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Investigation of Nutrient Feeding Strategies in a Countercurrent Mixed-Acid Multi-Staged Fermentation: Experimental Data

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Abstract Nutrients are essential for microbial growth and metabolism in mixed-culture acid fermentations. Understanding the influence of nutrient feeding strategies on fermentation performance is necessary for optimization. For a four-bottle fermentation train, five nutrient contacting patterns (single-point nutrient addition to fermentors F1, F2, F3, and F4 and multi-point parallel addition) were investigated. Compared to the traditional nutrient contacting method (all nutrients fed to F1), the near-optimal feeding strategies improved exit yield, culture yield, process yield, exit acetate-equivalent yield, conversion, and total acid productivity by approximately 31%, 39%, 46%, 31%, 100%, and 19%, respectively. There was no statistical improvement in total acid concentration. The traditional nutrient feeding strategy had the highest selectivity and acetate-equivalent selectivity. Total acid productivity depends on carbon–nitrogen ratio.

Keywords Countercurrent mixed-acid fermentation · MixAlco process · Fermentation optimization · Nitrogen behavior · Carbon–nitrogen (C/N) ratio · Nutrient feeding strategies

Nomenclature

A	Mass of carboxylic acid, g
$[A]$	Total carboxylic acid concentration, g/$L_{liq.}$
aceq	Acetic acid equivalents concentration, g/$L_{liq.}$
B_i	Fermentor i bottle plus centrifuge cake, g
C	Conversion, g NAVS consumed/g NAVS in feed
C/N	Carbon–nitrogen ratio, g C_{NA}/g N
C_{NA}	Non-acid carbon, g

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F_i	Bulk fermentation mass in Fermentor *i*, g wet (as-is)
F*i*	Fermentor *i*
I	Ash content, g ash/g dry sample
K_{Fi}	The average mass of wet solid cake in Fermentor *i*, g
L	Transfer liquid stream flowrate, g wet (as-is)/day (or *T*)
LRT	Liquid retention time, day
LV_{Fi}	Liquid volume in Fermentor *i*, $L_{liq.}$
M	Moisture content, g moisture/g wet (as-is) sample
N	Nutrient-rich substrate feedrate, g wet (as-is)/day (or *T*)
NAVS	Non-acid volatile solids, g
P	Total acid productivity, g acid produced/($L_{liq.}$·day)
Q	Total inlet liquid flowrate, L/day (or *T*)
S	Transfer solid stream flowrate, g wet (as-is)/day (or *T*)
SC_{Fi}	Solid concentration of Fermentor *i*, g NAVS in Fermentor *i*/$L_{liq.}$ in Fermentor *i*
T	Time period between transfers, ~56 h
TLV	Total liquid volume in all fermentors, L
VS	Volatile solids, g
VSLR	Volatile solids loading rate, g NAVS/$L_{liq.}$
W_i	Solids-retained-plus-bottle-weight set point, g
X	May represent *F*, *S*, or *L*
Y_F	Feed yield, g acid in feed/g NAVS in feed
Y_E	Exit yield, g acid exiting fermentation/g NAVS in feed
Y_C	Culture yield, g acid produced/g NAVS in feed
Y_P	Process yield, g acid in product liquid/g NAVS in feed

Greek symbols

a	Acetic acid equivalents concentration, mol/$L_{liq.}$
η	Soluble nitrogen mass fraction, g soluble N/g total N
ν	Nitrogen content, g N/g wet (as-is) sample
ρ_w	Density of water, 1 g/mL
σ	Total acid selectivity, g acid produced/g NAVS consumed

Subscripts

For *M*, *I*, ν, and η	The subscript denotes the corresponding stream or material
For *F*, *S*, *L*, and [*A*]	The subscript denotes the fermentor from which the material or stream came
For *N*	The subscript denotes the fermentor the nutrient-rich substrate is fed
Subscript *i*	Is a number placeholder

Introduction

The MixAlco Process

The MixAlco process is a "biorefinery" that converts any biodegradable biomass into useful chemicals and fuel [1–3]. Although some substrates (e.g., food scraps and office paper) are easily digested, most lignocellulosic biomass must be pretreated with lime and oxygen/air to increase digestibility [4]. The biomass is then fermented by a mixed culture of acidogens to produce two- to seven-carbon carboxylic acids, which are buffered with calcium carbonate or ammonium bicarbonate. The fermentation broth is clarified, concentrated, and

dried to produce carboxylate salts, a "biocrude" that can be chemically converted to chemicals and fuels.

Four fermentors (Fig. 1) are used to create a countercurrent fermentation "train" [5–10]. The first fermentor is fed with the most reactive (fresh) biomass, but has the highest product carboxylic acid concentration (greatest product inhibition). The last fermentor has the most recalcitrant (digested) biomass (i.e., lignocellulose), but has the lowest product concentration (least product inhibition). This countercurrent strategy achieves both high product concentration and high conversion [5, 7, 11–13]. The goal of fermentation is to maximize carboxylic acid yield (product per reactant fed) and carboxylic acid concentration (not grow cells) [1].

At first glance, the fermentation system described in Fig. 1 appears similar to use of chemostats in series [14–16]. However, there are differences that preclude meaningful comparison: (1) flow is countercurrent rather than one-directional, (2) mixed-culture of non-competitive microorganisms (not a monoculture nor competitive microorganisms), (3) complex substrate (not a single substrate, e.g., glucose), (4) cell mass and growth rate are not quantified nor of interest, and (5) fermentation is a heterogeneous reaction with solid substrate (i.e., lignocellulose).

Mixed-culture acid fermentations of lignocellulose are long (20–60 days liquid retention) and dilute (20–40 gacid/L), thus requiring large fermentors. Improving fermentation performance will significantly reduce capital costs and increase productivity. Nitrogen is required for cell replication, maintenance, metabolism, and production of enzymes [17]. Because lignocellulose hydrolysis is the rate limiting step, maintaining sufficient nitrogen concentrations/proportions is necessary to ensure that production of critical hydrolysis enzymes (e.g., cellulase) is not restricted [18, 19].

Role of Nitrogen Transport in Countercurrent Staged Fermentations

For the purposes of this paper, "nutrient-rich substrate" refers to feedstocks rich in vitamins, N, P, and minerals (e.g., chicken manure and sewage sludge). "Carbohydrate-rich substrate" refers to feedstocks rich in carbohydrates—principally cellulose and hemicelluloses—that provide carbon-based energy (e.g., paper, bagasse, and corn stover).

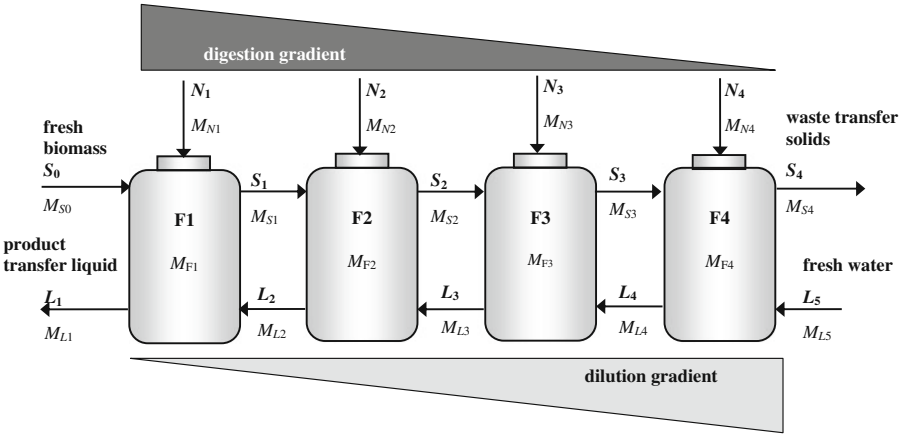

Fig. 1 Four-stage countercurrent fermentation train with digestion and dilution gradients. S_0, L_5, and N_i are the feed carbohydrate, water, and nutrient-rich substrate stream flow rates (mass/time), respectively. S_i, L_i, and F_i are the transfer solids stream flowrate (mass/time), transfer liquid stream flowrate (mass/time), and total fermentation mass in Fermentor i, respectively. M_{Xi} is the moisture content of the material in the stream of fermentor

Carbohydrate-rich (e.g., municipal solid waste, paper, and sugarcane bagasse) and nutrient-rich feedstocks (e.g., sewage sludge, and manure) ferment better when blended in an optimal ratio [6, 7, 20]. Transport of soluble and insoluble nutrients is significant and can lead to nutrient imbalances in high-solid countercurrent staged fermentations. For example, the majority of the nitrogen may be soluble and travel with the transfer liquid streams (L_i), whereas the lesser fraction insoluble nitrogen travels with the transfer solids (S_i). Uneven distribution can lead to premature loss of nitrogen before it can be incorporated into microbial cells and enzymes, thus reducing performance.

The goal of this study was to determine the influence of different nutrient-rich substrate feeding strategies (i.e., amount of nutrient-rich substrate (chicken manure) fed to each fermentor) on fermentation performance. It was hypothesized that (1) each nutrient feeding strategy would distribute nitrogen differently such that a unique carbon–nitrogen ratio (C/N) profile is observed, and (2) the best performance would coincide with the fermentation whose C/N profile was most near the optimal C/N ratio.

Methods

Fermentation Conditions

The fermentors, previously described [6, 7, 9, 10, 21], were incubated on a Wheaton Modular Cell Production Apparatus at 1.5 rpm and 40 °C. To keep the fermentors anaerobic when opened, the gas space was continually purged with N_2 or CO_2.

Substrates

Table 1 lists the feedstock properties. Shredded office paper (carbohydrate-rich substrate) from Texas A&M University's recycling center (College Station, TX, USA) and fresh (wet) chicken manure (nutrient-rich substrate) from Feathercrest Farm (Bryan, TX, USA) were used in a 4:1 carbohydrate-rich:nutrient-rich substrate ratio on a dry mass basis. Paper was selected because it is free of lignin and did not require pretreatment. No additional nutrients (bloodmeal, urea, etc.) were added. The C/N ratio of the feed was 39±1 g C_{NA}/g N.

Fermentation Media

Deoxygenated water was prepared by boiling deionized water to liberate dissolved oxygen gas. After cooling to room temperature in a covered vessel, 0.275 g sodium sulfide and 0.275 g cysteine (reducing agents) were added per liter of water.

Table 1 Feedstock properties

	Office paper	Fresh chicken manure
Moisture content, M (g H_2O/g wet (as-is) sample)	0.051±0.03	0.660±0.03
Ash content, I (g ash/g dry sample)	0.130±0.06	0.592±0.09
Carbon content, C (g C/100 g wet (as-is) sample)	36.3±0.8	6.91±0.7
Nitrogen content, N (g N/100 g wet (as-is) sample)	0.25±0.07	1.10±0.2
Carbon-nitrogen ratio (g C_{NA}/g N)	138.3±43	6.3±0.7

Error values represent one standard deviation

Methanogen Inhibitor

Each day, a small amount (80 μL) of methanogen inhibitor (20 g iodoform/L 200-proof ethanol) was added to each fermentor bottle [5, 6, 9, 12].

Inoculum

The inoculum was obtained from the MixAlco Pilot Plant (College Station, TX, USA), which was originally inoculated with environmentally sourced, undefined, mixed culture from beaches of Galveston, TX, USA.

Analytical Methods

Acid Concentration

Ultra-centrifuged (15,000 rpm) fermentation liquid was mixed with equal parts of internal standard (1.162 g/L 4-methyl-*n*-valeric acid) and 3-M H_3PO_4. The H_3PO_4 ensures that carboxylate salts are converted to carboxylic acid prior to analysis. The carboxylic acid concentration was measured using an Agilent 6890 Series Gas Chromatograph (GC) system equipped with a flame ionization detector (FID) and an Agilent 7683 automatic liquid sampler. A 30-m fused-silica capillary column (J&W Scientific Model No. 123-3232) was used. The column head pressure was maintained at 2 atm (absolute). After each sample injection, the GC temperature program raised the temperature from 40 °C to 200 °C at 20 °C/min. The temperature was subsequently held at 200 °C for 2 min, with a total run time per sample of 11 min. Helium was the carrier gas. The calibration standard was volatile acid mix (Matreya, LLC, Cat No. 1075). Acetic acid equivalents (aceq) equate the reducing potential of a carboxylic acid mixture to an energy-equivalent mass of acetic acid. Concentrations are converted to acetic acid equivalents using the equations outlined by Datta [22].

Gas Composition

Each fermentor was vented daily to relieve pressure and prevent rupture. The gas volume was measured by liquid. To monitor methane, 5-mL gas samples were taken through the fermentor septum then analyzed by the Agilent 6890 Series Chromatograph with a thermal conductivity detector (TCD). Samples were injected manually. A 4.6-m stainless steel packed column with 2.1-mm ID (60/80 Carboxen 100, Supelco 1-2390) was used. The inlet temperature was 230 °C, the detector temperature was 200 °C, and the oven temperature was 200 °C. The total run time was 10 min. Helium was the carrier gas.

Carbon and Nitrogen Contents

To quantify carbon–nitrogen ratios, the literature uses a variety of units (e.g., g total C/g total N, g starch C/g externally added N, and g carbohydrate chemical oxygen demand/total Kjedahl N) [18, 23, 24]. In this paper, *carbon–nitrogen ratio* is defined as the mass of total organic carbon minus the carbon contributed by the carboxylic acids (product; g non-acid carbon; g C_{NA}) per mass of nitrogen (g N). With respect to acidogens, this definition of C/N ratio characterizes the relative proportion of reactant (energy) per nitrogen. For this study, the organic acids represented 8–18% of the total carbon. If the carbon contributed by the

acids is not excluded, the C/N will be overstated, which could lead to suboptimal performance and over-addition of nitrogen supplement (e.g., urea) and/or nutrient-rich substrate (added cost).

The C/N ratio was characterized using total carbon and total nitrogen contents (g/100 g), both of which were measured in a single test using an Elementor Variomax CN (analyzed by Texas A&M University Soil, Water, and Forage Testing Lab (College Station, TX, USA)). The C/N ratio was used to compare trends among the different nutrient-rich substrate feeding strategies. No external buffer, such as calcium carbonate, was added because minerals in the feed self-regulated the pH between 5.5 and 6.5. The nitrogen compounds were undefined; only the total nitrogen content was of interest.

Moisture and Ash Contents

Moisture contents (M_{Xi}) and ash contents (I_{Xi}) were measured in series. First, the sample was dried in a 105 °C forced-convection oven (>12 h) and then ashed in a 550 °C furnace (>3 h). Before drying, 3 g $Ca(OH)_2$/100 g sample was added to ensure all volatile acids were converted to salts and retained during drying. This practice disproportionately overstates the ash content. To overcome this problem, the consumption of non-acid volatile solids (NAVS) was determined using the inert-ash approach (*Determination of NAVS Consumed* section).

Definition of Terms

Figure 2 shows the conversion of biomass using mixed-acid fermentation. The feed consists of initial volatile solids $VS_{initial}$, which are composed of undigested VS, other VS (e.g., "scum," energy storage compounds, and proteins), cells, and carboxylic acids (typically in the nutrient-rich substrate (e.g., chicken manure)). Enzymes produced by the mixed-culture of acid-forming microorganisms hydrolyze polymers (e.g., cellulose and hemicellulose) into sugars, which are subsequently fermented into carboxylic acids, gases, cells, and other VS. Ash is assumed to be inert.

Previous works defined *conversion* as volatile solids (VS) digested per VS fed [5–7, 10, 25]. This paper presents a simpler definition of conversion that can be applied to any fermentation. *Reactants* are defined as non-acid volatile solids (NAVS). Thus, undigested

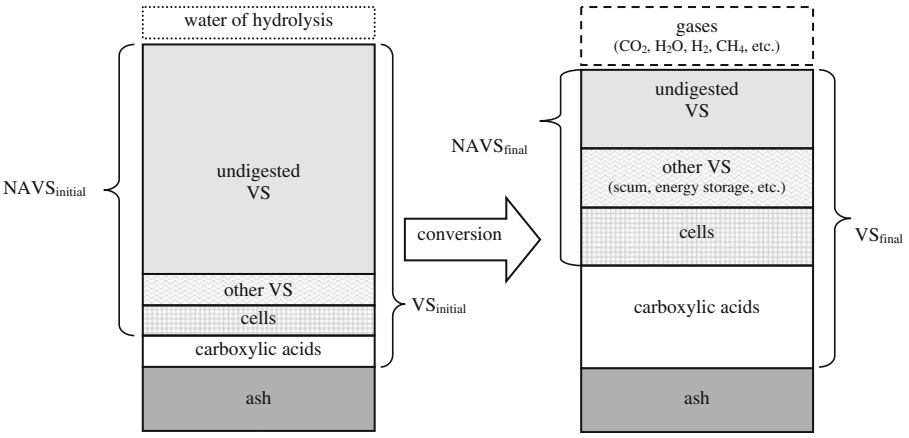

Fig. 2 The conversion of biomass

biomass, cells, extracellular proteins, energy-storage compounds, "scum," and all other NAVS are considered reactants. This definition simplifies a complicated reaction system into four quantifiable and industrially meaningful terms: water (solvent), ash (inert), acid (product), and NAVS (reactant). The water of hydrolysis may be estimated by assuming the biomass is predominately cellulose (monomer weight of 162 g/mol). When a cellulose monomer is hydrolyzed, it gains 1 mol of water.

$$water\ of\ hydrolysis(g) = NAVS_{consumed}(g) \times \frac{18}{162} \tag{1}$$

Analysis of the chicken manure used in this experiment shows that the feed contains a significant concentration of organic acids (~45 g/L_{liq}), which contributes 0.022 g acid/g NAVS fed. Without accounting for the carboxylic acids in the feed, selectivity is overstated, and yield is ill defined. This paper introduces four definitions of yield (Eqs. 10, 12, and 13) with respect to different points in the fermentation system: feed, exit streams, microbial culture, and product transfer liquid stream. The *exit yield* is identical to the yield calculated in previous works [5–8, 10, 12, 25, 26].

Referring to the labels defined in Fig. 1, the following terms are used in this paper:

$$\mathrm{NAVS_{feed}(g)} \equiv \text{sum of NAVS in } S_0, N_1, N_2, N_3, N_4, \text{and } L_5 \tag{2}$$

$$\mathrm{NAVS_{exit}(g)} \equiv \text{sum of NAVS in } S_4 \text{ and } L_1 \tag{3}$$

$$\mathrm{NAVS_{consumed}(g)} \equiv \mathrm{NAVS_{feed}} - \mathrm{NAVS_{exit}} \tag{4}$$

$$A_{\mathrm{feed}}(\mathrm{g}) \equiv \text{sum of carboxylic acid in } S_0, N_1, N_2, N_3, N_4, \text{and } L_5 \tag{5}$$

$$A_{\mathrm{exit}}(\mathrm{g}) \equiv \text{sum of carboxylic acid in } S_4, L_1, \text{and any liquid samples removed from F2 to F4} \tag{6}$$

$$A_{\mathrm{produced}}(\mathrm{g}) \equiv A_{\mathrm{exit}} - A_{\mathrm{feed}} \tag{7}$$

$$A_{L_1}(g) \equiv total\ carboxylic\ acid\ in\ L_1 \tag{8}$$

$$conversion \equiv C \equiv \frac{NAVS_{consumed}}{NAVS_{feed}} \tag{9}$$

$$yield_{feed} \equiv Y_{\mathrm{F}} \equiv \frac{A_{feed}}{NAVS_{feed}} \tag{10}$$

$$yield_{exit} \equiv Y_{\mathrm{E}} \equiv \frac{A_{exit}}{NAVS_{feed}} = Y_{\mathrm{F}} + Y_{\mathrm{C}} \tag{11}$$

$$yield_{culture} \equiv Y_C \equiv Y_E - Y_F \equiv \frac{A_{produced}}{NAVS_{feed}} = C \cdot E \tag{12}$$

$$yield_{process} \equiv Y_P \equiv \frac{A_{L_1}}{NAVS_{feed}} \tag{13}$$

$$total\ acid\ selectivity \equiv \sigma \equiv \frac{Y_C}{C} \tag{14}$$

$$total\ acid\ productivity(train) \equiv P \equiv \frac{A_{produced}}{TLV \times time} \tag{15}$$

where TLV (total liquid volume) is defined in Eq. 24.

Exit yield includes the carboxylic acid removed in product liquid (L_1), waste transfer solids (S_4), and liquid samples taken from Fermentors F2–F4. The cumulative amount of carboxylic acid in the samples is less than 1% of the total acid produced. NAVS is removed when sampling liquids and bulk material (unfiltered solids and liquid) were not quantified. The amount of NAVS in the sampling liquid is negligible, and bulk samples (~5 g) were taken only two times for C/N ratio analysis.

Measuring Performance

Slope Method

During the steady-state period, the flowrates (amount/day) of acid, ash, NAVS, water, and gas were determined. The fermentations trains were semi-continuous with material transfers performed three times per week. To determine the flowrate of a component, the moving cumulative sum of that component was plotted with time. The component flowrate (amount/day) was determined from the slope of the line. All performance variables were calculated from component flowrates determined by the slope method [27].

Determination of NAVS Consumed

The $NAVS_{consumed}$ is the difference between the NAVS in the inlet and exit streams. Assuming ash is inert, the ash flowrates in and out are equal. Based on this assumption, the difference between the dry material in the inlet and outlet streams results from the change in VS, not a change in ash. The $NAVS_{consumed}$ rate (g $NAVS_{consumed}$/day) may be determined by Eq. 16:

$$\begin{aligned} &NAVS_{consumed}\ \text{rate} = NAVS_{feed}\ \text{rate} - NAVS_{exit}\ \text{rate} \\ &= (\Sigma \text{dry solids}_{in} - \Sigma \text{ash}_{Iin} - \Sigma \text{acid}_{in}) - (\Sigma \text{dry solids}_{out} - \Sigma \text{ash}_{in} - \Sigma \text{acid}_{out}) \\ &= (\Sigma \text{dry solids}_{in} - \Sigma \text{acid}_{in}) - (\Sigma \text{dry solids}_{out} - \Sigma \text{acid}_{out}) \end{aligned} \tag{16}$$

where:

$$\text{dry solids in stream}\, X_i(g) = X_i(1 - M_{Xi}) \tag{17}$$

$$acid\ in\ stream\, X_i(g) = A_{X_i} = \frac{X_i[A]_{X_i} M_{X_i}}{\rho_W} \left(\frac{1\,L}{1,000\,mL} \right) \tag{18}$$

Operating Parameters

Liquid retention time (LRT; Eq. 19) quantifies the average time for liquid to travel through the system. LRT influences the product concentration ($[A]_{L1}$). Longer residence times allow for higher product concentrations.

$$LRT = \frac{TLV}{Q} \tag{19}$$

TLV is the total liquid volume expressed as

$$TLV = \sum_i \left(\frac{K_{Fi} M_{Fi}}{\rho_w} \left(\frac{1\,L}{1,000\,mL} \right) + L_{Fi} \right) \tag{20}$$

where

L_5, S_0, and N_i	Rates determined by the slope method (g/day)
K_{Fi}	The average mass of wet solid cake in Fermentor i (g)
L_{Fi}	The average volume of free liquid in Fermentor i (L)

Q is determined using Eq. 21 and the slope method:

$$\begin{aligned} Q &= \left(L_5 M_{L_5} + S_0 M_{S_0} + \sum_i N_i M_{N_i} \right) \frac{1}{\rho_w} \left(\frac{1\,L}{1,000\,mL} \right) \\ &= total\ inlet\ liquid\ flowrate(L/day). \end{aligned} \tag{21}$$

Previous works define the liquid flowrate as the product liquid exit F1 (L_1) and ignoring the liquid that exits with the waste solids (S_4). The LRT defined by Eq. 21 more accurately quantifies the average time liquid retained in the staged fermentation.

Volatile solids loading rate (VSLR) quantifies the reactant feed rate relative to the total liquid volume (i.e., normalized feed rate) and is defined as

$$VSLR = \frac{NAVS_{feed}\ rate}{TLV}. \tag{22}$$

VSLR is inversely related to conversion and yield [5, 6, 10]. As VSLR increases, NAVS have less time to digest, which lowers conversion and yield.

The NAVS concentration (SC_{Fi}; Eq. 23) is defined as the ratio of reactant in Fermentor Fi ($NAVS_{Fi}$) to the liquid volume in Fermentor Fi (LV_{Fi})

$$SC_{Fi} \equiv NAVS_{Fi}/LV_{Fi}. \tag{23}$$

Acid concentration is directly proportional to SC [28].

Steady-State Strategy

Transfer solids physically appear solid but have moisture contents of 0.70–0.85 g moisture/g total (wet; as-is) with all moisture fully absorbed in the biomass. Transfer liquids physically appear fluid but may have 1–5% suspended solids. Table 2 summarizes the operating parameters of the five trains described in this paper.

Before a transfer, each fermentor and its contents were centrifuged at 4,000 rpm. The liquid layer was decanted into a graduated cylinder and measured. The bottle and remaining

solid cake were weighed (B_i), where i equals the fermentor number. For F1, the amount of transfer solids fed (S_0) was constant. For subsequent fermentors (Fi), the transfer solids fed was equal to the transfer solids removed (S_{i-1}) from the previous fermentor plus the nutrient-rich substrate fed to that fermentor (N_i). The transfer solids retained in each fermentor were controlled by a solids-retained-plus-bottle-weight set point (W_i). The mass of transfer solids removed (S_i) was determined by a simple material balance ($S_i = B_i + S_{i--1} + N_i - -W_i$). For each train, the solids-retained-plus-bottle-weight set point for F1 was 200 and 300 g for F2 to F4. The set point for F1 was lower because fresh paper absorbed free transfer liquid added to F1. All decanted transfer liquid was transferred to the previous fermentor, as shown in Fig. 1.

Statistical Methods

To compare steady-state acid data, the two-tailed heteroscedastic Student's t test ("TTEST" function in Microsoft Excel 2007) with a confidence level of 5% was used to calculate P values. Unless otherwise stated, error bars represent a 95% confidence interval (two standard deviations). Sum-of-squared-errors techniques were used to determine the error of calculated values.

Results and Discussion

Experiment Overview

Five four-bottle trains were run with identical operating parameters (Table 2), each with a different nutrient-rich substrate-contacting pattern (Table 3). Many design parameters (e.g., SC, VSLR, LRT, substrates, solid–liquid separation efficiency, and number of stages) influence nutrient transport and fermentation performance. The interaction between these variables is complex; thus, the results of this study must be carefully interpreted and applied in context with operating parameters used.

Table 2 Operating parameters for nutrient-rich substrate contacting fermentations

	Fermentation train	1	2	3	4	P	AVG
Controllable	Temperature (°C)	40	40	40	40	40	40
	Frequency (T)	3 per week; every 56 h					
	$NAVS_{feed}$ rate (paper and manure) (g VS/T)	30.4	30.4	30.4	30.4	30.4	30.4
	Liquid feed rate (L_5) (mL/T)	300	300	300	300	300	300
	Solid-cake-plus-bottle-weight set point, F1 (g)	200	200	200	200	200	200
	Solid-cake-plus-bottle-weight set point, F2–F4 (g)	300	300	300	300	300	300
	Centrifuge liquid retained in F1–F4 (mL)	0	0	0	0	0	0
	Methane inhibitor (μL/T)	80	80	80	80	80	80
Normalized	VSLR (g NAVS/(L_{liq}·day))	7.5	6.7	6.8	7.1	7.0	7.0
	LRT (day)	13.6	15.2	15.0	14.2	14.6	14.5
	Avg. solid concentration (g NAVS/L_{liq})	57	49	48	55	53	52
	TLV (L)	1.75	1.96	1.93	1.83	1.87	1.87

T time period between transfers (~56 h)

Carbon–Nitrogen Ratio (C/N)

In biomethane fermentations, the carbon–nitrogen (C/N) ratio influences performance [17, 29]. Too much nitrogen may result in ammonium toxicity [30, 31], and too little nitrogen limits cellular activity; therefore, nitrogen control is necessary for optimum performance. For countercurrent mixed-acid fermentations, no models currently exist that describe nitrogen behavior.

For similar fermentations (methane and hydrogen), the literature cites a wide range of optimal C/N (10–90 g/g); 30 is the most cited optimum for producing carboxylic acids [24, 29, 30]. Because the C/N ratio is reported in a variety of units and there are conflicting scopes of research, a new study should be done to determine the optimum C/N ratio for mixed-acid fermentations. For the purpose of discussion and reference, this paper assumes 30 g C_{NA}/g N is the optimal C/N ratio.

Figure 3a shows the C/N ratio profile produced by each nutrient-rich substrate-loading pattern. Overall C/N ratio is defined as the sum of non-acid carbon (g C_{NA}) in all fermentors divided by the sum of total nitrogen (g N) in all fermentors. Train 1 produced the most even C/N profile with ratios slightly increasing in successive stages. Train 2 had a high C/N ratio (90 g C_{NA}/g N) in F1, but F2–F4 had C/N ratios very close to the optimum of 30 g C_{NA}/g N. Train 4 had the most uneven C/N profile. Trains 3, 4, and P had overall C/N ratios greater than the feed (39±1 g C_{NA}/g N), indicating distribution inefficiencies and/or gaseous nitrogen loss. Each train had one or more bottles with a C/N ratio above 30 g C_{NA}/g N indicating nitrogen limitations; thus, no train was fully optimized. Because nitrogen exists in soluble and insoluble forms traveling in both the transfer solids and transfer liquid streams, controlling C/N ratios in a countercurrent system is critical to maximizing performance.

Total Acid Productivity

Total acid productivity P is defined as the acid *produced* per liquid volume per day; thus, the acid contributed by the nutrient-rich substrate (chicken manure) is *not* included. Figure 3b shows the productivity profile of each train. Overall productivities are weighted averages with the total liquid volume of each bottle.

Although Trains 1 and 2 have virtually identical overall C/N ratios (37.6 and 38.5 g C_{NA}/g N, respectively), Train 2 had a much higher overall productivity (0.77 vs. 0.64 g acid produced/(L_{Liq}·day)). This resulted because Train 2 distributed nitrogen such that a greater percentage of its fermentation mass was closer to the optimum C/N ratio than Train 1. In contrast, Train P had a higher C/N profile (42.2 g C_{NA}/g N, overall) and a higher productivity (0.73 g acid produced/(L_{liq}·day)) than Train 1. This indicates the importance of non-nitrogen nutritional factors (e.g., phosphorus and minerals) and/or "freshness" of nutrient-rich substrate. F1 and F2 of Train 4 had similar C/N ratios around 170 g C_{NA}/g N

Table 3 The nutrient-rich substrate-loading pattern for Trains 1, 2, 3, 4, and P with the amount of wet chicken manure (g CM/*T*; on a dry basis) added to each fermentor (N_1–N_4)

	N_1	N_2	N_3	N_4
Train 1	8.0	–	–	–
Train 2	–	8.0	–	–
Train 3	–	–	8.0	–
Train 4	–	–	–	8.0
Train P	2.0	2.0	2.0	2.0

T time period between transfers (~56 h)

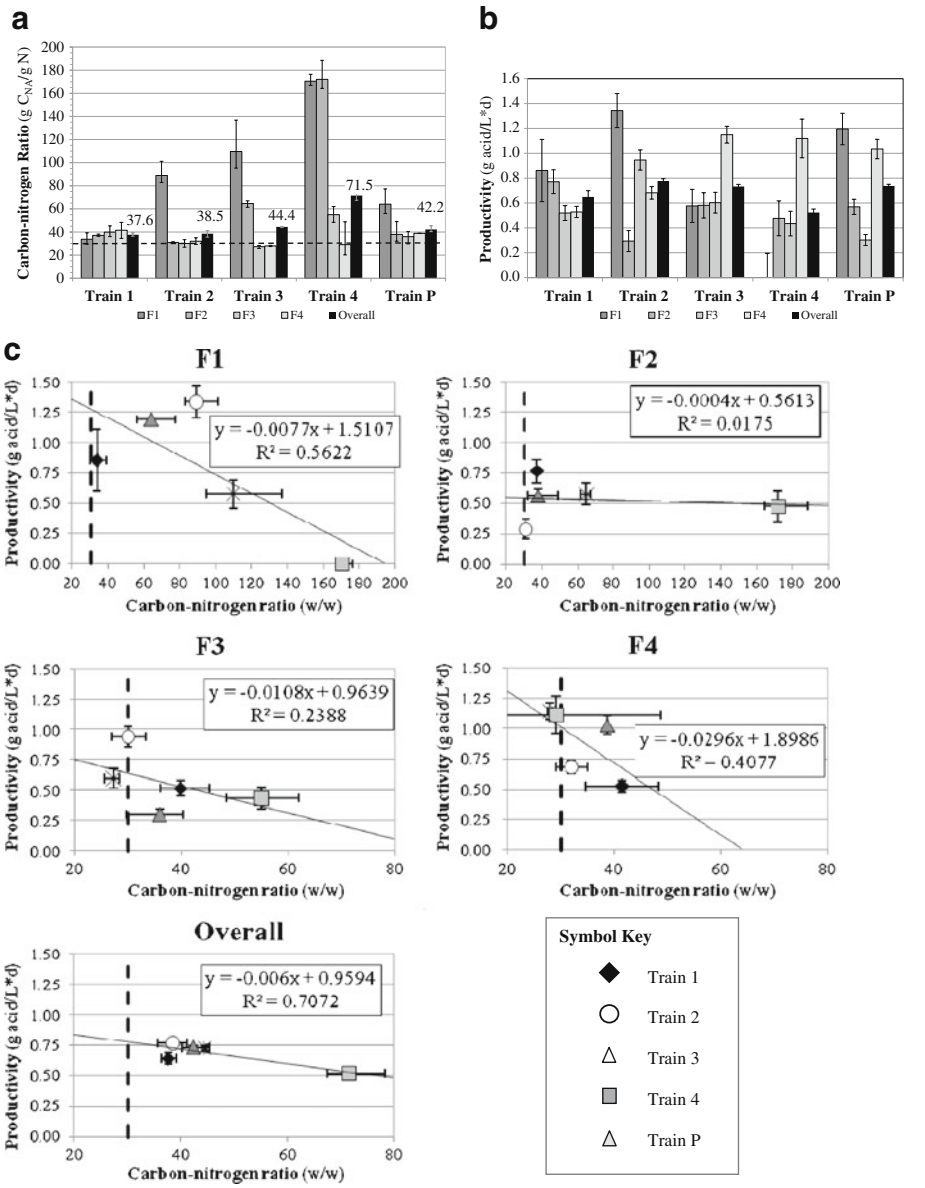

Fig. 3 **a** Carbon–nitrogen ratio profiles for each train. Carbon contributed by organic acid was excluded. Two profile samples were taken (days 138 and 162). The *error bars* represent the range. The average C/N ratio was determined by dividing the average non-acid carbon content by the average nitrogen content. The *dotted line* references the assumed optimum carbon–nitrogen ratio of 30 g C_{NA}/g N. **b** Productivity profiles for each train. Overall values represent the composite productivity of the train. **c** Correlation between productivity and C/N ratio for individual fermentors and train. The productivity *error bars* represent a 95% confidence interval (two standard deviations). The C/N ratio *error bars* represent the range. The *dotted line* references the assumed optimum C/N ratio of 30 g C_{NA}/g N

and similar steady-state acid concentrations around 13.8 g acid/L_{liq}. Despite receiving fresh paper, F1 of Train 4 had a productivity of zero, which indicates severe nitrogen and non-nitrogen nutrient limitations.

When comparing individual fermentors from each train, those that received the full amount of fresh nutrient-rich substrate *did not* have the highest productivity. A possible explanation for this phenomenon is the carboxylic acid content (not the nutrients) of the chicken manure caused product inhibition that reduced productivity. Figure 3c shows that total acid productivity depends on C/N ratio and increases as the C/N ratio approaches the optimum. The slope of the linear trend line indicates how sensitive a fermentor is to C/N ratio. F2 had the flattest slope indicating it was the least sensitive, whereas F4 had the steepest slope indicating the greatest sensitivity. This trend is understandable considering F4 contains the most recalcitrant biomass; thus, nitrogen is critical for digestion.

Further improvements in performance can be realized if optimal C/N ratios can be maintained in each fermentor. Using Fig. 3c to predict the productivity of each fermentor at a C/N of 30 g C_{NA}/g N suggests that overall productivities ranging from 0.83 to 0.99 g acid/($L_{liq.}$·day) could be obtained (VSLR=7 g NAVS/(L_{liq}·day) and LRT=15 day). If obtained, these productivities translate into culture yield improvements of 67–99% (0.138–0.165 g acid produced/g NAVS fed) versus Train 1.

Acid Concentration

Figure 4 shows the total acid concentration and aceq concentration in the product transfer liquid (L_1). Initially, the operating parameters did not produce transfer liquid from F1 because the paper-loading rate (S_0) was too high relative to the water throughput (L_5); there was no free liquid because all liquid was absorbed in the fresh paper. To correct this, the solids-retained-plus-bottle-weight set point for F1 (W_1) of each train was decreased from 300 to 200 g (day 20), and the water fed per transfer was increased from 175 to 300 mL per (day 27). Thus, the noise/peak prior to steady state resulted from very high initial solids concentrations.

To compare acid concentrations with different compositions of different molecular weight volatile fatty acids, acid concentrations are converted to acetic acid equivalents (aceq). Calculation of performance variables on an aceq basis allows comparison of the energy content of the carboxylic acid products. Train 2 had the highest average steady-state acid concentration (21.3 g/$L_{liq.}$) with Trains 1, 3, 4, and P having concentrations of 20.9, 18.7, 13.9, and 20.2 g/$L_{liq.}$, respectively. The Student's *t* test showed that Train 2 was not significantly different than Train 1 (*P* value=0.162). Train 1 had the highest average steady-state aceq concentration (28.0 g/$L_{liq.}$) with Trains 2, 3, 4, and P having concentrations of 27.2, 25.6, 18.2, and 26.1 g/$L_{liq.}$, respectively. Trains 1, 2, 3, and P had similar total acid and aceq product concentrations, indicating that the nutrient-rich substrate-loading pattern did not significantly affect product concentration.

The ratio of aceq concentration to total acid concentration for Trains 1, 2, 3, 4, and P is 1.33, 1.28, 1.37, 1.31, and 1.29, respectively. Train 3 has a higher ratio than the other four trains, indicating it produced more high molecular weight acids.

Yield

The exit, culture, and process yields were greatly influenced by the nutrient-rich substrate-loading pattern (Fig. 5a). The exit yield Y_E (Eq. 11) includes acid in the product transfer liquid, waste transfer solids, and liquid samples taken from F2 to F4. The exit aceq yield for

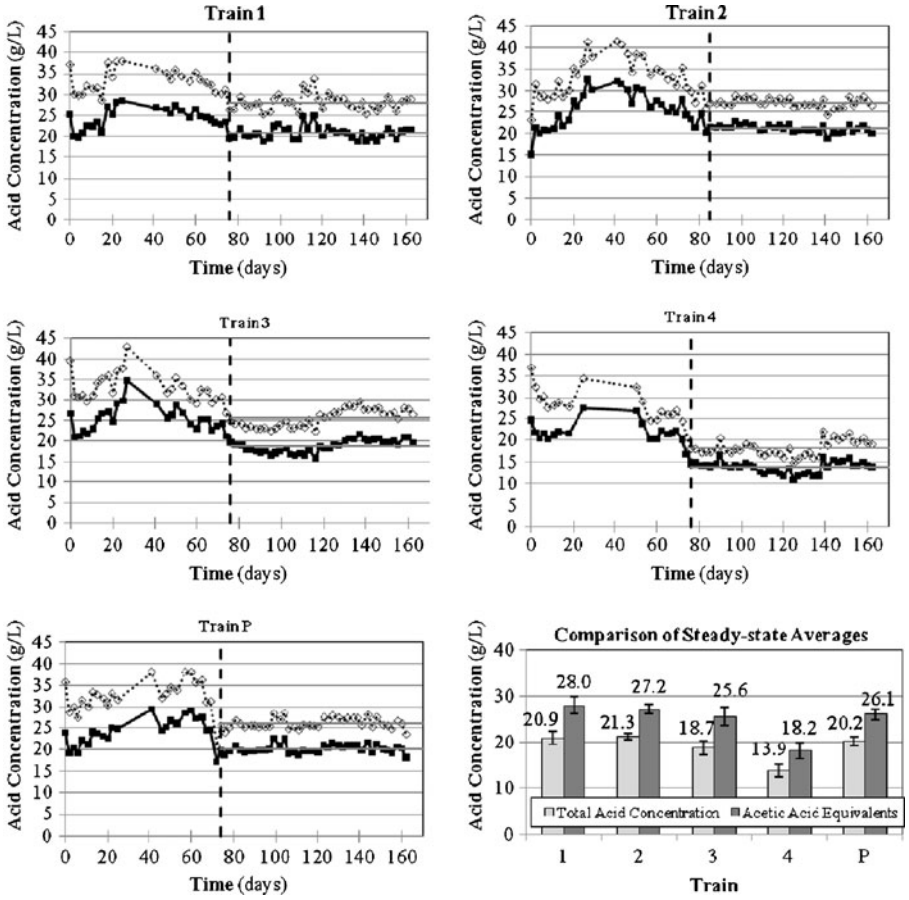

Fig. 4 Total acid concentration (*squares*) and acetic acid equivalence (*diamonds*) concentration plots. The steady-state region begins at the *vertical dotted line* with the steady-state average indicated by the *horizontal gray line*. *Error bars* represent one standard deviation

Trains 1, 2, 3, 4, and P were 0.140, 0.177, 0.183, 0.129, and 0.166 g aceq/g NAVS fed, respectively. Trains 2, 3, and P had exit aceq yields higher than Train 1 by 27%, 31%, and 19%, respectively.

Trains 2 and 3 had statistically identical exit yields (0.138 and 0.137 g acid produced/g NAVS fed, respectively) with Trains 1, 4, and P having yields of 0.106, 0.109, and 0.125 g acid/g NAVS fed, respectively. Trains 2, 3, 4, and P had exit yields higher than the traditional nutrient-rich substrate addition method (Train 1) by 31%, 30%, 3%, and 19%, respectively.

The culture yield Y_C (Eq. 12) represents the acid *produced* by the microbial cultures, which is equal to the exit yield minus the feed yield. The culture yield Y_C for Trains 1, 2, 3, 4, and P were 0.083, 0.116, 0.114, 0.087, and 0.103 g acid produced/g NAVS fed, respectively. Trains 2, 3, 4, and P had higher culture yields than Train 1 by 39%, 38%, 4%, and 24%, respectively.

This paper introduces *process yield* Y_P (Eq. 13), which quantifies *only* the acid in the product transfer liquid (L_1) but *not* the acids in the waste transfer solids (S_4). The process

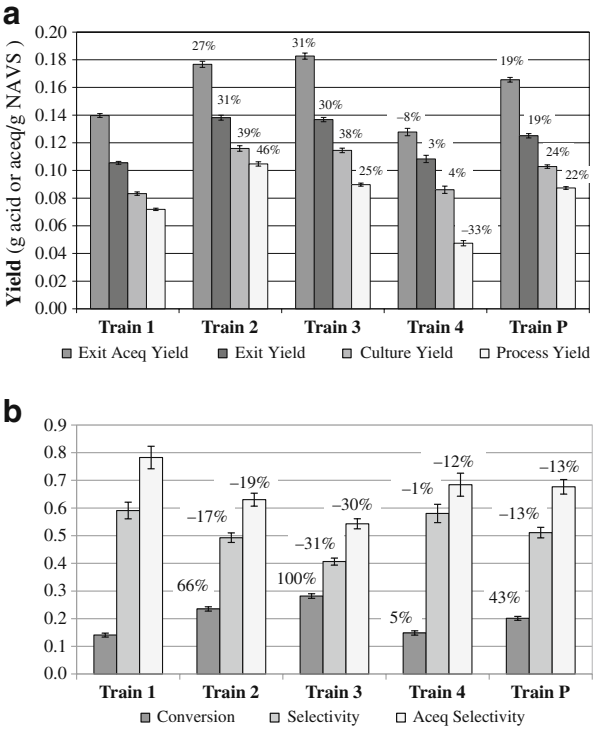

Fig. 5 **a** Comparison of yield values for each train. The *error bars* represent a 95% confidence interval (two standard deviations). The *percentage above each bar* is the improvement relative to Train 1. The feed yield Y_F equals 0.022 g acid/g NAVS fed. **b** Overall conversion and total acid selectivity for steady-state region. The *error bars* represent a 95% confidence interval (two standard deviations)

yield is of interest because it quantifies the net yield of acid that is sent downstream for concentration and further processing. In a commercial operation, recovering acids from waste transfer solids requires a countercurrent wash. Because the recovered acid is dilute, it will be returned to Fermentor F4. The liquid flows countercurrently relative to the solids, so the recovered acids eventually exit Fermentor F1 and become part of the product transfer liquid (L_1), thus increasing the process yield. In this experiment, no steps were taken to recover acid in the waste transfer solids (S_4) and return it to the fermentation; thus, the reported process yields represent the lower process yield limit. The process yield for Trains 1, 2, 3, 4, and P are 0.072, 0.105, 0.090, 0.048, and 0.088 g acid/g NAVS fed, respectively. Trains 2, 3, and P had higher process yields than Train 1 by 46%, 25%, and 22%, respectively.

The *exit yield* Y_E represents *all* the acid exiting the fermentation. If the acids in the waste transfer solids (S_4) are countercurrently washed with 100% recovery and the acids are returned to Fermentor F4 but impose no additional product inhibition, then all the acids will exit in the product transfer liquid (L_1). In this ideal scenario, the exit yield represents the theoretical upper limit of process yield.

The *process–exit yield ratio* (PE ratio; Eq. 27) quantifies the fraction of acid recovered in the product transfer liquid.

$$\text{Process–exit(PE)yield ratio} \equiv \frac{Y_P}{Y_E} \tag{27}$$

If all acid is recovered from the waste transfer solids, the PE ratio equals 1. The PE ratio for Trains 1, 2, 3, 4, and P were 0.682, 0.761, 0.658, 0.440, and 0.699, respectively. The PE

ratios of Trains 2 and 4 were significantly different than Trains 1, 3, and P; thus, PE ratio depends on the nutrient-rich substrate-loading pattern. This behavior results from (1) acid in the nutrient-rich substrate fed and (2) changes in solid–liquid separation, which is affected by the extent of digestion. Additionally, the PE ratio (without recovery of acid in waste transfer solids) depends on the solid–liquid separation efficiency and the relative flow rates of solids and liquids.

Although Trains 2 and 3 had the best yields, no single-loading pattern should be used generically as an optimum pattern. The nitrogen properties of the feedstocks, the operating parameters, the solid–liquid separation efficiency, and the nutrient-rich substrate-loading pattern influence the behavior of nitrogen in a countercurrent fermentation, which dictates performance. Based on this paper, Smith and Holtzapple [32] introduced a segregated-nitrogen model that may be used to predict/optimize nitrogen concentration profiles for a four-bottle train.

Nutrient-rich feedstocks (e.g., sewage sludge, and manure) can contain significant concentrations of organic acids. Characterizing the yield with respect to the feed, exit streams, microbial culture, and product transfer liquid provides greater insight and context to fermentation performance.

Conversion and Selectivity

Conversion and selectivity are shown in Fig. 5b. Trains 1, 2, 3, 4, and P had conversions (Eq. 9) of 0.141, 0.235, 0.282, 0.149, and 0.201 g NAVS consumed/g NAVS fed, respectively. Trains 2, 3, and P had conversions much higher than Train 1 by 66%, 100%, and 43%, respectively. The greatest digestion occurs when both F3 and F4 had near-optimum C/N (25–35 g C_{NA}/g N), which provided nitrogen necessary to digest the most recalcitrant biomass. Train 3 had the highest conversion because it benefits from both near-optimum C/N ratios in F3 and F4, and fresh nutrient-rich substrate fed to F3. Train 2 had the second highest conversion and benefitted from near-optimum C/N in F2–F4. Train P had higher C/N ratios (~38 g C_{NA}/g N) in F2–F4, but each fermentor received fresh manure.

Selectivity (Eq. 14) quantifies the microbial efficiency by reporting the ratio of acid *produced* in fermentation per mass of NAVS consumed; thus, it is equal to the culture yield (Eq. 12) divided by conversion (Eq. 9). Trains 1, 2, 3, 4, and P had selectivities of 0.590, 0.492, 0.406, 0.583, 0.511 g acid produced/g NAVS consumed, respectively. Trains 1 and 4 had the highest selectivities, which were statistically similar. Trains 1, 2, 3, 4, and P had aceq selectivities of 0.782, 0.632, 0.544, 0.688, and 0.677 g aceq/g NAVS consumed, respectively. No train had a selectivity or aceq selectivity higher that Train 1. Note, the higher selectivities and aceq selectivities *do not* correspond with the trains that had the highest yields or highest conversion. This observation supports the hypothesis that nutrient-limited environments increase selectivity because stoichiometric ratios are unavailable to create carbon-rich products (e.g., cells and enzymes) that are non-metabolites.

Conclusions

Acid in the feed proportionately reduces the fermentations total acid productivity. Performance improves as the C/N ratio of each fermentor approaches the optimum (~30 g C_{NA}/g N). Non-nitrogen nutrients and/or freshness of nutrient-rich component are

critical to optimum performance. Nutrients are most critical in the latter stages. To achieve maximum yields, a model is needed that describes nitrogen flow such that optimal C/N ratios may be controlled.

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