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# Fermentation of Sugarcane Bagasse and Chicken Manure to Calcium Carboxylates under Thermophilic Conditions

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**Abstract** Sugarcane bagasse and chicken manure were anaerobically fermented to carboxylic acids using a mixed culture of marine microorganisms at 55 °C. Using the MixAlco process— an example of consolidated bioprocessing— the resulting carboxylate salts can be converted to mixed alcohol fuels or gasoline. To enhance digestibility, sugarcane bagasse was lime pretreated with 0.1 g $Ca(OH)_2$/g dry biomass at 100 °C for 2 h. Four-stage countercurrent fermentation of 80% sugarcane bagasse/20% chicken manure was performed at various volatile solids (VS) loading rates and liquid residence times. Calcium carbonate was used as a buffer during fermentation. The highest acid productivity of 0.79 g/(L day) occurred at a total acid concentration of 21.5 g/L. The highest conversion (0.59 g VS digested/g VS fed) and yield (0.18 g total acids/g VS fed) occurred at a total acid concentration of 15.5 g/L. The continuum particle distribution model (CPDM) predicted the experimental total acid concentrations and conversions at an average error of 10.14% and 12.68%, respectively. CPDM optimizations show that high conversion (>80%) and total acid concentration of 21.3 g/L are possible with 300 g substrate/(L liquid), 30 days liquid residence time, and 3 g/(L day) solid loading rate. Thermophilic fermentation has a higher acetate content (~63 wt%) than mesophilic fermentation (~39 wt%).

**Keywords** Sugarcane bagasse · Consolidated bioprocessing · CBP · Carboxylic acids · Bioconversion · MixAlco · CPDM · Thermophilic fermentation · Acetic acid

**Nomenclature**

| | |
|---|---|
| Aceq | acetic acid equivalent concentration (grams of acetic acid equivalents/liter) |
| *a* | parameter constant (grams of acetic acid equivalents/liter) |
| *b* | parameter constant (grams of acetic acid equivalents/(liter day)) |

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| | |
|---|---|
| $c$ | parameter constant ($day^{-1}$) |
| $e$ | parameter constant (grams of acetic acid equivalent/grams of VS day) |
| $f$ | parameter constant (dimensionless) |
| $g$ | parameter constant (liter/grams of total acid)$^{1/h}$ |
| $h$ | parameter constant (dimensionless) |
| LRT | liquid residence time (day) |
| $p$ | total acid productivity (grams of total acid/(liter day)) |
| $r$ | reaction rate (grams of acetic acid equivalents/(liter day)) |
| $\hat{r}$ | specific rate (grams of acetic acid equivalents produced/(grams of VS day)) |
| $\hat{r}_{pred}$ | predicted specific rate (grams of acetic acid equivalents produced/(grams of VS day)) |
| $S_o$ | initial substrate concentration (grams of VS/liter) |
| $s$ | selectivity (grams of total acid produced/grams of VS digested) |
| $t$ | time (day) |
| VSLR | volatile solids loading rate (grams of VS/(liter day)) |
| $x$ | conversion (grams of VS digested/grams of VS fed) |
| $\alpha$ | acetic acid equivalent concentration (mole of acetic acid equivalents/liter) |
| $\phi$ | the ratio of total grams of carboxylic acid to total grams of acetic acid equivalents (grams of total acid/grams of acetic acid equivalents) |
| $\sigma$ | selectivity (grams of acetic acid equivalents produced/grams of VS digested) |

## Introduction

The inevitable depletion of the world's petroleum supply and growing energy demands have increased interest in biorefineries that make a variety of fuels and chemicals from biomass. Current commercial biomass-to-ethanol technologies use corn grain (starch) and sugarcane (sucrose), which are expensive and compete with food. In contrast, lignocellulosic biomass is renewable, sustainable, and inexpensive. A recent DOE/USDA report concluded that the USA has the potential to produce over 1.3 billion dry tons of biomass per year in addition to current agricultural and forestry production [1]. Lignocellulosic biomass includes agricultural residues (e.g., sugarcane bagasse and corn stover), industrial waste (e.g., sawdust and paper pulp), and energy crops (e.g., sorghum and energy cane).

Sugarcane bagasse, the fibrous by-product from sugarcane extraction, is plentiful in tropical and subtropical regions (e.g., Brazil, Hawaii, and the southern USA). Sugarcane bagasse is rich in carbohydrates (hemicellulose, cellulose, and lignin) but low in nutrients. In contrast, animal manure is deficient in carbohydrates, but contains large amounts of nutrients (protein, vitamins, and minerals). Utilizing animal manure not only provides an inexpensive nutrient source for anaerobic fermentations, but also has significant environmental benefits. The preferred ratio for anaerobic fermentation is 80% biomass/20% animal manure [2, 3].

Biological processes that convert lignocellulosic biomass to fuels and chemicals typically require three major sequential steps: *pretreatment* improves digestibility by removing lignin and/or hemicellulose and modifying cellulose microfiber structure, *saccharification* hydrolyzes cellulose and hemicellulose into monosaccharides (e.g., glucose) using enzymes (e.g., cellulase), and *fermentation* microbially converts fermentable sugars to fuels or chemicals [4, 5]. Consolidated bioprocessing (CBP) combines enzyme production, saccharification, and fermentation into a single step.

For enzyme-based biomass conversion, enzyme cost is one of the major technical barriers. For instance, enzymes contribute roughly 7% in designing a minimal ethanol

selling price (MESP) of $1.33/(gallon of ethanol) in 2009 [6]. The relatively low enzyme cost ($0.10/gallon of ethanol) was believed to be underestimated and is not available for commercial use [6]. In contrast, enzymes were estimated to account for over 20% of the total production cost by Lynd et al. [7]. Furthermore, strict aseptic conditions are required to prevent contamination in pure-culture fermentations [8]. For instance, the widely used simultaneous saccharification and fermentation (SSF) gives high reported product yields, but requires expensive enzymes and sterility [9].

The MixAlco process [10, 11] requires no enzymes or sterility, making it an attractive alternative for biomass utilization. Figure 1 summarizes the MixAlco process for converting biomass into chemicals and fuels. Biomass is pretreated with lime to enhance digestibility, and then, is fermented anaerobically using a mixed culture of carboxylic acid-forming microorganisms. Calcium carbonate is added to neutralize the produced acids and maintains a desired pH in the fermentation broth. The resulting carboxylate salt solution is concentrated and chemically converted to a variety of chemicals (ketones, carboxylic acids, esters, and ethers) and fuels (primary alcohols, secondary alcohols, and hydrocarbons).

Batch fermentations begin with highly reactive biomass and a low product concentration. As they proceed, the inhibitory product accumulates and the biomass becomes less reactive. As a result, both conversion and final product concentration are low. In contrast, high conversions and high product concentrations in the fermentation are possible using countercurrent operation [12]. Countercurrent fermentation (Fig. 2) allows the least reactive biomass to contact the lowest carboxylic acid concentration. As the solids are transferred from one fermentor to the next upstream fermentor (i.e., from F1 to F2, F2 to F3, and F3 to F4), the biomass becomes less reactive and the carboxylate salt concentration becomes lower. Therefore, fresh biomass contacts the highest acid concentration in fermentor F1 and fresh liquid contacts the lowest acid concentration in fermentor F4. This countercurrent flow arrangement reduces the inhibitory effect from the accumulation of product carboxylate salts by adding fresh liquid to the most digested biomass in F4.

Countercurrent fermentations in the laboratory are time consuming and take several months to achieve steady state [13, 14]; therefore, optimizing a single biomass feedstock would require years. To overcome this, continuum particle distribution model (CPDM) is used to predict the product concentration and biomass conversion using batch fermentation data [3, 15].

Mesophilic fermentations (20–40 °C) are stable and easy to control [16] and have been applied to a variety of biomass feedstocks, including rice straw [17], sugarcane bagasse

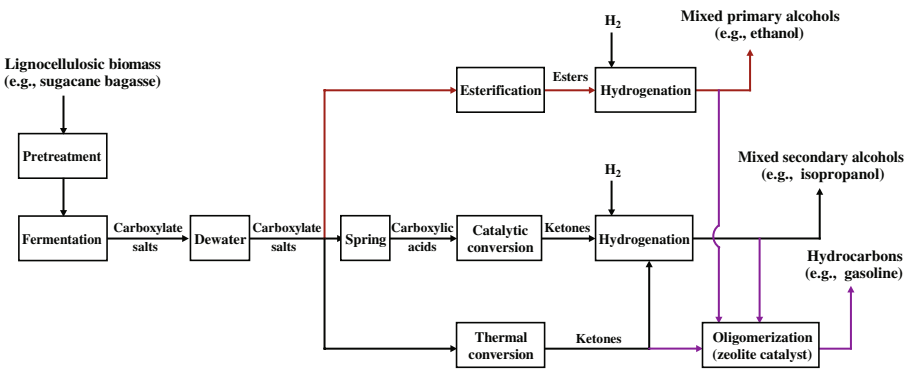


**Fig. 1** Overview of the MixAlco process

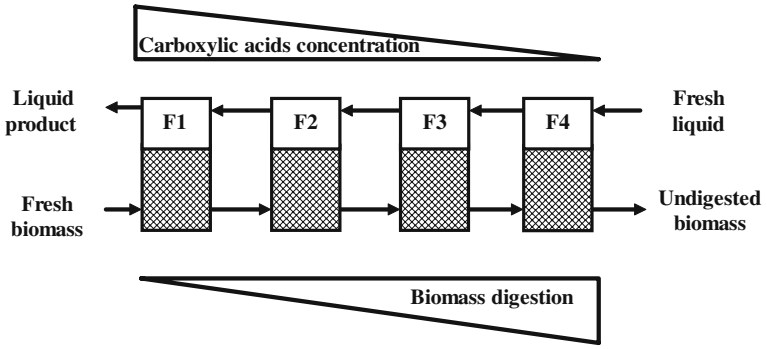


**Fig. 2** Four-stage countercurrent fermentations. *F1* fermentor 1, *F2* fermentor 2, *F3* fermentor 3, *F4* fermentor 4

[18], corn stover [14], municipal solid waste (MSW) [13], and office paper [15]. Thermophilic fermentations (50–60 °C) have higher production rates [19] and a product spectrum that favors acetate [3]. Corn stover and MSW [3] have been examined under thermophilic conditions; however, sugarcane bagasse has not been previously investigated.

This study focuses on countercurrent fermentations of 80% sugarcane bagasse and 20% chicken manure at 55 °C. Pretreatment employed lime, whereas fermentation employed calcium carbonate as a buffer. Different volatile solids loading rates (VSLR) and liquid residence times (LRT) were explored to determine their effect on acid concentration, yield, conversion, and total acid productivity. The acid concentrations and biomass conversions predicted by CPDM method were also compared with experimental data.

## Materials and Methods

### Raw Material

Fresh sugarcane bagasse from the Texas Lower Rio Grande Valley was dried and then ground in a Thomas-Wiley laboratory mill (Thomas Scientific, Swedesboro, NJ) equipped with a 1-mm mesh screen. Hot lime–water treatment was used to enhance the digestibility of sugarcane bagasse [20]. The loading of lime and water were 0.1 g $Ca(OH)_2$ and 10.0 mL per gram of dry bagasse, respectively [21]. The lime pretreatment of bagasse slurry was performed at 100 °C for 2 h. Chicken manure was collected from the Poultry Science Center (Texas A&M University, College Station, TX). Chicken manure was dried at 105 °C in an oven and stored in air-tight container for further use. The average volatile solids content for lime-treated sugarcane bagasse and chicken manure was 83.8% and 74.4%, respectively.

### Medium and Nutrient

The liquid medium was deoxygenated water prepared by boiling distilled water under nitrogen purge for 5 min. After cooling the medium to room temperature, 0.275 g/L sodium sulfide and 0.275 g/L cysteine hydrochloride were added under continuous nitrogen purge to further reduce the oxygen content of the medium. The recipes for dry nutrient mixture are reported elsewhere [20, 22].

Inocula Source

The marine inocula used in this study were previously collected from the sediments of four coastal zones at Galveston, Texas [20]. The sediment samples were taken from 0.5-m-deep holes, and stored in 1-L centrifuge bottles filled with deoxygenated water. Equal amounts of sediment liquid from each bottle were mixed and used as the initial fermentation inocula.

Methane Inhibitor

Methanogens were inhibited using iodoform ($CHI_3$) solution of 20 g iodoform/L ethanol in this study. Because of light and air sensitivity, the iodoform solution was kept in amber-colored glass bottles and capped immediately after use. It was added to each fermentor every 2 days.

pH Control

Calcium carbonate ($CaCO_3$) was added to the fermentors as a neutralizing agent to control the pH between 5.8 and 6.2. Urea was added only if the pH fell below 6.0.

Fermentor

Figure 3 shows the rotary fermentor that holds and mixes high-solid biomass slurries. Rotary fermentors were made from Beckman 1-L polypropylene centrifuge bottles (Thermo Scientific, Waltham, MA). The bottle tops were sealed with a Number 11 rubber stopper with a hole drilled in the middle. A glass tube was inserted through the hole and capped with a rubber septum for gas sampling and release. Two 0.25-in-diameter stainless steel tubes with welded ends were also inserted into holes in the stopper. Both tubes were used as stir bars to mix the biomass slurry inside the fermentors. Rotary fermentors were placed in a Wheaton Modular Cell Production Roller Apparatus (Fisher Scientific, Pittsburgh, PA) located in an incubator. The rollers rotated horizontally at 2 rpm.

Because the maximum pressure limit of the fermentors is 2 atm, daily gas venting from the fermentors was necessary to prevent fermentor breakage or explosions. The rubber septum was replaced if there was a visible hole.

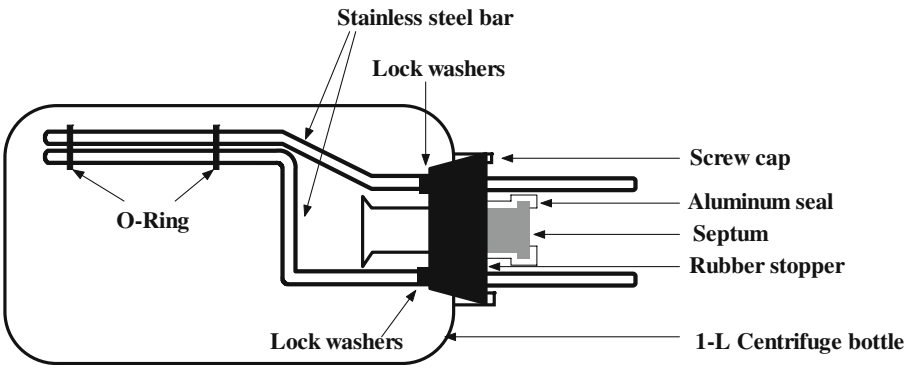


**Fig. 3** Design of rotary fermentor

## Countercurrent Fermentations

The countercurrent fermentations were performed at 55 °C. Anaerobic conditions were maintained by purging with nitrogen whenever the fermentors were opened. To establish the culture for 2 weeks, four fermentors were in batch mode with 80% lime-treated sugarcane bagasse and 20% chicken manure, then countercurrent fermentation was started. Every 2 days, liquids and solids were transferred (Fig. 2) and 2 g of calcium carbonate was added to each fermentor to neutralize the produced carboxylic acids.

A series of four countercurrent fermentation experiments were performed at various combinations of VSLR and LRT. Table 1 shows the operating parameters for the fermentation trains. The single-centrifuge procedure was used to transfer liquids in a single step [22]. After the system reached steady state (±2 g/L average total acid concentration), fermentation data were collected for about 30 days to determine acid productivity, carboxylic acid concentration, yield, selectivity, conversion, biotic $CO_2$ productivity, and $CH_4$ productivity.

## Analytical Methods

To measure concentrations of total carboxylic acids, liquid samples were analyzed using an Agilent 6890 series gas chromatograph (GC) system (Agilent Technologies, Palo Alto, CA) equipped with a flame ionization detector, an Agilent 7683 series injector, and a DB-FFAP capillary column (J&W Scientific, Folsom, CA). Liquid samples were centrifuged at 5,000 rpm for 20 min. The supernant was mixed with 1.162 g/L of internal standard solution (4-methyl-*n*-valeric acid) and acidified with 3-M phosphoric acid. A standard carboxylic acids mix solution (Matreya Inc., Pleasant Gap, PA) was injected prior to

**Table 1** Operating parameters for countercurrent fermentations of 80% lime-treated sugarcane bagasse/20% chicken manure.

| Fermentation trains | A | B | C | D |
|---|---|---|---|---|
| LRT (day) | 25.9 | 28.1 | 42.3 | 27.3 |
| VSLR (g VS/(L liquid in all fermentors day)) | 3.26 | 4.50 | 6.24 | 4.85 |
| VS feed at each transfer (g VS) | 6.30 | 9.44 | 12.59 | 9.44 |
| Solid feed at each transfer (g) | 8.00 | 12.00 | 16.00 | 12.00 |
| Treated bagasse (g) | 6.40 | 9.60 | 12.80 | 9.60 |
| Chicken manure (g) | 1.60 | 2.40 | 3.20 | 2.40 |
| Liquid fed to F4 at each transfer (L) | 0.10 | 0.10 | 0.10 | 0.10 |
| VS/liquid feed ratio (g VS/g liquid) | 0.06 | 0.09 | 0.13 | 0.09 |
| Liquid volume in all four fermentors (L) | 0.97 | 1.05 | 1.01 | 0.97 |
| $F_1$ retained weight (wet g) | 292 | 288 | 284 | 288 |
| Temperature (ºC) | 55 | 55 | 55 | 55 |
| Frequency of transfer | E 2 d | E 2 d | E 2 d | E 2 d |
| $F_2$–$F_4$ retained weight (wet g) | 300 | 300 | 300 | 300 |
| Iodoform addition rate (mg iodoform added/L liquid fed to F4) | 24 | 24 | 24 | 24 |
| Nutrients addition rate (g dry nutrients added/L liquid fed to F4) | 2.0 | 2.0 | 2.0 | 2.0 |

*E 2 d* every 2 days

injecting the samples as an external standard. The oven temperature in the GC increased from 50 °C to 200 °C at 20 °C/min and was held an additional 1 min at 200 °C.

Methane and carbon dioxide composition of gas samples were determined by the Agilent 6890 series GC system equipped with a thermal conductivity detector and a Carboxen 1004 Supelco packed column (J&W Scientific, Folsom, CA). Gas samples were taken directly through the middle rubber stopper of a rotary fermentor using a 5-mL syringe. A standard gas mixture of carbon dioxide (29.99 mol%), methane (10.06 mol%), and the balance nitrogen was routinely used to calibrate the gas chromatograph.

The volume of produced gas was measured by displacing water in a self-constructed inverted glass graduated cylinder apparatus filled with 300 g/L $CaCl_2$ solution [20]. To ensure accurate measurements, the rotary fermentors were cooled to room temperature before measuring the gas volume. A hypodermic needle was inserted through the fermentor septum and the released gasses displaced liquid in the glass cylinder until the pressure in the fermentor equaled the headspace pressure of the cylinder.

$CO_2$ produced during fermentation is the sum of biotic and abiotic $CO_2$. Abiotic $CO_2$ is produced by neutralizing the carboxylic acids with calcium carbonate and biotic $CO_2$ is produced directly from the fermentation. For every 2 mol of acid produced in the fermentor, 1 mol of abiotic $CO_2$ is produced. The biotic $CO_2$ produced directly from the fermentation was calculated by subtracting abiotic $CO_2$ from total $CO_2$. Only biotic $CO_2$ was used in the mass balance calculations.

During each countercurrent transfer (Fig. 2), liquids from fermentor F1 and solids from fermentor F4 were collected and stored in the freezer for future analysis. The liquid collected from fermentor F1 after each transfer was analyzed for residual volatile solids. The solids collected from fermentor F4 were analyzed for undigested volatile solids. The volatile solids (VS) content of a solid sample was determined by first drying at 105 °C in an oven and then ashing at 575 °C in a furnace for another 3 h. The VS weight was calculated as the difference between the dry weight and the ash weight. The VS of a liquid sample was determined by adding lime prior to drying to ensure that carboxylic acids would not volatilize and alter the measurement.

## Mass Balance Closure and Performance Definitions

Biomass is composed of VS and ash. Most of the volatile solids are reactive except lignin, whereas the ash content is nonreactive. Mass balances closure was performed during steady-state countercurrent fermentations. Figure 4 illustrates a typical fermentation process, which converts VS into gas and liquid products, with some solids

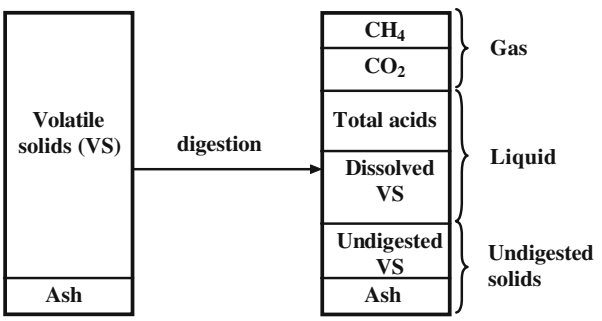


**Fig. 4** Digestion of biomass

remaining undigested. The following definitions were used to describe the fermentation performance:

$$\text{Volatile solids (VS) content} = \frac{\text{Oven dry weight} - \text{Ash weight}}{\text{Oven dry weight}} \tag{1}$$

$$\text{Conversion} = \frac{\text{VS digested}}{\text{VS fed}} \tag{2}$$

$$\text{Yield} = \frac{\text{Total carboxylic acids produced}}{\text{VS fed}} \tag{3}$$

$$\text{Total acid selectivity} = \frac{\text{Total carboxylic acids produced}}{\text{VS digested}} \tag{4}$$

$$\text{Total acid productivity} = \frac{\text{Total carboxylic acids produced}}{\text{L liquid in all reactors} \times \text{time}} \tag{5}$$

$$\text{Liquid residence time (LRT)} = \frac{\text{Total liquid in all fermentors}}{\text{Flow rate of liquid out of the fermentor train}} \tag{6}$$

$$\text{Volatile solids loading rate (VSLR)} = \frac{\text{VS fed to the system}}{\text{Total liquid in all fermentors} \times \text{time}} \tag{7}$$

For each countercurrent fermentation, a complete mass balance was calculated on the entire train during a steady-state period. The mass balance equations are defined as follows:

$$\text{VS in} + \text{water of hydrolysis} = \text{undigested VS} + \text{dissolved VS} + \text{carboxylic acids produced} + \text{biotic } CO_2 + CH_4 \tag{8}$$

To calculate the water of hydrolysis, the volatile solids in biomass could be represented as cellulose, which has a monomer weight of 162 g/mole [23]. When cellulose is hydrolyzed, it gains a molecule of water per monomer; therefore, the water of hydrolysis is calculated as

$$\text{Water of hydrolysis} = \text{VS digested} \times \frac{18}{162} \tag{9}$$

The mass balance closure over the steady-state period was calculated as follows:

$$\text{Closure} = \frac{\text{Mass(out)}}{\text{Mass(in)} + \text{Water of hydrolysis}} \tag{10}$$

$$= \frac{\text{Undigested VS} + \text{Dissolved VS} + \text{Carboxylic Acids} + \text{Biotic } CO_2 + CH_4}{\text{VS(in)} + \text{Water of hydrolysis}} \tag{11}$$

The mass balance closure represents the difference between the mass entering and the mass exiting the fermentation system. In theory, the mass balance closure should be 100%.

Deviations from the expected closure value are due to unavoidable errors in the transfer or measurement process.

Continuum Particle Distribution Model

The CPDM was used to simulate countercurrent fermentations based on data collected from batch fermentations. A "continuum particle" is defined as 1 g of initial volatile solids with a composition identical to the biomass being fed to the fermentor [23]. The model tracks the progress of the continuum particle as it flows through the fermentor, digests, and releases products. Batch experiments with varying initial substrate concentrations (40, 70, 100, and 100+ g dry substrate/L liquid) were used to obtain the data. The 100 and 100+ fermentations had the same initial substrate concentrations, but the 100+ fermentor contained a medium with a mixture of carboxylate salts at a concentration of 20 g carboxylic acids/L of liquid. The inocula for CPDM were taken from the adapted microorganisms in steady-state countercurrent fermentations. Deoxygenated water was used for this fermentation and other components (urea, dry nutrient, and calcium carbonate) were added initially to the fermentors. Iodoform was added daily to prevent methane production. Liquid samples were taken daily from batch fermentations and analyzed for carboxylic acid concentrations. The acid concentrations were converted to acetic acid equivalent ($\alpha$):

$$\begin{aligned}\alpha\ (\mathrm{mol/L}) = {} & \mathrm{acetic}\ (\mathrm{mol/L}) \\ & +1.75 \times \mathrm{propionic}\ (\mathrm{mol/L}) \\ & +2.5 \times \mathrm{butyric}\ (\mathrm{mol/L}) \\ & +3.25 \times \mathrm{valeric}\ (\mathrm{mol/L}) \\ & +4.0 \times \mathrm{caprioc}\ (\mathrm{mol/L}) \\ & +4.75 \times \mathrm{heptanoic}\ (\mathrm{mol/L})\end{aligned} \tag{12}$$

On a mass basis, the acetic acid equivalent can be expressed as

$$\mathrm{Aceq}\ (\mathrm{g/L}) = 60.05\ \times [\alpha(\mathrm{mol/L})] \tag{13}$$

The concentrations of acetic acid equivalents (Aceq) in each batch experiment were fit to Eq. 14.

$$\mathrm{Aceq}\ (t) = a + \frac{bt}{1+ct} \tag{14}$$

where $a$, $b$, and $c$ are constants, and $t$ is the fermentation time in days. Initial value for the parameters $a$, $b$, and $c$ were guessed. The parameters $a$, $b$, and $c$ were obtained by a least square method, which involved minimizing the following equation:

$$\mathrm{Residuals} \quad = \quad \sum_{\mathrm{data}} \left(\mathrm{Aceq}_{\,\mathrm{exp}} - \mathrm{Aceq}_{\,\mathrm{calculate}}\right)^2 \tag{15}$$

The reaction rate for the fermentation was then determined by the equation

$$r = \mathrm{rate} = \frac{d\,(\mathrm{Aceq})}{dt} = \frac{b}{(1+ct)^2} \tag{16}$$

The specific reaction rate ($\hat{r}$, the reaction rate per *continuum particle*) is calculated by the reaction rate in Eq. 16 divided by the initial substrate concentration ($S_o$) in the respective batch fermentor.

$$\hat{r} = \frac{r}{S_o} \tag{17}$$

In a batch fermentor, $S_o$, the substrate concentration (grams of VS/liter) is defined as the initial volatile solid mass $m_o$ per volume of liquid $V$ ($S_o = m_o/V$). In a four-stage countercurrent fermentation, $m_o$ is the mass of fresh volatile solids added to fermentor 1, and $V$ is defined as the fresh liquid volume added to fermentor 4.

The biomass conversion $x$ is calculated for each batch fermentor using Eq. 18.

$$x(t) = \frac{\text{Aceq}(t) - \text{Aceq}(t=0)}{S_o \sigma} \tag{18}$$

where $\sigma$ is the selectivity (grams of Aceq produced/grams of VS digested). In the CPDM method, the selectivity $\sigma$ is assumed as constant and calculated from the selectivity $s$ by Eq. 19.

$$\sigma = \frac{s}{\phi} \tag{19}$$

where the average value of selectivity $s$ (grams of total acid produced/grams of VS digested) is determined from the countercurrent experiments. The constant $\phi$ is the ratio of total grams of carboxylic acid to total grams of acetic acid equivalents.

Equation 20 is the governing equation deployed in the CPDM method. It relates the specific reaction rate $\hat{r}(x, \text{Aceq})$ with acetic acid equivalent concentration (Aceq) and conversion ($x$).

$$\hat{r}_{pred} = \frac{e(1-x)^f}{1 + g(\phi \cdot \text{Aceq})^h} \tag{20}$$

Where $x$ is the fraction conversion of volatile solids

$e$, $f$, $g$, and $h$ are empirical constants

Equation 20 is an empirical equation. The (1–$x$) term in Eq. 20 was described as the *conversion penalty function* and shows that the reaction rate decreases as the substrate is converted [24]. The denominator term in Eq. 20 describes the inhibitory effect of products on the microorganisms, which decreases the reaction rate. The parameter $\phi$ was introduced to avoid the inhibitory effects of higher acids that would overestimate the specific rate [23].

The batch fermentations produced values for Aceq, $\hat{r}$, and $x$ using Eqs. 13, 17, and 18, respectively. Parameter values of $e$, $f$, $g$, and $h$ in Eq. 20, were fit by nonlinear regression to minimize the difference between the experimental value and the predicted value of the specific reaction rate $\hat{r}$.

## Statistical Analyses

The nonlinear regression for CPDM was performed using software Systat Sigmaplot version 10.0 (Systat Software, San Jose, CA). The one-way analysis of variance (ANOVA) followed by mean comparison using Turkey's post test was conducted at the 0.05 level using software GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA).

## Results and Discussion

### Countercurrent Fermentations

Table 1 summarizes the operating conditions for countercurrent fermentation trains of 80% lime-treated sugarcane bagasse/20% chicken manure. Table 2 shows the fermentation results for these countercurrent fermentations. Figure 5 shows the mass balance closures were 98.9–105.4% and Fig. 6 shows the product concentration profiles.

The highest acid productivity of 0.79 g/(L day) occurred at a concentration of 21.5 g/L in fermentation train D [LRT=27.3 day and VSLR=4.85 g/(L day)]. Fermentation train A [LRT=25.9 day and VSLR=3.26 g/(L day)] with a concentration of 15.5 g/L had the highest conversion (0.59 g VS digested/g VS fed) and highest yield (0.18 g total acids/g VS fed). Fermentation train A had the highest conversion and yield because it had the lowest VSLR, which made more complete use of the biomass [14]. The highest selectivity of 0.41 g total acids/g VS digested was found in fermentation train B [LRT=28.1 day and VSLR=4.50 g/(L day)].

Thermophilic (55 °C) anaerobes were obtained from a marine environment (~22 °C). Figure 6a, b show that these naturally occurring microorganisms adapted to the new environment (higher temperature) and stably produced acids for many months under thermophilic conditions with no signs of degeneration. It is possible that the thermophiles were naturally present in the marine environment and were selected in the thermophilic laboratory environment. Alternatively, the thermophiles could have evolved from the naturally occurring microorganisms. Metabolic evolution is a powerful tool for selecting mutants and allows microorganisms to evolve in a series of fermentation transfers under a proper selective pressure (e.g., higher inhibitor levels) [25]. In this case, the natural

**Table 2** Summary of fermentation results from countercurrent fermentations of 80% lime-treated bagasse/20% chicken manure.

| Fermentation trains | A | B | C | D |
|---|---|---|---|---|
| Average pH in all fermentors | 6.03±0.27 | 6.07±0.26 | 5.88±0.16 | 5.88±0.09 |
| Total carboxylic acid concentration (g/L) | 15.5±0.7 | 20.5±0.9 | 28.0±0.8 | 21.5±0.7 |
| Acetic acid (wt%) | 59.1±1.8 | 60.5±2.1 | 67.4±1.0 | 65.5±1.1 |
| Propionic acid (wt%) | 2.74±1.06 | 1.40±0.23 | 1.23±0.08 | 1.48±0.14 |
| Butyric acid (wt%) | 33.9±1.5 | 34.7±2.0 | 27.2±0.8 | 27.9±1.1 |
| Valeric acid (wt%) | 0.41±0.47 | 0.04±0.10 | 0.00±0.00 | 0.00±0.00 |
| Caproic acid (wt%) | 3.69±0.34 | 3.32±0.46 | 4.14±0.26 | 5.13±0.42 |
| Heptanoic acid (wt%) | 0.22±0.49 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| Conversion (g VS digested/g VS fed) | 0.59 | 0.40 | 0.34 | 0.47 |
| Yield (g total acids/g VS fed) | 0.18 | 0.16 | 0.11 | 0.16 |
| Selectivity (g total acids/g VS digested) | 0.31 | 0.41 | 0.31 | 0.35 |
| Total carboxylic acid productivity (g total acids/ (L liquid day)) | 0.60 | 0.73 | 0.66 | 0.79 |
| Biotic carbon dioxide productivity (g $CO_2$/(L liquid day)) | 0.194 | 0.068 | 0.157 | 0.016 |
| Methane productivity (g $CH_4$/(L liquid day)) | 0.0177 | 0.0092 | 0.0083 | 0.0963 |
| Mass balance closure (g VS out/g VS in) | 1.049 | 1.027 | 0.989 | 1.054 |

All errors are ±1 standard deviation

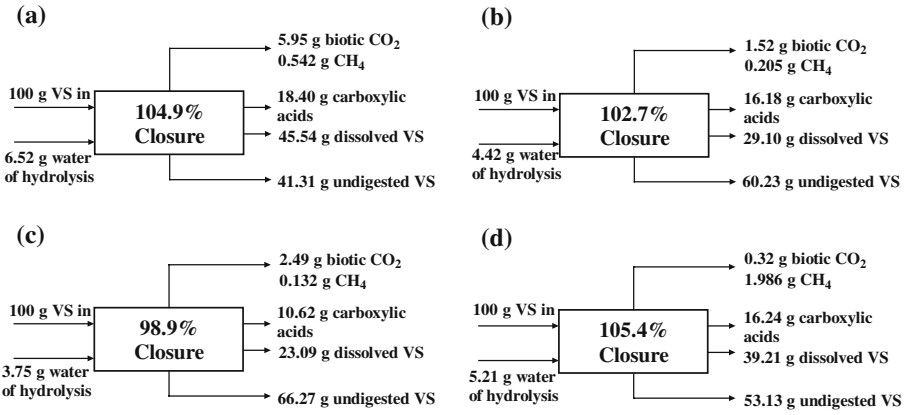


**Fig. 5** Mass balances for the countercurrent fermentations of 80% lime-treated sugarcane bagasse/20% chicken manure. **a** Fermentation train A [LRT=25.9 day and VSLR=3.26 g/(L day)]. **b** Fermentation train B [LRT=28.1 day and VSLR=4.50 g/(L day)]. **c** Fermentation train C [LRT=42.3 day and VSLR=6.24 g/(L day)]. **d** Fermentation train D [LRT=27.3 day and VSLR=4.85 g/(L day)]

microorganisms may have evolved under the pressure of high temperature by transferring every 2 days in the long-term countercurrent fermentations. The acid-forming thermophiles—whether selected or evolved—from fermentation trains A and B were used as seed inocula for thermophilic fermentations with other operating conditions.

For pure-culture fermentations, costly sterility is required to protect against potential contamination [8]. In contrast, without sterile operating conditions, the mixed culture of microorganisms produced carboxylic acids for many months (Fig. 6a, b) with no signs of contamination.

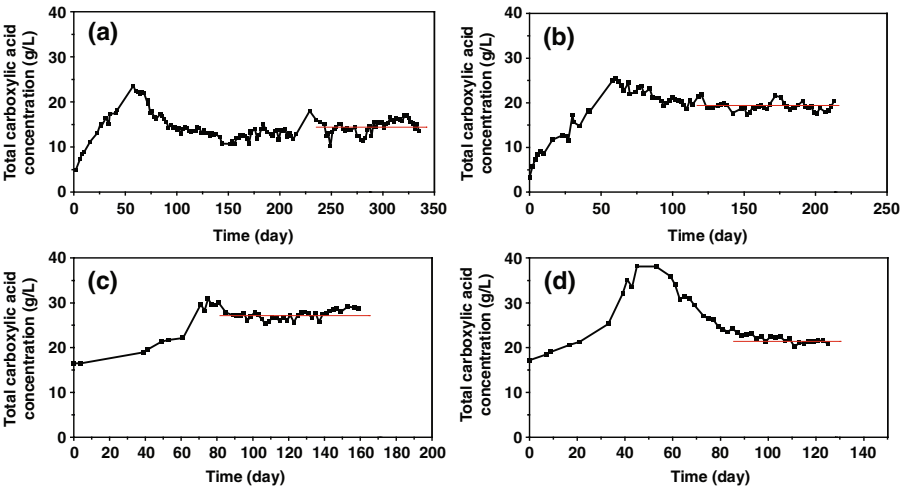


**Fig. 6** Time course of total carboxylic acid concentrations in countercurrent fermentations. Solid line indicates steady state. **a** Fermentation train A [LRT=25.9 day and VSLR=3.26 g/(L day)]. **b** Fermentation train B [LRT=28.1 day and VSLR=4.50 g/(L day)]. **c** Fermentation train C [LRT=42.3 day and VSLR=6.24 g/(L day)]. **d** Fermentation train D [LRT=27.3 day and VSLR=4.85 g/(L day)]

## Verification of Continuum Particle Distribution Model

As described earlier, batch experiments with 80 wt% lime-treated sugarcane bagasse/20 wt % chicken manure were performed to obtain model parameters for the CPDM method. The produced carboxylic acid concentrations were converted to Aceq. Table 3 shows the fitted parameters $a$, $b$, and $c$. Parameters $e$, $f$, $g$, and $h$ in the predicted rate equation (Eq. 21) were calculated by nonlinear regression.

$$\hat{r}_{\text{pred}} = \frac{0.49(1-x)^{3.28}}{1+3.22(0.85\text{Aceq})^{0.95}} \tag{21}$$

For batch fermentations, Fig. 7 compares the predicted specific rate with experimental specific rate.

For continuous countercurrent fermentations, Table 4 lists the system-specific variables required by CPDM prediction. Figure 8 shows a CPDM "map" for fermentations of 80% lime-treated sugarcane bagasse/20% chicken manure at a substrate concentration of 124 g VS/(L liquid), which was typical of the experimental procedure. The "map" predicts a total acid concentration of 20.5 g/L at LRT of 30 day, VSLR of 8 g/(L day) and a conversion of 34.0%. Table 5 compares the experimental total carboxylic acid concentration and conversion to the CPDM prediction (Fig. 8). As shown in Table 5, the CPDM predictions of total carboxylic acid concentrations and conversion agreed with the experimental data, with an average absolute error of 10.14% and 12.68%, respectively. This is sufficiently accurate to design industrial fermentors. The CPDM method assumes that the selectivity ($\sigma$) is constant; however, the selectivity varies with VSLR (Table 2). The variation in selectivity accounts for some of the error between the experimental values and the predicted values.

## Continuum Particle Distribution Model Predictions of High-Solids Fermentations

High-solids fermentations lower capital costs because of the reduced volume, they lower operating costs because of less energy for heating and cooling, and they lower downstream processing costs because of higher product concentrations [26]. High substrate concentration is possible in fed-batch or countercurrent operations. In SSF, solids concentration of 30% ($w/w$) steam-exploded corn stover have been reported [27]. High substrate

**Table 3** Values of the parameters $a$, $b$, and $c$ in CPDM for 80% lime-treated sugarcane bagasse/20% chicken manure.

| Initial substrate concentration (g/L) | $a$ (g/L liquid) | $b$ (g/(L liquid day)) | $c$ ($\text{day}^{-1}$) |
|---|---|---|---|
| 40 | 6.93 | 0.54 | 0.42 |
| 70 | 7.70 | 1.03 | 0.14 |
| 100 | 8.48 | 1.23 | 0.08 |
| 100+[a] | 24.23 | 1.72 | 0.24 |
| 100+[b] | 26.17 | 1.02 | 0.14 |

100 and 100+ fermentors had the same initial substrate concentrations, but the 100+ fermentor contained a medium with a mixture of carboxylate salts at a concentration of 20 g carboxylic acids/L liquid

[a] An 85 wt% calcium acetate, 5 wt% calcium propionate, and 10 wt% calcium butyrate

[b] An 70 wt% calcium acetate, 20 wt% calcium propionate, and 10 wt% calcium butyrate

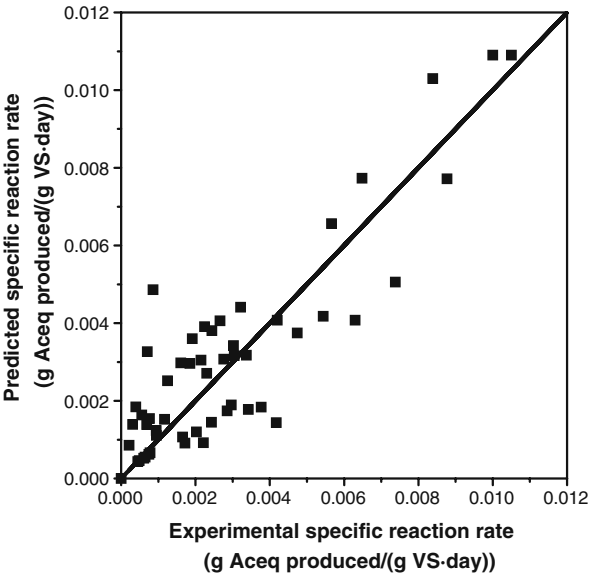


**Fig. 7** The experimental value and the CPDM prediction value for the specific reaction rate in five batch fermentations of 80% lime-treated sugarcane bagasse/20% chicken manure. *Filled square* CPDM predicted value; *line* reference line $y=x$

concentrations (>300 g VS/L liquid) is allowed if the MixAlco process is operated on a large scale [10].

The laboratory countercurrent fermentors were serviced every 2 days, so enough liquid had to be present to store 2 days worth of production. Typical solids concentrations were 124 g substrate/L liquid. Although it is not practical to achieve high solids in the laboratory, it can be achieved industrially by continuously processing the solids and liquids. The CPDM method is able to predict and optimize countercurrent fermentations at high substrate concentrations. The CPDM prediction "map" (Fig. 9) was used to simulate the industrial application with a solids concentration of 300 g substrate/L liquid (or 300 g/1,300 g slurry=23.1 *w*/*w*%). Total acid concentrations as high as 21.3 g/L can be reached at LRT of 30 days, VSLR of 3 g/(L day), and 80.1% conversion. Although there is good agreement between CPDM prediction and experimental values (Table 5), the selected design conditions should be verified in a pilot plant before implementation at the industrial scale.

With the calcium carbonate buffer used in these experiments, the predicted acid concentration (~22 g/L) is not in the targeted range (40–50 g/L). Recent experiments with

**Table 4** Parameter constant values in CPDM for countercurrent fermentations of 80% lime-treated bagasse/20% chicken manure.

| Parameter constant | Value |
|---|---|
| Holdup (g liquid/g VS cake) | 3.18 |
| Moisture (g liquid/g solid feed) | 0.03 |
| Selectivity (g Aceq/g VS digested) | 0.35 |
| $\phi$ (g total acid/g Aceq) | 0.85 |
| $e$ (g Aceq/(g VS·day)) | 0.49 |
| $f$ (dimensionless) | 3.28 |
| $g$ (L/g total acid)$^{1/h}$ | 3.22 |
| $h$ (dimensionless) | 0.95 |

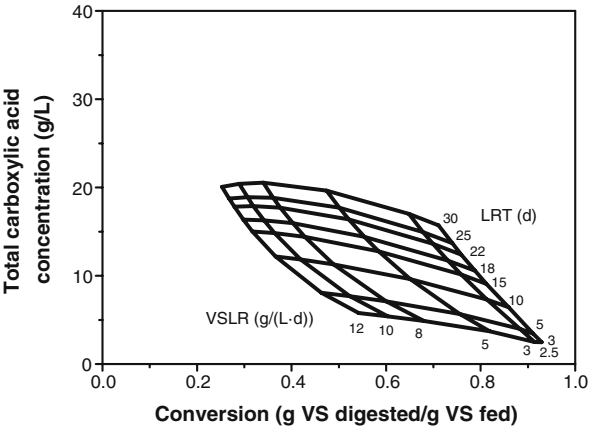


**Fig. 8** Predicted CPDM "map" for countercurrent fermentations of 80% lime-treated sugarcane bagasse/20% chicken manure (124 g VS/L liquid)

ammonium bicarbonate buffer show a 65–98% increase in product concentration [20], which achieves the targeted product concentration.

## Comparison of Acetate Contents under Different Temperatures

The percentage of acetic acid in the total produced carboxylic acids (i.e., acetate content) changes with temperatures. With countercurrent fermentations of 80% sugarcane bagasse/20% chicken manure, Table 2 shows the average acetate content was 63.1±4.0 wt% at 55 °C. At 40 °C, the average acetate content was 38.9±4.5 wt% using marine inocula, and 38.0±2.3 wt% using terrestrial inocula [18, 22]. The one-way ANOVA ($P<0.05$, $n=30$) showed there was a significant increase in acetate content under thermophilic conditions (~63 wt%) compared with that under mesophilic conditions (~39 wt%). Under mesophilic condition, there was no significant difference in acetate content between marine and terrestrial inocula.

The high acetate content (~63 wt%) in the product can be helpful if acetic acid is the desired product or in the biomass-to-ethanol pathway of the MixAlco process (Fig. 1).

**Table 5** Comparison of experimental and predicted results of countercurrent fermentations of 80% lime-treated bagasse/20% chicken manure.

| Fermentation trains | Train A | Train B | Train C | Train D | Average (%)[b] |
|---|---|---|---|---|---|
| LRT (day) | 25.9 | 28.1 | 42.3 | 27.3 | |
| VSLR (g VS/(L liquid in all fermentors day)) | 3.26 | 4.50 | 6.24 | 4.85 | |
| Experimental carboxylic acid concentration (g/L) | 15.5 | 20.5 | 28.0 | 21.5 | |
| Predicted carboxylic acid concentration (g/L) | 15.9 | 18.5 | 24.0 | 18.5 | |
| Error (%)[a] | 2.58 | −9.76 | −14.29 | −13.95 | 10.14 |
| Experimental conversion (g VS digested/g VS fed) | 0.59 | 0.40 | 0.34 | 0.47 | |
| Predicted conversion (g VS digested/g VS fed) | 0.64 | 0.52 | 0.36 | 0.50 | |
| Error (%)[a] | 8.47 | 30.0 | 5.88 | 6.38 | 12.68 |

[a] Error (%)=(predicted value–experimental value)×100/experimental value

[b] Average absolute error (%)= $\left(\sum_{i=1}^{4} |\text{Error}_i|\right) \Big/ 4$

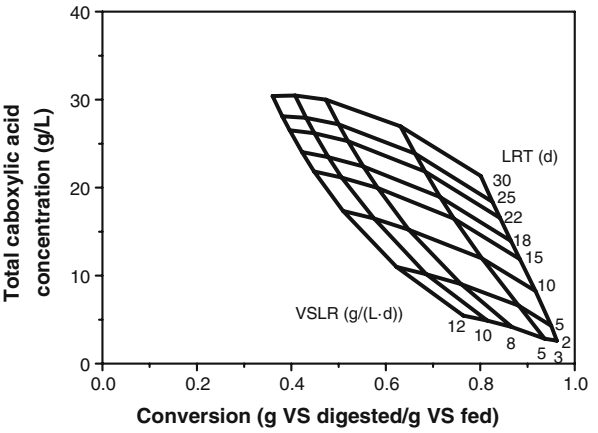


**Fig. 9** Predicted CPDM "map" for countercurrent fermentations of 80% lime-treated sugarcane bagasse/20% chicken manure (300 g VS/L liquid)

## Consolidated Bioprocessing

CBP integrates enzyme production, substrate hydrolysis, and fermentation into a single biological processing step and is a low-cost processing strategy [28, 29]. In a typical CBP biomass-to-ethanol process (Fig. 10a), recombinant microorganisms (pure culture) produce enzymes that hydrolyze polysaccharides and coferment hexoses and pentoses to produce ethanol in a single step. Despite intensive attempts, currently, there is no industrial application of CBP for the biomass-to-ethanol process because of technical limitations in identifying appropriate cellulolytic and ethanologenic bacteria [30]. Thermophilic bacteria (e.g., *Clostridium thermocellum* and *Thermoanaerobacter thermosaccharolyticum*) are typically investigated because of their rapid and effective ability to metabolize cellulose and hemicellulose under thermophilic conditions. However, carboxylic acids, a common by-product of the thermophilic bacteria, reduce ethanol yield and inhibit the overall fermentation [28]. Carboxylic acids, which are undesired by-products in the conventional CBP biomass-to-ethanol process, are desired products in the MixAlco process.

The MixAlco process is an example of CBP (Fig. 10b) that uses mixed cultures of microorganisms rather than pure cultures and accommodates the mixed composition of various biomass feedstocks. A living example is animal rumen in which feedstocks are fermented within only 25–30 h by a mixed culture of metabolically related microorganisms that produce the myriad of enzymes necessary to digest complex plant tissues (e.g., protein, cellulose, hemicellulose, pectins, starch, and fats) [31]. The MixAlco process is modeled after the rumen; however, the rumen maintains a low carboxylate concentration (~8 g/L) so the system is not significantly inhibited. In contrast, the MixAlco process uses a much higher carboxylate concentration (40–50 g/L), so salt-tolerant microorganisms are preferred.

## Conclusions

The anaerobic fermentation of 80% lime-treated bagasse/20% chicken manure was performed with marine inocula and calcium carbonate buffer. The data were modeled with the CPDM method, which predicted the experimental total acid concentrations and conversions within 10.14% and 12.68%, respectively. This is sufficiently accurate to design industrial fermentors. At VS conversions of 75%, an industrial-scale fermentor (300 g VS/L

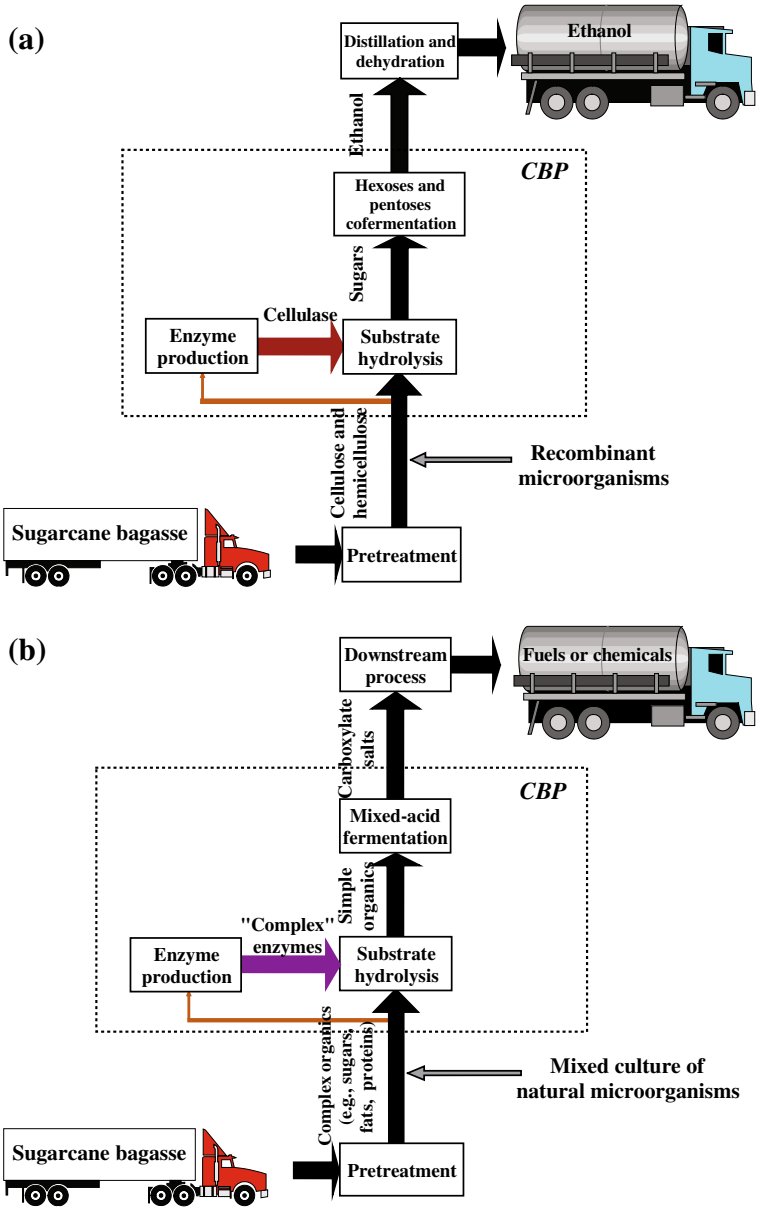


**Fig. 10** Comparison of consolidated bioprocessing (*CBP*) in different bioconversion technologies. **a** A typical biomass-to-ethanol process. **b** The MixAlco process

liquid) is predicted to produce 21.3 g/L carboxylic acids using LRT of 30 days and VSLR of 3 g/(L day). However, this selected design condition should be verified in a pilot plant before implementation at the industrial scale. For acetic acid production or the biomass-to-ethanol pathway of the MixAlco process, thermophilic fermentations are preferred because of higher acetate content (~63 wt%) than mesophilic fermentations (~39 wt%). The

MixAlco process is an example of CBP that has no sterility requirements and can digest any biodegradable biomass feedstocks.

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