COMPARING ETHANOL PRODUCTION OF CARBOXYDOTROPHIC *CLOSTRIDIUM* STRAINS DURING SYNGAS FERMENTATION WITH A TWO-STAGE CONTINUOUS CULTURE

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ABSTRACT

Several strains of anaerobic bacteria in the genus *Clostridium* can convert syngas, which consists of CO, H_2, and a small amount of CO_2, into ethanol *via* the Wood-Ljungdahl pathway in a process called syngas fermentation. Process optimization to increase production rates, improve gas/liquid mass transfer, and to control product ratios is important to render the process efficient and economically viable.

Strain differences in growth and product concentrations have been reported in the literature, yet experiments varied in reactor design, medium, batch *vs.* continuous fermentation, pH, syngas composition, and other parameters. To directly compare the productivity of these biocatalysts, some of which are indistinguishable based on 16S rRNA phylogeny, *C. ljungdahlii* ERI-2, *C. ljungdahlii* PETC, and *C. autoethanogenum* JA1-1 were separately cultured in a long-term continuous syngas fermentation system optimized for ethanol production. The pH was lowered during the fermentation runs in a controlled manner in an attempt to induce solventogenesis.

For each strain, duplicate fermentation runs were conducted, and for all strains the results of these duplicates runs were reproducible. A shift to solventogenesis was not achieved with JA1-1 and lowering the pH adversely affected its growth and CO consumption. ERI-2 and PETC performed similarly. Ethanol production rates for ERI-2 and PETC were correlated to biocatalyst density more so than pH, whereas acetate production rates for these strains decreased when pH was lowered. The highest average ethanol production rate of 0.301 g/L/h was generated with PETC at pH 4.5 with a corresponding 19 g/L ethanol concentration and 5.5:1 ethanol/acetate ratio.

Carbon conversion to ethanol for ERI-2, PETC, and JA1-1 was 70%, 75%, and 28% of theoretical maximum, respectively. A minimum mass transfer coefficient (K_La) of 40 h^{-1} was calculated for our second-stage bubble column when considering the maximum possible gas/liquid driving force; however, an apparent K_La value of 190 h^{-1} was found to be representative of normal continuous operation. The results provide information on long-term (>700 h), two-stage continuous syngas fermentation using a bubble column reactor, and on the dissimilar effect of pH 4.5 on *C. ljungdahlii* and *C. autoethanogenum* growth and ethanol/acetate production.

Keywords: second-generation biofuel, biomass, renewable energy, syngas fermentation, continuous fermentation, two-stage fermentation, *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, ethanol, acetate, 2,3 butanediol, carbon monoxide, mass transfer

BIOGRAPHICAL SKETCH

Michael Martin has spent half of his life in Ithaca, New York, including middle school and high school in nearby Lansing, college at Cornell University where he majored in the interdisciplinary subject of Biology & Society, and now graduate school in the Department of Biological & Environmental Engineering at Cornell University.

During the period between undergraduate and graduate school, Michael worked as a litigation legal assistant at Davis Polk and Wardwell LLP in Manhattan for a couple of years and then backpacked for just shy of a year around Southeast Asia, Myanmar, India, and Nepal. Traveling abroad spurred him to develop his analytical and technical skills in engineering and applied science to be more capable of addressing global problems.

He is passionate about sustainability and inspired by the elegance of biology. Harnessing the abilities of microbes to design biotechnological solutions for energy, agricultural, and urban development issues is his inspiration for pursuing a Master of Science degree in Biological & Environmental Engineering.

Michael will be moving to Chicago in August 2014 where he will continue his research on syngas fermentation as a fermentation scientist at LanzaTech.

DEDICATION

To my mother and father for their loving support of my formal and informal educational endeavors.

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CHAPTER 1: INTRODUCTION TO AND GENESIS OF RESEARCH PROJECT

Africa's population is projected to more than double in the next 40 years to 2.4 billion people. Sustainable enterprises to support livelihood and improve standard of living will be essential in the coming decades. The Village-Scale Pyrolysis to Liquid Fuels project at Cornell University aims to address this issue by developing a technology platform that will sustainably produce biochar for use as a soil amendment in addition to liquid fuel for use in household cooking and lighting. Numerous potential benefits include increased agricultural sustainability, reduced demand for synthetic fertilizers, cleaner cooking methods with less particulate matter and soot, and reduced demand for petroleum liquid fuels, such as kerosene, with high carbon footprints.

The goal is to develop a slow pyrolysis kiln that can convert biomass and organic waste matter into biochar and gases. The gas mixture that is produced is a specific type of producer gas called synthesis gas or 'syngas'. The term 'producer gas' refers to a mixture of combustible and non-combustible gases including carbon monoxide (CO), hydrogen (H_2), methane (CH_4), nitrogen (N_2), and carbon dioxide (CO_2), whereas the name syngas is used for a producer gas mixture that only consists of the combustible gases CO and H_2 (although a small amount of CO_2 may sometimes be present). This syngas can be processed *via* fermentation into ethanol and, with further research, butanol and other fuels and chemicals.

A collaboration at Cornell University consisting of academics in the departments of Applied Economics and Management, Biological and Environmental Engineering, Crop and Soil Sciences, and Mechanical and Aerospace Engineering, are working together on various aspects of this ambitious project in such areas as household surveys and economic impact modeling,

biomass sourcing and feedstock modeling, pyrolysis kiln design and combustion modeling, and soil fertility impact modeling with biochar characterization. Understanding, optimizing, and constructing the fermentation process for conversion of syngas into fuels is the responsibility of the Angenent Lab in the Department of Biological and Environmental Engineering.

Our initial objective was to develop a platform for syngas fermentation that yielded ethanol as the major product and to adapt the strains of interest to grow in a totally defined, cheap medium. Next, we compared several of the most prominently used strains for syngas fermentation under the same conditions to determine the most promising biocatalyst with which to continue working. Various carboxydotrophic, homoacetogenic *Clostridium* strains have been used to research syngas fermentation, but a side-by-side comparison of different strains in an optimized fermentation system had not been conducted, and generally only ethanol concentrations are reported in the literature, with few papers reporting specific ethanol production rates. Here, we used ethanol concentrations, ethanol-to-acetate ratios, specific productivities, and stoichiometry of CO conversion to ethanol to compare strains at pH 4.5-5.5.

Finally, with the results from the performance comparison, we sequenced, assembled, analyzed, and compared the genomes to determine possible leads for explaining differences in observed growth and product formation (on-going at time of thesis defense).

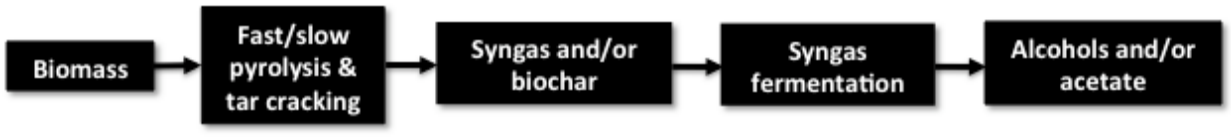

Figure 1: Overall biomass to liquid fuel conversion process from pyrolysis to distillation.

CHAPTER 2: BACKGROUND AND LITERATURE REVIEW ON SYNGAS FERMENTATION TO ETHANOL

Introduction

Harnessing microbial biotechnology to produce liquid fuels and chemicals from biomass has a long history, dating to the 19th century with Louis Pasteur researching acetone-butanol-ethanol (ABE) fermentation in 1861 (Kumar and Gayen 2011). During World War I, ABE fermentation using *Clostridium acetobutylicum* was used as an industrial process for producing acetone and butanol for military purposes and continued to be the main source for these products until the 1950s, when cheaper petroleum-derived equivalents prevailed (Worden et al. 1991). Interest in biofuel research again rose in the 1970s as a reaction to the Organization of Petroleum Exporting Countries (OPEC) oil embargo on the United States and other Western countries. In 1974, with petroleum prices at record highs, scientists in Germany started to discuss microbial routes for converting biomass into fuels similar to those derived from petroleum (Dürre and Richard 2011).

Today, biomass conversion to liquid fuels is again an active area for research due to current ambiguity over the extent of crude oil available in the world, increasing expense and risk mining for oil in extreme locations, interest in diversifying energy supply with renewable sources, and growing environmental awareness about climate change due to CO_2 emissions. Scientists are researching microbial metabolic routes for fermenting biomass substrates into products such as ethanol, butanol, biodiesel, methane, and hydrogen (Dürre and Richard 2011).

In addition, governmental policies are pressing for change toward a renewable, sustainable energy infrastructure. In the United States, Congress passed the Energy Independence and

Security Act of 2007, which requires renewable fuel, namely bioethanol, to be blended into transportation fuel as a fuel extender, with a mandate to blend 36 billion ethanol-equivalent gallons by 2022. Of these, 21 billion gallons must come from non-cornstarch products. On June 2, 2014, under executive order from President Obama, the Environmental Protection Agency (EPA) proposed the Clean Power Plan that would create state-specific rate-based goals for CO_2 emissions cuts from the fossil-fuel power sector, particularly from coal power plants. The European Union's Renewables Directive, 2009/28/EC, mandates 10 percent of the EU transport sector's energy come from renewable sources by 2020. In Brazil, the minimum ethanol content for transportation fuel is currently 20 percent.

First and Second Generation Ethanol Fermentation

Ethanol is a high-octane (Research Octane Number of 109 *vs.* 91 for gasoline) and renewable fuel that can be used on its own to run automobiles. When used as a fuel additive, ethanol eliminates the need to use the environmental pollutant methyl tertiary butyl ether (MTBE) as an oxygenate to raise the octane number of gasoline and prevent engine knocking (Abubackar et al. 2011). First-generation ethanol production uses yeast to ferment sugars (monosaccharides) and starches into ethanol, which is the same fermentation process used for centuries to produce alcoholic beverages. Unlike aerobic bioconversion, in an anaerobic fermentation no reducing equivalents are lost to molecular oxygen (Worden et al. 1991). The most common sources for the required sugar for first-generation biofuel production are corn and sugarcane. First generation ethanol production using yeast fermentation is a commercialized process and ethanol yields are high (425 L/tonne biomass) (Naik et al. 2010). However, because of the massive amounts of sugar and starch needed for commodity biofuel production, competition for land to

grow food and animal feed *vs*. biomass for fuel production has become a concern, as have the water, fertilizer/pesticide, and energy inputs for growing these monoculture crops.

Traditionally, second-generation biofuel was a label used to describe ethanol fermentation from sugars enzymatically derived from cellulosic materials. By using cellulosic material in addition to sugars and starches, a much greater proportion of a plant's biomass can be used and there is greater flexibility in feedstock. This is a desirable advancement, which has been commercialized as of 2013 (Brown and Brown 2013), because lignocellulose is the most abundant renewable organic matter on earth (Abubackar et al. 2011). Approximately 200 million dry tons of lignocellulosic biomass is produced in the U.S. alone per year, which is enough to produce 16 billion gallons of ethanol (Geddes et al. 2011).

However, the biochemical processes using yeast or engineered *E. coli* to ferment cellulosic material still requires access to the sugars. Therefore, the process requires extensive pretreatment steps to free fermentable monosaccharides in addition to mechanical separation of lignin that cannot be fermented and this is a major cost component of the overall process (Sims et al. 2010). One common pretreatment method is with dilute sulfuric acid or phosphoric acid, which demands large quantities of water and even exotic metal bioreactors resistant to corrosion (Geddes et al. 2011). Some other feasible pretreatment methods are steam explosion and ammonia fiber expansion (AFEX) (Brown and Brown 2013; Lynd et al. 2002). A major issue is that inhibitory compounds are formed during pretreatment that impede enzymatic activity and downstream fermentation (Geddes et al. 2011; Sanchez and Cardona 2008). Pretreatment is followed by cellulose hydrolysis requiring a mix of expensive cellulase enzymes (Geddes et al.

2011; Piccolo and Bezzo 2009) and, finally, ethanol fermentation. Importantly, this process can still not utilize the lignin content of biomass for ethanol production, which can account for up to 40% of plant biomass (Table 1) (Abubackar et al. 2011; Sun and Cheng 2002).

Table 1
The contents of cellulose, hemicellulose, and lignin in common agricultural residues and wastes[a]

Lignocellulosic materials	Cellullose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40–55	24–40	18–25
Softwood stems	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Paper	85–99	0	0–15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15–20	80–85	0
Cotton seed hairs	80–95	5–20	0
Newspaper	40–55	25–40	18–30
Waste papers from chemical pulps	60–70	10–20	5–10
Primary wastewater solids	8–15	NA[b]	24–29
Swine waste	6.0	28	NA[b]
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

[a] Source: Reshamwala et al. (1995), Cheung and Anderson (1997), Boopathy (1998) and Dewes and Hünsche (1998).
[b] NA – not available.

Table 1: Contents of cellulose, hemicellulose, and lignin in common agricultural residues

Recent technologies that can utilize the lignin matter of biomass in addition to cellulose and hemicellulose are also being categorized as second-generation biofuels (Naik et al. 2010; Sims et al. 2010). These emerging technologies might be better described as third-generation biofuels to distinguish them from processes that only make use of cellulose, although this label has been alternatively ascribed to algal biofuels and processes using CO_2 as feedstock. Therefore, second-generation ethanol production may be considered to comprise several technologies that, besides just enzyme-derived sugars from cellulose and hemicellulose, have the benefit of being able to use almost all of lignocellulosic biomass (cellulose, hemicellulose, and lignin). These technologies include biochemical, thermochemical, and hybrid processes (Daniell et al. 2012; Munasinghe and Khanal 2010a).

Gasification and Pyrolysis

One method that enables utilization of all the carbon in plant matter or any organic matter is to gasify it *via* gasification or pyrolysis. Gasification of biomass is a thermochemical process where the organic matter is converted into a mixture of fuel gasses by heating to high temperatures (600-1300°C) with a limited amount oxygen, steam, or air (oxidizing agent) (Griffin and Schultz 2012). It involves several steps: 1) moisture removal (dehydration) at 100-200°C; 2) pyrolysis at 200-300°C to produce char, volatiles, and tars; 3) oxidation (combustion) where carbon volatiles react with oxidizing agent to produce CO_2, steam, and considerable heat; and 4) reduction (gasification) where organic material (char and carbonaceous gasses) reacts with steam in the absence of oxygen to produce CO, H_2, and ash (Daniell et al. 2012).

Pyrolysis is a thermochemical process that converts biomass or any organic matter into char (solid), bio-oils (liquid), tars, and fuel gasses by heating to a relatively low temperature in the absence of oxygen as compared to gasification (Naik et al. 2010). Pyrolysis can be achieved at varying temperatures and residence times, including slow (275-675°C), fast (575-975°C), and flash pyrolysis (775-1025°C), to produce different ratios of biochar to fuel gasses and different gas compositions (Naik et al. 2010). Slow pyrolysis of biomass to produce syngas, as opposed to gasification, offers the advantages of lower process temperature, lower capital costs, and of concurrently producing biochar, which has value as a soil amendment to improve plant productivity, sequester carbon, and reduce nitrous oxide emissions (Cayuela et al. 2013). One disadvantage is less gas production than gasification. Often a high temperature tar-cracking step is necessary to convert tars into gasses, followed by gas cleaning (Abubackar et al. 2011; Griffin and Schultz 2012). The most important difference between gasification and pyrolysis is that

gasification occurs in an oxygen-limited environment whereas pyrolysis occurs in an environment completely absent of oxygen.

The producer gas from pyrolysis, after tar cracking, is comprised of CO, H_2, and only a small proportion of CO_2, and is called syngas. Syngas offers great feedstock flexibility because it can also be produced from gasification of different substrates such as organic waste, coal, or steam reforming natural gas. Its main components, CO and H_2, are also the main off-gasses of steel mills (CO only), petroleum refineries, and other industrial processes such as coke, methanol, and carbon black production (Gaddy 2002). Syngas can then be converted to liquid fuels through chemical or biological means. Unlike ethanol fermentation processes that only use sugars from starches (first generation) or from cellulosic material (second generation), processes using syngas as a feedstock are able to use biomass in its entirety, including lignin.

Fischer-Tropsch Synthesis

The thermochemical second-generation biofuel process that is based on Fischer-Tropsch (F-T) synthesis relies on chemical reactions to convert CO and H_2 into liquid hydrocarbons in the presence of a metal catalyst. The process is well understood and has been used since the 1930s to produce liquid hydrocarbon fuels from non-biomass syngas (Daniell et al. 2012; Naik et al. 2010). An advantage of F-T is that it has higher conversion rates than bioconversion. It allows for conversion of syngas into a variety of products in addition to ethanol such as methanol, methane, and heavy waxes (Abubackar et al. 2011). Because the F-T cycle has lower selectivity than a biocatalyst, various undesirable by-products are formed along with hydrocarbons, which often mandates expensive separation (Daniell et al. 2012). F-T is energy-intensive because it

requires high temperatures and pressure, so it greatly benefits from economies of scale. F-T depends on expensive and sensitive metal catalysts including iron, cobalt, and rhodium, that can be deactivated or 'poisoned' by tars and contaminants like sulfur if the syngas is not well cleaned (Abubackar et al. 2011; Henstra et al. 2007). For maximum productivity, a fixed composition ratio is normally required for the syngas, which can be difficult when using multi-sourced and changing lignocellulose feedstock. A water-gas shift step can correct the gas ratio but it is energy intensive (Daniell et al. 2012).

Syngas Fermentation

A hybrid technology of thermo- and bio-chemical processes for conversion of biomass to liquid fuel is syngas fermentation. Syngas fermentation to ethanol depends on acetogens that use the syngas substrate as a sole source of electrons and carbon to produce acetate and ethanol. In addition to its potential for producing ethanol from biomass, syngas fermentation shows promise as a platform for producing higher-value bio-based chemicals such as *n*-butanol and 2,3 butanediol. With improvements in reactor design, gas/liquid mass transfer, and advances in genetically engineering the biocatalysts, syngas fermentation has the potential to be used in industrial applications, and several companies including LanzaTech, Coskata, and INEOS Bio are working to commercialize the process.

Because syngas fermentation can utilize lignin, biomass to ethanol energy conversion efficiency of about 50-57 percent is possible (Clausen and Gaddy 1992; Griffin and Schultz 2012), compared to about 30-35 percent for acid hydrolysis/fermentation processes (Clausen and Gaddy 1992; Sims et al. 2010). The actual yield depends on the syngas composition, mass transfer, and

on energy required for gasification, distillation, and other processing. Syngas fermentation is also more tolerant than F-T to syngas impurities such as hydrogen sulfide (Klasson et al. 1993; Klasson et al. 1992b), although syngas contaminants such as nitrogen oxide, ammonia, and tars can still adversely effect growth and product formation in syngas fermentation (Xu et al. 2011). Other syngas fermentation advantages include high biocatalyst specificity, production of only acetate and ethanol, which simplifies downstream separation, tolerance to varying H_2/CO ratios, constant regeneration of the biocatalyst, and ambient process pressure and near ambient process temperature (Clausen and Gaddy 1992). Roadblocks in process optimization, mass transfer, product inhibition, and low production rates must still be overcome.

Organisms

The bacteria that have the ability to ferment syngas are called carboxydotrophic homoacetogens. Carboxydotrophs consist of aerobic and anaerobic bacteria that have the ability to obtain energy by oxidation of CO, and include acetogens, sulfate-reducing, and methanogenic bacteria that produce acetate, hydrogen sulfide, and methane, respectively (Meyer and Schlegel 1983; Svetlichny et al. 1991). Drake et al. offered a definition of acetogen as an anaerobe that use the reductive acetyl-CoA pathway as a mechanism for the synthesis of acetyl-CoA from CO_2, the fixation (assimilation) of CO_2 in the synthesis of cell carbon, and as a terminal electron-accepting and energy-conserving process (2006). It is common for the term 'homoacetogen' to refer to bacteria that utilize the reductive acetyl-CoA pathway regardless of end-products. Although the term implies that acetate is the only end-product, homoacetogens have been shown to synthesize other reduced compounds (e.g., ethanol, butyrate, lactate, succinate) under various conditions (Drake et al. 2006).

Syngas fermentation requires microorganisms that can grow chemolithoautotrophically with CO to produce organic compounds. Mesophilic microorganisms that have been used for syngas fermentation include *Acetobacterium woodii, Eubacterium limosum (Butyribactericum methylotrophicum), Clostridium aceticum, Clostridium autoethanogenum, Clostridium leatocellum SG6, Clostridium ljungdahlii, Mesophilic bacterium P7 (Clostridium carboxidivorans), Oxabactor pfennigii,* and *Peptostreptococcuc productus* (Henstra et al. 2007; Munasinghe and Khanal 2010a). Reports of six species of bacteria that produce ethanol with CO as their sole carbon source have been published; they are *C. ljungdahlii*, C. *carboxidivorans, C. autoethanogenum, E. limosum*, *C. ragsdalei*, and *Alkalibaculum bacchi* (Henstra et al. 2007; Wilkins and Atiyeh 2011).

Of these anaerobic carboxydotrophic microorganisms, the most common for syngas fermentation to ethanol are in the genus *Clostridium*. All are strict anaerobes, gram-positive, rod-shaped, and motile (Munasinghe and Khanal 2010a). *C. ljungdahlii* and *C. autoethanogenum* exclusively produce acetate and ethanol from syngas fermentation, unlike *C. carboxidivorans*, which also produces butanol (Henstra et al. 2007; Rajagopalan et al. 2002). In addition, proprietary strains have also been used for syngas fermentation to ethanol, including *C. ragsdalei* (Huhnke et al. 2008; Kundiyana et al. 2010), *C. coskatii* (Zahn and Saxena 2012), and *C. ljungdahlii* C-01 (Gaddy et al. 2004).

Wood-Ljungdahl Pathway

In syngas fermentation to ethanol, carboxydotrophic homoacetogens like those described above ferment CO and/or CO_2 and H_2 to acetyl-CoA *via* the reductive acetyl-CoA pathway, also called the Wood-Ljungdahl pathway. This biochemical pathway was likely used 3.8 billion year ago by the first autotrophs in an oxygen-limited environment and is still likely restricted to anaerobes (Henstra et al. 2007). As mentioned, CO can serve as the sole source of carbon and electrons in this fermentation, or combinations of CO/H_2 or CO_2/H_2 can serve as substrates. The stoichiometric equations for ethanol production *via* the Wood-Ljungdahl pathway are given below, with equations (2) and (3) demonstrating the increasing theoretical carbon conversion to ethanol with increasing H_2/CO ratio (though less energetically favorable):

$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2$ $\Delta G^{\circ\prime}$= -224 kJ/mol (1)
[1/3 CO conversion to EtOH]

$3CO + 3H_2 \rightarrow C_2H_5OH + CO_2$ $\Delta G^{\circ\prime}$= -164 kJ/mol (2)
[2/3 CO conversion to EtOH]

$2CO + 4H_2 \rightarrow C_2H_5OH + H_2O$ $\Delta G^{\circ\prime}$= -144 kJ/mol (3)
[1/1 CO conversion to EtOH]

$2CO_2 + 6H_2 + \rightarrow C_2H_5OH + 3H_2O$ $\Delta G^{\circ\prime}$= -104 kJ/mol (4)
[1/1 CO conversion to EtOH]

The stoichiometry equations for acetate production *via* the Wood-Ljungdahl pathway are:

$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2$ $\Delta G^{\circ\prime}$= -175 kJ/mol (5)

$2CO + 2H_2 + \rightarrow CH_3COOH$ $\Delta G^{\circ\prime}$= -135 kJ/mol (6)

$2CO_2 + 4H_2 + \rightarrow CH_3COOH + 2H_2O$ $\Delta G^{\circ\prime}$= -95 kJ/mol (7)

$\Delta G^{\circ\prime}$ values from (Daniell et al. 2012)

The Wood-Ljungdahl pathway (Figure B) consists of two branches, referred to as the 'methyl' and 'carbonyl' branches, and has been described in detail in the literature (Drake et al. 2006; Latif et al. 2014; Ragsdale and Pierce 2008). Carbon monoxide can enter the carbonyl branch directly, where it serves as the carbonyl group for acetyl-CoA synthesis. Carbon dioxide can be reduced to CO by a bifunctional carbon monoxide dehydrogenase (CODH). This route requires electrons from H_2 *via* a hydrogenase reaction. This resulting CO then serves as the carbonyl group for acetyl-CoA synthesis.

Conversely, CO can be oxidized to CO_2 by a CODH *via* a biological water-gas shift reaction that also serves to reduce ferrodoxin (Köpke et al. 2011a). This CO_2 molecule then enters the pathway at the methyl branch. Otherwise, CO_2 can directly enter the methyl branch if H_2 is present. In this case, a hydrogenase reaction releases electrons to reduce ferrodoxin necessary for enzymatic function in the methyl branch. Importantly, electron production from CO is more energetically favorable than from H_2, and CO also reversibly inhibits the hydrogenase, so the preference is to use CO if available (Daniell et al. 2012; Wilkins and Atiyeh 2011).

The CO_2 that enters the methyl branch is reduced to formate by a formate dehydrogenase. One ATP is required to activate formate and attach it to tetrahydrofolate (THF) by 10-formyl-THF synthetase. The formyl-THF is reduced to methyl-THF by a series of reductive steps carried out by methylene-cyclohydrolase, methylene-THF dehydrogenase, and methylene-THF reductase. Finally, the methyl group is transferred to a corrinoid-FeS protein by a methyl transferase. The multiunit enzyme complex called carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) accepts the carbonyl group provided by CO from the carbonyl branch and the

methyl group from the methyl branch and joins these moieties together with coenzyme-A (CoA) to form acetyl-CoA (Henstra et al. 2007).

This acetyl-CoA can be converted to acetate by phosphotransacetylase and acetate kinase reactions, which is the preferred route during optimal growth conditions. The acetate kinase reaction produces one ATP *via* substrate level phosphorylation; thus, there is no net ATP gain, and a coupled membrane gradient is required for ATP generation. (Köpke et al. 2011a). Alternatively, acetyl-CoA can be converted to acetaldehyde *via* an aldehyde dehydrogenase reaction, and then to ethanol *via* an alcohol dehydrogenase reaction. Another alternative is for acetate to be further reduced to ethanol by an aldehyde:ferrodoxin oxidoreductase (AOR) reaction to acetaldehyde and then the described route from acetaldehyde to ethanol (Köpke et al. 2010).

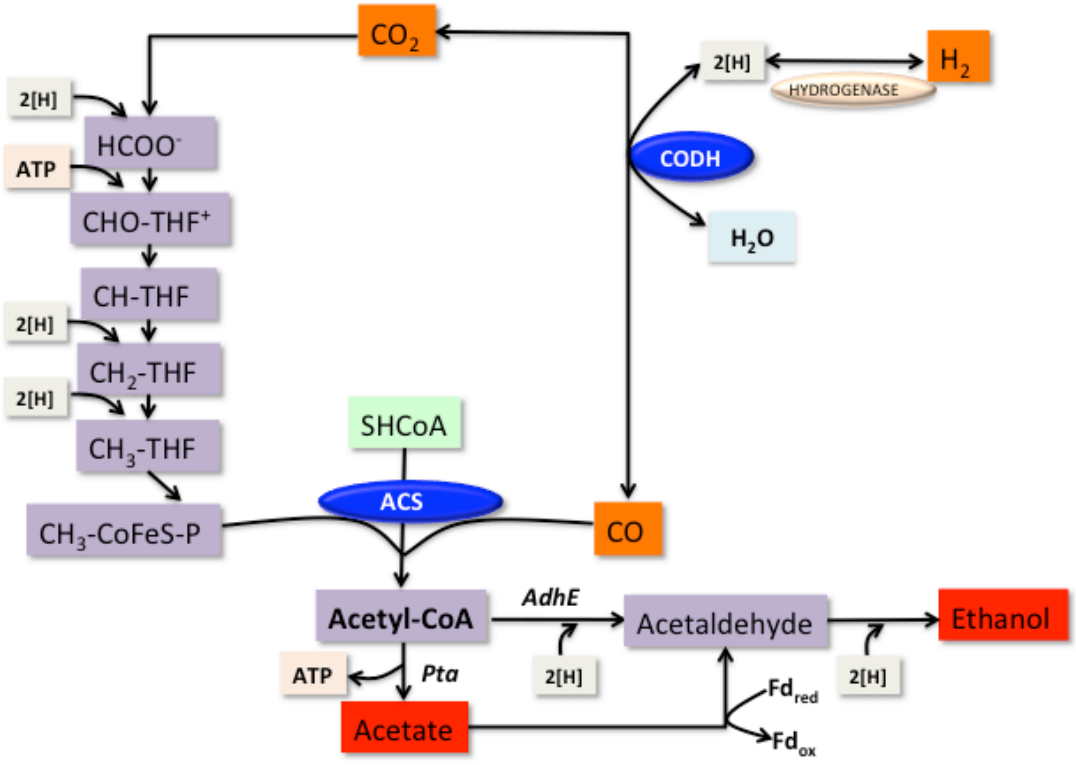

Figure 2: Wood-Ljungdahl pathway

Important for energy generation, *C. ljungdahlii* has genes for an *Rnf* complex that is present in many clostridial species (Biegel et al. 2011; Tremblay et al. 2013). Unlike other anaerobic homoacetogens, *C. ljungdahlii* does not use cytochromes or sodium ions for generating energy (Köpke et al. 2010). It is proposed that it couples electron flow from reduced ferrodoxin to NAD^+ with proton translocation across the cell membrane to generate a proton gradient (Köpke et al. 2010; Tremblay et al. 2013). This proton gradient can then be utilized by the membrane-bound ATP synthase complex to produce energy.

Routes to higher-value chemicals are being researched and better understood. For example, 2-propanol has also been reported as a product from syngas fermentation with *C. ragsdalei* (Kundiyana et al. 2010). 2,3 butanediol has been reported as a syngas fermentation product and it is proposed that acetyl-CoA can be converted to 2,3 butanediol by first converting acetyl-CoA to pyruvate *via* pyruvate:ferrodoxin oxidoreductase (Köpke et al. 2011b). Furthermore, systems for genetic manipulation of *C. ljungdahlii* are being developed to knock out acetate production (Berzin et al. 2012b; Latif et al. 2014; Leang et al. 2013). It should be noted that several research publications from the group of Tyurin, Berzin, and Kiriukhin at Syngas Biofuels Energy, Inc., involving genetically engineering *Clostridium* strains (e.g., for elimination of acetate production by inactivating the phosphotransacetylase gene and increased ethanol production by adding a synthetic acetaldehyde dehydrogenase gene (Berzin et al. 2012a; Berzin et al. 2012b; Kiriukhin and Tyurin 2013)) have been shown to be seriously flawed in methodology and reporting of data (Bengelsdorf et al. 2013).

Reactor Design and Mass Transfer

Various reactor designs have been used for syngas fermentation. The chief consideration for selecting a reactor design is maximizing mass transfer of CO and H_2 from gas phase to liquid phase while minimizing power consumption (Klasson et al. 1993). Carbon monoxide and H_2 have low solubilities (~0.82 and 0.69 mmol gas/L H_2O at 37°C, respectively) and gas/liquid mass transfer is often assumed as the rate-limiting step in syngas fermentation (Bredwell et al. 1999; Henstra et al. 2007). Hence, mass transfer is a factor that may control possible reactor size (Klasson et al. 1992a). High gas flow rates and gas recycling that increases gas residence time (Richter et al. 2013), micro-bubble sparging that increases the interfacial area between gas and liquid (Bredwell et al. 1999), impeller style and agitation speed (Ungerman and Heindel 2007), pressurization, and detergents and solvents can all improve mass transfer. The mass transfer coefficient (K_La) is used to evaluate and compare mass transfer in reactors.

The majority of studies on syngas fermentation employ continuous stirred tank reactors (CSTRs) at a bench-top scale (Daniell et al. 2012). Many studies have been conducted in serum bottles up to 1-L working volume CSTRs. Using a CO-myoglobin assay and an abiotic reactor, Ungerman et al. found that dual Rushton-type impellers in a CSTR provided the highest volumetric mass transfer of all operating conditions tested, but was coupled with the lowest mass transfer performance defined as K_La per unit power input (Ungerman and Heindel 2007). At an agitation speed of 400 rpm, K_La values ranged from 97-155 h^{-1} depending on gas flow rate. Few published studies report working with volumes larger than 2-L, however Kundiyana et al. published a pilot-scale syngas fermentation setup using a 100-L (75-L working volume) CSTR (Kundiyana et al. 2010).

Power consumption, correlated with improved mass transfer, is a particularly important consideration at a commercial scale (Bredwell et al. 1999), and designs that decrease energy input make the entire process more economically viable. Bioreactor designs that have been experimented with for improved mass transfer are multitudinous, and include bubble-column reactors (Bredwell et al. 1999; Richter et al. 2013), packed column reactors (Klasson et al. 1992a), reactors with immobilized cells (biofilm) grown on membranes/hollow fibers (Clausen and Gaddy 1983; Munasinghe and Khanal 2012; Robert et al. 2011)), trickle-bed reactors (Cowger et al. 1992), and micro-bubble reactors (Daniell et al. 2012; Klasson et al. 1993; Munasinghe and Khanal 2010b). Munasinghe and Khanal compared K_La values for various reactor styles and reported a range of 0.4-91.08 h^{-1} (Munasinghe and Khanal 2010b). The highest K_La reported used an air-lift reactor with a 20-μm bulb diffuser. Advantages of bubble-column reactors include lower power consumption by elimination of mechanical agitation, long residence time of gas through the culture and thus high interfacial area, and a slight inherent pressurization depending on the height of the column (Klasson et al. 1992a). In the absence of agitation, liquid recycling prevents coalescence and settling of cells (Richter et al. 2013).

In addition to the type of bioreactor, another primary consideration is whether to run it as a batch, semi-batch, or continuous culture. With syngas fermentation to ethanol, there are clear advantages for employing a continuous culture system at an industrial commodity scale, including less down time and less need for feedstock storage since syngas has to be supplied continuously (Richter et al. 2013). Reactor size can be considerably reduced with a continuous-flow system *vs.* batch (Clausen and Gaddy 1983). At a bench-top scale, a continuous culture allows for much longer run times and experimental versatility where, when the fermentation is at

steady state, a single variable can be changed and the influence assessed while other factors remain constant (Hoskisson and Hobbs 2005).

Another important consideration is whether to employ a one or two stage fermentation system. A two-stage continuous-flow system for syngas fermentation has several advantages. It allows for flexibility in parameters where the two stages can be optimized for different products (Worden et al. 1991). For example, a system can be optimized for fast growth and acidogenesis in the first stage followed by growth stagnation, nutrient limitation, and solventogenesis in the second stage (Kundiyana et al. 2011a; Richter et al. 2013). A two-stage system with two CSTRs was described by Gaddy et al. with the first stage optimized for growth and the second stage optimized for production (Gaddy et al. 2007). This design prevents the culture from adapting to its environment or limiting condition, which could result in acetate production instead of ethanol. It also allows for two different working volumes to control dilution and growth rates, as well as two different styles of bioreactors (Richter et al. 2013). In a single-stage system, the culture may eventually decline in its stressed solventogenic phase, adapt to solventogenesis-inducing conditions and return to acidogenesis, or oscillate unpredictably. A two-stage system allows for a long-term (indefinite) continuous fermentation.

Cell recycling allows for retention of biocatalyst and is often used in bioprocesses to maintain high cell concentrations (Worden et al. 1991). The greater density of biocatalyst allows for a higher total product concentration in the reactor (Klasson et al. 1993; Worden et al. 1991). With high cell concentrations, acetate production has been reported to be nearly eliminated (Clausen

and Gaddy 1992), and many of the highest ethanol concentrations have been reported in fermentations employing cell recycling.

Parameters that can be altered to induce solventogenesis include nutrient contents and concentrations in the medium, medium feed rate, pressure, pH, gas substrate composition, gas feed rate, agitation speed, removal of product to decrease inhibition, and cell density (Clausen and Gaddy 1992; Cotter et al. 2009a; Cotter et al. 2009b; Gaddy et al. 2007; Guo et al. 2010; Kundiyana et al. 2011a; Kundiyana et al. 2011b; Phillips et al. 1993; Richter et al. 2013; Saxena and Tanner 2011; Younesi et al. 2005). Removal of product is important because ethanol may have an inhibitory effect on *C. ljungdahlii* at concentrations above 20 g/L (Phillips et al. 1993).

In particular, medium optimization has been dutifully researched. Besides using a defined medium, or basal medium with yeast extract or beef extract, other economical medium sources have been researched, such as corn steep liquor (Kundiyana et al. 2011a; Maddipati et al. 2011). Nutrients that are suspected to have an important role in ethanol production and the Wood-Ljungdahl pathway are calcium pantothenate, vitamin B_{12}, and cobalt chloride, and limitation of these nutrients has been reported to improve the ethanol/acetate ratio (Gaddy et al. 2007; Kundiyana et al. 2011a). Specifically, reduction of B vitamins diminished growth slightly but improved ethanol/acetate ratio (Phillips et al. 1993). Individually increasing the trace metal ion concentrations of nickel (Ni^{2+}), zinc (Zn^{2+}), selenate (SeO_4^-), and tungstate (WO_4^-) has shown to increase ethanol production with *C. ragsdalei* (Saxena and Tanner 2011).

Concentrations and Production Rates

Information on the source and highest ethanol concentrations and production rates, when available, are given below for common species of *Clostridium* used for syngas fermentation (all strains I worked with from 2011-2014 in the Angenent Lab).

C. ljungdahlii PETC

C. ljungdahlii PETC (formerly ATCC 49587, redeposited as ATCC 55383, DSMZ 13528) was isolated from chicken yard waste in 1988 at the University of Arkansas (Barik et al. 1988; Tanner et al. 1993). It is a gram-positive, motile, spore-forming rod, and a strict anaerobe. Of all the species used in syngas fermentation to ethanol, the highest concentration of 48 g/L ethanol has been reported using *C. ljungdahlii*, with a corresponding 3 g/L concentration of acetate (Phillips et al. 1993). This concentration was achieved after 560 h in a 1-L CSTR at pH 4.5 using a totally defined (no yeast extract) medium, agitation at 450 rpm, and cell recycling. The dilution rate for this particular fermentation is unclear. Surprisingly, I am not aware of any peer-reviewed published concentrations in the 20 years since then that have reported more than half of this concentration of ethanol from syngas fermentation. More recently, an ethanol concentration 6.5 g/L was published for PETC in a 2-L CSTR with 500 rpm agitation (Mohammadi et al. 2012).

C. ljungdahlii ERI-2

C. ljungdahlii ERI-2 (ATCC 55380) was discovered in a natural water source in 1992 (Gaddy 1997; Gaddy 2002). Gaddy et al. described it as a "rod-shaped, gram positive, non-thermophilic anaerobe with superior acetic acid yields [that] operates at low pH." Furthermore, it infrequently

forms spores. An ethanol concentration of 9.7 g/L and corresponding 2.0 g/L acetate concentration was achieved using a fed-batch bench-scale reactor, 1000 rpm agitation, and pH 4.5 (Gaddy 1997). With a straight-through (no cell recycle) CSTR at 1000 rpm agitation, 10 g/L ethanol and 1 g/L acetate was reported (Table 3 in Patent) (Gaddy et al. 2007).

A continuous ethanol production rate of 0.303 g/L/h and ethanol concentration of 428 mM (19.7 g/L) was published for ERI-2 in a two-stage continuous fermentation system consisting of a 1-L CSTR and 4-L bubble column at pH 4.4-4.8 and a dilution rate of 0.039 h^{-1} and syngas containing 60% CO (Richter et al. 2013).

C. autoethanogenum JA1-1

C. autoethanogenum JA1-1 (DSMZ 10061) was isolated from rabbit gut and feces in 1994 (Abrini et al. 1994). Abrini et al. published an ethanol concentration of 0.36 g/L using CO as substrate in batch mode. Ethanol concentration of 0.43 g/L was later published by Cotter et al. (2009a) using sugar (xylose) substrate rather than syngas. In a continuous culture at pH 5, an ethanol production rate of 0.49 mol/L/day (0.94 g/L/h) with corresponding 0.24 mol/L/day (0.60 g/L/h) acetate production was recently published (Wang et al. 2013). This strain is in commercial use by LanzaTech, Roselle, IL, USA (Bengelsdorf et al. 2013). In an oral presentation that I attended at the 2013 Society for Industrial Microbiology and Biotechnology annual meeting, LanzaTech announced a concentration of 70 g/L ethanol with an adapted strain of *C. autoethanogenum* (Köpke 2013).

C. ljungdahlii C-01

Gaddy reported 33 g/L ethanol using *C. ljungdahlii* C-01 (ATCC 55988) with a corresponding production rate of 0.6 g/L/h in a steady-state CO-rich (65%) gas fermentation using a straight-through (no cell recycle) CSTR and 750 rpm agitation (Table 4 in Patent) (Gaddy et al. 2007). In the same patent, Gaddy also reported ethanol concentrations and production rates in a system under pressure and with H_2-rich syngas. He reported 25.96 g/L ethanol and an ethanol production range from 8.96-10 g/L/h using a pressurized (2.61 atm) CSTR with cell recycling, pH 4.5, 800 rpm agitation and 81% H_2 in the syngas (Table 2 in Patent) (Gaddy et al. 2007). Even more impressive, Gaddy reported concentrations of 25 g/L ethanol and 3 g/L acetate, and a 15.38 g/L/h ethanol production rate in a high-pressure (6 atm) CSTR with cell recycling and 55% H_2 in the syngas (Example 10 in Patent) (Gaddy et al. 2007). *C. ljungdahlii* C-01 is a proprietary strain of INEOS Bio, Inc., Lisle, IL, USA.

C. coskatii

Zahn et al. obtained 24 g/L ethanol using *C. coskatii* ('PS02') (ATCC PTA-10522) in a CSTR at pH 5.2 with 900 rpm agitation (Zahn and Saxena 2012). The concentration was achieved in a steady-state long-term (>1000 h) fermentation with syngas containing 37% CO and 35% H_2. An ethanol production rate of 5.4 mmol/L/h (0.25 g/L/h) was also reported with a 28.27 mmol/L/h CO uptake rate. *C. coskatii* is a proprietary strain of Coskata, Inc., Warrenville, IL, USA.

C. ragsdalei

C. ragsdalei ('P11') (ATCC BAA-662) was isolated from a duck pond sediment by researchers at the University of Oklahoma and Oklahoma State University (Huhnke et al. 2008). In semi-

continuous batch culture, the patent reports an ethanol concentration of 11.6 g/L. A concentration of 25.26 g/L ethanol and ethanol production rate of 0.036 g/L/h was published by Kundiyana et al. in a 75-L working volume CSTR pilot scale fermentor using *C. ragsdalei* (2010). The medium for this study was corn steep liquor. Additional products detected were 2-propanol (9.25 g/L) and 1-butanol (0.47 g/L). Maddipati et al. published a concentration of 9.6 g/L ethanol in a 7.5-L fermentation using corn steep liquor in the medium (2011).

Conclusion

Research on syngas fermentation and the bacteria that serve as biocatalysts has increased in the last five years as evidenced by the number of publications. In the literature there is little published on ethanol production rates (Bengelsdorf et al. 2013), or direct comparison of strain performance in a long-term continuous medium and syngas flow system. The highest reported ethanol production rate, which was achieved with *C. ljungdahlii* C-01 and is stated above, was not published in a peer-reviewed publication and was achieved with an H_2-rich syngas. Syngas fermentation for production of ethanol and as a platform technology for producing other valuable liquid fuels and chemicals has enormous potential. The technology could benefit from strain development to produce higher yields with improved efficiency and from bioreactor and fermentation optimization. A side-by-side comparison of commonly used strains in a long-term fermentation system would be useful as a baseline for evaluating changes in process parameters and newly engineered or adapted strains.

The objectives of this thesis were: 1) to build and develop a protocol for running a two-stage, continuous-flow syngas fermentation system with product yields comparable to those published

in the literature. This system was optimized over the past few years by Richter and Martin; 2) to grow the strains described above purely on syngas (using a syngas mix that reflected a realistic composition from a biomass slow pyrolysis kiln as substrate) and defined medium, with no addition of sugar or yeast extract. The strains that successfully were adapted to grow with no yeast extract or sugar continued to be researched in a comparison study; 3) to run duplicate long-term continuous syngas fermentation experiments with each strain, lowering the pH in the production stage to determine differences in growth, ethanol production rates, and ethanol/acetate ratios between the strains and at different pH levels; and 4) to calculate a mass transfer coefficient for CO in our Stage 2 bubble column reactor.

CHAPTER 3: COMPARING ETHANOL PRODUCTION OF CARBOXYDOTROPHIC *CLOSTRIDIUM* STRAINS DURING SYNGAS FERMENTATION WITH A TWO-STAGE CONTINUOUS CULTURE

Introduction

Gasification/pyrolysis in combination with syngas fermentation to ethanol is a promising thermo- and biochemical hybrid process for producing liquid fuel from biomass, offering, among other advantages, a higher theoretical efficiency for carbon conversion to ethanol than lignocellulosic acid hydrolysis/enzymatic fermentation (Clausen and Gaddy 1992; Sims et al. 2010) or syngas to liquid fuels *via* Fischer-Tropsch synthesis (Griffin and Schultz 2012). It allows for great feedstock flexibility because, in addition to biomass, it can utilize carbon from any organic waste stream or off-gasses from industrial processes in varying gas compositional proportions as the substrate for fermentation. Several reviews have been published on syngas fermentation (Abubackar et al. 2011; Bengelsdorf et al. 2013; Daniell et al. 2012; Mohammadi et al. 2011; Munasinghe and Khanal 2010a), with microbial species in the genus *Clostridium* being the most regularly used as biocatalysts: *C. ljungdahlii* (ERI-2, PETC, and C-01), *C. autoethanogenum* JA1-1*, C. ragsdalei* P11, and *C. coskatii*. All except *C. autoethanogenum* JA1-1 have intellectual property interests attached to them, indicating their commercial potential.

These anaerobic bacteria, which are categorized as carboxydotrophic homoacetogens, can use the reductive acetyl-CoA pathway, also known as the Wood-Ljungdahl pathway, to partially oxidize hydrogen and transfer electrons to CO_2. The pathway enables them to fix carbon and harvest energy for growth and maintenance purposes. A major product of this metabolic pathway is acetate, but other byproducts including ethanol are possible, depending on

environmental conditions and genetic/enzymatic capabilities. A substantial amount of literature has reported on ethanol production with these strains, however, the research efforts often focused on product concentrations, with few publications reporting ethanol production rates (Martin 2014). Rates are important to consider when scaling up a continuous system.

The concentration of ethanol in the fermentation broth is important when considering the energy required for product recovery. There is a dramatic rise in the distillation energy requirement as ethanol concentration decreases, especially below 5 wt % ethanol (Vane et al. 2012). Other methods for product recovery include membrane pervaporation (Mulder et al. 1983), membrane separation and membrane assisted vapor stripping (Huang et al. 2008; Vane et al. 2012), and molecular sieve-distillation hybrid systems using an adsorbent such as zeolite (Huang et al. 2008; Vane et al. 2012). Distillation has been optimized and is regularly used for first generation ethanol production, whereas membrane technology can be more expensive and require more maintenance. An alternative to ethanol recovery, which is being researched in the Angenent laboratory, is to upgrade the dilute ethanol and acetate from syngas fermentation to *n*-caproate, which is a higher value compound, through chain elongation by an open culture that includes *Clostridium kluyveri* (Vasudevan et al. 2014). However, *n*-caproate recovery requires a liquid-liquid extraction step and it is undetermined whether such separation is economical and scalable, albeit it is much less energy intensive.

Furthermore, experimental setups from publications are difficult to compare because of differences in design and efficiencies of fermentation (e.g., batch *vs.* continuous-flow, reactor style and size, agitation, pH, pressure, medium contents, syngas composition and flow rate, and

dilution rates). It is useful for differences in performance between existing biocatalyst strains to be documented on a comparable basis under similar operating conditions. Metrics of interest include ethanol concentration and production rate, ethanol/acetate product ratio, growth and density, stability of production, optimal pH, and efficiency of gas consumption.

Here, we present observations and results with three commonly used strains for syngas fermentation: *C. ljungdahlii* ERI-2, *C. ljungdahlii* PETC, and *C. autoethanogenum* JA1-1. Each strain was used as the biocatalyst in a two-stage, continuous-flow syngas fermentation system with continuously fed with syngas, under the same conditions in duplicate runs that each lasted around 700 h. The pH was decreased gradually in the production stage with the expectation that ethanol production would increase, acetate production decrease, and the overall ethanol/acetate ratio would increase while the culture was increasingly stressed by low pH.

Materials and Methods

Bacterial Strains

All strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) except for *C. autoethanogenum* JA1-1, which was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The six *Clostridium* strains that were originally investigated were *C. ljungdahlii* PETC (ATCC 55383), *C. ljungdahlii* ERI-2 (ATCC 55380), *C. ljungdahlii* C-01 (ATCC 55988), *C. autoethanogenum* JA1-1 (DSMZ 10061), *C. ragsdalei* P11 (ATCC BAA-622), and *C. coskatii* PS02 (ATCC PTA-10522).

Medium and Growth Conditions

Chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) or VWR International (Radnor, PA, USA). The simulated syngas supplied to cultures was a mixture composed of 60% CO, 35% H_2, and 5% CO_2 (Airgas East, Ithaca, NY, USA) that reflects a realistic syngas composition from a biomass pyrolysis kiln, although it contained no impurities. Strains were grown anaerobically in medium used to grow *C. carboxidivorans* P7 (Datar et al. 2004; Rajagopalan et al. 2002) and referred to here as '1x P7 medium' (Martin 2014, Appendix 3).

All cultures were grown anaerobically at 36±1 °C. Sterile techniques were applied for transferring cultures and inoculating reactors. Bottles and CSTRs were sterilized by autoclaving at 120°C for appropriate times and vitamins were added to ambient temperature medium with a 0.2 micron sterile syringe filter after autoclaving. The bubble column could not be autoclaved because of its size and was instead filled and flushed with 5% bleach solution for at least 30 min before filling with medium.

Initially, 160-mL serum bottles (VWR International, Radnor, PA, USA) with butyl stoppers (VWR International, Radnor, PA, USA) containing 10 mL of 1x P7 medium at pH 5.5 with 0.5 g/L Diffco yeast extract, 5 g/L MES (2-(N-morpholino)ethanesulfonic acid) buffer, and a syngas headspace pressurized to 1.9 bar were inoculated with 5% (vol/vol) inoculum from frozen stock. Serum bottles contained only 10-mL of medium in order to supply a sufficient amount of substrate. Resazurin was used as an oxygen indicator and l-cysteine was added to scavenge any residual oxygen in the medium. The l-cysteine was a significant source of sulfur in the medium (1 mM) in addition to magnesium sulfate (1.7 mM). Reactors and serum bottles with medium

were either prepared in an anaerobic hood or sparged with nitrogen before adding l-cysteine. Next, cultures were adapted to grow with less yeast extract. When the serum bottle cultures were visibly dense, 2% or 5% (vol/vol) inoculum was used to inoculate similar serum bottles but with 0.05 g/L yeast extract instead of 0.5 g/L. This transfer procedure using serum bottles with 0.05g/L yeast extract was used to maintain actively growing cultures as a seed train.

Stage 1 was inoculated with 5% (vol/vol) inoculum from dense (late exponential phase) serum bottle cultures. The initial medium in Stage 1 was 1x P7 medium with 0.05 g/L yeast extract, no MES buffer, and pH 5.5. The only change from the serum bottle medium was the absence of MES buffer because the acetate produced during growth was found to be a sufficient buffer and the CSTR was equipped with a pH controller and pump for adding 2 M KOH on demand. It was found that beginning the fermentation with a small amount of yeast extract (0.05 g/L) in Stage 1 greatly improved initial growth and decreased the lag phase after inoculation. Once this initial yeast extract was consumed, the bacteria grew with no yeast extract. An exception was required for *C. autoethanogenum* and an additional 0.05 g/L yeast extract was added after 338 h of the operating period to stimulate growth. This was repeated for the duplicate run after 93 h to spur growth. After this second one-time addition of yeast extract for *C. autoethanogenum* the bacteria grew with no additional yeast extract for the remaining run. Thus, *C. autoethanogenum* grew without yeast extract for the remainder of the operating period with a residence time of 25 h and continuous medium feed conditions.

After Stage 1 was inoculated, it grew up in batch mode until it reached an OD_{600} of 1.0 at which point the system was turned to continuous-flow mode (40 mL/h) and 2x concentrated P7 medium

was used for the duration of the fermentation. The dilution rate for Stage 1 was 0.96 day^{-1} for the duration of the run. The '2x P7 medium' had double the concentrations of minerals, vitamins, and trace metals compared to 1x P7 medium. Like 1x P7 medium, 2x P7 medium contained no yeast extract nor MES buffer. The 2x P7 medium was prepared in 10-L carboys holding 8-L of medium and this medium reservoir was sparged continuously with syngas to maintain an anaerobic environment. In all six runs, silicon antifoam at a concentration of 100,000x volumetric dilution (Sigma Antifoam 204) was added into the 2x P7 medium starting with the second reservoir tank and all subsequent tanks until the end of the operating period to address the foaming in Stage 1 that occurred at an OD_{600} above 1.0.

Stage 2 was inoculated when the system was set to continuous-flow mode by flowing Stage 1 effluent into the Stage 2 bioreactor. It received 40 mL/h of fresh 2x P7 medium through a bypass in addition to 40 mL/h of culture from Stage 1. Thus, the dilution rate in Stage 2 was 0.48 day^{-1}. This design allowed for additional nutrients to be provided to the denser second stage without increasing the medium flow rate in Stage 1 that would have flushed the cell population out of the first stage. The first stage provided fresh biocatalyst to the second stage as well as acetate for conversion into ethanol. Antifoam probes and a controller (Cole Parmer, Vernon Hills, IL, USA) were used in Stage 2 to allow for automatic pumping of antifoam into the second stage as needed, which was crucial for preventing clogged gas-recycle lines and spargers.

Reactor Setup

The two-stage continuous syngas fermentation system used for the fermentation runs and the analytical procedures have been described in detail (Richter et al. 2013) but a brief description is

provided here for clarity. The entire system and medium reservoir was maintained anaerobically by sparging syngas through cylindrical spargers made of stainless steel, with pore size of 0.5 microns (More Flavor, Concord, CA, USA) at the bottom of each fermentor and medium reservoir. The fermentation system consisted of two fermentors. A 2-L (1-L working volume) Braun Biostat M continuous stirred tank reactor (CSTR) (Braun, Allentown, PA, USA) was used as a growth stage and is referred to as Stage 1. It was well-mixed with dual impellers at 200 rpm agitation to disperse syngas through the culture to aid in mass transfer. This is a considerably slower agitation speed compared to other syngas fermentation CSTR studies.

Stage 1 was coupled to a custom-built 6-L (4-L working volume) glass bubble column with conical bottom that was used as a production stage and is referred to as Stage 2. Stage 2 was equipped with an acid/base pH auxostat using 2M HCl and KOH. Both fermentors were water-jacketed and maintained at 36±1 °C. The system incorporated cell recycling (400 mL/min) in Stage 2 using a Cellflo polyethersulfone hollow fiber module with 500-cm^2 membrane surface and 0.2 micron pore size (Spectrum Laboratories, Rancho Dominguez, CA, USA) to increase cell density in Stage 2. Gas recycling was applied in both stages (180 mL/min and 400 mL/min) to increase the contact area and retention time of the gas in the liquid phase.

Experimental Design and Rationale

All strains grow optimally at a pH in the range of 5.5-6.0, but lowering the pH has been shown to improve the ethanol/acetate ratio. Previous experiments with *C. ljungdahlii* showed lowering the pH to 4-4.5 coupled with nutrient limitation and high mass transfer results in a drastic shift in product formation in favor of ethanol over acetate (Phillips et al. 1993). The pH value in Stage 2

was changed in a controlled manner during the fermentation as part of the experiment. The two-stage system ran continuously at a pH of 5.5 in both stages until Stage 2 reached the target OD_{600} of 10.0. At that point, the pH in Stage 2 was gradually decreased by 0.15 units per day until a pH of 4.5 was reached. An exception was needed for the *C. autoethanogenum* duplicate runs: this stepping down of pH was begun at an OD_{600} of near 7 because of slower apparent growth rate and generally lower final OD_{600} reached in Stage 2 compared to the *C. ljungdahlii* strains.

Analytical Procedures

Stage 1 and Stage 2 were sampled daily for product concentrations and gas composition in the headspace. The volume of liquid effluent was measured in a graduated cylinder to verify daily medium flow rate. Microscopic controls (phase-contrast) of both stages were conducted regularly to check for signs of possible contamination. Antifoam and acid/base consumption of each stage was recorded daily by weighing the respective reservoirs.

Sample ports for Stage 1 and Stage 2 allowed sampling of the cultures. These samples were centrifuged for > 5 min at 12,000 rpm after which the supernatant was stored at -20°C. These aliquots were analyzed for metabolites by HPLC (Waters, Milford, MA, USA) with an Aminex HPX87H column (Bio-Rad, Hercules, CA, USA) heated to 65°C and an RI-detector. The HPLC buffer was 5 mM sulfuric acid in MilliQ water and the flow rate was 0.6 mL/min. Optical density in both stages was measured using a Milton Roy Spectronic 1201 spectrophotometer at a wavelength of 600 nm. Cell dry weight was determined by using a previously calculated correlation-coefficient of 242 mg dry weight/($L \cdot OD_{600}$) and the universal proportion of elements in microbial biomass, $C_5H_7O_2N$ (Perez et al. 2013).

Inlet and outlet volumetric gas flow rates were measured for Stage 1 and Stage 2 with custom-made in-line graduated glass bubble flow meters. Gas inlet and outlet pressures were measured by a digital pressure gauge (Cole Parmer) through septa ports on the gas lines. A 500-mL air-lock syringe (Hamilton, Reno, NV, USA) was used to sample the bioreactors and to inject into one of two gas chromatography instruments (Gow Mac, Bethlehem, PA, USA) equipped with thermal conductivity detectors (TCDs). Carbon monoxide and carbon dioxide were quantified using helium as a carrier gas and a 1.8 m Supelco 80/100 Hayesep Q column at 25°C. Hydrogen was quantified using nitrogen as a carrier gas and a 4.5 m Supelco60/80 Carboxen 100 column at 25°C. Consumption rates of CO and H_2, and production rates of CO_2 were calculated from individual concentrations, pressures, and total gas-flow rates relative to the known simulated syngas tank composition.

Results and Discussion

Cell Growth

A prerequisite for continuing to work with a strain was the ability to cultivate it in a completely defined medium (with no yeast extract). Yeast extract is a relatively expensive additive and it has been reported that elimination of yeast extract increases the ethanol/acetate product ratio (Klasson et al. 1993). All research was conducted with fresh culture collection strains before each fermentation run, and we were not successful in adapting cultures of *C. ljungdahlii* C-01, *C. ragsdalei* and *C. coskatii* to grow in serum bottles or 1-L CSTR without yeast extract (batch-mode CSTR and serum bottle data not shown) (Martin 2014). Therefore, we did not include them in this comparison study. *C. ljungdahlii* ERI-2, *C. ljungdahlii* PETC, and *C. autoethanogenum* JA1-1 were successfully grown without yeast extract, and therefore were

included. All three of these strains have been previously shown to produce ethanol via syngas fermentation (Abrini et al. 1994; Gaddy 2002; Klasson et al. 1992b).

Duplicate fermentation runs with ERI-2, PETC, and JA1-1 were remarkably similar to each other in growth, product concentrations, and production rates, which indicates a stable and reproducible fermentation system. Stage 1 growth for all three strains was comparable (Appendix 1). All cultures were able to sustain a near steady-state OD_{600} of between 1.5 and 3 over the duration of the run. For ERI-2 Run 1, an overnight power outage at 255 h of the operating period caused the OD_{600} in Stage 1 to drop from 2.4 to 0.1 and the culture then recovered to a steady OD_{600} of ~1.1. ERI-2 Run 2 maintained a steady OD_{600} near 2. For PETC Run 1, a steady OD_{600} of ~2.7 was maintained. For PETC Run 2, an accident in Stage 1 at 414 h of the operating period resulted in no sparging for one night and caused the density to drop from 2 to 0.4 but it then recovered fully to an OD_{600} above 2.

There were no accidents or power outages with JA1-1, however, slower growth was observed with this strain, with the first run finally reaching a steady OD_{600} around 2 only after adding an additional 0.05 g/L of yeast extract at 338 h of operation. Data for this run was shifted by trimming the beginning 194 h of no growth so that it better aligned with Run 2. The duplicate JA1-1 Stage 1 was very similar in growth; an addition of 0.05 g/L yeast extract was added at 94 h of operation. Several unsuccessful runs were terminated early-on because unusual, large lemon-shaped cells were observed along with poor growth in Stage 1. A black powder that developed on the headspace walls of the inside of Stage 1 was examined under the microscope and determined to be non-biological and was likely iron sulfide.

In Stage 2, both ERI-2 and PETC had similar growth rates, reaching an OD_{600} of 10 after approximately 215 h and 155 h, respectively (Figure 3). In contrast, JA1-1 had reached less than OD_{600} 7 in both runs after 200 h of growth. With the continual influx of fresh cells from Stage 1, the cell density in Stage 2 for all strains was not adversely affected by decreasing the pH in Stage 2, and all fermentation runs reached an OD_{600} of greater than 11 eventually, with the ERI-2 and PETC runs exceeding an OD_{600} of 20 (4.8 g DW/L) by the end of the operating periods.

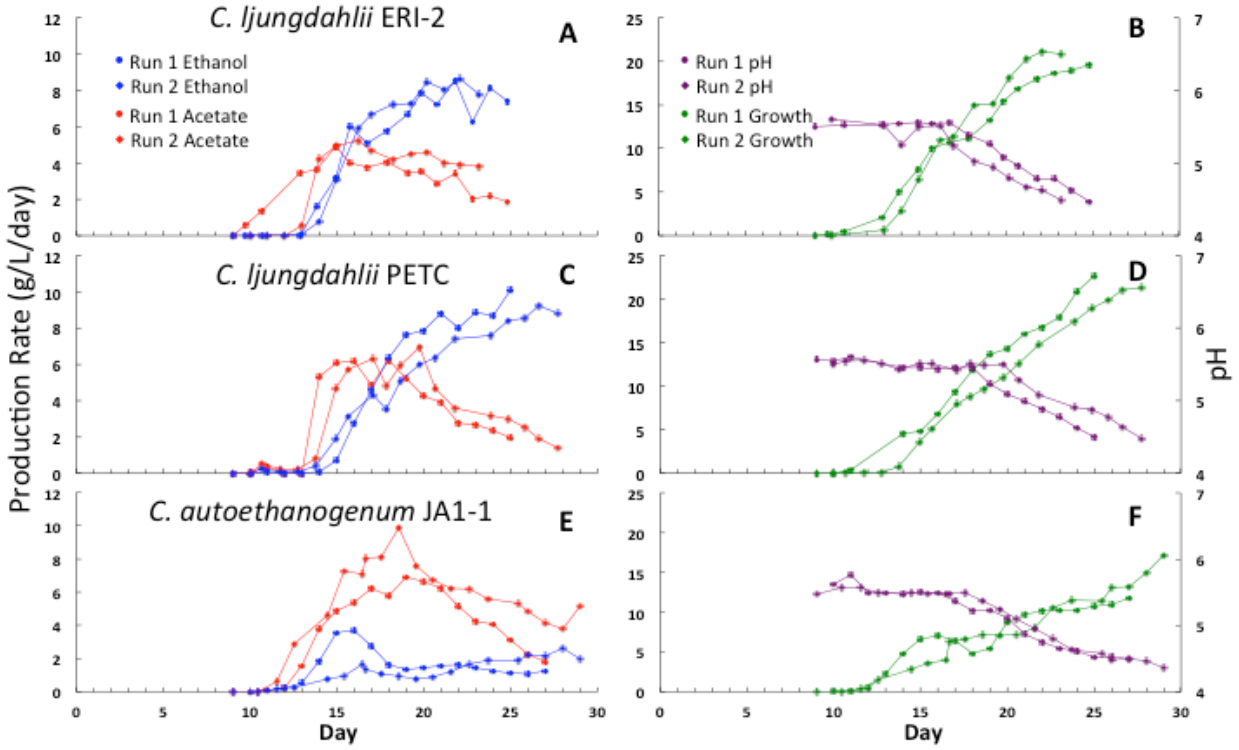

Figure 3. Stage 2 ethanol and acetate productivities and corresponding pH and OD_{600} for duplicate runs with *C. ljungdahlii* ERI-2 (A,B), *C. ljungdahlii* PETC (C,D), and *C. autoethanogenum* (E,F). For all runs, the pH was decreased from 5.5 to 4.5 over the course of about 10 days, while culture density continued to increase over the entire span of fermentation runs.

Concentrations and Ethanol/Acetate Ratios

In Stage 1 (the growth stage), the strains were acetogenic, and thus produced acetate as their main product. In Stage 2 (the production stage), the pH value was manipulated to stress the cells and induce solventogenesis. The continually increasing cell density in Stage 2, and potential accompanying nutrient limitation, was a confounding variable. The bypass of fresh 2x P7 medium directly to Stage 2 was implemented to address a possible nutrient limitation.

The effect of pH on ethanol production and ethanol/acetate ratios has been discussed in the literature and is considered one of the most important factors for improving ethanol production (Daniell et al. 2012; Mohammadi et al. 2012). However, the relationship remains ambiguous. An inverse correlation between pH and ethanol production by *C. carboxidivorans* in a 20 day bubble column reactor fermentation was discussed by Datar et al. (2004) but because the source of syngas was switched from bottled to producer gas right before increased ethanol production it is unclear if increased ethanol production can be attributed to decreased pH. With *C. ljungdahlii*, low pH (4.0-4.5) along with high CO mass transfer and nutrient limitation was associated with favorable ethanol production (Klasson et al. 1993), however, in those experiments pH was held constant so it is difficult to assess its relative influence on product concentrations. In a month-long 2-L CSTR reactor experiment with *C. ljungdahlii* and medium at pH 6.8 but uncontrolled during fermentation, cell dry weight was drastically reduced and pH was readjusted to 6.5 after falling to 4.18 (Mohammadi et al. 2012). Mohammadi et al. concluded pH was one of the most important factors affecting substrate metabolism and that the lower pH resulted in a shift from acidogenesis (growth-associated) to solventogenesis (stationary- or stressed- cell associated).

However, the relationship is likely complex, and acetate and ethanol production were observed in both growth and production phases in our system.

The rationale for gradually stepping down the pH over a course of days in our experimental design was to lessen the toxic and inhibitory affect of undissociated acetate by acclimating Stage 2 and giving the culture time to convert the acetate fed from Stage 1 into ethanol and prevent a buildup of undissociated acetate. Organic acids have inhibitory effects in biorefining processes and undissociated acetic acid can diffuse across the cell membrane where it dissociates due to the cell's higher pH, disrupting pH balance in the cytoplasm and dissipating the membrane potential (Mohammadi et al. 2012; Warnecke and Gill 2005).

To analyze the effect of pH on ethanol, acetate, and total C_2 product concentrations in Stage 2, data from two three-day periods from each run were compared (Figure 4). The first period used was the three days before the pH step-down began. The data from this period provide average product concentrations for the strains at pH 5.5. The second period was the last three days of each fermentation run. The data from this period provide average product concentrations for the strains at pH 4.5.

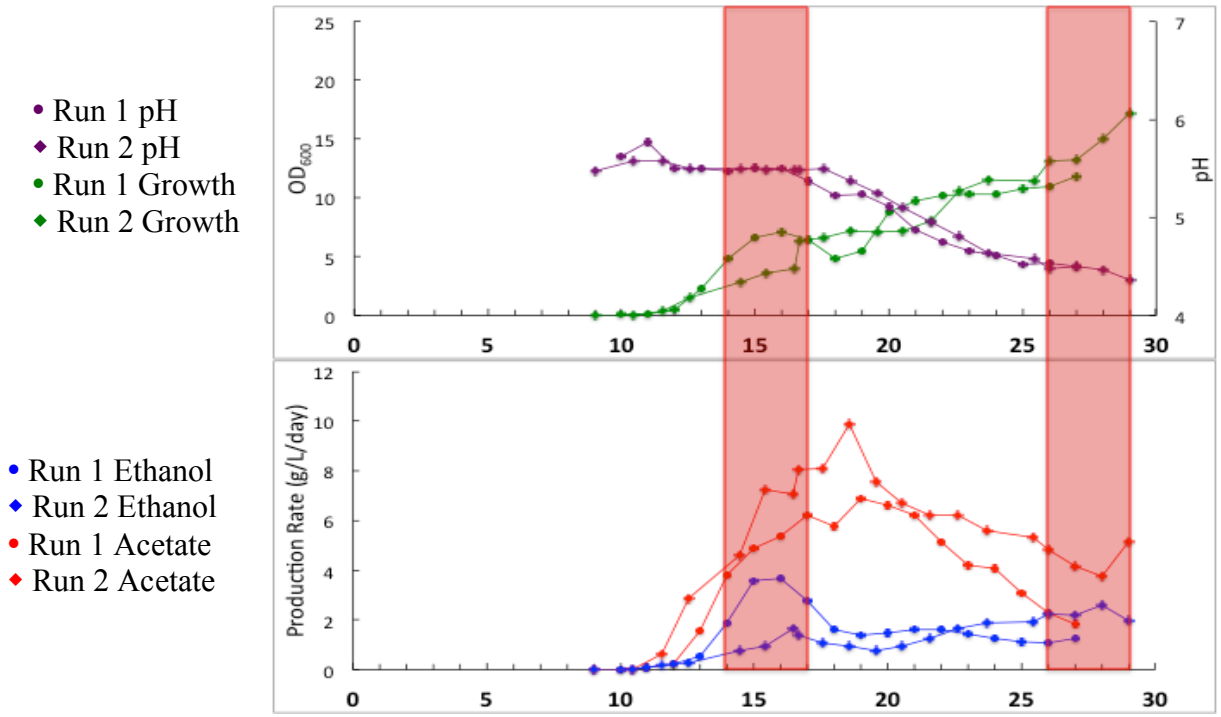

Figure 4. An example using *C. autoethanogenum* data of the two three-day periods used for analyzing the fermentation culture at pH 5.5 and 4.5

Because the culture density in Stage 2 was constantly increasing for all runs due to continuous influx of fresh cells from Stage 1, cell recycling in Stage 2, and some cell growth in Stage 2, I normalized the product concentrations to OD_{600} (Figure 5).

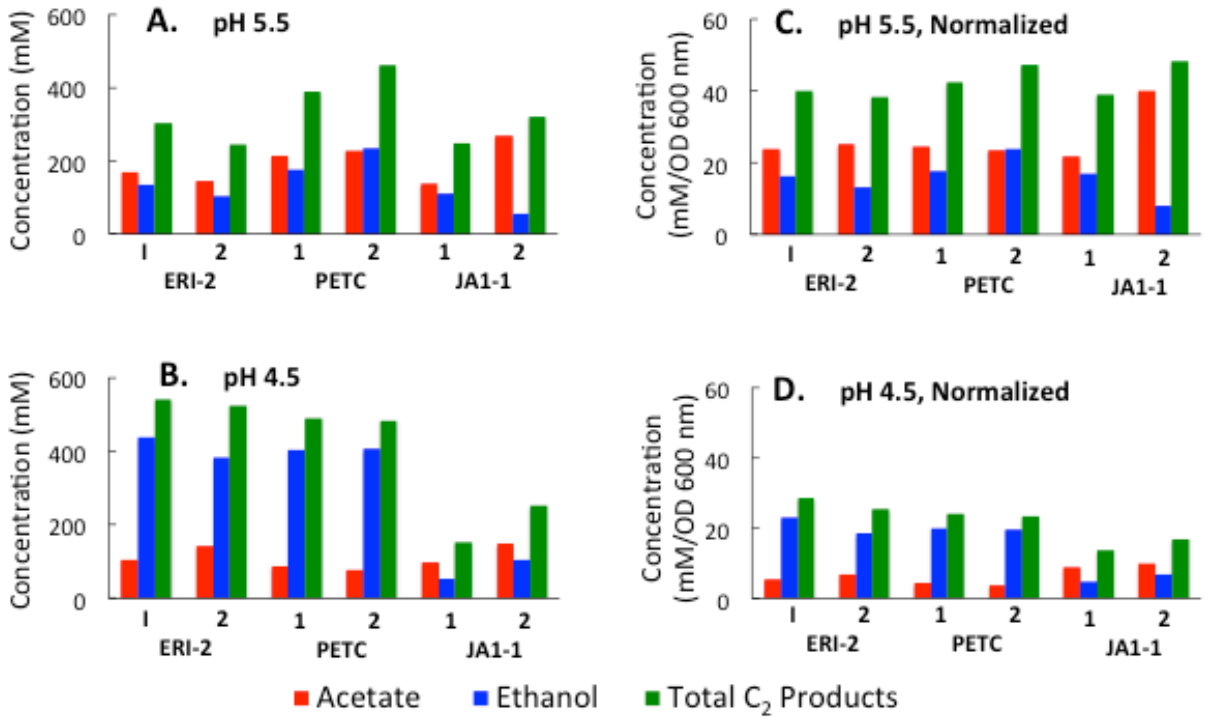

Figure 5. Average product concentrations in fermentation Stage 2 are shown for duplicate runs (1,2) for the three compared strains at: (A) pH 5.5 during a three day period before stepping down the pH; (B) pH 4.5 during a three day period at the end of each fermentation run; and (C,D) same data as (A,B) with concentrations normalized to optical density.

It is evident that concentrations for total C_2 products, normalized to OD_{600}, decreased by nearly 50% from pH 5.5 to 4.5 for ERI-2 and PETC, and an even bigger decrease was observed for JA1-1, suggesting that the lower pH has an inhibitory effect on all strains (Table 2).

	ERI-2		PETC		JA1-1	
pH	**5.5**	**4.5**	**5.5**	**4.5**	**5.5**	**4.5**
Acetate	23.2	5.9	22.8	3.6	33.6	9.0
Ethanol	22.8	20.7	23.8	20.0	14.0	6.0
Total C_2 Products	46.0	26.6	46.6	23.6	47.8	15.0

Table 2. Normalized average product concentration values for duplicate runs (mM/OD_{600})

For ERI-2 and PETC, the specific ethanol/acetate ratio greatly improved at pH 4.5 compared to pH 5.5 (Table 3). Interestingly, the effect is due to a decrease in the specific acetate concentration; the specific ethanol concentration stays almost constant between the different pH values (Table 2). This decrease in specific acetate concentration accounted for the decrease in total C_2 product concentration for these two strains. Overall, JA1-1 had a poorer ethanol/acetate ratio under the fermentation conditions of this comparison and was more severely affected by lower pH than ERI-2 and PETC. Not only did the total product specific concentration drastically decrease at pH 4.5, but both acetate and ethanol specific concentrations declined (unlike with ERI-2 and PETC). Notably, at a pH 5.5, the specific total product concentration was similar for ERI-2, PETC, and JA1-1, suggesting that at pH 5.5 all strains have similar rates of metabolism, but reducing equivalents are directed toward acetate more than ethanol formation in JA1-1.

Strain	pH 5.5	pH 4.5	Ethanol Concentration pH 4.5
ERI-2	0.98 ± 0.10	3.66 ± 0.96	412 mM (1.89%)
PETC	1.05 ± 0.02	5.52 ± 0.40	412 mM (1.89%)
JA1-1	0.51 ± 0.34	0.66 ± 0.02	79 mM (0.36%)

Table 3. Average ethanol/acetate concentration ratios (n=2)

A poorer ethanol/acetate ratio for *C. autoethanogenum vs. C. ljungdahlii* (1:13 *vs.* 1:8 respectively) was also reported by Cotter et al. (2009b) when grown on syngas (but not observed when grown on fructose, 1:8 *vs.* 1:7 respectively). Cotter et al. looked at the effects of pH, syngas flow rate (with 20% CO), and substrate (sugar or syngas only), on growth and ethanol production of *C. ljungdahlii* PETC and *C. autoethanogenum* JA1-1. It was conducted in 250-mL batch reactors with continuous syngas flow and no stirring. For *C. autoethanogenum*, the basal medium was at pH 6.0 and contained 1 g/L yeast extract. Based on the highest product

concentrations of 1.45 mM ethanol and 23.3 mM acetate for *C. autoethanogenum*, it does not appear that solventogenesis was ever achieved.

Gas Consumption and Carbon Recovery

In Stage 1, the syngas inflow rate ranged from 0.9-2.4 L/h; and in Stage 2, from 3-6 L/h. Syngas was fed separately to the two stages and always in considerable excess of what was consumed so that we would not unintentionally limit the carbon and electrons needed for growth or production. For this reason, calculating carbon efficiencies for carbon fed in the form of CO and CO_2 would not be meaningful. However, recovery of the carbon from consumed CO in the two stages into products, biomass, and CO_2 was calculated as Σ of carbon in products/Σ of carbon substrate consumed (Table 4).

Strain	ERI-2		PETC		JA1-1	
pH	5.5	4.5	5.5	4.5	5.5	4.5
Compound	Concentrations, Stage 2 Outlet					
Acetate (mM)	166 +/- 3	118 +/- 22	218 +/- 11	75 +/- 5	217 +/- 61	119 +/- 33
Ethanol (mM)	163 +/- 19	412 +/- 32	228 +/- 14	412 +/- 4	90 +/- 42	79 +/- 24
Total Products (mM)	329 +/- 22	530 +/- 10	446 +/- 24	487 +/- 1	308 +/- 18	197 +/- 57
Ethanol/Acetate Ratio	0.98 +/- 0.10	3.66 +/- 0.96	1.05 +/- 0.02	5.52 +/- 0.40	0.51 +/- 0.34	0.66 +/- 0.02
OD600	7.15 +/- 0.41	19.90 +/- 0.84	9.58 +/- 0.24	20.61 +/- 0.14	6.45 +/- 0.25	13.16 +/- 1.98
Bacteria (g DW/L)	1.73 +/- 0.10	4.82 +/- 0.20	2.32 +/- 0.06	4.99 +/- 0.03	1.56 +/- 0.06	3.19 +/- 0.48
	Rates for Total System [mmol/(L * min)]					
CO Consumption	0.449 +/- 0.088	0.505 +/- 0.019	0.545 +/- 0.015	0.544 +/- 0.025	0.493 +/- 0.090	0.285 +/- 0.043
H2 Consumption	0.259 +/- 0.050	0.264 +/- 0.015	0.282 +/- 0.018	0.308 +/- 0.014	0.171 +/- 0.012	0.089 +/- 0.003
CO2 Production	0.110 +/- 0.000	0.224 +/- 0.002	0.196 +/- 0.014	0.233 +/- 0.005	0.207 +/- 0.043	0.111 +/- 0.041
Ethanol Production	0.041 +/- 0.002	0.093 +/- 0.005	0.067 +/- 0.008	0.109 +/- 0.002	0.025 +/- 0.011	0.021 +/- 0.006
Acetate Production	0.042 +/- 0.003	0.028 +/- 0.009	0.053 +/- 0.002	0.020 +/- 0.002	0.062 +/- 0.018	0.031 +/- 0.009
	Carbon Recovery for Total System [Σ of Carbon Products/Σ of Carbon Substrate Consumed]					
Run 1	0.80	0.90	0.84	0.91	0.72	0.60
Run 2	0.56	0.95	0.79	0.93	0.88	0.90

Table 4. Concentrations in production stage (Stage 2), consumption/production rates for entire five-liter system, and recovery of consumed carbon are compared for when Stage 2 was at pH 5.5 *vs.* pH 4.5. A representative three day period was used to calculate averages for each fermentation run at pH 5.5 and 4.5. The average values for the duplicate runs are reported in the table with standard error (n=2).

Carbon recoveries calculated for pH 4.5 were generally very high (>90%) and were almost always higher than those calculated for pH 5.5. The reason for this is unclear. The maximum theoretically possible carbon conversion to ethanol from the syngas composition used ($CO:H_2:CO_2$ = 60:35:5), assuming no acetate production, is 52.6% according to the theoretical stoichiometric fermentation balance:

$$3.80\ CO + 2.20\ H_2 + 0.80\ H_2O \rightarrow 1.00\ C_2H_5OH + 1.80\ CO_2 \quad (8)$$

Average carbon conversion to ethanol at pH 4.5 were calculated for the total system. For ERI-2, PETC, and JA1-1, we achieved 36.8% (70% of theoretical max), 39.4% (75% of theoretical max), and 14.7% (28% of theoretical max), respectively. Carbon conversion efficiency to ethanol was worse at pH 5.5 than at pH 4.5 for all strains. Low CO consumption observed at low pH for JA1-1 is another indication that it tolerates low pH less well than ERI-2 and PETC. Hydrogen consumption was minimal because the excess syngas fed provided energetically favorable CO in excess. Carbon monoxide also reversibly inhibits the hydrogenase that is required for using electrons from H_2 in the pathway. Carbon monoxide consumption rates are not normalized to OD_{600}. Since they stay relatively constant for ERI-2 and PETC it means specific CO consumption declines as cell density increases, suggesting mass transfer limitation or a negative effect of the decreasing pH, the latter certainly in the case of JA1-1.

Production Rates

A balance must be struck between maximizing the ethanol concentration in the culture relative to the ethanol production rate of the system. Logically, when the dilution rate is increased, the

ethanol concentration decreases and the production rate increases. Less energy is required for distillation of effluent with a higher ethanol concentration, but if the ethanol concentration is too high it is toxic to the culture. Increasing the dilution rate increases the ethanol production rate but requires more medium usage and lowers the ethanol concentration in the effluent. Therefore, it is important to compare the corresponding concentration and production rate data from a fermentation run, which is rare to find in the literature.

For all runs in this comparison, the dilution rate for the entire system was 0.384 day^{-1}. Ethanol production rates in Stage 2 for duplicates of ERI-2 and PETC are closely correlated with cell density (Figure 3). Ethanol production rates for these two strains continued to rise throughout the runs unaffected by decreasing pH. In Stage 2 only, the ethanol production rate for ERI-2 reached 0.358 g/L/h and for PETC 0.421 g/L/h. For the whole 5-L system, average ethanol production rates for ERI-2 and PETC were similar for the last three days at 0.093 mmol/(L·min) (0.257 g/L/h) and 0.109 mmol/(L·min) (0.301 g/L/h), respectively (Table 4). Average acetate production rates for the same time points were also similar, at 0.028 mmol/(L·min) (0.101 g/L/h) and 0.020 mmol/(L·min) (0.072 g/L/h), respectively. The general trend for these two strains was that the ethanol production rate approximately doubled, and the acetate production rate approximately halved, at pH 4.5 compared to 5.5. Again, it must be taken into account that the OD_{600} at these two pH levels approximately doubled as well (from 10 to 20) for the strains.

JA1-1 performed noticeably different. Our production rates confirm that JA1-1 was an inferior producer of ethanol compared to ERI-2 and PETC in our system. In duplicate runs, the ethanol production rate never surpassed the acetate production rate and also did not correlate as strongly

with optical density (Figure 3), indicating that a shift to solventogenesis was never achieved. The ethanol production rate in Stage 2 remained relatively steady throughout the operation period at 0.062 g/L/h for both runs. For the overall 5-L system, the ethanol production rate averaged 0.021 mmol/(L·min) (0.058 g/L/h) for the last three days of the runs (Table 4). The average acetate production rate for this same period was 0.031 mmol/(L·min) (0.112 g/L/h). Whereas an increase in ethanol production rate was observed for ERI-2 and PETC at pH 4.5 compared to pH 5.5, the ethanol production rate for JA1-1 stayed relatively level at around 0.021 mmol/(L·min) at both pH levels (Table 4), indicating that lower pH does not increase the ethanol production rate for JA1-1.

Mass Transfer

Both CSTRs and bubble column reactors have been shown to provide good mass transfer of gas substrate. The bubble column reactor is advantageous in that it requires relatively little energy to disperse the syngas (no agitation with impellers) and allows for longer syngas retention time in the culture. The volumetric mass transfer coefficient (K_La) is a useful metric for modeling a process that might be gas/liquid mass transfer limited and it is an important number to consider when scaling up. 'K_L' is the gas-liquid phase mass transfer coefficient and 'a' is the interfacial area per unit volume of liquid (it is difficult to separate the two terms). The standard K_La equation requires measuring dissolved donor gas in the liquid phase. It is usually measured in an abiotic reactor. Measuring mass transfer by traditional methods is difficult in a biological system at steady-state where disruption of gas flow could be detrimental to the culture. Furthermore, probes that measure dissolved CO are not as readily available as dissolved O_2 probes.

Below is the equation for calculating a mass transfer coefficient:

$$dC/dt = K_La \cdot (C_i - C) \qquad (9)$$

where

dC/dt = change in concentration of dissolved CO over time (CO transfer rate)

K_La = mass transfer coefficient [h^{-1}]

C_i = saturated dissolved CO concentration at liquid-gas interface, at infinite time (Dependent on CO headspace pressure; at room temperature and ambient pressure, C_i = 28 mg/L (Munasinghe and Khanal 2010b)

C = local dissolved CO concentration in bulk liquid at any time *t* [mg/L]

It is helpful to think of the CO transfer rate (dC/dt) as equal to the transfer area (K_La) multiplied by the driving force ($C_i - C$). We can calculate a minimum K_La value for our system with this equation. A CO consumption rate of 40 mmol CO/L/h was observed at a sample point representative of typical CO consumption in Stage 2 at a high cell density. If we assume that gas/liquid mass transfer is the limiting step, we can assume that the CO transfer rate is equal to the CO consumption rate. To calculate a minimum K_La value, we maximize the driving force by taking from the literature, C_i = 28 mg/L and assuming C = 0. The local dissolved CO concentration equaling zero is plausible in a dense culture where the bacteria is likely taking up all accessible CO. Under these assumptions a K_La of 40 h^{-1} is calculated and can be considered a minimum K_La value for the bubble column reactor (Martin 2014, Appendix 8).

As indicated above, with a fermentation close to steady-state and with a high cell density as we find in Stage 2, one may assume the potential rate of microbial CO uptake is higher than the rate of CO transport through the gas/liquid interface (Vega et al. 1989), which means that the

concentration of dissolved CO is negligible. This would point toward a mass-transfer limited situation and the following rate expression can be used for CO uptake (Q_{CO}):

$$Q_{CO} = (K_L a/H)_{CO} \cdot P^G_{CO} \quad (10)$$

where

H = Henry's Law constant

P^G_{CO} = partial pressure of CO in the Stage 2 headspace

(Vega et al. 1989)

Under these assumptions, we use the same CO transfer/uptake rate as before, 40 mmol CO/L/h, set P^G_{CO} = 18% or 0.18 atm, which was the measured headspace proportion for CO at a sample time when the fermentation was running smoothly, and set H_{CO} = 0.85 (L·atm/mmol CO) (adjusted to 37°C using Van't Hoff equation), to calculate a $K_L a$ of 190 h^{-1}. This compares favorably to $K_L a$ values published for syngas fermentation with various reactor styles and sparging systems where the highest $K_L a$ of 91.08 h^{-1} was recorded for an air-lift reactor combined with a 20-um bulb diffuser (Munasinghe and Khanal 2010b). The bubble column reactor reported on in their study had a $K_L a$ of 72 h^{-1} (Table 5).

Table 2. Volumetric Mass Transfer Coefficients (k_La) for Various Reactor Configurations and Hydrodynamic Conditions

Reactor configurations	N†(rpm)	Microorganisms	Gaseous substrates	Volumetric mass transfer coefficient (k_La)(h^{-1})
Trickle bed	n/a‡	n/a‡	Syngas	22
CSTR	n/a‡	n/a‡	Syngas	38
CSTR	200	*B. methylotrophicum*	CO	14.2
CSTR	300	*SRB*mixed culture*	Syngas	31 for CO, 75 for H_2
CSTR	300	*C. ljungdahlii*	Syngas	35 for CO
CSTR	300	*R. rubrum*	Syngas	28.1 for CO
CSTR	450	*R. rubrum*	Syngas	101 for CO
Stirred tank - microbubble sparger	200	*B. methylotrophicum*	CO	90.6
Stirred tank - microbubble sparger	300	*SRB*mixed culture*	Syngas	104 for CO, 190 for H_2
Packed bubble column	n/a‡	*R. rubrum*	Syngas	2.1
Trickle bed	n/a‡	*R. rubrum*	Syngas	55.5
Trickle bed	n/a‡	*SRB*mixed culture*	Syngas	121 for CO, 335 for H_2
Trickle bed	n/a‡	*C. ljungdahlii*	Syngas	137 for CO
Batch stirred tank	n/a‡	*P. productus*	CO	7.15
Stirred tank	300	*C. ljungdahlii*	CO	14.9
Stirred tank	400	*C. ljungdahlii*	CO	21.5
Stirred tank	500	*C. ljungdahlii*	CO	22.8
Stirred tank	600	*C. ljungdahlii*	CO	23.8
Stirred tank	700	*C. ljungdahlii*	CO	35.5
Bubble column	n/a‡	n/a‡	CO	72
Stirred tank	400	n/a‡	CO	10.8 to 155
Stirred tank	500	*R. rubrum*	Syngas	71.8
Column diffuser	n/a‡	n/a‡	CO	2.5 to 40.0
20-μm bulb diffuser	n/a‡	n/a‡	CO	31.7 to 78.8
Sparger only	n/a‡	n/a‡	CO	29.5 to 50.4
Sparger with mechanical mixing	150	n/a‡	CO	33.5 to 53.3
Sparger with mechanical mixing	300	n/a‡	CO	34.9 to 55.8
Submerged CHFM module	n/a‡	n/a‡	CO	0.4 to 1.1
Air-lift combined with a 20-μm bulb diffuser	n/a‡	n/a‡	CO	49.0 to 91.1
Air-lift reactor combined with a single point gas entry	n/a‡	n/a‡	CO	16.6 to 45.0

† Agitation speed. ‡ Not applicable. * Sulfate reducing bacteria.

Table 5. Volumetric mass transfer coefficients (K_La) for various reactor configurations sparging syngas or carbon monoxide (Munasinghe and Khanal 2010b)

Furthermore, using this equation, it is likely we achieved a much greater apparent K_La in our bubble column reactor, of up to 373 h^{-1}, when the CO in the headspace was minimal (Martin 2014, Appendix 8). One reason we achieve such good mass transfer is because the solvents produced by the bacteria decrease the surface tension in the culture. This can be observed by comparing the much larger bubbles sparging through the bubble column reactor pre-inoculation to the micro-bubbles sparging through the reactor a few days post-inoculation.

2,3 Butanediol Production

Evidence of 2,3 butanediol was observed when analyzing fermentation samples by HPLC. 2,3 butanediol is a valuable chemical used as a precursor to chemicals such as gamma-butyrolactone (GBL) and 1,3 butadiene, which is in turn a precursor to a synthetic rubber. A peak with a retention time of 18.8 min was often observed but HPLC was only run with standards for acetate and ethanol calibration until the last run with JA1-1, when a 2,3 butanediol standard was included. For this second fermentation with JA1-1, we recorded 2,3 butanediol production of 5 mM (0.0024 mol/L/day). 2,3 butanediol has also been reported, and a pathway proposed, with *C. autoethanogenum* at a concentration of 1.4-2 mM (Köpke et al. 2011b). More recently, 0.06 mol/L/day 2,3 butanediol was published (Wang et al. 2013).

Conclusions

ERI-2 and PETC performed markedly better than JA1-1 for ethanol production in our syngas fermentation system. These two strains performed very similarly and were faster to grow up and reached a higher biocatalyst density than JA1-1. We generated 0.301 g/L/h ethanol with PETC in a long-term continuous fermentation and ERI-2 performed almost as well. This rate is one-third of the recently reported 0.49 mol/L/day (0.941 g/L/h) with *C. autoethanogenum* (Wang et al. 2013), however the dilution rate used in that fermentation was almost five times as high as ours (1.8 day^{-1} compared to our 0.38 day^{-1}). We surmise that we could sustainably increase our ethanol production rate by increasing our dilution rate, as was previously suggested (Richter et al. 2013).

It was determined that with *C. ljungdahlii* the ethanol production rate was more closely related to cell density than to pH, but that lowering pH can have a positive effect on the ethanol/acetate concentration ratio because the acetate production rate decreases with low pH. Cell-specific total product concentrations and CO consumption declined with increased cell density, but it is unclear if the reason is related to the cell density (mass transfer, nutrient limitation) or the pH, which was lowered from pH 5.5 to 4.5 over the same period, or both. It was determined that pH 4.5 was too low for JA1-1, causing low overall product concentrations and diminished CO consumption.

Our calculated mass transfer coefficient of 190 h^{-1} for Stage 2 compares well with the highest published values. From the data collected during this study it is impossible to conclude whether our system is mass-transfer limited, nutrient limited, or both. With these comparison runs serving as a baseline, future experiments can further elucidate which parameters need to be optimized for higher ethanol production rates.

CHAPTER 4: RECOMMENDATIONS FOR FUTURE RESEARCH

In addition to the conclusions described in Chapter 3, some further experiments to run with our syngas fermentation are suggested below and could significantly increase our ethanol yields and would provide more clarity on the effects of pH.

1. Increase dilution rate for system to achieve higher ethanol production rate. Assuming a doubling time of 6 h for *C. ljungdahlii* (Dürre No Date), we could increase our flow through our 1-L Stage 1 from 40 mL/h (240 mL/6 h) to 160 mL/h (960 mL/6 h) without flushing out the cells. Flushing out Stage 2 is not a concern because it employs cell recycling. In addition, specific ethanol concentrations have shown to be higher at lower cell densities. Because we can adjust medium flow independently in the two stages with our bypass, flow through our 4-L Stage 2 could be increased from 80 mL/h (480 mL/6 h) up to 320 mL/h (1920 mL/6 h). This would match the 160 mL/h Stage 1 medium with fresh medium and bring the dilution rate in Stage 2 up from 0.48 day^{-1} to 1.92 day^{-1}, just above the dilution rate used by Wang et al. (2013), who showed higher ethanol production rates.

2. Confirm significance of the two-stage system by reaching normal continuous-flow steady state operation and then bypassing Stage 1 completely while maintaining same dilution rate in Stage 2 with fresh medium only.

3. Run system the same way as in comparison but continually bleed Stage 2 once it reaches an OD_{600} of 10.0. This will prevent cell density from confounding the effects of pH on growth, CO consumption, and ethanol production.

4. Pressurize the reactors and determine if this improves gas/liquid mass transfer by calculating the new K_La value and measuring the ethanol production rate.

5. Increase the gas recycle rate in Stage 2 to improve CO mass transfer and utilization. Decrease syngas inflow rate to just above what is consumed to increase efficiencies.

6. Run a control by running the system for 700 h exactly as described in the comparison study, but control the pH at 5.5 for the entire duration. Observe ethanol and acetate production and ethanol/acetate ratio.

7. Switching to working with actual syngas instead of simulated syngas is a necessary next step in scaling up the system to ensure the growth and production can be maintained in contact with gas impurities.

8. Monitoring power consumption used by our system and conducting a life-cycle analysis would be interesting to get information for modeling power demands and determining practicality of scaling up.

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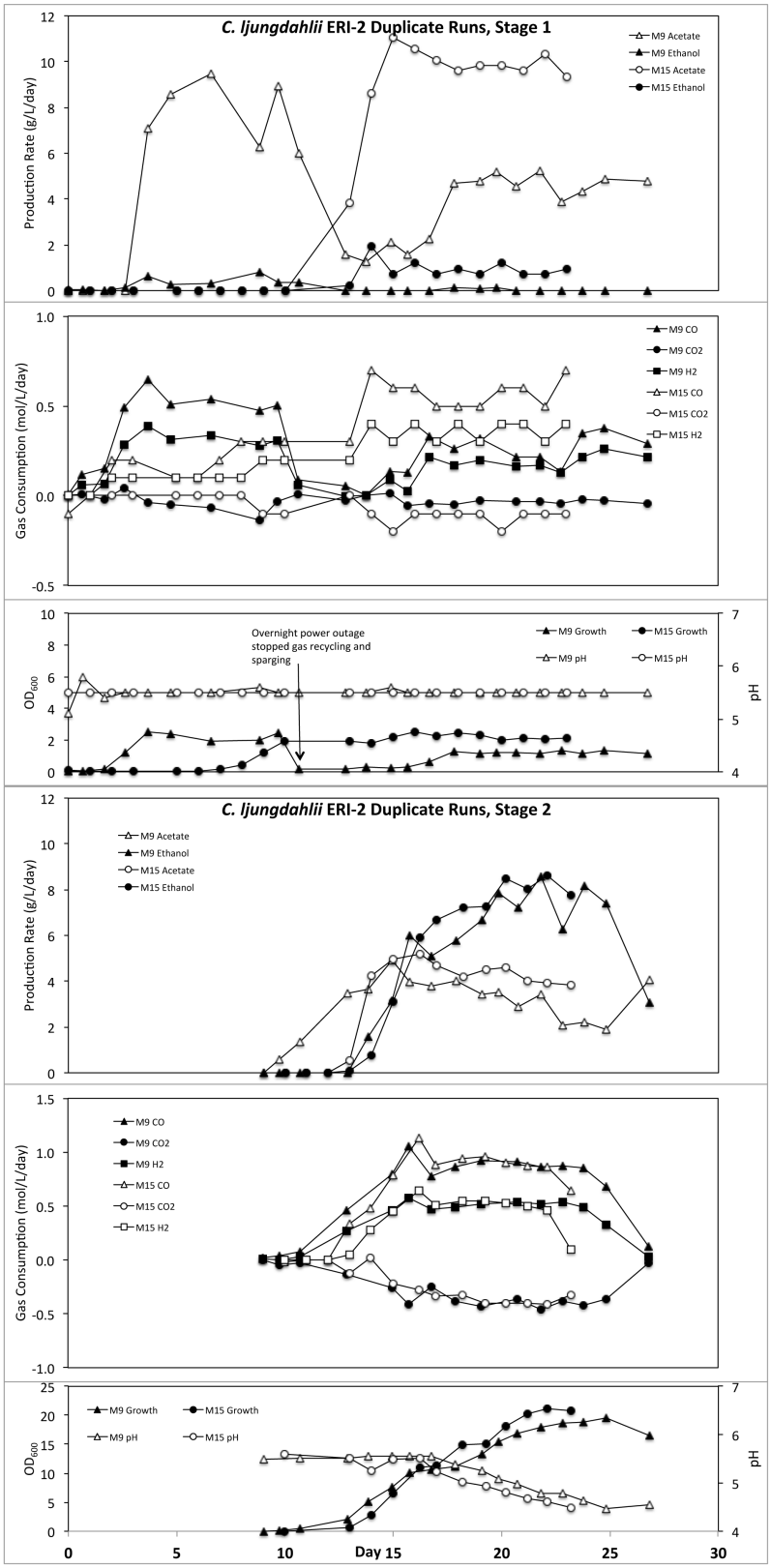
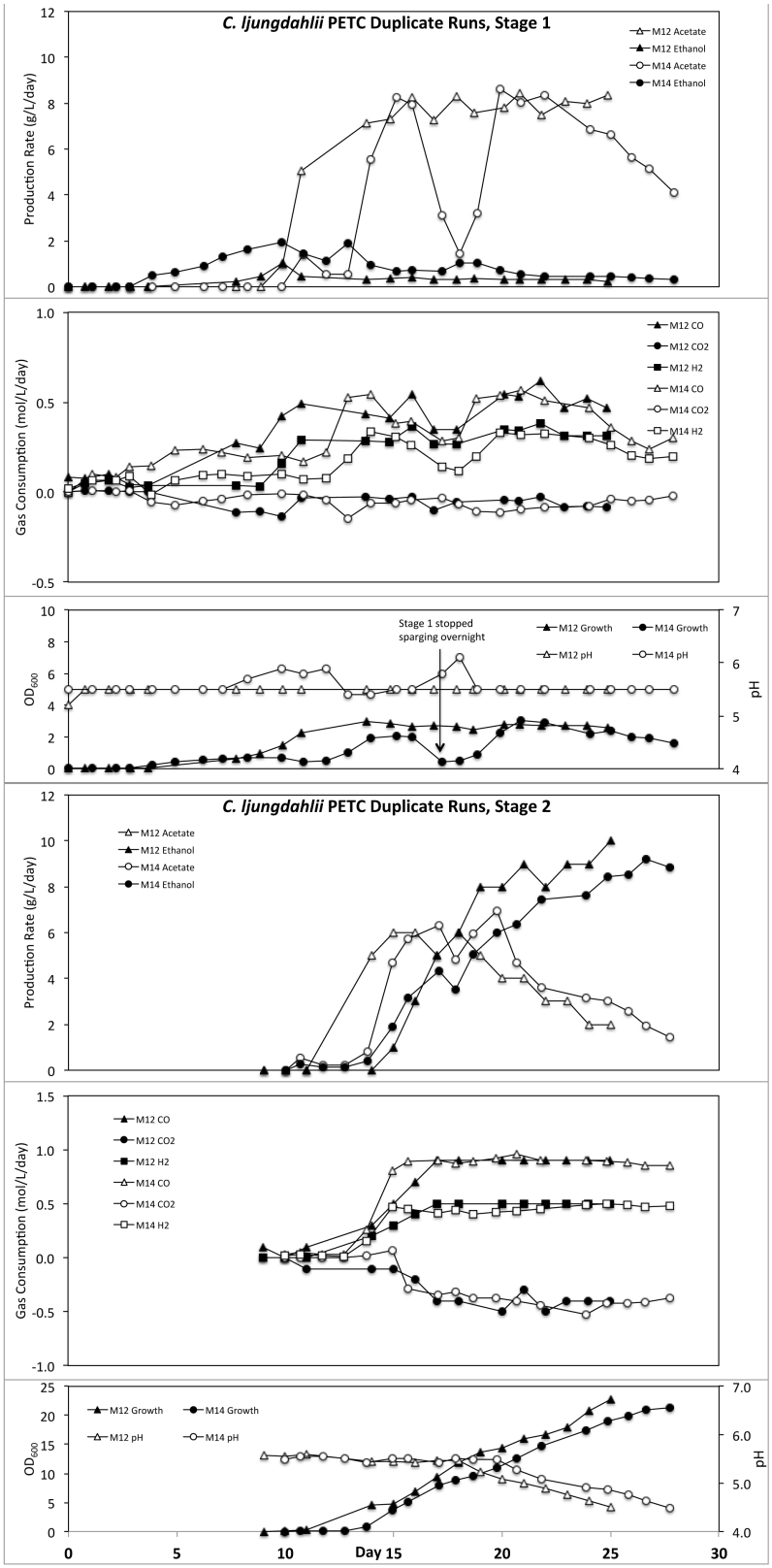
APPENDIX

Full time series data for *C. ljungdahlii* ERI-2 duplicate runs



Full time series data for *C. ljungdahlii* PETC duplicate runs



Full time series data for *C. autoethanogenum* JA1-1 duplicate runs

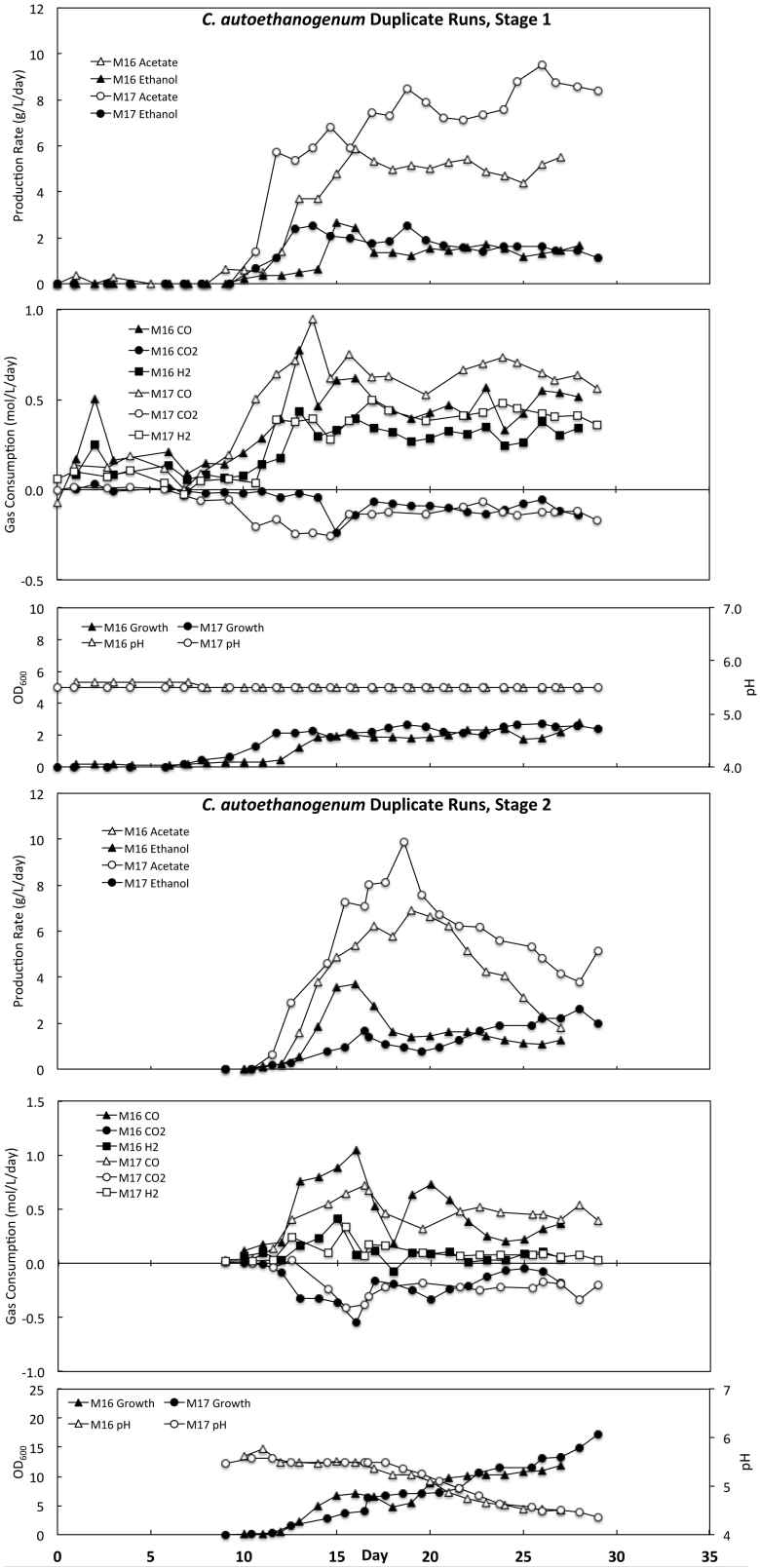


Photo of syngas fermentation system in Riley-Robb Hall, Room B45, Cornell University, Ithaca, NY, USA. Stage 1 (right) has been inoculated, while Stage 2 (left) has not been inoculated.

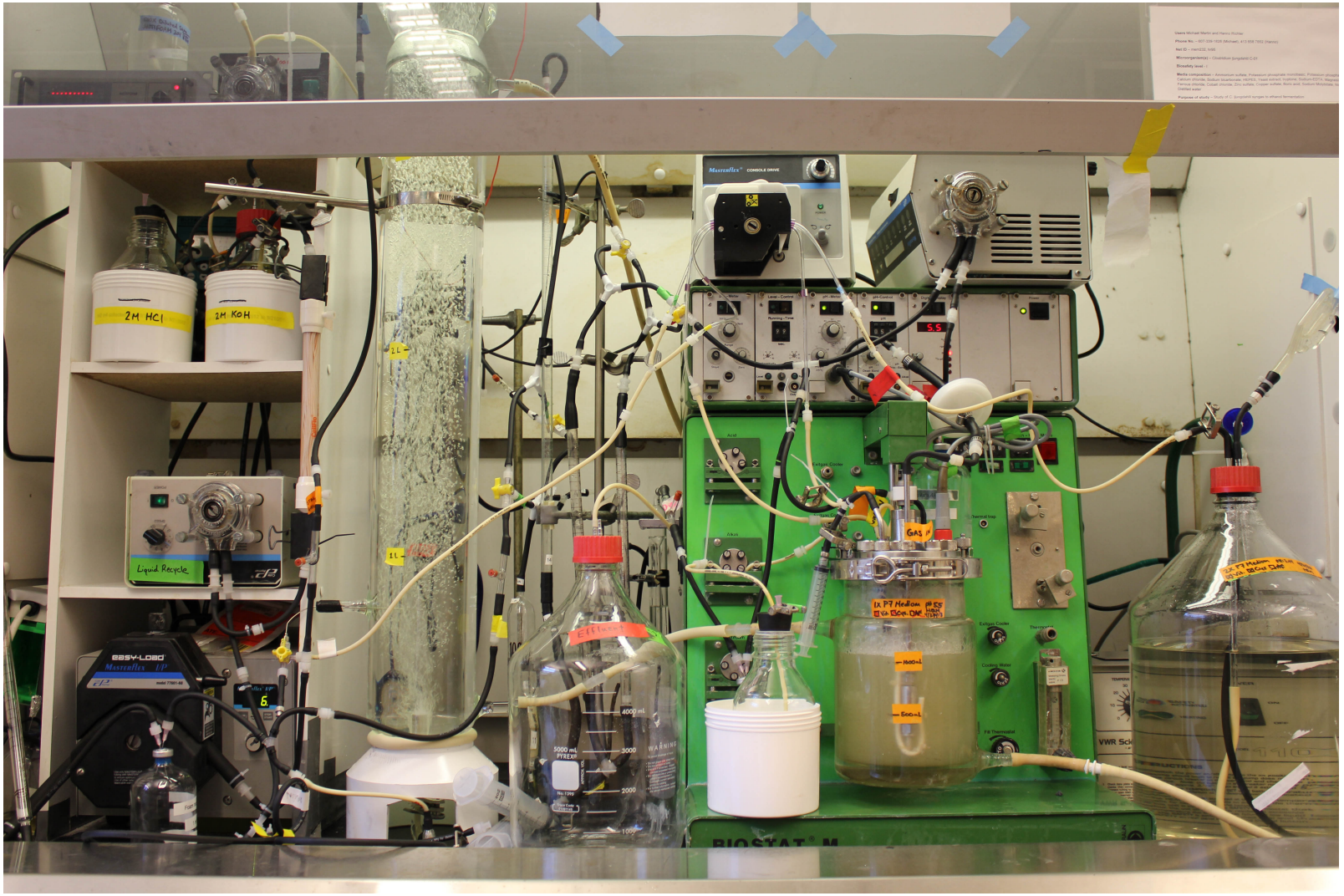
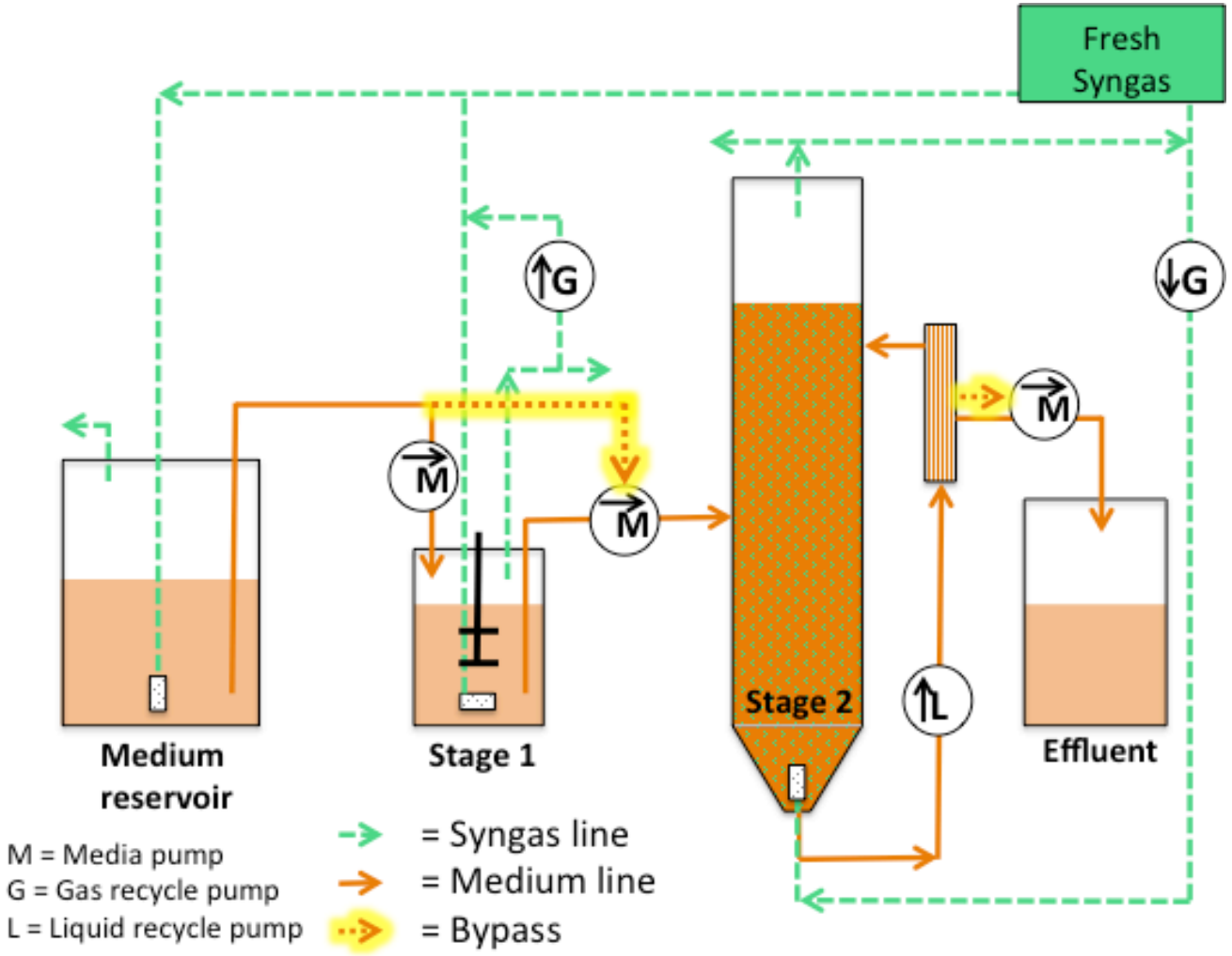


Diagram of two-stage syngas fermentation system with bypass



1x P7 Medium (Initial batch CSTR medium):

Name	Chemical Formula	Final Concentration in Medium (mg/L)
Minerals		
Sodium chloride	NaCl	800.00
Ammonium chloride	NH_4Cl	1000.00
Potassium chloride	KCl	100.00
Potassium phosphate monobasic	$KH_2PO_4•H_20$	100.00
Magnesium sulfate	$MgSO_4$	200.00
Calcium chloride	$CaCl_2$	40.00
Vitamins		
Pyridoxine		0.10
Thiamine		0.05
Riboflavin		0.05
Calcium pantothenate		0.05
Thiocitic acid		0.05
Nicotinic acid		0.05
Vitamin B12		0.05
Biotin		0.02
Folic acid		0.02
Mesna		0.10
Aminobenzoic acid		0.05
Trace metals		
Nitriloacetic acid		20.00
Manganese sulfate	$MnSO_4$	10.00
Ferrous ammonium sulfate	$(NH_4)_2Fe(SO_4)_2•6H_2O$	8.00
Cobalt (II) chloride	$CoCl_2•6H_20$	2.00
Zinc sulfate	$ZnSO_4$	2.00
Copper chloride	$CuCl_2$	0.20
Nickel chloride	$NiCl_2$	0.20
Sodium molybdate	Na_2MoO_4	0.20
Sodium selenite	$Na_2SeO_3(H_2O)_5$	0.20
Sodium tungstate	$Na_2WO_4•2H_2O$	0.20
Resazurin		1.00
L-cysteine		121.16
Yeast extract		50.00

Differences for continuous fermentation medium ('2x P7 Medium'):

-No yeast extract
-2x Minerals
-2x Trace metals
-2x Vitamins

Medium for *Clostridium carboxidivorans* P7 (Datar et al. 2004), modified

For vitamin and trace metals modifications see Rajagopalan et al. (2002). Biomass & Bioenergy, Vol 23, 487-493 (Rajagopalan et al. 2002)

ATCC medium 1754, Tanner, R. S., Miller, L. M. & Yang, D. (1993). *Clostridium ljungdahlii* sp. nov., an acetogenic species in clostridial rRNA homology group I.
Int J Syst Bacteriol 43, 232–236.

Note: Differences between Datar and ATCC medium are in composition of $CaCl_2$, Vitamin B_{12} and $ZnSO_4$ Also, we use Na_2SeO_3 instead of Na_2SeO_4.

Yeast extract* (Bacto)	0.5 g/L
MES (Morpholinoethanesulfonic acid)	5 g/L

Add mineral solution 30 mL/L, and 10 mL/L each of vitamin, trace metal and 1 mM cysteine solution.

Mineral solution for *Clostridium carboxidivorans* medium from (Datar et al. 2004):

Sodium chloride	80 g/L
Ammonium chloride	100 g/L
Potassium chloride	10 g/L
Potassium phosphate monobasic	10 g/L
Magnesium sulfate	20 g/L
Calcium chloride	4 g/L

100x Vitamin solution for *Clostridium carboxidivorans* medium:

Pyridoxine	0.01 g/L
Thiamine	0.005 g/L
Riboflavin	0.005 g/L
Calcium pantothenate	0.005 g/L
Thioctic acid	0.005 g/L
Amino benzoic acid	0.005 g/L
Nicotinic acid	0.005 g/L
Vitamin B12	0.005 g/L

Biotin	0.002 g/L
Folic acid	0.002 g/L
Mesna (Mercaptoethanesulfonic acid sodium salt)	0.01 g/L

100x Trace metals solution for *Clostridium carboxidivorans* medium:

Nitrilo triacetic acid	2 g/L
Manganese sulfate	1 g/L
Ferrous ammonium sulfate	0.8 g/L
Cobalt chloride	0.2 g/L
Zinc sulfate	0.2 g/L
Copper chloride	0.02 g/L
Nickel chloride	0.02 g/L
Sodium molybdate	0.02 g/L
Sodium selenate	0.02 g/L
Sodium tungstate	0.02 g/L

Protocol for Continuous Fermentation Systems
° Updated 01/02/13

Glycerol stocks

Prepare glycerol stocks for new strains (original protocol in blue notebook toward end, 2/24/12)
Mix:
1ml of 100% glycerol
2ml of P7 media
1ml of happy cells in exponential phase

Prepare P7 media in 150ml volume serum bottle:

For 100ml P7 media	**Concentration**	**Amount to add to 100ml media**	**Amount to add to 100ml media (using 2x stock)**
Mineral Solution	10ml/L	1ml	0.5ml
Trace Metal Solution	10ml/L	1ml	0.5ml
Resazurin	1mg/L	0.1ml of 1000mg/L solution OR 0.1mg powder	
MES buffer	5g/L	0.5g	
Glucose	5mM	0.25ml of 2M glucose into 100ml	
NO Yeast Extract			
*Cysteine (100mM)	10ml/L	1ml	
*Add Cysteine in the anaerobic hood, **after adjusting pH to 6.0**. Mix and then cap bottle with butyl stopper. Autoclave for 25 minutes.			
**Vitamins	10ml/L	1ml	0.5ml

** Add Vitamins to the P7 media serum bottle after autoclaving.
Next distribute 1mL glycerol into small butyl stoppered glass vials. Autoclave for 15 minutes.
Flush vials with sterile syngas and pressurize to 14psi.
Add 2mL P7 media into each vial.
Add 1mL of happy cells.
Gently mix and store in labeled box in -80℃ freezer.

Pre-cultures

Prepare pre-culture serum bottles (original protocol on p.37 of brown lab book)

Prepare 150ml of P7 media:

For 150ml P7 media	**Concentration**	**Amount to add to 150ml media**	**Amount to add to 150ml media (using 2x stock)**
Mineral Solution	10ml/L	1.5ml	0.75ml
Trace Metal Solution	10ml/L	1.5ml	0.75ml
Resazurin	1mg/L	0.15ml of 1000mg/L solution OR 0.15mg powder	
MES buffer	5g/L	0.75g	
Yeast Extract	**1g/L**	0.15g	
*Cysteine (100mM)	10ml/L	1.5ml	
*Add Cysteine in the anaerobic hood **after adjusting pH to 6.0**. Mix and then distribute 150ml evenly into 15 serum bottles (10ml in each). Cap with butyl stoppers. Autoclave for 25 minutes. Flush with syngas for 5 minutes with sterile filter and 2 green needles and then fill to 14psi.			
Vitamins (per bottle**)	10ml/L	0.1ml into 10ml media	0.05ml

Grow 2 precultures bottles (from frozen stock) in premade 10ml serum bottles.
**Vitamins need to be added to individual bottles! Inoculate with 2ml of frozen culture.
Incubate at 37° (on shaker?) until turbid (~2-5 days).

Growth cultures

Prepare 300ml of P7 media for 2 growth bottles (150ml media in each 250ml volume growth bottle):

For 300ml P7 media	**Concentration**	**Amount to add to 300ml media**	**Amount to add to 300ml media (using 2x stock)**
Mineral Solution	30ml/L	9ml	4.5ml
Trace Metal Solution	10ml/L	3ml	1.5ml
Resazurin	1mg/L	0.3ml of 1000mg/L solution OR 0.3mg powder	
MES buffer	5g/L	1.5g	
Yeast Extract	**0.5g/L**	0.15g	
Include stir bar in each bottle. **Adjust pH to 6.0**. Pour 150ml into each growth bottle setup. Autoclave bottles for 30 minutes. Flush with nitrogen to make anaerobic environment.			
Vitamins (per bottle**)	10ml/L	1.5ml into 150ml media	0.75ml
*Cysteine (100mM) (**per bottle**)	10ml/L	1.5ml into 150ml media	
***Antifoam 1000x dilution **(per bottle)**	1ml/L	.15ml into 150ml media	

*Add Cysteine to each bottle.
**Add Vitamins to each bottle.
***Add Antifoam to each bottle (*optional*)
When media turns from pink to clear, place bottles into 37℃ warm water bath and hooked up to be sparged with Syngas. Bottles will turn pink again.
Add another shot of Cysteine (1.5ml) after 15 minutes of sparging with Syngas. Wait until media turns from pink to clear (about 30 minutes).
Inoculate the 150ml of media with 7.5ml (5%) of turbid pre-culture.

Stage A

Initial P7 media (1x) for growing up culture and weaning it off Yeast Extract

Prepare 1L of P7 (1x) media:

For 1L P7 media	**Concentration**	**Amount to add to 1L media**	**Amount to add to 1L media (using 2x stock)**
Mineral Solution	30ml/L	30ml	15ml
Trace Metal Solution	10ml/L	10ml	10ml
Resazurin	1mg/L	1ml of 1000mg/L solution OR 1mg powder	
No MES buffer			
Yeast Extract	**0.05g/L**	0.05g	
Adjust pH to 5.5. Autoclave Stage A with all top fittings and syngas filter for 45 minutes (prepare outlets/inlets with appropriate clamps and aluminum foil).			
**Vitamins	10ml/L	10ml	5ml
*Cysteine (100mM)	10ml/L	10ml	

Prepare 1L of 1x P7 media with 0.05g/L yeast extract and no MES buffer. Install into Biostat and sparge with syngas until anaerobic (overnight). Add Vitamins and Cysteine. Turn on water heater circulation loop and set to 37℃.

Take base OD measurement of the P7 media and conduct baseline gas GC. Microscope check to make sure there is no contamination of Stage A. Take pH with external probe to confirm Biostat pH display is correct.

Inoculate Stage A with 100mL (10%) from the 150mL growth culture (take growth culture OD). Grow Stage A up as a batch culture to an OD between 0.5-1.0.

2x Concentrated P7 Media tank prep

First medium tank will be a **2x concentrated**, No Yeast Extract tank, once OD >1.0 and system turns to continuous. Begin pumping from Stage I to Stage 2 at Stage I OD of 1.0

Prepare 8L of P7 (2x) media:

For 8L P7 media	**Concentration**	**Amount to add to 8L media**	**Amount to add to 8L media (using 2x stock)**
Mineral Solution	60ml/L	480ml	240ml
Trace Metal Solution	20ml/L	160ml	80ml
Resazurin	1mg/L	8ml of 1000mg/L solution OR 8mg powder	
No MES buffer			
No Yeast Extract			
Take pH (should be near pH of 3.3). Clamp sparger tubing and liquid outflow tubing while leaving third (gas outlet) tubing open. Apply foil to luer lock fittings. Autoclave tank for 75 minutes on liquid cycle.			
*Vitamin Solution	20ml/L	160ml	80ml
**Cysteine (100mM)	10ml/L	80ml	
***Sigma Antifoam 204	1ml/L of 1000x diluted Antifoam	8ml	

When ready to replace tank,
Add:
*Aerobic Vitamin Solution
***Antifoam

Sparge with nitrogen for at least 3 hours.

**Finally, add L-Cysteine and wait until media turns from pink to light orange or clear.

Protocol for Continuous Fermentation System:
°*Updated 1/24/2013 → 4/23/13*

Precultures
Grow 2 precultures (from frozen stock or old preculture) in premade 10ml serum bottles with 0.5g/L yeast extract, 14psi syngas headspace, ph=5.5 or 6; *Vitamins need to be added! Incubate at 37°C until turbid (~2-7 days). If already adapted strain, use 0.05g/L yeast extract only. Wean strains down to 0.05 g/L yeast extract in serum bottles. Transfers weekly using 2% inoculum.

Growth Bottles
250ml growth bottle containing 150ml of P7 media with 0.05g/L yeast extract, 5g/L MES, 1x P7 media [Minerals (30ml/L, Trace Metals solution (10ml/L), Resazurin, P7 Vitamins (10ml/L), Cysteine (10ml/L)] and pH of 5.5.

Growth bottles continuously sparged with syngas in 37°C water bath with stir bar. Inoculate with 7.5ml from preculture (takes ~3-5 days to grow up).

Stage A
Prepare 1L of initial 1x P7 media with 0.05 g/L yeast extract and no MES buffer.
Calibrate pH probe!
Install into Biostat and sparge with syngas until anaerobic (overnight).
Add cysteine and P7 Vitamins. Microscope check for contamination.
Connect water jacket and warm Stage A to 37°C.
Turn on agitation, 200rpm.
Connect pH control and adjust pH to 5.5 (*I noticed pH will decrease (to ~5.3) in Stage 1 once syngas begins sparging through*)

Take base OD measurement of the P7 media and conduct baseline gas GC.
Inoculate Stage A with 40mL from 4 of the 10mL serum bottles or 50ml from growth bottle.
Grow Stage A up as a batch culture to an OD of at least 1.0

Begin continuous mode: At OD of around 1.0-1.5, begin media flow through and Bypass to Stage B. Media tank will be 2x concentrated P7 media with no yeast extract.

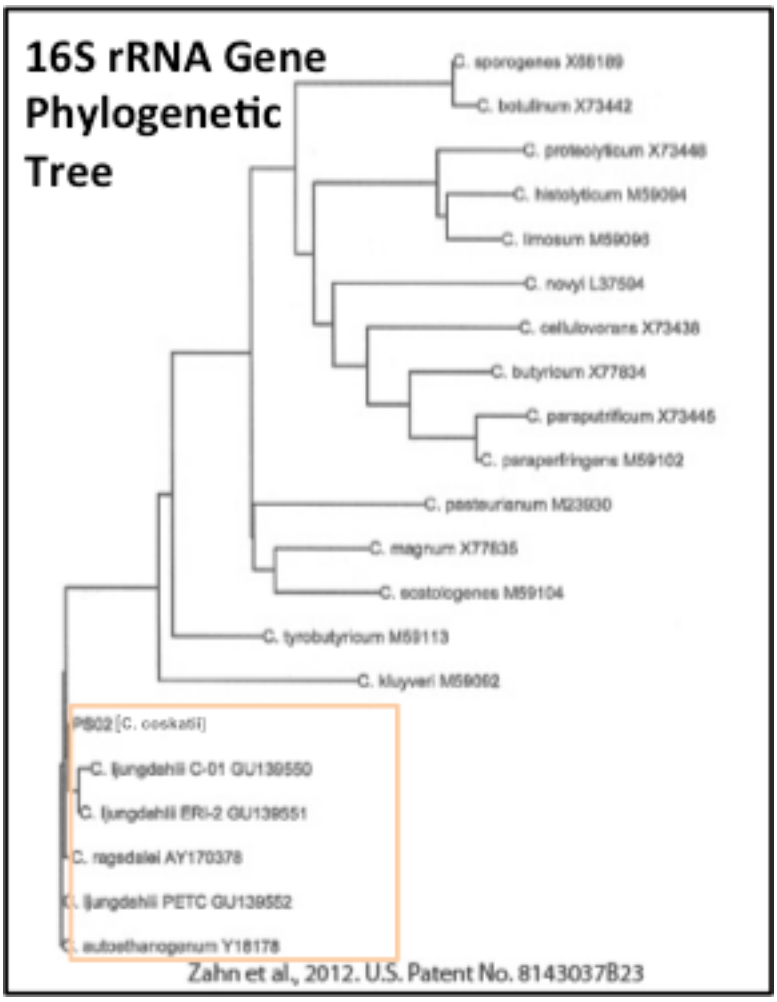
Stage B
Using a 1-sparger setup for the glass column now.
Begin at pH of 5.5. Set pH Controller to a range of 5.45-5.60 (M12) or 5.50-5.70 (M9).
Fill with 2x concentrated P7 media.
Once media flow and bypass begin, run system until Stage B reaches an OD of 10.

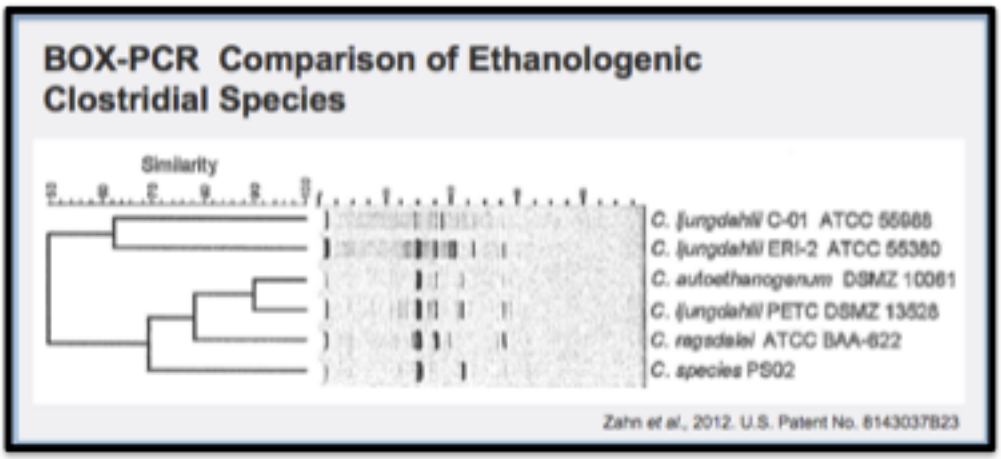
At OD 10, gradually decrease pH in Stage 2 by 0.15 (every day) to induce solventogenesis until pH 5.0 is reached. Reduce pH to optimal pH for given strain (~4.8), to induce solventogenesis. The ending pH range will be 4.40-4.80**.

Antifoam:
At beginning use 500x dilution.
At OD 3 swap to 100x dilution.
At OD 6 swap to 10x dilution.

See diagram for media flow rates, pump recycle speeds, and gas flow rates.

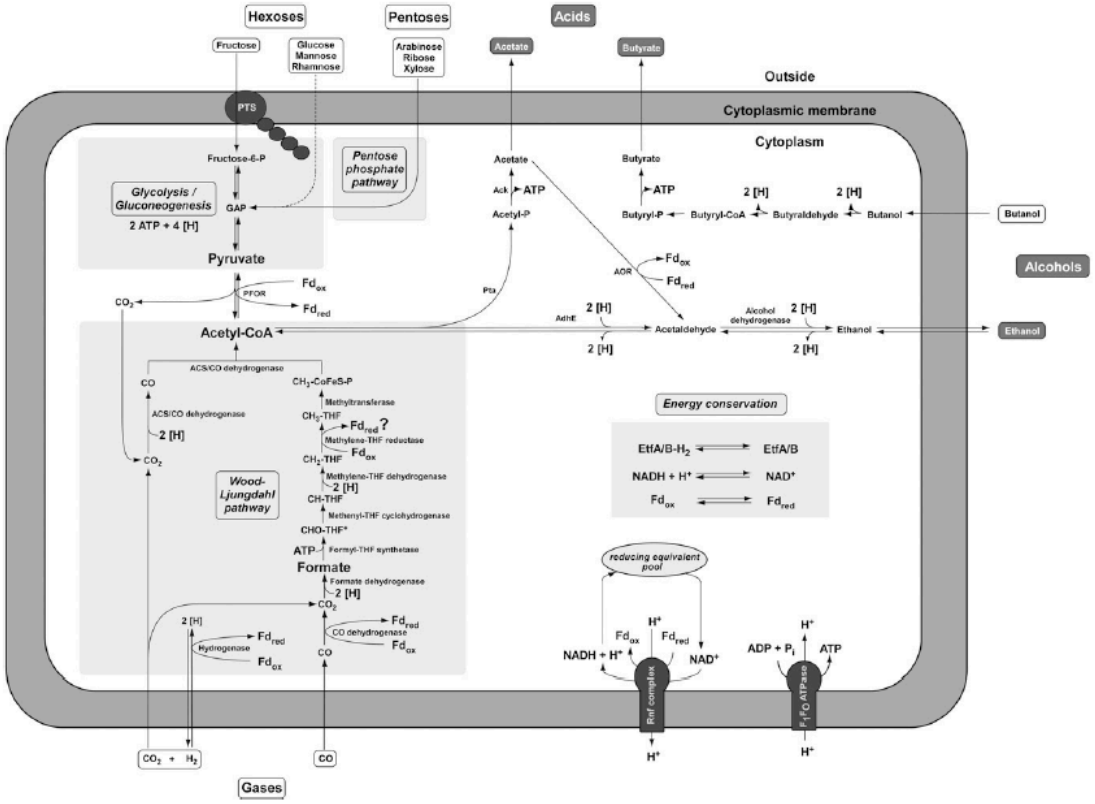
**When Stage 2 is at OD 10, take a 10mL sample in weighed Falcon tube, dehydrate overnight at 100degC and weigh again to ascertain Dry Weight in mg of cells. (This will not necessarily give weight of live cells?) Calculate coefficient to use to convert OD to mgDW, using new quartz cuvette that was used for OD measurement in M12, and old plastic cuvette that was used for OD calculation in M9.


16S rRNA Gene Phylogenetic Tree
C. sporogenes X68189
C. botulinum X73442
C. proteolyticum X73448
C. histolyticum M59094
C. limosum M59093
C. novyi L37594
C. cellulovorans X73438
C. butyricum X77834
C. paraputrificum X73445
C. paraperfringens M59102
C. pasteurianum M23930
C. magnum X77835
C. scatologenes M59104
C. tyrobutyricum M59113
C. kluyveri M59092
PS02 [C. cooskatii]
C. ljungdahlii C-01 GU139550
C. ljungdahlii ERI-2 GU139551
C. ragsdalei AY170378
C. ljungdahlii PETC GU139552
C. autoethanogenum Y18178
Zahn et al., 2012. U.S. Patent No. 8143037B23


BOX-PCR Comparison of Ethanologenic Clostridial Species
Similarity
C. ljungdahlii C-01 ATCC 55988
C. ljungdahlii ERI-2 ATCC 55380
C. autoethanogenum DSMZ 10061
C. ljungdahlii PETC DSMZ 13528
C. ragsdalei ATCC BAA-622
C. species PS02
Zahn et al., 2012. U.S. Patent No. 8143037B23

Detailed Wood-Ljungdahl Pathway

Köpke, M., et al., *Clostridium ljungdahlii represents a microbial production platform based on syngas.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(29): p. 13087-13092.



C. ljungdahlii C-01 serum bottle and Stage 1 growth data

The preculture serum bottles were 125ml volume with 10ml of P7 media (refer to our recipe) and 14 psi syngas (60% carbon monoxide, 35% hydrogen, 5% carbon dioxide) headspace. After inoculation, bottles wer
The goal of the adaptive laboratory evolution was to grow the strains in medium with no yeast extract.

C. ljungdahlii C-01

				New serum bottles used			Number of serum bottles inoculated: Growth (-/0/+)					Notes
Generation #	**Inoculum culture**	**Transfer Date**	**Inoculation amount (mL)**	**Date**	**pH**	**Yeast extract (g/L)**	**1**	**2**	**3**	**4**	**5**	
		1/16/13	5% (0.5)		5.5	0.5	-	-				Discarded 2 c
	1 Frozen stock	**1/30/13**	5% (0.5)		6.0	1.0	-					Discarded on
		1/16/13	5% (0.5)		5.5	0.05	-					Discarded on
	1 Frozen stock	**7/16/13**			5.5	0.5		n/a	n/a	n/a		Inoculated 2
	1 Frozen stock	**8/6/13**	4% (0.4)	7/15/13	5.5	0.5	-	-	n/a	n/a	n/a	No growth
	2 8/6/13	**8/20/13**	4% (0.4)	8/7/13	5.5	1.0	-	n/a	n/a	n/a	n/a	No growth
	2 8/6/13	**8/28/13**	4% (0.4)	8/7/13	5.5	1.0	-	n/a	n/a	n/a	n/a	No growth
	1 Frozen stock	**9/9/13**	2% (0.2)	8/7/13	5.5	1.0	-	-	n/a	n/a	n/a	little growth,
	2 9/9/13	**10/9/13**	4% (0.4)									

Updated 9/18/13 MEM

Experiment M6 (*C. ljungdahlii C-01*) ATCC 55988

2-stage fermentation with Clostridium ljungdahlii CO-1. Medium in stage 1 is P7 1x concentration + 0.05 g/L YE, pH 5.5.
Stage A original OD600 blank medium, 1x concentrated = 0.115 0.115

OD600 blank medium, 2x concentrated = 0.102

Sample #	Date	time	hours		OD600	Dilution factor	OD phosphate buffer	calc blank		Final OD600	mgDW/L	pH
0	1/3/13	12:00	0.00		0.115	1		0.115		**0**	0.0	5.5
1	1/3/13	14:30	2.50		0.128	1		0.115		**0.013**	3.1	5.6
2	1/4/13	17:00	29.00		0.132	1		0.115		**0.017**	4.1	5.6
3	1/5/13	17:15	53.25		0.126	1		0.115		**0.011**	2.7	5.6
4	1/7/13	10:00	94.00		0.122	1		0.115		**0.007**	1.7	5.6
5	1/8/13	9:45	117.75		0.124	1		0.115		**0.009**	2.2	5.5
6	1/9/13	10:00	142.00		0.127	1		0.115		**0.012**	2.9	5.6
7	1/10/13	13:30	169.50		0.122	1		0.115		**0.007**	1.7	5.5
8	1/11/13	17:30	197.50		0.126	1		0.115		**0.011**	2.7	5.6
9	1/12/13	17:00	221.00		0.133	1		0.115		**0.018**	4.4	5.6
10	1/14/13	11:00	263.00		0.138	1		0.115		**0.023**	5.6	5.6
11	1/15/13	12:00	288.00		0.159	1		0.115		**0.044**	10.6	5.6
12	1/16/13	8:30	308.50		0.156	1		0.115		**0.041**	9.9	5.6
13	1/17/13	9:15	333.25		0.156	1		0.115		**0.041**	9.9	5.6
				-990660.00								
				-990660.00								
				-990660.00								
				-990660.00								

C. ragdalei serum bottle growth data

The preculture serum bottles were 125ml volume with 10ml of P7 media (refer to our recipe) and 14 psi syngas (60% carbon monoxide, 35% hydrogen, 5% carbon dioxide) headspace. After inoculation, bottles were incubated at 37 degrees Celsius. The goal of the adaptive laboratory evolution was to grow the strains in medium with no yeast extract.

C. ragsdalei P11

					New serum bottles used			Number of serum bottles inoculated: Growth (-/0/+)					Notes
Generation #	Inoculum culture		Transfer Date	Inoculation amount (mL)	Date	pH	Yeast extract (g/L)	1	2	3	4	5	
1	Frozen stock		7/15/13			5.5	0.5	+	+	n/a	n/a	n/a	Inoculated 2
2		7/15/13	8/6/13	2% (0.2)	7/15/13	5.5	0.5	+	+	n/a	n/a	n/a	Transferred i
3		8/6/13	8/20/13	2% (0.2)	8/7/13	5.5	1.0	-					No growth
3		8/6/13	**8/23/2013?**	4% (0.4)	8/7/13	5.5	1.0	+		n/a	n/a	n/a	

Updated 9/18/13 MEM

C. coskatii Stage 1 growth data

Experiment M7 (*C. coskatii*)

2-stage fermentation with *Clostridium coskatii*. Medium in stage 1 is P7 1x concentration + 0.05 g/L YE, pH 5.5.
Stage A original OD600 blank medium, 1x concentrated = 0.115 0.115

OD600 blank medium, 2x concentrated = 0.118

Sample #	Date	time	hours	OD600	Dilution factor	OD phosphate buffer	calc blank	Final OD600	mgDW/L
0	1/18/13	17:00	0.00		1		0.115	**-0.115**	-27.8
1	1/29/13	10:30	0.00	0.115	1		0.115	**0.000**	0.0
2	1/29/13	14:30	4.00	0.123	1		0.115	**0.008**	1.9
3	1/30/13	12:30	26.00	0.150	1		0.115	**0.035**	8.5
4	1/31/13	14:30	52.00	0.181	1		0.115	**0.066**	16.0
5	2/1/13	17:00	78.50	0.243	1		0.115	**0.128**	31.0
6	2/2/13	15:30	101.00	0.544	1		0.115	**0.429**	103.8
7	2/4/13	9:00	142.50	0.240	4	0.085	0.093	**0.590**	142.8
8	2/5/13	15:45	173.25	0.254	4	0.085	0.093	**0.646**	156.3
9	2/6/13	11:00	192.50	0.232	5	0.085	0.091	**0.705**	170.6
10	2/7/13	15:00	220.50	0.277	4	0.085	0.093	**0.738**	178.6
11	2/8/13	14:00	243.50	0.160	5	0.086	0.092	**0.341**	82.5
12	2/10/13	17:30	295.00	0.293	1		0.115	**0.178**	43.1
13	2/11/13	15:30	317.00	0.386	1		0.115	**0.271**	65.6
14	2/12/13	14:00	339.50	0.420	1		0.115	**0.305**	73.8
15	2/13/13	14:30	364.00	0.365	1		0.115	**0.250**	60.5
16	2/14/13	10:30	384.00	0.335	1		0.115	**0.220**	53.2
17	2/15/13	13:00	410.50	0.441	1		0.115	**0.326**	78.9
18	2/17/13	16:00	461.50	0.411	1		0.115	**0.296**	71.6
19	2/18/13	15:00	484.50	0.307	1		0.115	**0.192**	46.5
20	2/19/13	11:30	505.00	0.348	1		0.115	**0.233**	56.4
21	2/20/13	14:00	531.50	0.410	1		0.115	**0.295**	71.4
22	2/21/13	16:00	557.50	0.386	1		0.115	**0.271**	65.6
23	2/22/13	9:30	575.00	0.358	1		0.115	**0.243**	58.8
24	2/24/13	23:00	636.50	0.328	1		0.115	**0.213**	51.5
25	2/25/13	16:00	653.50	0.403	1		0.115	**0.288**	69.7
26	2/26/13	11:00	672.50	0.588	1		0.115	**0.473**	114.5
27	2/27/13	18:00	703.50	0.380	1		0.115	**0.265**	64.1
28	3/1/13	17:00	750.50	0.398	1		0.115	**0.283**	68.5
29	3/4/13	18:00	823.50	0.320	1		0.115	**0.205**	49.6

K_La calculations

Henry's Law constant for CO: K_H° for CO at 298K = 9.9×10^{-4} M/atm

Sander, R. *et al*. (1999). Compilation of Henry's Law Constants for Inorganic and Organic Species of Potential Importance in Environmental Chemistry. Available at http://www.mpch-mainz.mpg.de/~sander/res/henry.html

Van't Hoff Equation to adjust K_H° from 25°C to 37°C:

$k_H = (K_H^\circ) \exp [-\Delta solnH/R((1/T)-(1/T^\circ))]$ and $-d\ln(k_H)/d(1/T) = -\Delta solnH/R = 1300K$

therefore,

$\mathbf{k_H}$ = $(9.9\times10^{-4}$ M/atm) exp [-1300K((1/310K)-(1/298K))] = 0.00117 M/atm at 37°C = 1.17 mmol/L·atm = **0.8547 L·atm/mmol CO**

Minimum K_La
Representative sample: PETC Run 2, sample M14-B-21

Carbon monoxide consumption: 2.67 mmol CO/min in 4L culture = 40 mmol CO/L/h

Headspace: 18 mol % CO → P^G_{CO} = 0.18 atm

$dC/dt = K_La \cdot (C_i - C)$

40 mmol CO/L/h = $K_La \cdot$ (28 mg CO/L – 0)
40 mmol CO/L/h = $K_La \cdot$ (.999 mmol CO/L – 0)
$K_La = 40\ h^{-1}$

Representative K_La

$Q_{CO} = (K_La/k_H)_{CO} \bullet P^G_{CO}$

where,

k_H = Henry's Law constant

P^G_{CO} = partial pressure of CO in the Stage 2 headspace

40 mmol CO/L·h = $(K_La/k_H)_{CO} \times$ (0.18 atm)
$(K_La/k_H)_{CO}$ = 222.22 mmol CO/(L·h·atm)
$(K_La)_{CO} = 222.22$ mmol CO/(L·h·atm) × $(k_H)_{CO}$
$(K_La)_{CO} = 222.22$ mmol CO/(L·h·atm) × (0.8547 L·atm/mmol CO)
$K_La = 189.9\ h^{-1}$

Maximum K_La
(PETC Run 1, sample M12-B-11)

Carbon monoxide consumption: 1.27 mmol CO/min in 4L culture = 19 mmol CO/L/h

Headspace: 4.35 mol % CO → P^G_{CO} = 0.0435 atm

$Q_{CO} = (K_La/k_H)_{CO} \bullet P^G_{CO}$

19 mmol CO/L·h = $(K_La/k_H)_{CO}$ × (0.0435 atm)
$(K_La)_{CO}$ = 475 mmol CO/(L·h·atm) × (0.8547 L·atm/mmol CO)
K_La = 373.3 h^{-1}

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Article

A Two-Stage Continuous Fermentation System for Conversion of Syngas into Ethanol

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Abstract: We have established a two-stage continuous fermentation process for production
of ethanol from synthesis gas (syngas) with *Clostridium ljungdahlii*. The system consists of a 1-L continuously stirred tank reactor as a growth stage and a 4-L bubble column equipped with a cell recycle module as an ethanol production stage. Operating conditions in both stages were optimized for the respective purpose (growth in stage one and alcohol formation in stage two). The system was fed with an artificial syngas mixture, mimicking the composition of syngas derived from lignocellulosic biomass (60% CO, 35% H_2, and 5% CO_2). Gas recycling was used to increase the contact area and retention time of gas in the liquid phase, improving mass transfer and metabolic rates. In stage two, the biocatalyst was maintained at high cell densities of up to 10 g DW/L. Ethanol was continuously produced at concentrations of up to 450 mM (2.1%) and ethanol production rates of up to 0.37 g/(L·h). Foam control was essential to maintain reactor stability. A stoichiometric evaluation of the optimized process revealed that the recovery of carbon and hydrogen from the provided carbon monoxide and hydrogen in the produced ethanol was 28% and 74%, respectively.

Keywords: syngas; ethanol; *Clostridium ljungdahlii*; mass transfer; continuous culture

1. Introduction

Pyrolysis of organic materials, including lignocellulosic feedstocks, to obtain producer gas (synthesis gas or syngas) and subsequent fermentation of the syngas into ethanol and other short-chain hydrocarbon compounds is a strategy for production of bioalcohols that has been developing since the 1980s. Syngas derived from biomass is a mixture of carbon monoxide, hydrogen, and carbon dioxide, with other impurities, such as methane, hydrogen sulfide, nitrogen compounds, tars, and traces of other hydrocarbon and aromatic compounds. Microbes with promising capabilities to ferment carbon monoxide, hydrogen, and carbon dioxide into ethanol and other products have been isolated, and the biochemistry of these so-called acetogenic carboxydotrophic bacteria is well explained in the literature [1,2]. Several comprehensive reviews on syngas fermentation have recently been published [3–5].

Still, there are only a handful of scientific publications that focus on the technical and energetic feasibility of syngas fermentation. Some of the studies employ continuously stirred tank reactors (CSTRs) with one or two stages and cell recycling to increase mass transfer of substrate gases, density of biocatalyst and productivity [6–10]. Most reports focus on the concentration of products, and on the ratio of ethanol:acetic acid produced. Few reports actually provide information about specific rates of formation of the product(s) of interest, specifically ethanol. To our knowledge, the highest ethanol productivities of 1.6 g/(L·h) and 15 g/(L·h) have been reported in a patent for CSTR systems with a headspace pressure of 1 or 6 atmospheres, respectively, for *Clostridium ljungdahlii* strain C-01 [6].

The main product of syngas fermenting carboxydodrophic clostridia is usually acetic acid. Formation of acetic acid or ethanol from syngas has been reported for *C. ljungdahlii*, analogous to formation of butyric acid or butanol during acidogenesis or solventogenesis, by ABE fermenting clostridia [11]. In the following, we will use the terms “acidogenesis” and “solventogenesis” for formation of either acetic acid or ethanol, while we want to emphasize that the molecular mechanisms for production of carboxylic acids and conversion of carboxylic acids into alcohols are much different in ABE fermentation *vs*. syngas fermentation. The key mechanisms for ABE fermentation involve glycolysis, thiolase, and CoA-transferase, while this is the Wood-Ljungdahl pathway, and aldehydeoxidoreductase for syngas fermentation [1,11]. During syngas fermentation, acidogenesis occurs at high growth rates at favorable growth conditions (sufficient supply with nutrients, such as vitamins and minerals, optimal pH and temperature, and no end product inhibition), but somewhat limited supply with electron donor (CO or H_2). Solventogenesis, on the other hand, is favored during slow growth, in the presence of abundant electron donor [12], but otherwise unfavorable growth conditions, such as lower temperature, nutrient limitation [6] and lower pH [13,14]. Another contributing factor is very likely end-product inhibition caused by high concentrations of (undissociated) acetic acid, analogous to butanol formation in solventogenic clostridia being triggered by similar factors and by undissociated butyric acid [11,15].

For many fermentation processes, a continuous culture is seen as advantageous compared to a batch culture because in a continuous culture in general, the biocatalyst, once grown, remains viable for a long time (theoretically indefinitely), while batch cultures have to be re-grown after each fermentation run, before they become productive again. In case of syngas fermenting organisms, re-growing can take a long time, because of the relatively long doubling times, which would cause downtimes in the order of weeks for a commercial batch system. Then, the syngas at least, has to be

supplied continuously, in contrast to sugar fermentations, where the substrate can be added to the liquid medium once, at the beginning of the run. In addition, a two-stage continuous culture with spatial separation of cell growth and ethanol production is likely to have numerous advantages over a single-stage continuous fermentation setup because: (1) the temperature and pH can be optimized separately in each stage; (2) the working volume of each stage can be adjusted to set different dilution and growth rates, to promote fast growth and acidogenesis in stage one, and growth stagnation, nutrient limitation, and solventogenesis in stage two (because of nutrient consumption in stage one and low dilution rates in stage two); (3) the acetic acid produced in stage one can be converted into ethanol in stage two during solventogenesis. The latter is even triggered by the additional acetic acid derived from stage one; (4) the accumulation of biocatalyst in stage two (because of lower dilution rate) is beneficial for high reactor productivity and can be further enhanced by cell recycling (filtering the effluent and keeping the cells in the reactor); and (5) the supply of nutrients into stage two can be individually altered (via a media bypass, see below) to adjust the balance between sufficient supply for cell viability and limitation of nutrients for maintaining the solventogenic state.

There are numerous studies suggesting that syngas fermentation rates can quickly become limited due to the supply of gaseous substrates because of the low solubility of carbon monoxide and hydrogen in water [3]. Continuously stirred tank reactors (CSTRs) and bubble columns, both with micro bubble generators, have been reported to be the most efficient reactor types for gas distribution. Bubble columns have the additional advantage that they require a lower volumetric energy input [16].

Here, we describe a continuous syngas fermentation system optimized for ethanol productivity, consisting of two stages operating at ambient pressure: (1) a growth stage (CSTR) operated at pH 5.5, allowing optimal growth of *C. ljungdahlii* while producing mainly acetic acid; and (2) a production stage (bubble column) operated at a lower pH to trigger solventogenesis and to achieve conversion of syngas and of acetic acid (from stage one) into ethanol. Stage two was equipped with a cell recycling module to accumulate biocatalyst, and a bypass to supply additional nutrient media from the reservoir, required by high cell densities. Both stages utilized gas recycling to improve residence time and mass transfer of the substrate gases in(to) the liquid media. Foam in stage two was controlled with an antifoam injection system, which was triggered by high foam levels.

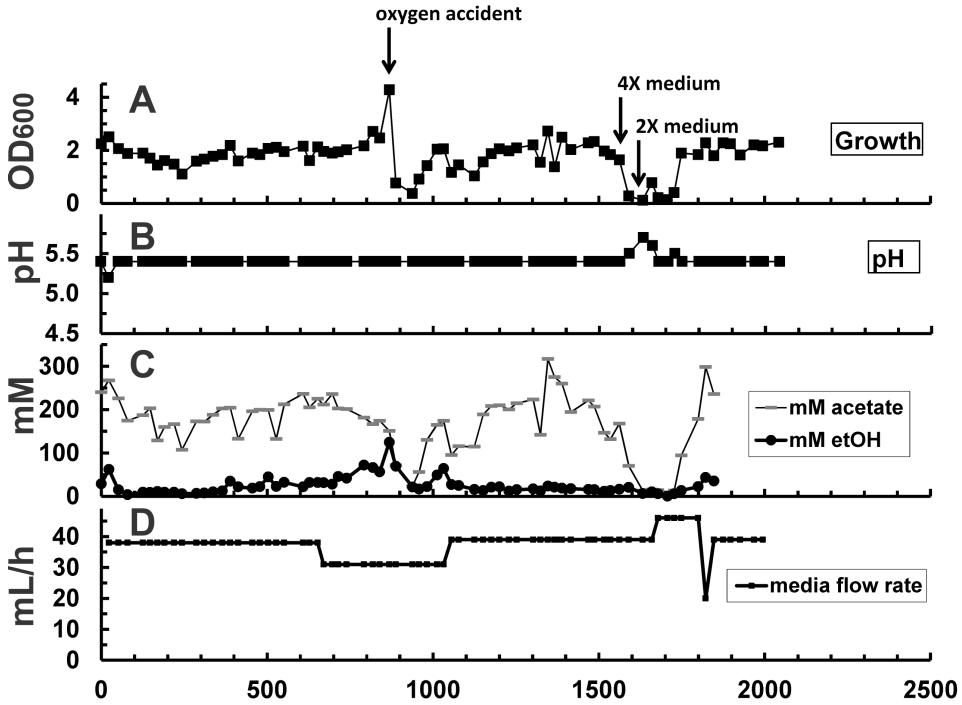
2. Results and Discussion

2.1. Cell Growth

Stage one (growth stage): Once inoculated with 40 mL of preculture, stage one was first operated in batch mode to allow for a maximum initial increase of cell density. The OD_{600} increased from 0.030 to 0.814 within 2 days. Next, continuous operation was started with the pumping of fresh medium at a flow rate of 39 mL/h (dilution rate $D = 0.039\ h^{-1}$). The cell density in stage one increased slowly until reaching an OD_{600} of ~2.0 after 4 more days, when equilibrium (growth rate = dilution rate) was reached. This timepoint is plotted as 0 h in Figure 1. Two incidents had severe negative impact on stage 1, which recovered after the issues were fixed: (1) a leak in the gas recycle loop at 888 h, causing oxygen to intrude; and (2) when 4× concentrated medium was supplied from hour 1562–1632. For estimation of the amount of acetic acid produced by a certain amount of cells, it is helpful to note that the concentration of acetic acid in stage one was always ~100 mM per OD_{600}. Knowing that the coefficient for milligram dry weight per Liter per OD_{600} [mg DW/(L·OD_{600})] is 242 for *C. ljungdahlii*

ERI-2 [17], one can estimate that 1 g DW of cell mass produced about 0.4 mol, or 24 g acetic acid. The ethanol concentration was generally a factor of 5–20 below the concentration of acetic acid throughout the entire run, illustrating that stage one was primarily acidogenic.

Figure 1. Stage one during continuous operation for 2014 h (83 days). Shown are the data for: (**A**) growth (OD_{600}); (**B**) pH value; (**C**) concentration of the fermentation products ethanol and acetate in mM; and (**D**) the average total daily feed rate of media (total media flow, mL/h). Arrows with comments indicate changes made to the system, explained in more detail in Table 1 and in the text.



Stage two (production stage): Once growth equilibrium had been reached in stage one, its effluent was directed into stage two for inoculation and constant supply with grown cells and acetic acid. The pH in stage two was always maintained within a range of 4.4 to 4.8 (Figure 2B).

Media dilution rate in stage two was initially 0.01 h^{-1} at a media flow rate of 39 mL/h. In Table 1, the operational changes made to stage one and stage two (during the continuous run) are summarized, as well as their effects on performance parameters. Overall, increasing the media flow and dilution rates resulted in lower OD_{600} and product concentrations in stage one because of a higher washout rate (not shown), but in a higher performance of stage two. For example, the maximum cell density (OD_{600}) increased from 9.9 to 17.8, when the dilution rate in stage two was increased from 0.010 to 0.016 (Figure 2A, and Table 1, time point 650 h).

Figure 2. Performance of stage two during continuous operation for 2014 h (83 days). Shown are the data for: (**A**) growth (OD_{600}); (**B**) pH value; (**C**) concentration of the fermentation products ethanol and acetate in mM; (**D**) the average daily ethanol production rate (mmol/min); (**E**) the average daily rates of consumption (mmol/min) for carbon monoxide and hydrogen and for production of carbon dioxide; (**F**) the average daily molar ratios of carbon monoxide and hydrogen consumed *vs*. ethanol produced; and (**G**) the average total daily feed rate of media (total media flow, mL/h). Arrows with comments indicate changes made to the system, which is explained in more detail in Table 1 and in the text. 1517 h is the timepoint at which stable performance at optimized conditions was achieved, and where performance data were taken for Table 2.

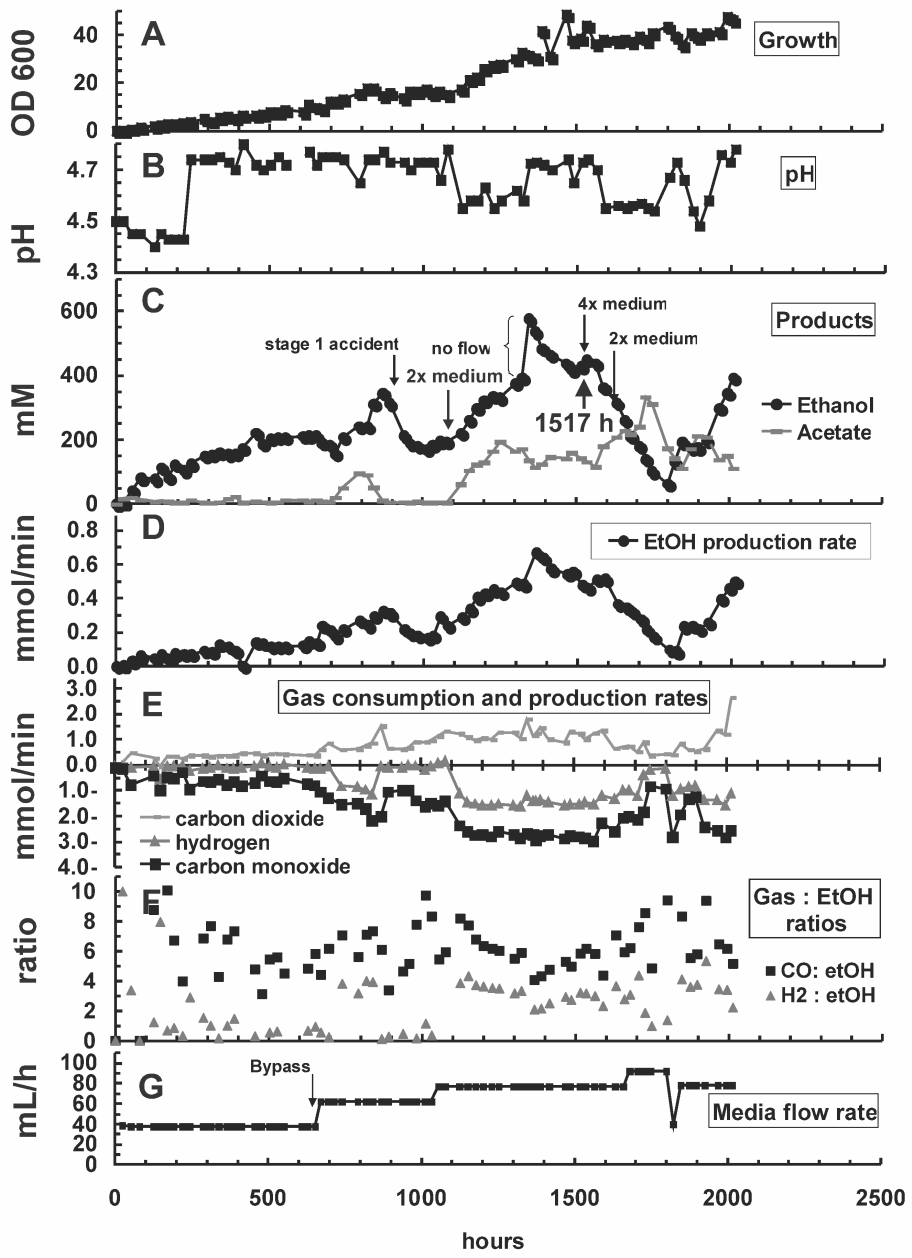


Table 1. Summary of changes in reactor operating conditions and their effect on reactor performance.

Hour	Change in reactor condition (Figure 2B,G)		Effect on reactor performance (Figure 2A, C–E)	
	Stage one	**Stage two**	**Stage one**	**Stage two**
0–650	$D = 0.039\ h^{-1}$ pH = 5.5	$D = 0.010\ h^{-1}$ pH = 4.4–4.8	Growth at equilibrium, OD_{600} at ~2.0	Inoculation; OD_{600} increased at an initial rate of 0.37/day to ~9.9
650–888	Activated media bypass channel (Figure 1), increased overall media flow rate by 50% to 62 mL/h; increased dilution rate in stage 2 relatively to stage 1 $D = 0.031\ h^{-1}$	$D = 0.016\ h^{-1}$	OD_{600} increased to ~2.4	OD_{600} increased to 17.8; higher reactor performance (Figure 2C–E)
888	ACCIDENT: ambient air drawn into stage 1		OD_{600} declined to 0.37, recovery completed at 1013 h	OD_{600} was not affected, but metabolic rates declined (Figure 2C–E)
1056	$D = 0.039\ h^{-1}$	$D = 0.020\ h^{-1}$	OD_{600} decreased to ~2.0	OD_{600} increased to ~30.0
1347	ACCIDENT: Media pump stopped for several hours			Rapid increase of ethanol concentration from 393 to 576 mM; stabilized at 400–450 mM ethanol within 100 h after repairing the pump
1366		Raised gas recycle rate from 180 to 430 mL/min to increase syngas retention time and mass transfer		Stage 2: sudden rise of OD_{600} to 41.7 (Figure 2A) due to stir up of settled cells. OD_{600} stayed at higher level until end of experiment.Syngas consumption did not improve significantly (Figure 2E)
1535–1632	2× medium was exchanged with 4× medium in the reservoir for 97 h to improve supply with nutrients		OD_{600} declined from 2.0 to 0.1. Recovery completed at 1749 h.	Salt shock did not affect OD_{600}, but metabolic rates declined. Recovery started at 1800 h
1679	$D = 0.046\ h^{-1}$	$D = 0.023\ h^{-1}$	New OD_{600} level settled at ~1.8	New OD_{600} level settled at ~43.4
1800	$D = 0.039\ h^{-1}$	$D = 0.020\ h^{-1}$	OD_{600} back at ~2.0	Recovery from salt shock started
2014	End of experiment			Final OD_{600} of 46.2; ethanol concentration of 394 mM

Table 2. Performance parameters of continuous operation of the 2-stage system at time point 1517 h at which stable operating conditions had been achieved. These parameters were used to calculate the fermentation balance explained in the text. The gas inlets for stage 1 and 2 contained the same gas, and are therefore summarized in one column. Abbreviations: (G) and (L) indicate gas or liquid state at ambient conditions; g DW/L: gram dry weight per liter.

Compound	Concentrations		
	Outlet stage 1	**Outlet stage 2**	**Inlet stages 1 & 2**
CO (G), (vol%)	53	19	60
H_2 (G), (vol%)	34	14	35
CO_2 (G), (vol%)	13	63	5
Ethanol (L), (mM)	11.5	428.4	NA
Acetic acid (L), (mM)	146.5	142.5	NA
Bacteria (g DW/L)	0.476	9.34	NA
Compounds	**Rates [mmol/(L·min)]**		
	Stage 1	**Stage 2**	**Total**
CO in	0.607	0.808	0.768
CO out	0.330	0.110	0.154
CO consumption	0.277	0.698	0.614
H_2 in	0.354	0.471	0.448
H_2 out	0.182	0.085	0.105
H_2 consumption	0.172	0.386	0.343
CO_2 in	0.051	0.067	0.064
CO_2 out	0.085	0.371	0.314
CO_2 production	0.034	0.303	0.250
Ethanol production	0.007	0.136	0.110
Acetic acid production	0.094	0.025	0.039
Compounds	**Efficiencies (%)**		
	Stage 1	**Stage 2**	**Total**
CO consumption	46	86	80
H_2 consumption	49	82	77

When the dilution rate was further increased, and the nutrient concentration in the media was raised to 2× (Figure 2, and Table 1, time point 1056 h), the OD_{600} increased to around 30. Higher dilution rates, and rates of nutrient supply sustained a higher OD_{600} (washout of grown cells was prevented in stage two). The OD_{600} did not rise infinitely in stage two (in spite of the cell filtration module) because at the maximum OD_{600}, the rate of cell lysis had reached the rate of new cells entering from stage one. An attempt to increase the nutrient *concentration* to 4× (Figure 2, Table 1, time point 1535 h), resulted in a rapid loss of reactor viability, which could only be restored when the nutrient concentration was reverted to 2×. It is likely that the increased osmolarity in the 4× concentrated medium, conveyed mainly by the minerals chloride (407 mM), ammonium (224 mM), and sodium (164 mM), caused the bacteria to undergo salt stress. In addition, ammonium is known to be toxic to bacteria at high concentrations. We believe we can exclude that trace metals acted inhibitory. Although several trace metals are known to be toxic or inhibitory to cellular processes at higher concentrations, a recent study with *Clostridium ragsdalei* showed that a 10-fold increase of trace metal concentration did not result in

decreased metabolic acitivities [18]. Another interesting detail is that the cell density did not decrease while the metabolic activity almost completely declined due to the salt shock. This could have occurred because of spore formation instead of cell death and lysis as a response to salt stress, but this was not further investigated.

Increasing the gas recycle rate independent from the media flow rate (Table 1, time point 1366 h) did not result in an increase in cell density beyond the increase caused by stirring up settled cells, which had accumulated throughout the fermentation run against the conical side walls at the bottom of the reactor. This indicates that cell growth (and productivity) at this high cell density were rather limited by the media dilution rate (nutrient supply and product removal), than by mass transfer of substrate gas into the liquid phase. This was surprising, given that mass transfer is considered a major limiting factor in the literature, and the cell densities reached in our system surpassed ones reported in literature by far. It is, therefore, likely that a large proportion of cells in our reactor was inactive, due to nutrient limitation. However, in our experimental setup, it was not feasible to test if higher dilution rates would further improve reactor performance.

2.2. Substrate Consumption and Product Formation

Our data demonstrate that acetic acid from stage one was converted into ethanol during solventogenesis in stage two. This is particularly evident during hour 0–600, when the acetic acid concentration in stage one oscillated around 200 mM, while in stage two the acetic acid concentration remained around 5–20 mM, while the ethanol concentration increased continuously up to around 200 mM. Under continuous optimal conditions, constant ethanol concentrations in stage two were between 400 and 450 mM (20.7 g/L). The time point 1517 h of the operating period is used as a reference at which the ethanol concentration was 428 mM, while the ethanol production rate of the entire system was 0.549 mmol/min (Table 2). This corresponds to an overall ethanol production rate of 0.303 g/(L·h). The ethanol production in stage two alone was 0.374 g/(L·h). This is below the rate of 1.6 g/(L·h) achieved by Gaddy *et al*. [6], although the cell density in our reactor was higher (10 *vs*. 2 g DW/L), and our fermentation was not mass transfer limited. As outlined above, it is likely that a large proportion of cells in our stage two reactor was inactive due to nutrient limitation. Higher rates of nutrient supply (increasing the rate of supply rather than the concentration) can be achieved *via* increased media flow (dilution) rates in stage 2, and this will be a future strategy to improve rates of product formation.

The molar ratio of ethanol:acetic acid in stage two at our reference point was 2.8. The relative proportion of acetic acid increased when dilution rate and cell density were increasing (Figure 2A,C,G). The ratio of acetic acid was much lower during times when the system had been operated undisturbed at a steady dilution rate, and when the OD_{600} was at maximum (e.g., at time points 628 h and 869 h) with molar ratios of ethanol:acetic acid of 28 and 29, respectively. The molar ratios of CO and H_2 consumed *vs*. ethanol produced (Figure 2F) were not constant throughout the operation. During times when cell density increased, the ratios went up to levels significantly higher than theoretical values, because growth and production of acetic acid required additional carbon, ATP, and reducing equivalents. During times of optimum ethanol production and no growth, the ratios were closer to ideal stoichiometries reported in the literature [3]. The overall fermentation stoichiometry calculated for our

system during optimum performance at 1517 h is described by the following equation derived using data from Table 1:

$$5.58CO + 3.12H_2 \rightarrow 1.00ethanol + 0.35acetate + 2.27CO_2$$

From this equation, carbon recovery and redox balance can be calculated as 89% and 0.93, respectively. A perfectly balanced fermentation would have resulted in values of 100% carbon recovery and a redox balance of 1.00 [19]. It was verified by HPLC that fermentation byproducts common for homoacetogenic bacteria, such as formic, lactic, or *n*-butyric acid, *n*-butanol [2], and isopropanol [20], were not produced. We did observe a peak in the HPLC with a retention time corresponding to 2,3-butanediol, which is a known fermentation product of *C. ljungdahlii* [21]. However, its concentration could not be exactly quantified, but always remained below 1 mM, which would contribute a less than 1% change to the fermentation balance. Another potential loss of carbon could have occurred by stripping of ethanol *via* the gas flow, but this had not been tested for. We explain the slightly unbalanced fermentation by formation of 2,3-butanediol, ethanol stripping, and fluctuations in performance during the continuous operation and by the fact that carbon flux into biomass was not considered (but estimated to be ~0.5% of the CO consumed). Considering the loss of syngas that was not consumed (Table 1), 28% of the carbon contained in carbon monoxide provided, and 74% of the hydrogen provided were recovered in ethanol. With the syngas composition of $CO:H_2:CO_2$ of 60:35:5, a theoretical carbon recovery of 53% from carbon monoxide, and a hydrogen recovery of 100% in ethanol is expected, according to the theoretical stoichiometric fermentation balance:

$$3.79CO + 2.21H_2 + 0.79H_2O \rightarrow 1EtOH + 1.79CO_2$$

Therefore, the current setup achieved 53% and 74% of the theoretical possible recovery of carbon from carbon monoxide and hydrogen from hydrogen, respectively, in the product ethanol, suggesting that some improvement of performance is still possible by optimizing parameters such as rates of dilution, nutrient supply, and gas supply, and the ratio of ethanol: acetic acid.

The experiment was not replicated. However, we anticipate that we would obtain similar results for product concentrations and rates of formation if we repeated the experiment with the same operational parameters as those applied around timepoint 1517 h. Indeed, comparable reactor performance was achieved after the culture had recovered from the salt shock at the end of the operational run (Figure 1D).

3. Experimental Section

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) except for syngas for which a blend of 60% (vol/vol) CO, 35% H_2, and 5% CO_2 was used (Airgas East, Ithaca, NY, USA).

3.1. Biocatalyst and Growth Conditions

C. ljungdahlii ERI-2 (ATCC 55380) was used as a biocatalyst, since it had proven to be a good ethanol producer [17]. Bacteria were always grown anaerobically at 35 °C in medium designed for efficient syngas fermentation [22], which is referred to here as 1× medium. Precultures were grown in 160-mL serum bottles containing 10 mL of 1× medium adjusted to pH 5.5, and syngas in the headspace at a pressure of 1.93 bar. Precultures were maintained by weekly transfer of 2% (vol/vol). The concentration of MES (2-(N-morpholino)ethanesulfonic acid) buffer was 5 g/L in the precultures,

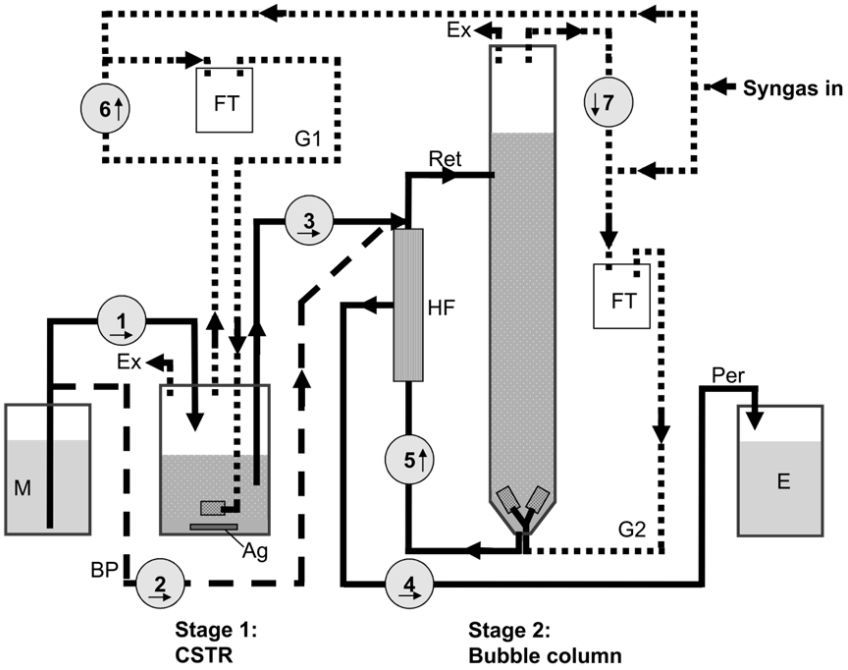
and in the initial startup medium in the 1-L CSTR fermentor, where yeast extract was added at 0.05 g/L to promote initial growth. Yeast extract and MES were omitted from medium in stage two, and from the continuous feed medium in which the pH was controlled *via* addition of 2 M KOH or HCl. Prior to inoculation of stage one, stage one and two were filled with 1 L and 4 L of 1× concentrated growth medium, respectively. The pH setpoints in stage one were 5.5 (low) and 5.7 (high), with the actual medium pH always being at the low end of the range due to acidogenesis. In stage two, the pH setpoints were 4.4 (low) to prevent acid crash in case the culture turned acidogenic, and 4.8 (high) to prevent the culture from turning acidogenic in the first place. In the sourcemedium for continuous operation, the concentration of all minerals, trace elements, and vitamins was doubled (2× medium) or quadrupled (4× medium), after a maximum OD_{600} had been reached in stage two with 1× medium. Antifoam 204 (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium reservoir at 10 µL/L, which prevented foaming in stage one. In stage two, because of high cell densities, a foam controller (Cole Parmer, Vernon Hills, IL, USA) was installed to deliver antifoam 204 solution (100× diluted) on demand. The antifoam amounts and concentrations had been carefully determined in previous experiments to provide efficient foam control without killing the cells by adding too much of the agent, which seems to be toxic to *C. ljungdahlii.* In stage two, a total of 462.5 mL of 100× diluted Antifoam 204 was consumed during the entire run at an average rate of 0.236 mL/h.

3.2. Reactor Setup

The two-stage continuous system was set up according to Figure 3. The stage one fermentor was a 2-L Braun Biostat M CSTR (Braun, Allentown, PA, USA) with 1 L working volume. The agitation speed was 200 rpm. Stage two was a custom-made 6-L bubble column with 4 L working volume. Both systems were equipped with temperature (water jacket), and pH control. Stage two was equipped with a foam control system (Cole Parmer, Vernon Hills, IL, USA) that injected 100× diluted antifoam 204 solution upon detection of high foam levels.

Peristaltic media pumps (Cole Parmer) #1–4 and gas-recycle pump #7 were operated at variable flow-rate, while the cell recycle pump #5 and gas recycle pump #6 were set to 180 mL/min. Microbubble spargers (MoreFlavor, Concord, CA, USA) were made of stainless steel with a pore size of 0.5 µm. Foam traps in the gas recycle lines prevented clogging of microspargers. The rates of syngas supply into both stages were maintained at levels that exceeded the consumption by at least 10% to avoid limitation of gaseous substrate, which has been reported to be detrimental for ethanol production [6]. Flexible tubing (Cole Parmer) was norprene for liquid lines, and viton for the gas lines, respectively, since viton has a low gas permeability. The cell recycle module was a Cellflo polyethersulfone hollow fiber module with 500-cm^2 membrane surface area and 0.2-µm pore size (C22E-011-01N, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA).

Figure 3. Setup of two-stage continuous fermentation with cell and gas recycle. Solid lines: flow of liquid media; dotted lines: flow of substrate and exhaust gases. Abbreviations: 1–7 pumps; Ag, agitation; BP, bypass; E, effluent reservoir; Ex, exhaust; FT, foam trap; G1, G2, gas recycle loops; HF, hollow fiber module for cell recycle; M, media reservoir; Per, permeate; Ret, retentate.



3.3. Analytical Procedures

Liquid media aliquots of both stages were analyzed daily for ethanol, acetic acid, and other possible metabolites by HPLC (Waters, Milford, MA, USA) with an Aminex HPX87H column (Bio-Rad, Hercules, CA, USA) kept at 65 °C and an RI-detector. HPLC buffer was 5 mM sulfuric acid in water, and the flow rate was 0.6 mL/min. Volumetric gas flow rates and gas pressures in the fermentation system were measured with in-line volumetric flow meters (custom-made), bubble flow meters, and digital pressure gauges (ColeParmer) at the gas inlets and outlets of both stages. Concentrations of carbon monoxide, hydrogen, and carbon dioxide, and cell dry weight were determined as previously described [17]. Both fermentation stages were checked for possible contaminants *via* daily controls of liquid samples using a phase-contrast microscope. The flow rate of media was adjusted and monitored by measuring the volume of effluent. The amount of 2 M aqueous solutions of KOH and HCl spent for pH adjustment was measured daily by weighing the reservoir bottles. This was useful to immediately evaluate if the stage-two reactor was solventogenic: pumping of HCl always correlated with high rates of ethanol formation. This can be explained by the consumption of acetic acid by the bacteria, and the resulting increase in the pH.

4. Conclusions and Outlook

The ethanol productivity of 0.374 g/(L·h) (in stage two) is promising. Compared to typical average ethanol production rates during hexose fermentation by yeast in commercial bioethanol plants 1.25–3.75 g/(L·h) [23], syngas fermentation has potential to reach or even succeed these rates, and we

are optimistic to further improve the performance, especially since current efforts in commercializing the process by companies such as Lanzatech (Roselle, IL, USA), Coskata (Warrenville, IL, USA), and IneosBio (Lisle, IL, USA) suggest that this is possible. However, challenges for economic continuous syngas fermentation remain:

- The relatively low ethanol concentration of 2% in the effluent requires advanced strategies for distillation to keep the energy balance of the entire process positive. A promising method has been described recently [24];
- When feeding the fermentation with "real world" producer gas derived from pyrolysis of biomass, toxic contaminants that inhibit syngas fermentation [7,25] have to be removed by a gas cleaning process;
- The relatively high costs for media ingredients required to support growth of the biocatalyst [26,27] suggest that the operational costs of syngas fermentation will remain in an uneconomical range, unless cheaper sources of growth medium are found for this process.

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Conflict of Interest

The authors declare no conflict of interest.

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