of contents of the fermentor. The fermentors were placed in a Wheaton roller apparatus set at 2 rpm. The roller apparatus is kept in an incubator chamber maintained at constant temperature. The fermentors were monitored every 48 hours.

Batch fermentation monitoring procedure

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

5. Centrifuge (4,000 rpm, 10 min) the fermentors to separate the solid and liquid fractions.
6. After centrifuging, carefully move the bottles to ensure that the solid cake at the bottom is not disturbed.
7. Collect 1-mL sample of the liquid fraction and store it in a 2-mL microcentrifuge tube.
8. Measure and record pH using pH meter. Before each monitoring procedure, pH meter should be calibrated using three point calibration (Buffer pH = 4.01, 7.00, 10.01). Mixed-culture fermentations are performed at neutral pH to achieve high yield.
9. If recorded pH is less than the target pH, add appropriate buffer (e.g., $MgCO_3$) in steps of 0.1 g to the bottles and mix well. Keep adding $MgCO_3$ until the fermentor reaches the target pH.
10. Add methanogen inhibitor, if a methane peak is found in gas sample.
11. Mix contents of all bottles thoroughly and purge each fermentor with N_2.
12. Replace fermentor caps and place fermentors in the incubator.
13. Keep the liquid samples in freezer.

APPENDIX C

CARBOXYLIC ACID AND ALCOHOL ANALYSIS USING GC

This procedure describes analysis of carboxylic acids and alcohols in fermentation samples. At least 1 mL of liquid is sampled from fermentor, placed in a 2-mL microcentrifuge tube and stored in freezer at –10 °C. When analyzed, the samples were thawed and vortexed. If the acid concentration is high, it may require further dilution before using the method below.

GC liquid sample preparation

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

GC operation

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

Carboxylic acid analysis

1. Check the following setting conditions in the method:
    A. Inlet Conditions:
        i. Splitless mode (Splitless liner should be used)
        ii. Temperature: 230 °C
        iii. Pressure: 15 psig
        iv. Flow rate: 185 mL/min
    B. Detector conditions:
        i. Temperature: 230 °C
        ii. Air flow rate: 400 mL/min

iii. H_2 flow rate: 40 mL/min

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

C. Oven conditions:

i. Initial temperature: 40 °C

ii. Initial hold time: 2 min

iii. Ramp rate: 20 °C/min

iv. Final temperature: 200 °C

v. Final hold time: 1 min

D. Total run time per vial: 11 min

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

Simultaneous analysis of carboxylic acids and alcohols

1. Check the following setting conditions in method:

A. Inlet Conditions:

i. Split mode (Split liner should be used)

ii. Temperature: 230 °C

iii. Pressure: 15 psig

iv. Flow rate: 56.2 mL/min

v. Split flow: 50 mL/min

B. Detector conditions:

   i. Temperature: 230 °C

   ii. Air flow rate: 400 mL/min

   iii. H_2 flow rate: 40 mL/min

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

C. Oven conditions:

   i. Initial temperature: 70 °C

   ii. Initial hold time: 4 min

   iii. Ramp rate: 20 °C/min

   iv. Final temperature: 200 °C

   v. Final hold time: 1 min

D. Total run time per vial: 11.5 min

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

APPENDIX D

MOISTURE AND ASH CONTENT ANALYSIS

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

1. Record the label and weight of a clean, dry crucible ($W1$).
2. Place a representative sample of the material (liquid or solid) into the crucible and record the weight ($W2$).
3. Dry the crucible at 105 °C for 24 hours in the drying oven. In a desiccator, allow to cool to room temperature before weighing. Record the dry weight ($W3$).
4. Ash the crucible at 575 °C for at least 12 h. Remove and allow sample to cool to room temperature in a desiccator. Record the ash weight ($W4$).
5. The moisture content (MC) of the sample is calculated as

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

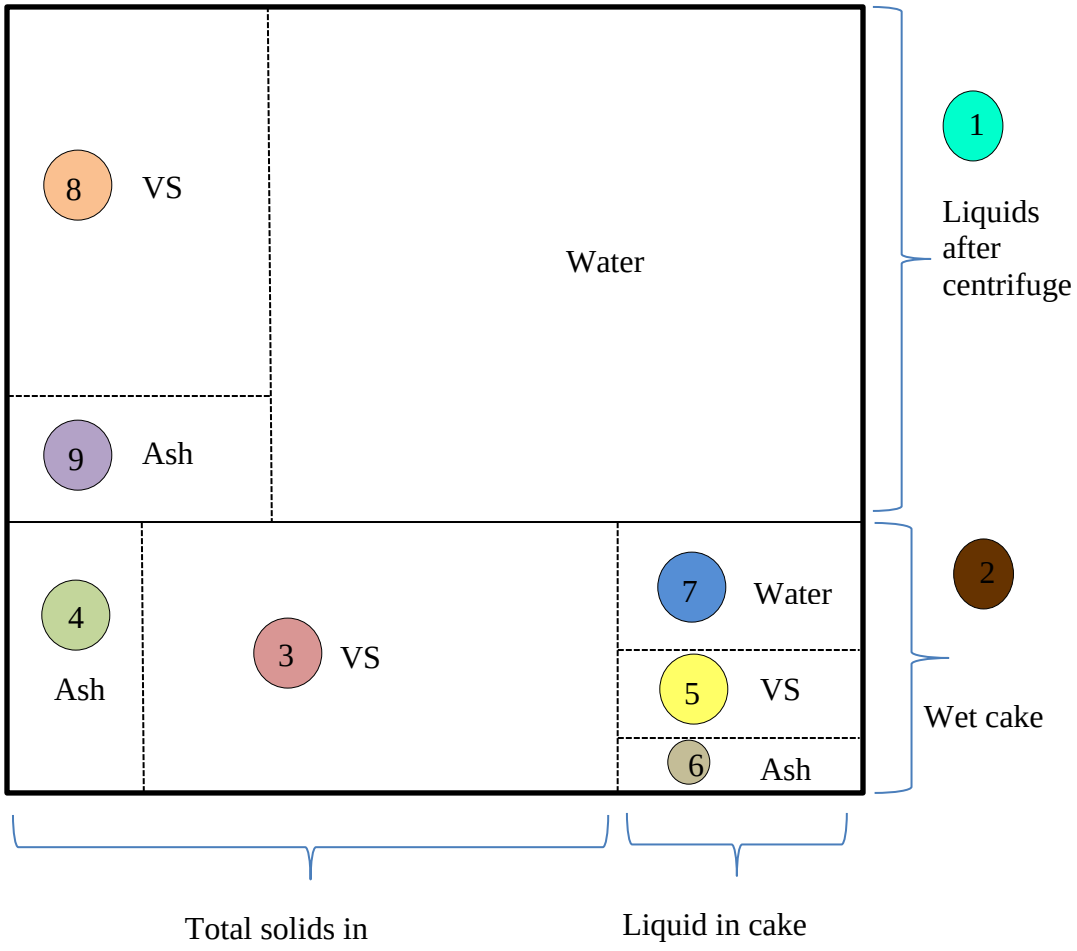
6. The ash content (AC) of the sample is calculated as

$$\text{AC} = \frac{W4 - W1}{W3 - W1}$$

APPENDIX E

FERMENTATION PARAMETER CALCULATIONS

The performance of fermentation is measured in terms of conversion, selectivity, and yield. After the fermentations are terminated, it has mainly two components: undigested solid and product liquid. The undigested solids are partly ash and partly volatile solids. Following formulae are used to measure the fermentation parameters.



Calculated fractions:

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

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

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

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

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

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

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

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

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

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

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

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

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

14) Total volatile solids = $\text{VS}_{\text{wet cake}} + \text{VS}_{\text{liquid}}$

15) Total ash = $\text{Ash}_{\text{wet cake}} + \text{Ash}_{\text{liquid}}$

14) $\text{Conversion}\ (x) = \dfrac{\text{Volatile solids digested (g)}}{\text{Volatile solids fed (g)}}$

15) $$\text{Selectivity} = \frac{\text{Total acids produced (g)}}{\text{Volatile solids digested (g)}}$$

16) $$\text{Yield} = \frac{\text{Total acids produced (g)}}{\text{Volatile solids fed (g)}}$$

APPENDIX F

SUBMERGED LIME PRETREATMENT

Submerged lime pretreatment (SLP) is used to remove lignin from lignocellulosic biomass. This appendix describes detailed procedure of SLP. Approximately 2.5 kg dry weight of biomass was mixed with weighed calcium hydroxide (0.1 g $Ca(OH)_2$/g dry biomass) and placed in a cylindrical jacketed steel vessel (volume = 65.3 L). The solids concentration was 0.05 kg dry biomass/kg water. Deionized water was used for pretreatment. A 6-inch headspace was left to avoid spills. The biomass treatment system was maintained at ~50 °C by circulation hot water through the jacket surrounding steel vessel (Figure F1). Heat exchanger was used to maintain hot water temperature. Air was scrubbed through a lime slurry container and then bubbled through the pile via an air scrubber in the bottom of the vessel.

1. Mix the raw biomass (e.g., 2.5 kg) with excess lime (0.1g $Ca(OH)_2$/g dry biomass). Mix well to ensure a complete contact between the lime and the biomass.
2. Fill the pretreatment steel vessel with the lime/biomass mixture. Add deionized water to the vessel until it reaches a concentration ~ 0.05 kg dry biomass/kg water.
3. Fill the heat exchanger with water and start the circulation pump.
4. Set the temperature controller to 49 °C.

5. Adjust the air valve connected to the diffusers until the air gently bubbles up through the mixture.
6. Add more water to the heat exchanger every day so it does not evaporate dry.
7. Add more water to the vessel and keep the 6 inch headspace.
8. Check the system daily for leaks and monitor the circulation pump to ensure it retains prime.
9. Monitor the pH of the lime slurry to ensure basic conditions are maintained (e.g., desired pH > 9).
10. Maintain conditions for 24–28 days. At the end of the time period, turn off the temperature controller, the circulation pump and the air valve.
11. Add 5-N HCl (~1.2 L) to adjust the final pH to ~ 4–5.
12. Remove the biomass slurry from the vessel and allow it to cool down to room temperature.
13. Centrifuge the biomass slurry and dispose the liquid.
14. To wash the biomass, add deionized water and make uniform slurry by mixing manually.
15. Centrifuge the biomass again and dispose the liquid.
16. Repeat Steps 14 and 15 three times to ensure lime is washed off from biomass.
17. Spread the centrifuged biomass on aluminum tray and air-dry at room temperature (5–7 days). Scrape and turn the biomass upside down and spread again every day to ensure uniform drying. Store the dried biomass in a labeled Ziploc bags.

18. Clean the interior of the steel vessel and flush with deionized water.

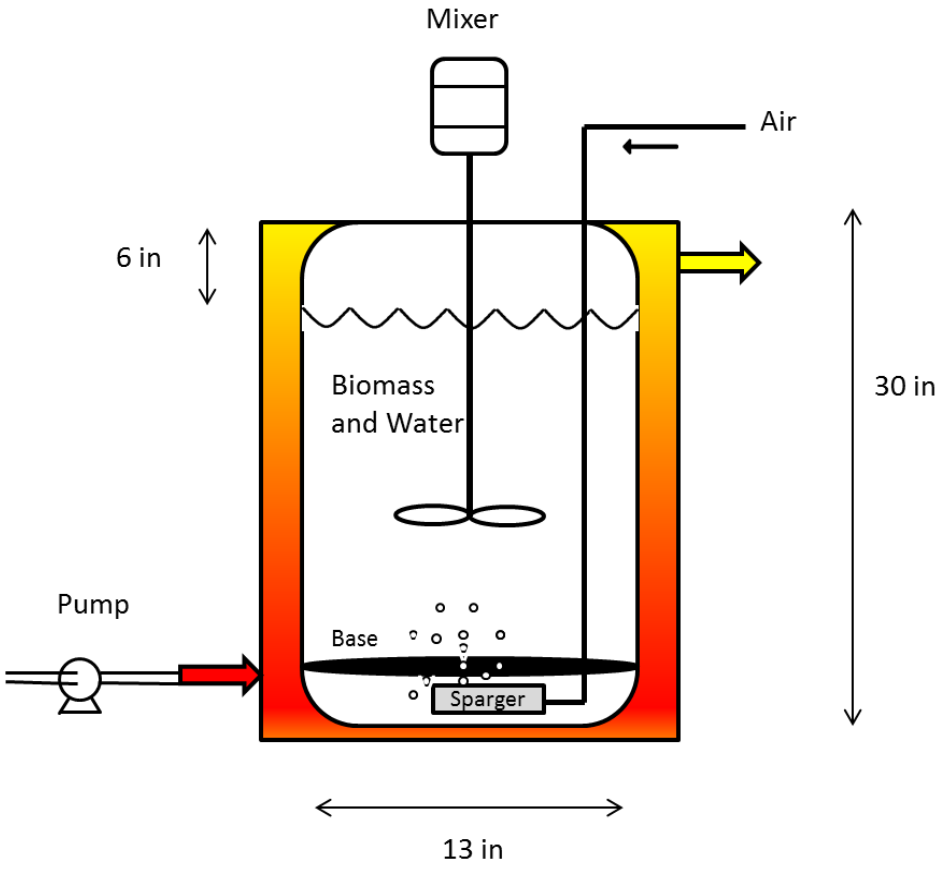


Figure F1. Schematic process flow diagram of pretreatment apparatus.

APPENDIX G

ENZYME DILUTION

Materials:

Novozymes CTec2 solution

Novozymes CTec3 solution

Novozymes HTec3 solution

DI water

Apparatus:

50-mL volumetric flask

Kimwipes

1000–5000 μL auto-pipette

Pipette tips

50-mL centrifuge tubes

Procedure:

1. Fill 50-mL volumetric flask with approximately 20–25 mL of DI water.
2. Take enzyme (CTec2, CTec3, or HTec3) out of refrigerator and shake well.
3. Take 5 mL enzyme solution with auto pipette.
4. Clean the enzyme residue that sticks on the outside of the pipette tip with Kimwipes.
5. Empty pipette into 50-mL volumetric flask. Keep the tip in the flask and remove it from auto pipette.

6. Rinse the inside of tip several times with DI water.
7. Add DI water to the flask to 50 mL mark and shake well.
8. Pour the diluted enzyme into 50-mL centrifuge tubes and store in 4°C refrigerator.

APPENDIX H

CITRATE BUFFER PREPARATION

Citrate buffer is used for enzymatic saccharification experiments to maintain optimum pH. Enzyme activity depends on pH. Cellulase and hemicellulase enzymes perform effectively at a pH around 4.8–5.0.

Materials:

Citric acid monohydrate

Citric acid, trisodium salt dihydrate

DI water

Apparatus:

1-L volumetric flask

pH meter

Analytic balance with 0.0001-g precision

Weighing boat

Weighing spatula

Procedure:

1. Fill a 1-L glass volumetric flask with approximately 800 mL of DI water.
2. Weigh 8.4000 ± 0.0005 g of citric acid monohydrate and 17.6500 ± 0.0005 g trisodium citrate dihydrate and add to 1-L volumetric flask.
3. Shake vigorously to dissolve all the solids.
4. Fill water to the 1-L mark and shake well.

5. Measure pH of the citrate buffer; it should be 4.8 ± 0.02.
6. Store the solution in 4°C refrigerator.

APPENDIX I

ANTIBIOTIC PREPARATION

Antibiotic solutions are used to prevent growth of microorganisms in saccharification reactors.

Reagents and equipment needed

Analytic balance w/ 0.1 mg precision

Weighing papers

Weighing spatula

Gloves (two pairs per antibiotic)

Dust mask or respirator

100-mL volumetric flask (1 per antibiotic)

Deionized distilled water (DI H_2O)

Ethanol (200 proof)

Tetracycline hydrochloride

Cycloheximide

Preparation of tetracycline solution (10 mg/ml)

1. Put on protective dust mask and two pairs of gloves.
2. Working in a ventilated area or hood, weigh 1.000 ± 0.005 g of tetracycline hydrochloride powder on weighing paper.
3. Carefully funnel into a 100-mL volumetric flask.
4. Immediately store tetracycline powder and discard outer layer of gloves.

5. Add 70 mL of 200-proof ethanol to flask and gently mix to dissolve powder.
6. Fill to mark with DI H_2O and mix well.
7. Store solution in sealed containers in freezer at –10°C.

Preparation of cycloheximide solution (10 mg/ml):

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

WARNING:

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

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

APPENDIX J

BATCH ENZYMATIC HYDROLYSIS PROCEDURE

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

Materials:

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

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

Citrate buffer (Appendix H)

Tetracycline solution, cycloheximide solution (Appendix I)

DI water

Apparatus:

Incubator capable of agitation at ~2 rpm

50-mL centrifuge tubes

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

Moisture content analyzer (Denver Instruments IR 120)

Analytic balance with 0.0001-g precision

100-mL beakers or flasks

2-mL microcentrifuge tubes

Vortex Mixer

Procedure:

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

2. Calculate the target air-dry substrate mass for 1 g dry biomass.
3. Measure protein concentration of CTec2, CTec3, and HTec3.

Procedures of test samples preparation:

1. Calculate required enzyme volume.
2. Calculate required DI water volume to make sure total reaction volume is 10 mL (assume substrate density ≈1 g/cm^3).
3. Weigh the target air-dry biomass of each sample and add to labeled tubes.
4. Add 5 mL citrate buffer, required water volume, 0.08 mL tetracycline solution, 0.06 mL cycloheximide solution in sequence to each tube and mix well with mixer.
5. Add required amount of enzyme to each tube, record the time and mix well.
6. Put the tubes in the incubator at 50°C and axial rotation speed 2 rpm for exact 5 days.

Procedures of substrate blank samples preparation:

1. Calculated required DI water volume to make sure total reaction volume 10 mL (assume substrate density ≈1 g/cm^3).
2. Weigh the target air-dry biomass of each sample and add to labeled tubes.
3. Add 5 mL citrate buffer, required water volume, 0.08 mL tetracycline solution, 0.06 mL cycloheximide solution in sequence to each tube and mix well with mixer.
4. Put the tubes in the incubator together with test samples at 50°C and axial rotation speed 2 rpm for exact 5 days.

Procedures of enzyme blank samples preparation:

1. Calculate required enzyme volume.
2. Calculated required DI water volume to make sure total reaction volume 10 mL.
3. Add 5 mL citrate buffer, required water volume, 0.08 mL tetracycline solution, 0.06 mL cycloheximide solution in sequence to each tube and mix well with mixer.
4. Put the tubes in the incubator together with test samples at 50°C and axial rotation speed 2 rpm for exact 5 days.

Termination procedures:

1. After exactly five days, remove the tubes from the incubator and place them in boiling water for 20 min to deactivate the enzymes.
2. When the samples cool to room temperature, pour nearly 1.5 mL of liquid into 2-mL microcentrifuge tubes and store in freezer.

Note: Every test sample should accompany with its corresponding substrate blank and enzyme blank samples. Test samples are repeated in triplicate. Substrate and enzyme blank samples are repeated in duplicate.

APPENDIX K

COUNTERCURRENT SACCHARIFICATION TRANSFER PROCEDURE

This procedure is adapted from "Batch enzymatic hydrolysis of pretreated corn stover and improvements with countercurrent saccharification" (Liang et al., 2014). The procedure describes monitoring of a countercurrent saccharification train.

Materials:

Tetracycline solution (Appendix I)

Cycloheximide solution (Appendix I)

Diluted Novozymes CTec3 (Appendix G)

Diluted Novozymes HTec3 (Appendix G)

Citrate buffer (Appendix H)

DI water

Lime-pretreated corn stover

Apparatus:

Weighing boats

50-mL centrifuge tubes

2-mL microcentrifuge tubes

250-mL graduated cylinder

50-mL graduated cylinder

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

Auto pipette (100–1000 µL)

Pipette tips

Weighing spatula

Centrifuge

pH meter

Preparation:

1. Calibrate the pH meter with 1.68, 4.01 and 7.00 buffer solutions.
2. Measure 10 g dry lime-pretreated corn stover in a weigh boat.
3. Remove all 16 bottles out of the incubator.
4. Weigh all bottles and record the weight of bottles.
5. Balance pair of Bottles before centrifuge.

Transfer procedure:

Bottle 1:

1. Centrifuge Bottles 1, 2, 3, 4 at 3000 rpm for 5 min.
2. Remove the Bottles 1 and 2 from centrifuge.
3. Pour the liquid of Bottle 1 into a 250-mL cylinder and record liquid mass and volume.
4. Measure pH of the liquid and take 1 mL sample with pipette and place in a 2-mL microcentrifuge tube.
5. Store approximately 45 mL of liquid in a 50 mL centrifuge tube.
6. Weigh bottle (without cap) + wet cake, and calculated the weight of wet cake.
7. Calculate the move target: wet cake + pre-weighed dry biomass – target weight (90 g).

8. Remove calculated move target from the bottle and add pre-weighed dry biomass to Bottle 1.
9. Weigh the bottle (without cap) and calculate the wet cake again to ensure its weight is close to 90 g.

Bottle 2 – 15

1. Pour liquid from bottle to 250-mL cylinder slowly, record the liquid mass and volume.
2. Measure pH of liquid fraction, and take 1-mL sample with pipette and place in 2-mL microcentrifuge tubes.
3. Transfer the liquid to previous bottle.
4. Measure the bottle without cap and calculate wet cake weight.
5. Calculate move target: wet cake weight + moved weight from previous bottle – target weight (90 g).
6. Remove move target from the bottle and add wet cake removed from previous bottle.
7. The amount of solid transferred between bottles is calculated as follows:

   Wet cake transferred from Bottle *N* to Bottle *N*+1 (g) =

   [Wet cake weight in Bottle *N* (g)] +

   [Wet cake transferred from Bottle *N*–1 to Bottle *N* (g)] –

   [target wet cake in Bottle *N* (g)]

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

Note: Before transferring solids to Bottle 5, centrifuge Bottles 5, 6, 7, 8 at 3000 rpm for 5 min; before transferring solids to Bottle 9, centrifuge Bottles 9, 10, 11, 12 at 3000 rpm for 5 min; before transferring solids to Bottle 13, centrifuge Bottles 13, 14, 15, 16 at 3000 rpm for 5 min.

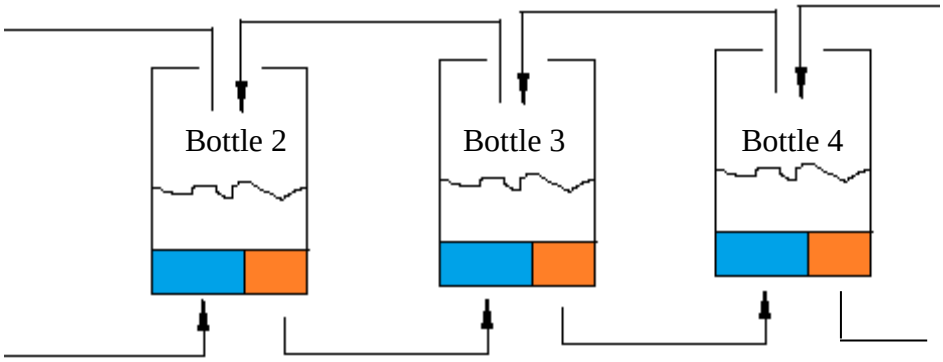


Figure K1. Schematic of countercurrent saccharification. (Use Bottles 2, 3, and 4 as examples)

Bottle 16

1. Pour liquid from Bottle 16 to 250-mL cylinder slowly, record the liquid mass and volume.
2. Measure pH of liquid fraction, and take 1-mL sample with pipette into 2-mL microcentrifuge tubes.
3. Pour liquid to previous bottle.

4. Measure the bottle without cap and calculate wet cake weight.
5. Calculate move target: wet cake weight + move weight from previous bottle - target weight (90 g).
6. Remove calculated move target from the bottle and take nearly 0.5 g moved wet cake to test moisture content.
7. Store the rest moved wet cake in 4°C refrigerator.
8. Add 50 mL of citrate buffer and 40 mL of DI water to Bottle 16.

Post-transfer procedure:

1. Add 0.4 mL of tetracycline solution and 0.3 mL of cycloheximide solution to every bottle.
2. Add calculated amount of enzyme dose (CTec3 or CTec3 + HTec3) to Bottle 4.
3. Record final weight of each bottle with cap.
4. Close every bottle very tightly and shake to homogenize slurry.
5. Put all 16 bottles back into the rolling incubator, set at 50°C and 2 rpm axial rotation.

APPENDIX L

SUGAR ANALYSIS USING HPLC

Samples of enzymatic hydrolysis are tested for sugars using HPLC. This procedure describes the HPLC sample preparation and testing for sugars.

1. If samples are frozen, allow to thaw completely.
2. Vortex for 10–15 seconds to avoid any concentration gradient.
3. Place 1.5–2 mL of liquid into labeled 2-mL Eppendorf tube.
4. Centrifuge Eppendorf tubes in a microcentrifuge at 13,000 rpm for 10 min.
5. Using a 1 mL disposable syringe, extract free liquid from Eppendorf tubes without disturbing the centrifuged solids at the bottom.
6. Attach a 0.2-µm cellulose acetate filter (VWR, Catalog # 28145-477) unit and filter liquid sample into labeled autosampler 12×32 mm snap-it vial (Thermo Scientific, C4011-5).
7. Secure vial caps (Thermo Scientific, Catalog # C4011-51) to vials.
8. Prepare sugar standards (1, 3, 5, 10, 25, 50, 75 g/L glucose concentration, with a 2:1 ratio of glucose:xylose) and use a 50 g/L glucose concentration sample as a control verification standard (CVS).
9. Analyze samples using an HPLC equipped with refractive index detector, auto-sampler, a pair of de-ashing guard columns (Bio-Rad Micro-Gurad de-ashing cartridges, 30 mm × 4.6 mm), and a HPLC carbohydrate analysis column (Bio-

Rad Aminex HPX-87P, 300 mm × 7.8 mm), using HPLC water as a carrier phase.

10. Maintain analytic column temperature at 85°C, with a HPLC water flow rate of 0.6 mL/min. The assay time is 21 min per sample.
11. After the sequence run is over, turn off the column heater and change the method to slowflow mode.

APPENDIX M

CPDM MATLAB PROGRAM

CPDM Matlab code to obtain conversion and acid concentration:

```
%MATLAB Code for CPDM Prediction
%This code is for a standard four-stage countercurrent fermentation
%Program predicts acid concentrations and conversion at varying VSLR and LRT.
%Department of Chemical Engineering, Texas A&M University, College St, TX
%CODE BY Sagar 02/19/2014
clear all
close all
global so taus e1 f1 g1 h1
global holdup moist ratio stages loading tauloverall
global acid nnot factr1
global x_1 nhat_1 x_2 nhat_2 x_3 nhat_3 x_4 nhat_4

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

VSLR_data=[4,6,8,10,12]';
LRT_data=[10,15,20,25,30]';
ACID = [];
CONVERSION = [];
VSLR_loop=10; %loop is for varying VSLR.
%To make map, set to lowest VSLR, otherwise, set to specific VSLR
while VSLR_loop<10.1 % if want loop, set to highest VSLR
   LRT_loop=25;   %loop is for varying LRT.
   %To make map, set to lowest LRT, otherwise set to specific LRT
   while LRT_loop<25.1 %if want loop, set to highest VSLR

      %%Basic parameters for Fermentation
      stages=4; %Fermentor stages
      so=0.4; %Aeq selectivity (gAEQ/g VS digested)
      %Please note that in older versions of the code (i.e. Loescher's)
      %this term referred to a VS selectivity of g VS/g total solids and
      %was carried over in the differential equations in Ross and Fu.
      holdup =2.0; %ratio of liq to solid in wet cake (g liq/gVS cake)
      %Note: holdup is the liq in the solid cake NOT the lig of the
      %total slurry
      moist =.07; %ratio of liquid to solid in feed (g liq/gVS cake)
```

```
SQ =1.0;
ratio=0.693; %phi ratio of g total acid to g AEQ
loading = VSLR_loop;
tauloverall = LRT_loop;
vol=[.48,.28,.28,.28]'; %Liquid volime in each fermentor
totvol=sum(vol);
liquidfeed = totvol/tauloverall;
nnotreal = [100,100,100,100]'; %VS concentration gVS/L (?in each fermentor?)
solidfeed = loading*totvol; %Solid Feed (g dry weight)
Convrsn = [.1,.2,.3,.4]'; %Initial value for conversion
nnot = nnotreal./(1-Convrsn);
taus = nnot.*vol/solidfeed;
L =0.1*ones(stages+1,1); %L initial value for liquid flow rate in every reactor
taul = tauloverall/stages*ones(stages,1);

e1=0.103; f1=2.404; g1=3.76e-4; h1=1.725; %CPDM parameters
rmodel = @(x1,acid) e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1);
syms x1 acid
drmodel_1 = diff(e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1),x1);
drmodel = @(x2,acid2) subs(drmodel_1,{x1,acid},{x2,acid2});

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

%Initial values for acid, acidold
%ans=ones(stages,1); % dont use ans it is a matlab variable.
acid=[35,30,28,25]';
acidold=ones(stages,1);
taulnew = 1000*ones(stages,1);  %column vector
nhatzero =100*ones(stages,1);  %CP concentration
creation = ones(stages,1);
destruction = ones(stages,1);
tauloverallnew = 20;

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

while done < 0.50
   taulnew = 1000*ones(stages,1);  %Obtain Flowrate for each fermentor
   taulover_error = 0.001;
   while abs(tauloverall-tauloverallnew) > taulover_error
      liquidfeed = liquidfeed*(1+(tauloverallnew-tauloverall)/tauloverall*0.5);
      L(5) = liquidfeed;
```

```
            L(4) = L(5) + solidfeed/1000*holdup*(Convrsn(4)-Convrsn(3));
            L(3) = L(4) + solidfeed/1000*holdup*(Convrsn(3)-Convrsn(2));
            L(2) = L(3) + solidfeed/1000*holdup*(Convrsn(2)-Convrsn(1));
            L(1) = moist*solidfeed/1000 + L(2) - solidfeed/1000*holdup*(1.0-
Convrsn(1));
            tauloverallnew = totvol/L(1);
         end

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

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

         %parameters for ODE45
         options = odeset('RelTol',1e-3,'AbsTol', 1e-3);
         x_low=0; x_high=0.99;

         %Reactor 1

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

         %Reactor 2

         i=2;
```

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

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

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

        %Reactor 3

        i=3;
        nnottoler = nnot(i)/500;
        while abs(taulnew(i)-taul(i))>liqtoler;
           ndone = 0;
           while ndone <0.50
```

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

         F_32 = @(x_3)interp1(x,nhat,x_3).*x_3;
         Convrsn(i)= quad(F_32,x_low,x_high)/nnot(i);
         robs = solidfeed*so/vol(i)*(Convrsn(i)-Convrsn(i-1));
         taulnew(i) = (L(i)*acid(i) + solidfeed/1000*(1-Convrsn(i))*holdup*acid(i)-
L(i+1)*acid(i+1))/(L(i)*robs);
         acid(i) = acid(i) + (taul(i)*robs-(L(i)*acid(i)+solidfeed/1000*(1-
Convrsn(i))*holdup*acid(i)-L(i+1)*acid(i+1))/L(i))*.5;
         disp([' taulnew(',num2str(i),')=',num2str(taulnew(i), '%15.5f'),'
taul(',num2str(i),')=',num2str(taul(i),'%15.5f'),]);
      end
      disp([' acid(',num2str(i),')=',num2str(acid(i),'%15.5f'),'
taulnew(',num2str(i),')=',num2str(taulnew(i),'%15.5f'),' robs=', num2str( robs,
'%15.5f')]);


      %Reactor 4

      i=4;
      nnottoler = nnot(i)/500;
      while abs(taulnew(i)-taul(i))>liqtoler;
         ndone = 0;
         while ndone <0.50
            nhat0 =nhatzero(i);
            options = odeset('RelTol',1e-3,'AbsTol',1e-3);
            [x,nhat] = ode15s(@Chan4,[x_low,x_high],nhat0,options); %was chan4
            x_4=x;  nhat_4=nhat;
```

```
            F_4 = @(x_4)interp1(x,nhat,x_4);
            nhattot=quad(F_4,x_low,x_high);
            disp([' nhatzero= ',num2str(nhatzero(i), '%15.5f'),';  nhattot=
',num2str(nhattot, '%15.5f'),';  nnot(',num2str(i),')= ',num2str(nnot(i), '%15.5f')]);
            if abs(nhattot - nnot(i))<nnottoler;
               ndone = 1;
            end
            if (nhatzero(i) + (nnot(i) - nhattot)*1.0)>0
               nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.7;
            else
               nhatzero(i)= nhatzero(i) + (nnot(i) - nhattot)*0.2;
            end
         end

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

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

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


   %Output results section

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

   for i3 = 1:(stages+1);
```

```
        disp(['  L(',int2str(i3),')= ',num2str(L(i3))]);
      end

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

      %Calculation of Destruction

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

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

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

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

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

Codes for function files used in CPDM code:

Chan 1

```
function dnhat = Chan1(x,nhat1)
global taus  e1 f1 g1 h1 i
global ratio acid

rmodel = @(x1,acid)e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1);
drmodel = @(x1,acid) -(e1.*f1.*(1 - x1).^(f1 - 1))./(g1.*(acid.*ratio).^h1 + 1);
i=1;
dnhatdt = -nhat1*(drmodel(x,acid(i))+1/taus(i))/rmodel(x,acid(i));
dnhat = [dnhatdt];
```

Chan 2

```
function dnhat = Chan2(x,nhat1)
global  taus e1 f1 g1 h1 i
global ratio acid nnot factr1
global x_1 nhat_1

rmodel = @(x1,acid) e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1);
drmodel = @(x1,acid) -(e1.*f1.*(1 - x1).^(f1 - 1))./(g1.*(acid.*ratio).^h1 + 1);
F_1m = @(x_m)interp1(x_1,nhat_1,x_m);

i=2;
dnhatdt = -nhat1*(drmodel(x,acid(i))+1/taus(i))/rmodel(x,acid(i)) +
F_1m(x).*nnot(i)./nnot(i-1)*factr1*1/taus(i)/rmodel(x,acid(i));

dnhat = [dnhatdt];
```

Chan 3

```
function dnhat = Chan3(x,nhat1)
global  taus e1 f1 g1 h1 i
global ratio acid nnot
global x_2 nhat_2

rmodel = @(x1,acid) e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1);
drmodel = @(x1,acid) -(e1.*f1.*(1 - x1).^(f1 - 1))./(g1.*(acid.*ratio).^h1 + 1);
F_2m = @(x_m)interp1(x_2,nhat_2,x_m);
```

```
i=3;
dnhatdt = -nhat1*(drmodel(x,acid(i))+1/taus(i))/rmodel(x,acid(i)) +
F_2m(x).*nnot(i)./nnot(i-1)*1/taus(i)/rmodel(x,acid(i));

dnhat = [dnhatdt];
```

Chan 4

```
function dnhat = Chan4(x,nhat1)
global  taus e1 f1 g1 h1 i
global ratio acid nnot
global x_3 nhat_3

rmodel = @(x1,acid) e1.*(1-x1).^f1./(1+g1.*(acid.*ratio).^h1);
drmodel = @(x1,acid) -(e1.*f1.*(1 - x1).^(f1 - 1))./(g1.*(acid.*ratio).^h1 + 1);
F_3m = @(x_m)interp1(x_3,nhat_3,x_m);

i=4;
dnhatdt = -nhat1*(drmodel(x,acid(i))+1/taus(i))/rmodel(x,acid(i)) +
F_3m(x).*nnot(i)./nnot(i-1)*1/taus(i)/rmodel(x,acid(i));

dnhat = [dnhatdt];
```

Code for CPDM prediction map:

```
VSLR=[14;14;14;14;14;14;12;12;12;12;12;12;10;10;10;10;10;10;8;8;8;8;8;8;6;6;6;6;6;6
;4;4;4;4;4;4];
LRT=[5;10;15;20;25;30;5;10;15;20;25;30;5;10;15;20;25;30;5;10;15;20;25;30;5;10;15;2
0;25;30;5;10;15;20;25;30];
CONVERSION=[0.454;0.445;0.439;0.434;0.429;0.425;0.499;0.49;0.483;0.477;0.47;0.4
67;0.552;0.544;0.537;0.53;0.524;0.519;0.62;0.613;0.606;0.599;0.591;0.585;0.702;0.698;
0.692;0.685;0.678;0.674;0.808;0.804;0.803;0.798;0.792;0.785];
ACID=[22.74;38.61;50.19;58.73;65.34;70.62;22.06;38.37;50.70;60.05;67.48;73.44;21.0
5;39.00;50.48;60.76;68.97;75.83;19.55;35.91;49.28;60.34;70.29;77.11;17.3;32.48;46.00;
57.48;67.51;75.55;13.92;26.85;39.03;50.20;60.12;68.88];
mapdata=[VSLR,LRT,CONVERSION,ACID];
VSLR_sorted=sortrows(mapdata,1);
LRT_sorted=sortrows(mapdata,2); %sort
[map_num,map_1]=size(mapdata);
VSLR_sort = sort(mapdata(:,1));
```

```
uniqueM = [diff(VSLR_sort);1] > 0;
VSLR_sort1 = VSLR_sort(uniqueM);
VSLR_number = diff(find([1;uniqueM]));
LRT_sort = sort(mapdata(:,2));
uniqueM = [diff(LRT_sort);1] > 0;
LRT_sort1 = LRT_sort(uniqueM);  %Unique LRT
LRT_number = diff(find([1;uniqueM]));
temp1=zeros(length(VSLR_sort1)+1,1);
for j1=1:length(VSLR_sort1)
temp1(j1+1)=temp1(j1)+VSLR_number(j1);
mapdata_1=VSLR_sorted(temp1(j1)+1:temp1(j1+1),:) ;
%for VSLR(j1)
F = @(x)interp1(mapdata_1(:,3),mapdata_1(:,4),x,'spline');
hold on;
plot(mapdata_1(:,3),F(mapdata_1(:,3)),'k');
if j1==1
for j3=1:length(mapdata_1(:,3))
text(mapdata_1(j3,3)-0.01,mapdata_1(j3,4)-0.2, ['  ', num2str(mapdata_1(j3,2))]
,'HorizontalAlignment','left');
end
text(mapdata_1(1,3)-0.345,mapdata_1(1,4)-0.6, ' VSLR (g/(L-day)) '
,'HorizontalAlignment','left');
end
end
%plot for LRT part
temp1=zeros(length(LRT_sort1)+1,1);
for j1=1:length(LRT_sort1)
temp1(j1+1)=temp1(j1)+LRT_number(j1);
mapdata_2=LRT_sorted(temp1(j1)+1:temp1(j1+1),:) ;
%for LRT(j1)
F2 = @(x)interp1(mapdata_2(:,3),mapdata_2(:,4),x,'spline');
hold on;
plot(mapdata_2(:,3),F2(mapdata_2(:,3)),'k');
if j1==1
for j3=1:length(mapdata_2(:,3))
text(mapdata_2(j3,3)+0.01,mapdata_2(j3,4)-2.25, ['  ',num2str(mapdata_2(j3,1))] ,
'HorizontalAlignment','right');
end
text(mapdata_2(1,3)+0.41,mapdata_2(1,4)+15, 'LRT (day) '
,'HorizontalAlignment','left');
end
end
hold off;
xlabel('Conversion');
```

```
ylabel('Total carboxylic acid concentration (g/L)');
axis([0 1 0 80]);
```