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Production of Ethanol from the Fermentation of Synthesis Gas

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PRODUCTION OF ETHANOL FROM THE FERMENTATION OF SYNTHESIS GAS

By

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Ethanol produced from lignocellulosic agricultural products and waste is an environmentally-friendly alternative to petroleum-derived fuel. Lignocellulosic biomass is gasified producing synthesis gas, which is composed of CO, CO_2, and H_2. Synthesis gas is fermented via anaerobic biocatalyst. The bacterium was grown in a fructose-rich medium then concentrated in ethanol production medium for synthesis gas fermentation. While the known ethanol-producing bacterium *Clostridium ljungdahlii* was used to provide baseline values for synthesis gas utilization and ethanol production, synthesis gas fermentation were conduced with a culture discovered at Mississippi State University. Additionally, efforts were made to isolate other anaerobic cultures capable of fermenting synthesis gas to ethanol.

DEDICATION

This thesis is dedicated to the person I admire most in the world: my little sister, Leila. Sissy, your inner glow makes all those who meet you love you. I'm so proud to be your Sissy!

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CHAPTER I

INTRODUCTION

Political, economic and environmental forces are driving Americans to seek alternatives to petroleum derived fuels. In 2002, Americans consumed about 25% of the world's oil, but only contributed 12% (EIA, 2004). Petroleum not produced in the United States was purchased largely from politically unstable countries that are bound together by membership in the Organization of Petroleum Exporting Countries (OPEC). Because of our dependence on their oil, OPEC's whimsical attitude concerning oil output and prices destabilizes the American economy. The United States first experienced OPEC's whimsy in 1973, when OPEC increased prices and cut production of crude oil to protest the Yom Kippur War (EIA, 2003). Initiatives to find alternative fuels began just one year later in 1974, as the Solar Energy Research, Development, and Demonstration Act provided support for research aimed at converting biomass into fuels. The political and economic need for alternative fuels has been reinforced again and again since 1974, with countless legislative measures and alternative fuel subsidies.

Concern for the environment, both individual and political, also drives Americans to explore alternatives to petroleum-derived fuels. Under natural conditions, carbon found in oil reserves inside the Earth does not enter the biosphere, the living part of the Earth stretching from the deepest tree roots far into the atmosphere. When oil is pumped up into the biosphere, there is a net increase in the amount of exotic carbon to the

biosphere. Burning petroleum based fuels converts carbon in oil into environmentally harmful greenhouse gases. These gases are blamed for global warming as well as holes in the protective ozone layer (Columbia, 2001).

Methyl-tert-butyl ether (MTBE), a petroleum-derived oxygenate, was added to gasoline in the 1990's to enhance octane ratings, thus creating a cleaner burning fuel. Since then, those exposed to gasoline with high levels of MTBE have reported dizziness, headaches, nausea, and irritation in the nose and throat (ATSDR, 1996). MTBE has been attributed with causing cancer in laboratory animals. The United States government passed the EPA Renewable Oxygen Standard (ROS) in 1994 that required 30% of oxygenates added to fuels to be derived from renewable sources (EIA, 2003). Ethanol from biomass was a main focus of this legislation.

America needs a renewable, clean-burning fuel source that recycles carbon already in the biosphere. Ethanol first showed promise as a fuel in 1876, when it was used in Otto Cycle combustion engines (EIA, 2003). Ethanol-burning internal combustion engines were included in the design of Henry Ford's Model T. Since the Model T, gasoline has replaced ethanol as the United State's primary automobile fuel. The need for lead-free, high-octane fuels prompted the addition of oxygenates like methyl-tert-butyl ether (MTBE) to gasoline (ATSDR, 1996). Concern over the health effects of MTBE prompted the use of ethanol as an oxygenate to replace MTBE in 1988 (ATSDR, 1996; EIA, 2003).

The U.S. Department of Energy's most developed "fuels from biomass" program is the production of fuel-grade ethanol from corn (Rajagopalan et al., 2002). Since only

the corn kernels from each 6-foot cornstalk are used in the actual fermentation, an incredible amount of biomass is wasted. Even then, only a fraction of the carbon in a single corn kernel is fermented into ethanol. The corn fermentation industry relies on the sale of by-products like cornstarch, corn syrup, and distiller's grain to make ethanol plants economically viable.

Few states in the U.S. can support a corn based fermentation plant. Many states, like Mississippi, that have very agrarian economies may be overlooking an opportunity to utilize agricultural wastes as a feedstock for fuel production. This lignocellulosic waste, also called biomass, consists of chicken litter, corn stover, peanut hulls, sawdust, cotton ginning waste, etc. In the 1970's, scientists and engineers were motivated by fuel shortages and government initiatives to create new technologies to convert this lignocelulosic waste into fuel grade ethanol (EIA, 2003). Though the carbon in this so-called waste needs to be utilized for fuels production to further increase production volumes, the lignin and cellulose holding the carbon cannot be fermented as is. Breaking these long chains down into smaller molecules by gasification transforms the carbon in a more easily fermented form. Gasifying biomass under oxygen-starved conditions yields synthesis gas that is composed of 25-30% H_2, 40-65% CO, 1-20% CO_2, 0-7% CH_4, and trace amounts of other gases (Phillips et al., 1993). Bacteria were discovered and patented that could convert the components of synthesis gas into commodity chemicals, most notably ethanol, by anaerobic fermentation (Gaddy and Clausen, 1992). The overall reaction of synthesis gas catalyzed by ethanol-producing bacteria is simplified below (Klasson et al., 1992b):

$$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2 \qquad (1)$$

$$2\ CO_2 + 6\ H_2 \rightarrow CH_3CH_2OH + 3\ H_2O \qquad (2)$$

Though organisms have been proven to produce ethanol, the processes were never optimized to the point of being economically feasible. Additionally, few bacterial sources have been screened for potential production of ethanol from synthesis gas. This project focused on isolating a novel organism or consortium for the production of ethanol from synthesis gas as well as development and optimization of a novel 2-step approach to synthesis gas fermentation. Additionally, once experiments were initiated, significant shortcomings in traditional microbiological techniques were noted. Thus, a substantial portion of this thesis was directed toward resolving the numerous challenges associated with these shortcomings. Therefore, method development coupled with data highlighting these shortcomings and other data exemplifying the success of the new method developed are presented.

1.1 Research Hypothesis and Objectives

The intent of this research was to test and optimize a two-step process for ethanol production from synthesis gas (Figure 1.1-1). The overall concept is that it may be metabolically easier and more productive to separate cell growth from product formation. Figure 1.1-1 illustrates this concept, in which one vessel is optimized for cell growth, while synthesis gas fermentation occurs in the second reactor, allowing for a reactor better suited for ethanol production. This concept was developed to remedy the mass

transfer limitations observed in other synthesis gas fermentation systems by providing large quantities of cells within optimized production systems (Klasson et al., 1991).

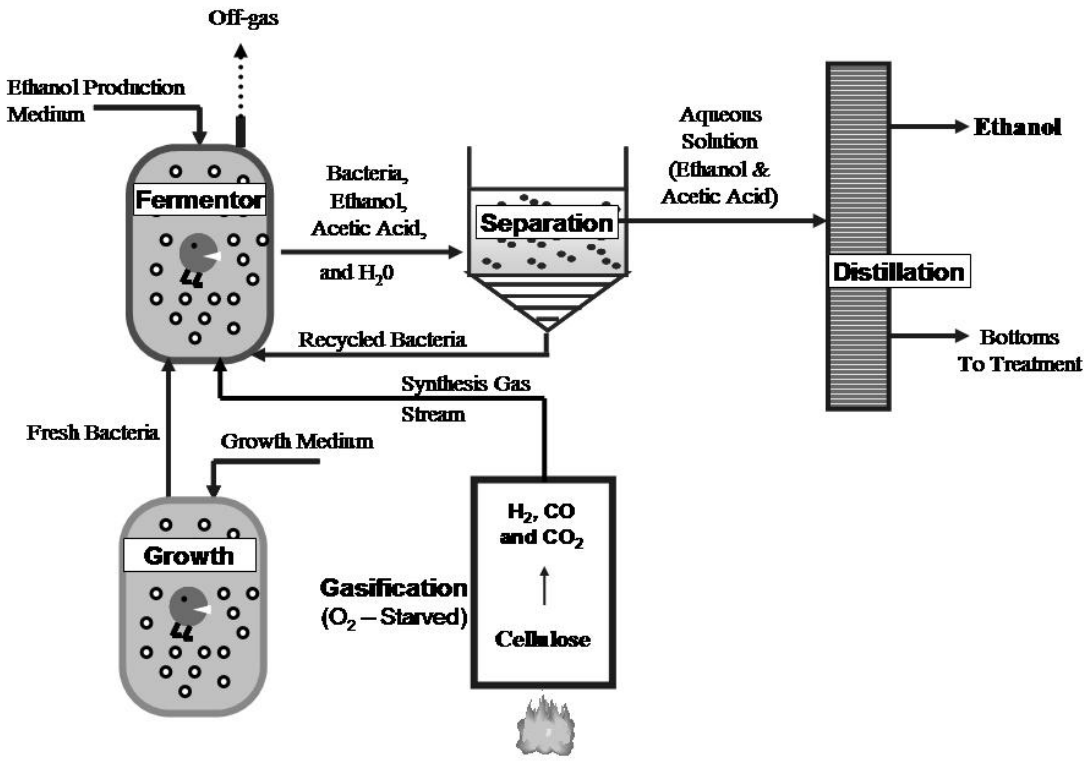


Figure 1.1-1. Schematic of proposed 2-step process for ethanol production from synthesis gas

The two steps that served as the focus of this research were cell growth in a sugar-rich growth medium and ethanol production from synthesis gas in Ethanol Production Medium.

CHAPTER II

LITERATURE REVIEW

The Solar Energy, Research, Development, and Demonstration Act of 1974 began the research and development rush toward finding a means of converting cellulosic material into fossil fuel substitutes (EIA, 2003). Legislative support for ethanol production from renewable resources prompted engineers, scientists, and farmers to attempt development and implementation of new means for converting biomass to fuel-grade ethanol. To date, there are no economically favorable means of large-scale ethanol production from lignocellulosic biomass (NAS, 2003). Though fermentation of corn to ethanol in the corn belt of the United States is a well-known means of converting biomass to ethanol, other processes are developing to include a wider range of feedstocks. Other methods of converting alternative feed stocks to chemicals include synthesis gas conversion by metal catalysis, synthesis gas fermentation, and acid hydrolysis.

2.1 Other Ethanol Production Methods

In addition to synthesis gas fermentation, acid hydrolysis of biomass and enzymatic conversion of biomass are under development as means of converting lignocellulosic feed stocks to ethanol. The acid hydrolysis process begins with the pretreatment of the lignocellulosic material to remove the lignin (Rajagopalan et al., 2002). The remaining cellulose and hemicellulose are converted to a hydrolyzate

composed of 5- and 6-carbon sugars. The resulting sugars are then fermented by yeast and bacteria to ethanol. This process is unable to convert all of the lignocellulosic biomass, particularly the 25% lignin component, to a fermentable feedstock since the lignin is removed initially (BBI Ethanol, 2003). The separated lignin also presents a disposal problem (Rajagopalan et al., 2002). The hydrolyzate produced by acid hydrolysis can contain substances toxic or inhibitory to fermentative bacteria (Leonard and Hajny, 1945). Additionally, the 5- and 6-carbon sugar hydrolyzate is acidic and must be neutralized before fermentation. Neutralization of the hydrolyzate results in the generation of large quantities of salts that present a disposal problem.

Enzyme hydrolysis employs isolated enzymes like cellulases and xylanases to break lignocellulosic biomass into 5- and 6-carbon sugars (Rajagopalan et al., 2002). Lignin is not converted to a fermentable feedstock by enzyme hydrolysis and poses a disposal problem. The high cost of enzymes has limited this process to laboratory scale testing only.

Even though ethanol made from agriculture and forestry waste requires less energy input per gallon of ethanol, corn fermentation is the most developed means of biomass-to-ethanol conversion (Kendell, 2000). Unlike the synthesis gas fermentation, enzymatic conversions, or acid hydrolysis, ethanol fermentation from corn is well past the laboratory stage of development. In 1998, approximately 1.4 billion gallons of corn-derived ethanol were produced in the United States using corn fermentation (DiPardo, 2002). The current 52¢ per gallon subsidy on corn-derived ethanol and by-product revenues from the wet milling and dry milling processes prop up the Corn Belt's ethanol

industry. Both the wet milling and dry milling require the separation of the corn kernels from the rest of the plant, leaving behind a large amount of unutilized biomass (BBI Ethanol, 2003).

Wet milling begins with the soaking of the corn kernels. The corn is then separated into its four major components: starch, protein, fiber, and germ. The germ is converted to corn oils. Starch from the wet milling process is processed further into sweeteners, food starch, industrial starch, and fuel-grade ethanol.

Dry milling begins when corn meal is treated with ammonia and enzymes creating a mash. This mash is then fermented to ethanol and the ethanol is distilled. The unused grain is collected, dried and sold as animal feed.

2.2 Synthesis Gas Production

The first step in synthesis gas conversion to ethanol is the gasification of biomass. Gasification is a thermal process that converts biomass, including lignocellulosic material, into synthesis gas (Maschio et al., 1994). Synthesis gas is composed of varying amounts of carbon monoxide, hydrogen, carbon dioxide, methane, and trace amounts of sulfur (Phillips et al., 1993). Gasification conditions and feedstock composition dictate exact synthesis gas composition (Klasson et al., 1992a).

Gasification begins with pyrolysis, the thermochemical decomposition, of the feedstock biomass at 400-600ºC yielding char, tar, gas and volatile compounds (Maschio et al., 1994). The pyrolysis step turns out 20-50 weight % synthesis gas. The char is further gasified at 700-950ºC in the presence of air, oxygen, or steam. Gasification in

oxygen or steam is preferable over gasification in air, which yields a syngas with undesirable nitrogen compounds. After quenching and purification, synthesis gas is composed of 25-35% H_2, 40-65% CO, 1-20% CO_2, 0-7% CH_4, and trace amounts of other compounds (Klasson et al., 1992a).

2.3 Metal Catalysis of Synthesis Gas

Synthesis gas can then be fed into a catalytic reactor for conversion into ethanol. Traditional, including catalysts nickel, ruthenium, copper, zinc, aluminum, iron, and cobalt, have been used to convert synthesis gas to methanol, ethanol, and higher order alcohols (Stiles et al., 1991). Metal catalysts are non-specific and the end product of synthesis gas conversion is a mixture of alcohols including methanol, ethanol, n-butanol, and other higher molecular weight alcohols as well as aldehydes and methyl ethyl ketone (Saha, 1997; Stiles et al., 1991). Traditional catalysis occurs at high temperatures near 400ºC and extreme pressures of 1500-3000 psi (Saha, 1997).

2.4 Biocatalysis of Synthesis Gas

Biocatalysis by microorganisms offers a low temperature, low pressure alternative to metal catalysis (Saha, 1997). Higher specificity and sulfur tolerance further characterize biocatalysts capable of converting synthesis gas to alcohols and commodity chemicals. Higher yields are seen from fermentations compared to metal catalysis (Klasson et al., 1992a). Poisoning is less of a problem when using microorganisms in synthesis gas conversion to alcohols and commodity chemicals because of bacterias'

ability to adapt to gas composition and the presence of contaminants. Additionally, because of complicated enzymatic pathways employed during bacterial metabolism, microbiological reactions are more difficult to reverse, which allows for the stable and complete conversion of the synthesis gas to the desired product.

Many anaerobic microorganisms utilize the components of synthesis gas (CO, CO_2, and H_2) as carbon and energy sources (Grethlein and Jain, 1992). The metabolic pathways in these bacteria produce compounds like ethanol, acetate, n-butanol, butyrate, and methanol that serve as terminal electron acceptors (Rao et al., 1987). This class of organisms includes *Clostridium thermoaceticum*, *Clostridium autoethanogenum*, *Peptostreptoccus productus*, *Eubacteriam limosum*, *Butyribacterium mehylotrophicum*, *Clostridium acetobutylicum*, and *Clostridium ljungdahlii* (Abrini et al., 1994; Grethlein and Jain, 1992; Rao et al., 1987).

Clostridium thermoaceticum was isolated by F. E. Fontaine in 1942 (Sugaya et al., 1986). It was grown on carbon monoxide with a doubling time of 16 hours, or on carbon dioxide and hydrogen with a doubling time of 18 hours (Grethlein and Jain, 1992). The optimal growth temperature for *C. thermoaceticum* is 55-60°C (Sugaya et al., 1986). Acetic acid is formed by the following reactions (Grethlein and Jain, 1992):

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \qquad (1)$$

$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH \qquad (2)$$

Clostridium autoethanogenum is a Gram-positive, motile, rod-shaped bacterium isolated from rabbit feces by Abrini et al. (1994). Optimal growth conditions occur at 37ºC and pH 5.8-6.0 (Abrini et al., 1994). *C. autoethanogenum* converts carbon

monoxide and carbon dioxide to ethanol and acetate. Under growth conditions, ethanol concentrations range from 1.56 mmol/l to 7.71mmol/l and acetate concentrations were detected in the 5.62 mmol/l to 7.96 mmol/l range. Conversion of CO to ethanol and acetate by *C. autoethanogenum* follows the stochiometry of Equations 3 and 4 shown below:

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + CO_2 \qquad (3)$$

$$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2 \qquad (4)$$

Peptostreptoccus productus was isolated from sewage digester sludge. It produced acetate from CO or CO_2 and H_2 (Grethlein and Jain, 1992). Additionally, *P. productus* has the fastest known doubling time at 1.5 hours of any organism using CO as the sole carbon and energy source. Cell growth and CO uptake are not affected by H_2S concentrations up to 20%. Acetate is produced as shown in Equation 5 (Vega et al., 1990):

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + CO_2 \qquad (5)$$

Eubacterium limosum produces acetate from CO by the same stochiometry as P. *productus* (Vega et al., 1990). Trace amounts of butyrate are formed in the presence of CO_2. *E. limosum* was isolated from sheep rumin (Grethlein and Jain, 1992).

Butyribacterium methylotrophicum converts 100% CO to acetate and butyrate, ethanol, and n-butanol (Saha, 1997). The dominant end product is determined largely by the fermentation pH. Lower pH results in higher ethanol and butyrate levels while a pH of 6.8 favors acetate production (Saha, 1997; Grethlein and Jain, 1992). Cell growth

occurs at pH values ranging from 5.0 to 6.8 with slower growth occurring in the lower end of this range (Grethlein et al., 1990). Growth is finally inhibited below pH 5.0.

End product composition from synthesis gas fermentation by *Clostridium ljungdahlii* is also highly dependent on pH (Gaddy and Clausen, 1992). *C. ljungdahlii* was isolated at the University of Arkansas from chicken waste and patented by J. L. Gaddy and E. C. Clausen in 1992, as a Gram-positive, rod-shaped, motile anaerobic bacterium capable of producing ethanol and acetate from CO, CO_2, and H_2 (US Patent 5,173,429). Growth, ethanol production, and acetate production occur at an optimal temperature of 37ºC (Gaddy and Clausen, 1992). Acetate production is favored between pH 5.0 and pH 7.0. Ethanol production is favored between pH 4.0 and pH 4.5. The stochiomety of synthesis gas fermentation to ethanol and acetate is as follows (Klasson et al., 1992a):

$$6\ CO + 3\ H_2O \rightarrow CH_3CH_2OH + 4\ CO_2 \qquad (6)$$

$$2\ CO_2 + 6\ H_2 \rightarrow CH_3CH_2OH + 3\ H_2O \qquad (7)$$

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \qquad (8)$$

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \qquad (9)$$

In batch fermentations, it is estimated that 3.5% of the carbon monoxide is converted into cell mass (Vega et al., 1989a). Under batch conditions at pH 5.0, ethanol concentrations were found to be less than 1 g/l with an ethanol to acetate molar ratio of 0.05 (Gaddy and Clausen, 1992). Lowering the medium pH in a batch reactor to pH 4.5 yielded an increase in ethanol production to concentrations of 7 g/l and a molar ratio of 9

moles ethanol per mole acetate produced. Continuous fermentations were also performed with the maximum ethanol concentration observed being 23 g/l (Phillips et al., 1993).

Microbiology is a fairly new science, having its beginnings circa 1857, when Louis Pasteur first showed that lactic acid fermentation was caused by microorganisms (Prescot, 2001). It is estimated that only 1% of all microorganisms on Earth have been cultured to date. Recall that microbiologists discovered hyperthermophilic organisms living in thermal vents in the 1980's (Wantabe, 1994). In addition to yet undiscovered microorganisms, the genome sequencing of *Ecsherichia coli* in 1997, opened the flood gates for the development of novel, engineered microorganisms (Prescot, 2001). Even before the genome of *E. coli* was sequenced, genetic material coding for ethanol production was transferred from ethanol producing bacteria into *E. coli* in an attempt to increase ethanol yields from simple sugars and to increase cell growth rates (Ingram et al., 1987).

2.5 Metabolic Mechanism of Synthesis Gas Fermentation

The Acetyl-CoA pathway has been described as the primary anaerobic metabolic pathway allowing microorganisms to utilize CO, CO_2 and H_2 as sole carbon and energy sources (Wood, 1991). Nickel/iron-sulfur CO dehydrogenase (CODH) is the key enzyme employed by carbon monoxide utilizing anaerobic microorganisms (Wood et al., 1986). CO dehydrogenase converts carbon monoxide to carbon dioxide (Wood, 1991). Acetyl-CoA is synthesized from the resulting CO_2 in the CODH cycle of the Wood-Ljungdahl pathway (Ferry, 1995). First, a methyl group is fixed to the reduced metal site of the

CODH. Next, carbonylation of the methyl-metal species of CODH occurs followed by the migration of the methyl group forming an acetyl-metal intermediate. Finally, Coenzyme A binds to the acetyl-metal intermediate forming acetyl-CoA. Acetyl-CoA is converted by the cell to cell mass, acetate and ATP, or ethanol and NADH (Klasson et al., 1992b). The acetyl-CoA pathway indicates that acetate is the terminal electron acceptor when ATP is produced. ATP is required for cell growth; therefore, acetate is the terminal electron acceptor under growth conditions. Under non-growth conditions, ATP is consumed to maintain cell function (Klasson et al., 1992a). When cells are not growing, NADH is formed and ethanol is the terminal electron acceptor (Klasson et al., 1992b). Therefore, non-growth conditions induce synthesis gas fermentation to ethanol and conversely growth conditions produce acetate.

2.6 MEDIA DESIGN FOR *CLOSTRIDIUM LJUNGDAHLII*

In addition to using pH to manipulate synthesis gas fermentation end-products, media constituents may be altered to achieve the desired effect. Klasson et al. (1992a) reported that yeast extract has no effect on the observed ethanol to acetate ratios. Phillips et al. (1993) attempted to develop an ideal medium for cell growth on synthesis gas and ethanol and acetate production from synthesis gas. Pfennig's Basal Medium (see Table 2.6-1) was used initially to culture *C. ljungdahlii* by Phillips et al. (1993). Attempts were made to optimize this medium by changing component concentrations. It was determined that yeast extract was not needed for growth and ethanol production. Doubling medium concentrations created a hypertonic solution that inhibited growth.

Trace metal concentrations were determined to be the growth-limiting factor. The reduction of B-vitamins initially added to the medium slowed growth potential while causing a marked increase in the ethanol to acetate production ratio. The formulation for the final designer medium developed by Phillips et al. (1993) is outlined in Table 2.6-1. This medium was used in efforts to culture *C. ljungdahlii* at Mississippi State University.

Reducing agents are used to direct electron flow in cell metabolism (Klasson et al., 1992a). Reducing agents induce *C. ljungdahlii* to direct cell metabolism to the production of NADH and ethanol, thus inducing non-growth conditions. The reducing agent methyl viologen was used as the electron acceptor in the isolation of CO dehydrogenase from *Clostridium thermocaeticum* (Wood, 1991). When added to production medium, methyl viologen increased ethanol to acetate ratios from 0.12 mol ethanol/mol acetate to 0.20 mol ethanol/mol acetate in the presence of 50 ppm reducing agent (Klasson et al., 1992a). Thirty parts per million methyl viologen increased the molar ethanol to acetate ratio from 0.24 to 0.40. Benzyl viologen increased the molar ethanol to acetate ratio from 0.12 to 0.21 with a concentration of 50 ppm and from 0.24 to 1.10 with a concentration of 30 ppm. Sodium thioglycolate and ascorbic acid were also evaluated as potential reducing agents.

2.7 Laboratory Testing Protocols

Several test methods for evaluating ethanol production from synthesis gas fermentation have been developed. These bench scale methods varied by reactor type, reactor size, media components, bacterial source, and gas composition.

The best reported ethanol concentration produced by *C. ljungdahlii* in a batch reactor was 1 g/l (Bredwell et al., 1999). Vega et al. (1989a) conducted batch studies with *Clostridium ljungdahlii*. Pfennig Basal Medium was prepared under an 80% N_2 and 20% CO_2 atmosphere and adjusted to pH 5.0. Serum bottles, each with a total volume of 1216 ml, were filled with 200 ml of medium and sealed with butyl rubber stoppers. The medium and bottles were autoclaved for 20 minutes at 121ºC. Before the culture was added, reducing agents L-cysteine and sodium sulfide were added. The bottles were sparged with synthesis gas and pressurized. Argon was added as an inert gas. The reactors were incubated at 37ºC on a shaker incubator at 100 rpm. These batch fermentations were reported to be "highly irreproducible" (Vega et al., 1989a)

Philips et al. (1994) conducted further batch studies on *C. ljungdahlii* in basal medium. The basal medium (see Table 2.6-1) was prepared under a nitrogen atmosphere and adjusted to pH 4.5 with 0.5 M NH_4OH. Fifty milliliters of medium was added to serum bottles, each with a total volume of 158 ml. The bottles were sealed with butyl rubber stoppers and autoclaved. Before *C. ljungdahlii* was added, L-cysteine was added to the medium as a reducing agent. The vials were flushed with synthesis gas composed of 24% H_2, 65% CO, and 11% CO_2. Fifty milliliters of methane were added as an inert reference gas and the bottles were incubated on a shaker incubator at 37ºC and 130 rpm. Ethanol production was not reported for these experiments.

Klasson et al. (1991) used the designed basal medium (see Table 2.6-1) in batch experiments with *C. ljungdahlii*. The medium was prepared and dispensed into 157.5 ml total volume serum bottles and sealed with butyl rubber stoppers. Reducing agents

(methyl viologen and benzyl viologen) were added to alter electron flow for ethanol production. The bottles were incubated on a shaker incubator in the dark at 100 rpm and 37ºC. The bottles were only removed from the dark incubator for a maximum of 3 minutes each time samples were taken. The addition of 30 ppm benzyl viologen caused an increase in the ethanol to acetate production ratio to 1.1. Without the addition of benzyl viologen, the highest achieved ratio of ethanol to acetate was 0.658 (Phillips et al., 1994).

Work with *C. ljungdahlii* in continuous stirred tank reactors (CSTRs) yielded much higher ethanol concentrations than the work done in batch reactors. Vega et al. (1990) conducted CSTR trials of *C. ljungdahlii* within a 750 ml New Brunswick Bioflo C.30 with 350 ml of liquid phase. The medium was maintained at pH 4.5 and 37ºC. L-cysteine was added to the basal medium just before the medium was inoculated. Synthesis gas composed of 18.5% H_2, 15.4 % Ar, 54.1% CO and 10% CO_2 flowed though the medium at a rate of 3.5 ml/minute for 36 days. During the first 4 days, only gas flowed through the reactor. Once a cell concentration of 300 mg/l was reached, basal medium with cells was removed and fresh medium was added to maintain the cell concentration. The final ethanol concentration reached was 2.8 g/l.

Phillips et al. (1994) used the designed basal medium (see Table 2.6-1) for CSTR fermentations. The medium was autoclaved in a carboy and poured into a New Brunswick Bioflo. The medium was made anaerobic by bubbling sterile nitrogen through the Bioflo. L-cysteine was added to the medium before inoculation with *C. ljungdahlii*. The medium was maintained at pH 4.5 and 36ºC and agitated at a rate of 400

rpm. Trials with the designed basal medium yielded ethanol concentrations of 23 g/l ethanol, while trials with basal medium yielded concentrations of 1.5 g/l ethanol.

Klasson et al. (1991) began using 2 CSTRs in series to produce ethanol from synthesis gas. Two chemostats were oriented in series. The first Chemostat was filled with basal medium (see Table 2.6-1) and synthesis gas was bubbled though the reactor. Fresh medium was added slowly causing the medium containing cells to flow via gravity into the second reactor. The second reactor was sparged continuously with synthesis gas. Ethanol was produced at a rate of 250-300 mmol ethanol/g cell/day. This rate was a 30-fold improvement over ethanol production rates observed with single CSTRs.

Low ethanol yields from synthesis gas observed in batch and CSTR experiments with *C. ljungdahlii* combined with the low solubility of CO and H_2 pointed to mass transfer as the rate-limiting step in the conversion of synthesis gas to ethanol (Bredwell et al., 1999). Reactors were designed to overcome this limit. Klasson et al. (1991) experimented with packed-bed bubble and trickle bed columns. Both columns were constructed in 5.1 cm inner diameter Plexiglas cylinders 63.5 cm long. The total volume of each cylinder was 1091 cm^3. The packed bed reactors were operated under counter-current flow: liquid medium was fed from the top and synthesis gas bubbled from the bottom. The trickle bed reactors were filled with 0.6 cm ceramic Intalox™ saddles which yielded a bed porosity of 0.59. Both the liquid medium and synthesis gas were fed from the top of the column. No ethanol production values were given for this study.

Further efforts to overcome the mass transfer limit included the use of microbubbles (Bredwell and Worden, 1998). Microbubbles were used to increase the

interfacial area for mass transfer between the synthesis gas and the medium.

Microbubbles were first modeled using O_2.

Table 2.6-1. Basal Medium (Phillips et al., 1993)

	Basal	Designed
Pfennig's Minerals	**mg/L**	**mg/L**
$CaCl_2 \cdot H_2O$	50	200
$MgCl_2 \cdot H_2O$	330	500
NaCl	400	200
NH_4Cl	400	-
KH_2PO_4	500	-
B-vitamin	**mg/L**	**mg/L**
Biotin	0.1	0.106
Folic acid	0.1	0.005
Pyridoxal-HCl	0.05	0.0025
Thioctic acid	0.3	0.015
Riboflavin	0.25	0.0125
Thiamine-HCl	0.25	0.266
Calcium-D-Pantothenate	0.25	0.413
Vitamin B_{12}	0.25	0.0125
P-Aminobenzoic acid	0.25	0.0125
Nicotinic acid	0.25	0.0125
Pfennig's Trace Metals	**mg/L**	**mg/L**
$ZnSO_4 \cdot 7H_2O$	0.1	1
$FeCl_2 \cdot 4H_2O$	1.5	15
$CoCl_2 \cdot 6H_2O$	0.2	2
$CuCl_2 \cdot H_2O$	0.01	0.1
H_3BO_3	0.3	3
$Na_2MoO_4 \cdot 2H_2O$	0.01	0.3
$NiCl_2 \cdot 6H_2O$	0.02	0.2
Na_2SeO_3	0.01	0.1
$MnCl_2 \cdot 4H_2O$	0.03	0.3
Supplements	**mg/L**	**mg/L**
Yeast Extract	1000	-
$(NH_4)_2HPO_4$	-	2000
H_3PO_4	-	1.5
NaH_2CO_3	2500	-
KCl	-	150

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

All experiments were performed within 40 ml VOA vials (Fisherbrand, Pittsburgh, Pennsylvania, or Wheaton Corporation, Mellville, New Jersey). Each vial acted as a small batch reactor with 20 ml of liquid medium and 20 ml of gaseous headspace. The VOA vials were capped with either polypropylene open caps with white silicon rubber septa (Fisherbrand) or with Mininert valve Teflon caps (Pierce Biotechnology, Rockford, Illinois [Figure 3.1-1]). The vials were incubated at 37°C on an incubator shaker at 150 rpm (New Brunswick Scientific, Edison, New Jersey).

Liquid media used were divided into two categories: media prepared with a carbon source and media prepared without a carbon source. D-fructose provided a means for rapid cell growth in media prepared with a carbon source. PETC 1754 Medium was the recommended medium for American Type Culture Collection (ATCC) culture *Clostridium ljungdahlii* number 55383 (ATCC, 2004). D-fructose, ATCC trace metals, and ATCC Wolfe Vitamins were added to Mineral Salts Medium (MSM) and yeast extract creating modified MSM for cell growth. Dr. Lewis Brown of Mississippi State University's Department of Biological Sciences provided the formulation for the Brown Media used. An ultra-rich medium of peptone, yeast extract, and D-fructose, called PYF,

was used to grow large quantities of cells quickly. The formulations for Brown Medium, PETC 1754 Medium, Modified MSM, and PYF are included in Table 3.1-1. Trace Elements (Table 3.1-2) and Wolfe's Vitamins (Table 3.1-3) were purchased from ATCC.

Carbon-free liquid media were used for ethanol production and culture enrichments. Brown Medium was made without D-fructose for use in enrichment studies. Ethanol production medium (EPM) and acetate production medium (APM) differ only by their pH. In one liter, production media contains 50 ml of mineral solution, 5 ml of trace elements solution, and 20 ml of B-vitamin solution (see Table 3.1-4). The pH of ethanol production medium was adjusted to 4.5 using 1 M potassium hydroxide or 30% hydrochloric acid. A glass-body AccuTupH pH Probe and either an Accumet pH Meter 915 or an Accumet Portable AP62 pH/mV Meter was used to measure media pH (AccuStandard Inc, New Haven, Connecticut). Glass pipettes were used with all titrations.

Solid media with and without a carbon source were used in colony isolation. Seventeen grams per liter of Difco gel agar from Difco Laboratories, Livonia, Michigan, was used to solidify the APM used in making APM agar slants. Plate Count Agar (PCA) was prepared and poured into 90 mm by 15 mm FisherBrand petri plates.

All media were mixed on, and if necessary heated by, a Corning Stir Plate/Hot Plate. A Steris SG920 Scientific Gravity Autoclave, by Steris Corporation, Mentor Ohio, sterilized all media and glassware (121°C at 15 psi).

Media, both solid and liquid, were allowed to become anaerobic in a Flexible Anaerobic Chamber, referred herein as the glove bag (Coy Laboratory Products, Grass Lake, Michigan). The bag was filled with gas mixed by NexAir composed of 95% nitrogen and 5% hydrogen. The pre-chamber cycled three times, evacuating and refilling the chamber with pure nitrogen twice and finally refilling the chamber with the gas mixture. An incubator at 37°C was kept in the glove bag for anaerobic petri plate incubation (Gaddy and Clausen, 1992). Two catalytic fan boxes in the glove bag converted any incidental oxygen into water. An Oxford BactiCenerator III in the glove bag was used for heat sterilization of culture loops used in the glove bag.

A gas mixer was constructed to create variable synthetic synthesis gas compositions based on percent volume (Figure 3.1-2). Gas Bottles, one each of carbon monoxide, carbon dioxide, and hydrogen, were secured to a mobile gas cylinder cart. Gilmont Accucal rotameters (Gilmont, Barrington, Illinois) were bolted to the gas cart that was purchased from Fisher Scientific. Stainless steel tubing, $^{1}/_{8}$-inch in diameter, connected the gas regulators to the rotameters with Swagelok stainless steel connectors (Swageloc, Solon, Ohio), forming a distribution manifold. All three regulators were set to have downstream pressure of 20 psi. The rotameters were then used to adjust the synthesis gas composition based on volumetric flow. The gas flowed though an inline vortex (Keflo, Cary, Illinois) into a $^{1}/_{8}$-inch ID PTFE tubing to a sterile gassing syringe with a 1-inch, single-use needle (Figure 3.1-3).

C. ljungdahlii Culture Number 55383, a proven ethanol- and acetate-producing bacterium, was purchased from ATCC (Gaddy and Clausen, 1992). The Mississippi

State University Mesophilic Culture 1 (MSU1) from Dr. Lewis Brown of Mississippi State University's Biological Sciences Department was tested for ethanol production capabilities. Consortia were acquired from Dr. Brown for further isolation studies. Several potential sources of ethanol-producing bacteria were screened including sludge from Farbest Farms, sludge from Bryan Foods, secondary sludge from Tuscaloosa, AL, sludge from a methane-producing bioreactor, and horse manure.

Gram stains and simple stains were used to characterize working cultures. Cultures were heat-fixed to Fisherfinest Superfrost glass microscope slides. Fisher Protocol Gram Stains sets included Gram crystal violet, stabilized iodine, decolorizer, and safranin. Methylene blue was used in simple stains. Stains were viewed under the Nikon Elipse E400 microscope at powers ranging from 4X to 100X. Type A Immersion Oil was used with the 100X oil immersion lens.

Transferring cells from the growth step to the ethanol production step required separation of the cells from the growth medium and mixing cells into EPM. A Sorvall RT 6000 D centrifuge operated at 4000 rpm and 20ºC formed a cell pellet in 50 ml Naglene Oak Ridge Teflon FEP tubes with airtight caps. A Fisher Vortex Genie 2 was used to agitate the cells back into solution.

Hamilton 100 µL Gas Tight Syringes were used to collect gas samples from cultures in production media gassed with synthesis gas and the biotic and abiotic controls. The same syringes were used to collect gas samples from enrichments gassed with CO or synthesis gas. Each gas sample was injected into the manual injection port of an Agilent 6890N Gas Chromatograph System equipped with a Thermal Conductivity Detector

(TCD). The system used a column selection method with two Supelco columns: a 45/60 Molecular Sieve 5A (10 ft x 118 in Stainless Steel) and an 80/100 Porapak Q (6ft x 1/8 in Stainless Steel).

Liquid samples were taken using 3 ml Leur-Lok Becton-Dickson syringes with Becton-Dickson Precisionglide 23 gauge, 1-inch needles (BD Scientific Laboratories, Bedford, Massachusetts). All liquid samples were filtered using Millipore Isopore 0.2 μm membrane filters in Millipore Swinnex filter holders (Millipore, Bedford, Massachusetts). To prevent disruption of reactor kinetics, only small samples, less than 0.5 ml, could be taken from vials of culture in production media. When placed in standard autosampler vials, the analytical equipment could not detect the small sample volume. The liquid was placed in 0.25 ml conical polypropylene inserts (Sun-SRI, Wilmington, North Carolina) to bring the meniscus of the liquid sample up to a level that the analytical equipment could detect. For ethanol analysis, the inserts were placed in amber, 2 ml autosampler vials with screw caps. The liquid sample vials were placed on the Agilent Technologies 7683 Series Injector Auto Sampler attached to an Agilent Technologies 6890N Network GC System with an Agilent Technologies Innowax Column (Agilent, Palo Alto, California). The Innowax column was 30 m by 0.250 mm with a 0.25 micron pore size. Once separated by the column, a Flame Ionization Detector (FID) detected the sample components.

When analyzing for acetate, the Sun-SRI inserts were transferred to HPLC vials. These vials were placed in the 717 Plus Autosampler made by Waters (Milford Massachusetts). Samples taken by the autosampler were transferred to the mobile phase

pumped by a Waters 515 HPLC pump. The 3.5 pH, 20 mmol NaH_2PO_4 mobile phase carried the sample though a YMC ODS-AQ 55 µ 120 Å, 4.0X23 mm threaded guard column. The fluid then passed through a YMC ODS-AQ 55 µ 120 Å column with 150X4.6 mm ID and S5, 12 nm packing.

All chemicals were purchased from Fisher Scientific, Pittsburgh, Pennsylvania, or Sigma-Aldrich, St. Louis, Missouri. Mass was measured using a Mettler Toledo AG204 scale (Mettler, Columbus, Ohio). All gasses were purchased from NexAir Corporation (Columbus, Mississippi).

Table 3.1-1. Growth Media Formulations

	Amount (per 1.0 L)			
Media Component	**Brown**	**1754 PETC**	**Modified MSM**	**PYF**
NH_4Cl	1.0 g	1.0 g	1.0 g	---
KCl	0.1 g	0.1 g	---	---
$MgSO_4 \cdot 7\ H_2O$	0.2 g	0.2 g	0.2 g	---
NaCl	1.1 g	0.8 g	---	---
KH_2PO_4	0.1 g	0.1 g	0.38 g	---
K_2HPO_4	0.02 g	---	---	---
$CaCl_2 \cdot 2\ H_2O$	---	0.02 g	---	---
$FeCl_3$	---	---	0.5 g	---
$NaHCO_3$	2.0 g	---	---	---
Peptone	---	---	---	5.0 g
Yeast extract	---	1.0 g	1.0 g	10.0 g
Fructose	5 g (optional)	5.0 g	5.0 g	5.0 g
Trace Elements[1]	20 ml	10.0 ml	10 ml	---
Wolfe's Vitamin solution[2]	10 ml	10.0 ml	---	---
4g/L L-cysteine solution	10 ml	---	---	---
Distilled water	960 ml	980 ml	990 ml	1000 ml
Solution final pH	---	5.9	7.0	6.0

1. See Table 3.1-2
2. See Table 3.1-3

Table 3.1-2. Trace Elements Solution

Nitrilotriacetic acid	2.0 g
$MnSO_4 \cdot H_2O$	1.0 g
$Fe(SO4)_2(NH4)_2 \cdot 6\ H_2O$	0.8 g
$CoCl_2 \cdot 6\ H_2O$	0.2 g
$ZnSO_4 \cdot 7\ H_2O$	0.2 mg
$CuCl_2 \cdot 2\ H_2O$	20 mg
$NiCl_2 \cdot 6\ H_2O$	20 mg
$Na_2MoO_4 \cdot 2\ H_2O$	20 mg
Na_2SeO_4	20 mg
Na_2WO_4	20 mg
Distilled water	1.0 L

Purchased from ATCC

Table 3.1-3. Wolfe's Vitamin Solution

Biotin	2.0 mg
Folic acid	2.0 mg
Pyridoxine HCl	10.0 mg
Thiamine HCl	5.0 mg
Riboflavin	5.0 mg
Nicotinic acid	5.0 mg
Calcium-D-pantothenate	5.0 mg
Vitamin B_{12}	0.1 mg
p-Aminobenzoic acid	5.0 mg
Thioctic acid	5.0 mg
Distilled water	1.0 L

Purchased from ATCC

Table 3.1-4. Production Medium Formulation

Medium component	**ml/1L medium**
Mineral solution	**50**
Trace minerals	**5**
B-vitamin solution	**20**
Distilled water	**925**
Mineral solution	**g/L**
$(NH_4)_2SO_4$	10
NH_4CL	10
KH_2PO_4	136
B-vitamin Solution	**mg/L**
Biotin	20
Folic acid	20
Pyridoxal HCl	10
Thioctic acid	60
Riboflavin	50
Thiamine HCl	50
Calcium-D-Pantothenate	50
Vitamin B_{12}	50
P-Aminobenzoic acid	50
Nicotinic acid	50
Trace mineral Solution	**g/L**
Nitrilotriacetate	1.5
$MgSO_4 \cdot 7\ H_2O$	6.1
NaCl	1.0
$FeSO_4 \cdot 7\ H_2O$	0.1
$CoCl_2 \cdot 6\ H_2O$	0.1
$CaCl_2 \cdot 2\ H_2O$	0.1
$ZnCl_2$	0.1
$CuCl_2 \cdot H_2O$	0.01
$AlK(SO_4)_2 \cdot 12\ H_2O$	0.01
H_3BO_3	0.01
$Na_2MoO_4 \cdot 2\ H_2O$	0.01
$NiCl_2 \cdot 6\ H_2O$	0.05
Na_2SeO_3	0.0005
$MnSO_4 \cdot H_2O$	0.5

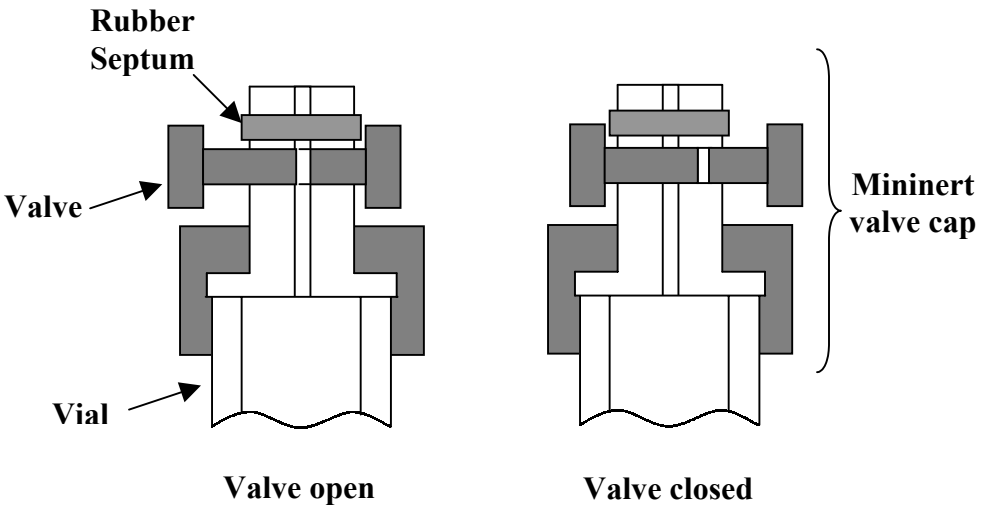


Figure 3.1-1. Mininert valve Teflon caps with valve open and with valve closed.

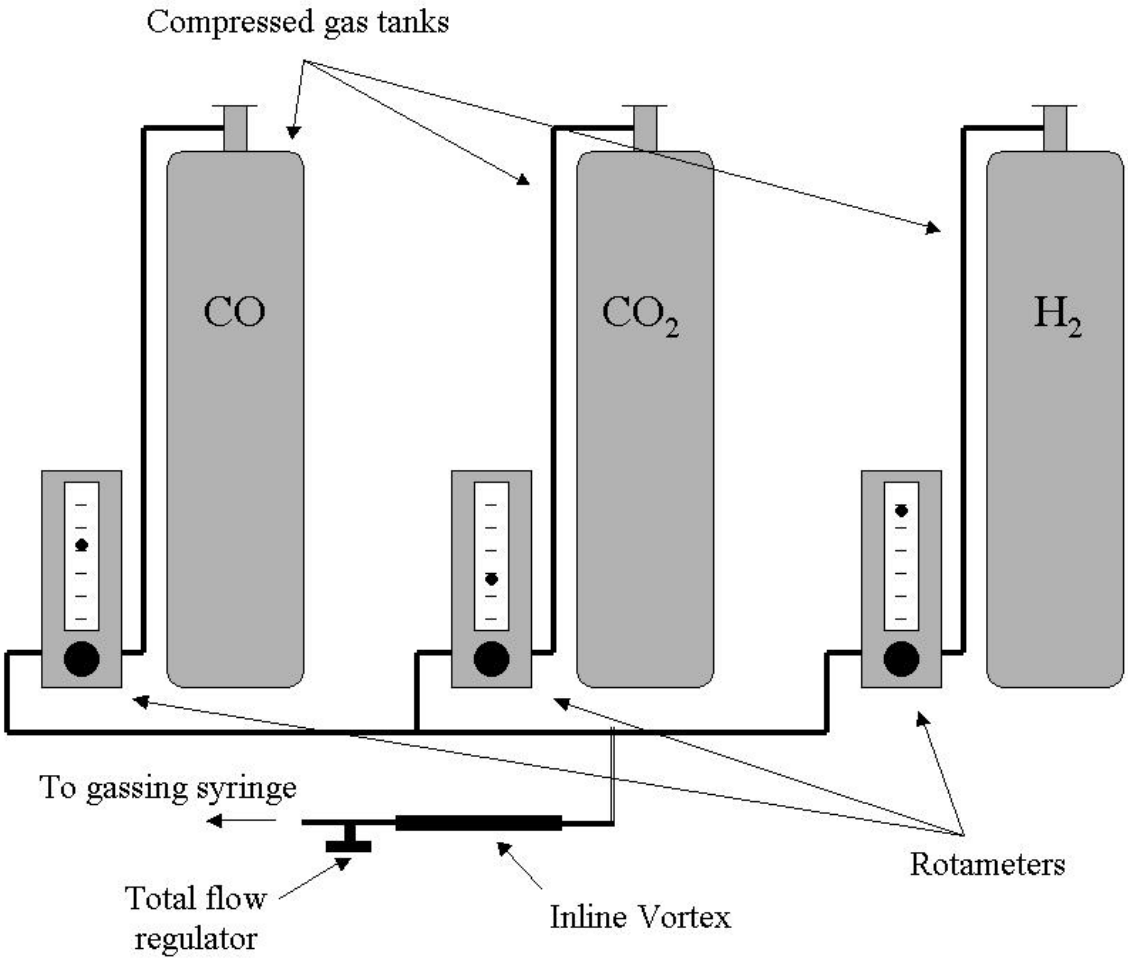


Figure 3.1-2. Synthesis gas mixing apparatus schematic.

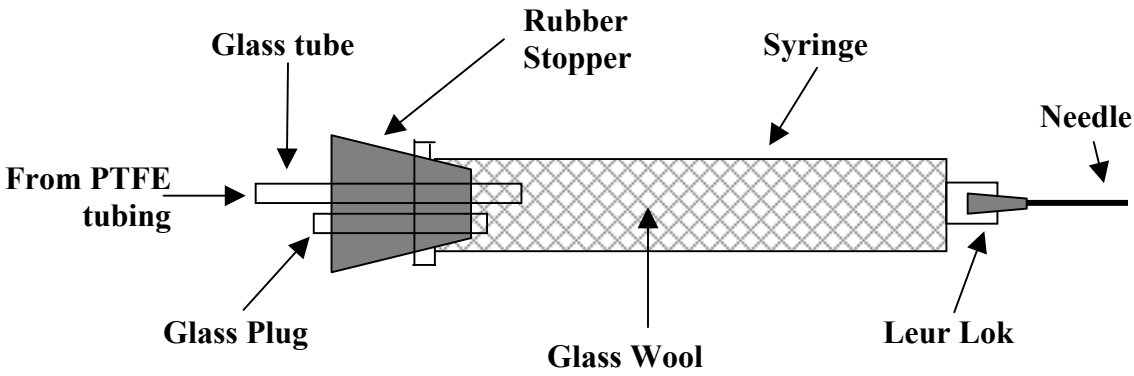


Figure 3.1-3. Gassing syringe

3.2 METHODS

Liquid Media Preparation

Different liquid media formulations were used depending on the organism employed and whether bacterial isolation, cell growth, or ethanol production was desired. Media used in culture isolation contained no sugar. Production media were composed of 50 ml Mineral Solution, 20 ml B-vitamin Solution, and 5 ml of Trace Elements Solution. Compositions of the Mineral Solution, the B-vitamin Solution, and the Trace Elements Solution are outlined in Table 3.1-4. Production media was differentiated as either Ethanol Production Medium (EPM) or Acetate Production Medium (APM). EPM had a pH of 4.5, while APM had a pH of 7.0. These media provided all the vitamins and minerals needed for bacterial growth and ethanol or acetate production without providing a carbon source. The carbon source was added later in the form of gaseous carbon monoxide and carbon dioxide. A medium formulated by Dr. Lewis Brown, outlined in Table 3.1-1, was also used without fructose to promote culture isolation.

ATCC (2004) recommended Medium PETC 1754 for *Clostridium ljungdahlii* growth (Table 3.1-1). Trace Elements Solution and Wolfe's Vitamin Solution were purchased from ATCC (Tables 3.1-2 and 3.1-3). One liter of water was mixed with 1 ml of Wolfe's Vitamin Solution and 10 ml of Trace Elements Solution. The remaining compounds were added and the medium was adjusted to pH 5.9 with 1M potassium hydroxide or 30% hydrochloric acid.

Fructose, trace elements, yeast extract, and Wolfe's Vitamins were added to traditional mineral salts medium (MSM) to promote bacterial growth (Table 3.1-1). Table 3.1-1 also includes the formulation for the ultra rich PYF media that is composed solely of peptone, yeast extract, and fructose.

All growth media were made in 1 liter batches. Twenty milliliters of growth media were dispensed into each of the 40 ml VOA vials. The vials were loosely capped to allow contact between the media and the autoclave's steam. The vials were then autoclaved in a Steris SG920 Scientific Gravity Autoclave. The media-filled vials were removed promptly at the end of the autoclave cycle and placed in the anaerobic glove bag to cool and equilibrate with the anaerobic glove bag atmosphere to become anaerobic. After photolytic degradation of the B-vitamins in the media was observed, APM and EPM were stored in shoeboxes in the glove bag to prevent degradation of B-vitamins.

Solid Media Preparation

Solid media were used in the isolation of microorganisms from the various bacterial consortium collected as seed sources. Seventeen grams of Difco granular agar were dissolved in a liter of APM and boiled to transparency. About 5 ml of hot agar was poured into each 40-ml VOA vial and autoclaved. The vials were laid on their side in the glove bag to cool, harden, and become anaerobic.

Difco Plate Count Agar (PCA) was mixed, poured into medial bottles, autoclaved and poured onto 95 mm x 15 mm Petri dishes. The cooled plates were put in the glove bag and allowed to become anaerobic.

Isolation of a Culture or Consortium

After collection, the potential bacterial sources were used to innoculate fresh APM. One gram of solid or 3 ml of liquid innoculant wwas used to start enrichments. The potential bacterial sources in APM were gassed with pure carbon monoxide and incubated in the shaker incubator set at 37ºC. Gas samples were collected weekly. When CO uptake or an increase in turbidity indicating cell growth was observed, liquid samples were taken and 3 ml of the consortium were transferred to fresh APM.

Enrichments of the cultures acquired from Dr. Brown were transferred into Brown Medium without fructose (Table 3.1-1). These cultures were gassed with 100% carbon monoxide. One vial of each enrichment was incubated on the shaker incubator at 37°C and one vial of each enrichment was incubated in a stationary incubator at 37°C. The vial headspaces were sampled weekly for CO uptake. After either CO uptake or increased turbidity indicating cell growth was observed, liquid samples were collected and 3 ml of the culture were transferred into fresh Brown Medium.

Cultures that consistently utilized carbon monoxide were streaked onto solid media, usually PCA. PCA was mixed according to the instructions on the package. The liquid PCA was boiled for 2 minutes or until the medium was transparent at which time the PCA was dispensed into 300 ml media bottles and autoclaved. The bottles were allowed to cool slightly before the medium was poured into pre-sterilized petri plates as follows:

1. A media bottle was opened and held at an angle to prevent dust contamination from the air.
2. The lip of the media bottle was flamed to prevent bacterial contamination.
3. A thin layer of PCA was poured into a sterile petri plate while the petri lid was held above the plate to prevent contamination.
4. The plates were allowed to cool and solidify
5. The plates were allowed to become anaerobic by equilibrating with the anaerobic atmosphere in the glove bag.

Once the agar was firm and anaerobic, a candidate culture was streaked onto the agar surface. In the glove bag, the candidate vial was opened carefully with the lid still covering the vial opening. The loop was sterilized in the BacteriCenerator III and dipped into the candidate vial. After swirling the loop through the liquid media, the loop was removed with a bubble of organism-filled medium. The candidate vial was re-capped, the droplet deposited on the agar, and the loop zigzagged across the media surface to spread the bacteria. The agar was incubated in the glove bag incubator at 37ºC.

Analysis of Culture Type and Purity

The microscope was used to view each culture allowing for the characterization and classification of each organism. It was also possible to suppose purity based on whether only one type of cell was visually observed.

In order to be visible by microscopy, either a Gram stain or a simple stain was performed of each culture. A small sample from an active vial of organisms was smeared onto a microscope slide and allowed to dry. Once dry, the slides were waved though a Bunsen burner flame to heat-fix the organisms to the slide. Gram stains were most commonly performed. The heat-fixed slide was covered in crystal violet for 1 minute then rinsed with water before the slide was covered with iodine for 1 minute and rinsed with water. The slide was rinsed with 2 droppers full of decolorizer and immediately rinsed with water. Safranin covered the slide for a minute, then was rinsed off before drying with bibulous paper. This stain differentiated between Gram-positive organisms and Gram-negative organisms. When viewed under the microscope, Gram-positive organisms appeared purple and Gram-negative organisms appeared pink.

When organisms could not be viewed under the microscope after Gram staining, a simple stain was performed. A simple stain required only one step. Once a sample of the culture was heat fixed to the slide, the slide was covered with methylene blue for one minute, rinsed, and blotted with bibulous paper before viewing.

Cell Growth

Each vial of a rack of 24 sterile vials containing 20 ml each of PETC 1754 medium was inoculated in the glove bag with 1 ml to 2 ml of *C. ljungdahlii* cultured in PETC 1754. The vials of *C. ljungdahlii* in PETC 1754 were incubated on the shaker incubator at 37ºC for 4 to 7 days. MSU1 was grown in batches of 24 vials. Each vial containing 20 ml of sterile modified MSM was inoculated with 1 ml to 2 ml of MSU1.

The vials were incubated on the shaker incubator at 37ºC for 4 to 7 days. The culture was then ready for ethanol production testing.

Cell Mass

The ethanol production step began with the separation of bacteria from growth media by centrifugation. Twenty of vials of growth media that were turbid with cells were combined in the glove bag to fill ten 50 ml centrifuge tubes. These tubes were centrifuged and then returned to the glove bag where the spent growth media is decanted. Thirty milliliters of sterile physiological saline are used to combine and re-suspend the cells. These cells are centrifuged again to form one large pellet. The physiological saline is decanted in the glove bag. The resulting pellet in the centrifuge tube was removed from the glove bag and weighed to determine cell mass.

Ethanol Production

After a pellet was formed by centrifugation, the centrifuge tube was returned to the glove bag where 35 ml of sterile EPM were used to re-suspend the organisms creating a cell concentrate. Five milliliters of the cell concentrate were used to inoculate each of 6 VOA vials containing 15 ml of fresh EPM. Three vials of 20 ml of sterile EPM were gassed with synthesis gas for use as abiotic controls. Three of the inoculated vials were gassed with synthesis gas creating the test vials. The remaining three inoculated vials served as biotic controls with only 95% N_2 and 5% H_2 in the headspace. All production vials were incubated on the shaker incubator at 37ºC.

Gas and liquid samples were collected daily from each vial. A 100 µL headspace sample was taken using a gastight syringe. The sample was injected into the Agilent

6890N Network Gas Chromatograph System with TCD. Volume percent values for CO, CO_2, and O_2 were recorded. The ethanol production vials were then moved into the glove bag where liquid samples were drawn for analysis for ethanol and acetate. One-inch needles attached to sterile 3 ml Leur-Lok syringes were used to remove ½-ml samples. Each liquid sample was filtered into a polypropylene insert in a GC autosampler vial to be analyzed on the Agilent 6890N Network Gas Chromatograph System with FID.

CHAPTER IV

RESULTS

4.1 Overview

The overall objective of this research was to optimize the separate growth and ethanol production steps that were proposed as a remedy to the mass transfer limitation observed during synthesis gas fermentation (Klasson et al., 1991). This proposal is outlined in the Research Hypothesis (Chapter I) and illustrated by Figure 1.1-1. *Clostridium ljungdahlii* was used to provide baseline ethanol production values for comparison with a culture developed at Mississippi State University. This organism provided by Dr. Lewis Brown of Mississippi State University and referred to herein as Mississippi State University Mesophilic Culture 1 (MSU1), was tested concurrently with *C. ljungdahlii*. The ultimate goal of this project was to scale-up the dual growth and fermentation system using novel ethanol producing cultures. Three major problems arose during experimentation that hindered the advancement of the proposed system:

1. Following commonly used microbiological protocol, ethanol was used as a sterilizing agent in the glove bag. Since the glove bag is a closed system, volatilized ethanol was not dissipated over time. The volatilized ethanol dissolved into media in the glove bag. After ethanol was observed in EPM blanks, which were analyzed using the GC/FID, the ethanol was removed from the glove bag.

2. The need for an alternative means of sterilizing objects in the glove bag after ethanol was eliminated as a sterilizing agent prompted the use of isopropanol. Isopropanol was chosen to replace ethanol because it was not the targeted product and therefore would not interfere with product analysis. Unfortunately, like ethanol, isopropanol also volatilized into the glove bag atmosphere and then adsorbed into media becoming anaerobic in the glove bag. Isopropanol also co-eluded with the ethanol peak during GC analysis. Unlike ethanol, isopropanol was not recognized as a contaminant in the media until all ethanol production trials were complete. The isopropanol peak on the chromatographs from the GC/FID analyses was identified only after efforts were made to identify other peaks routinely observed on chromatographs from the analysis of the liquid phase of the enrichments. Isopropanol was removed from the glove bag and the remaining enrichments were transferred into fresh, isopropanol-free media. Calibration curves for isopropanol and acetone were created and the chromatographs from past ethanol production trials were reintegrated.
3. Both cultures used in ethanol production trials were apparently easily out-competed by undesired microorganisms in the fructose-rich growth step, as witnessed by frequent Gram stains performed with each culture. The loss of both *C. ljungdahlii* and the MSU1 culture to bacterial contaminants prompted the search for new bacteria capable of producing ethanol from synthesis gas.

Serial enrichments were made over a five month period before isopropanol was discovered in the media.

Due to the problems outlined above, the focus of this research was adjusted. While optimization of cell growth and ethanol production from synthesis gas remained part of the focus, the search for novel organisms capable of producing ethanol from synthesis gas as well as optimization of media were incorporated into the scope. This chapter is divided into four main sections that reflect these changes: Cell Growth, Ethanol Production, Enrichments, and Media Optimization. Each section highlights experimental method difficulties, remediation methods attempted/employed, and experimental results generated that provide insight into the meeting of the experimental objectives.

4.2 Growth Experiments

The first step in the proposed synthesis gas fermentation process was the growth of large quantities of the desired microorganism in order to concentrate them for use in the production media for synthesis gas fermentation into ethanol. It was theorized that the bacteria could be grown in fructose-rich media, which would promote rapid cell growth yet retain ethanol production capability. Since *Clostridium ljungdahlii* and the Mississippi State University Mesophilic Culture 1 (MSU1) provided by Dr. Lewis Brown were both tested extensively for synthesis gas fermentation capabilities, determining the cell yield from fructose in the growth media for each of these organisms was considered key toward the development of the proposed two-step process.

Clostridium ljungdahlii

The American Type Culture Collection (ATCC) recommended the fructose-rich growth medium PETC 1754 for growth of *C. ljungdahlii* (ATCC, 2004). The original formula for PETC 1754 contained 5 g/l fructose, 1 g/l yeast extract, 1 ml/l ATCC Wolfe's Vitamins, 10 ml/l ATCC Trace Metals, and other minerals (Table 3.1-2). In an effort to determine an optimal carbon formulation for the growth of *C. ljungdahlii*, the original formula PETC 1754 as well as three formulations of PETC 1754 with varying concentrations of fructose, yeast extract, and peptone were inoculated with *C. ljungdahlii* and then incubated on the shaker incubator for 3 to 4 days (until the medium was so turbid that it was no longer translucent). The following formulations were tested:

- Two trials of PETC 1754 with 2.5 g/l fructose and 0.5 g/l yeast extract.

- Two trials of PETC 1754 with 2.5 g/l fructose, 0.5 g/l yeast extract, and 2.5 g/l peptone.
- Three trials of the original formula PETC 1754, which contained 5 g/l fructose and 1 g/l yeast extract.
- Two trials of PETC 1754 with 5 g/l fructose, 1 g/l yeast extract, and 1 g/l peptone.

To generate enough cells to conduct an ethanol production trial, cells from 20 vials of growth media were centrifuged and combined to form one large pellet. This pellet was weighed to determine cell mass. The cell growth rate was then calculated by dividing the cell mass produced per liter of growth media by millimoles of fructose initially included in the growth media and the number of days the cells were incubated in the growth media. This calculation resulted in a growth rate with the units of g cell/(mmol fructose x day).

Figure 4.2-1 shows that less cell mass per millimole fructose was obtained when *C. ljungdahlii* was grown in the PETC 1754 medium with only 2.5 g/l fructose versus 5g/l fructose. Because the fructose was more concentrated in PETC 1754 with 5 g/l fructose than with 2.5 g/l fructose, PETC 1754 with 5 g/l fructose was able to produce more cells. Figure 4.2-1 also illustrates that the addition of peptone (a protein mixture) significantly increased the *C. ljungdahlii* growth rate per millimole fructose. This means that *C. ljungdahlii* is able to utilize protein compositional components for cell growth.

Mississippi State University Mesophilic Culture 1

While extensive trials of *C. ljungdahlii* were performed in growth media with various concentrations of fructose, yeast extract, and peptone, only one medium formulation was used in growth trials with MSU1 before the culture was lost to bacterial contamination. Triplicate trials of MSU1 grown in modified Mineral Salts Media were performed with 5 g/l fructose and 1 g/l yeast extract. As Figure 4.2-2 shows, MSU1 was incubated for 3, 4, and 5 days. With each additional day of incubation, an increase in the cell growth rate was observed. The increase in cell growth rate as time passes suggests a plot of cell growth versus time will be an exponential plot. This indicates that in all three cases, MSU1 was removed from the growth medium while the cells were in the exponential growth phase. It is desired that the cells be transferred into EPM while still in the exponential phase of growth to prevent any initial lag in ethanol production from synthesis gas.

Data Presentation and Discussion

The level of turbidity visually observed in the vials of *C. ljungdahlii* incubated in PETC 1754 was indistinguishable from the level of turbidity observed with MSU1 incubated in Modified MSM. However, cell mass measurements indicated that *C. ljungdahlii* grew at a greater rate in PETC 1754 than did MSU1 in Modified MSM. Figure 4.2-3 shows that *C. ljungdahlii* incubated in PETC 1754 with 5 g/l fructose and 1 g/l yeast extract grew at nearly four times the rate of MSU1 in Modified MSM with 5 g/l fructose and 1 g/l yeast extract. Several possible reasons for *C. ljungdahlii* growing at a greater rate than MSU1 are that PETC 1754 was optimized for *C. ljungdahlii* growth and

the Modified MSM was not optimized for MSU1. Since this was a new organism, there were no data available concerning optimized growth, as was the case for *C. ljungdahlii*. The possibility exists that MSU1 was not well acclimated for utilizing fructose as a carbon and energy source. The use of another sugar, like dextrose or manose, or some other carbon source in the Modified MSM, may increase the cell growth rate of MSU1 by providing a carbon source better suited to the catabolic system of this organism.

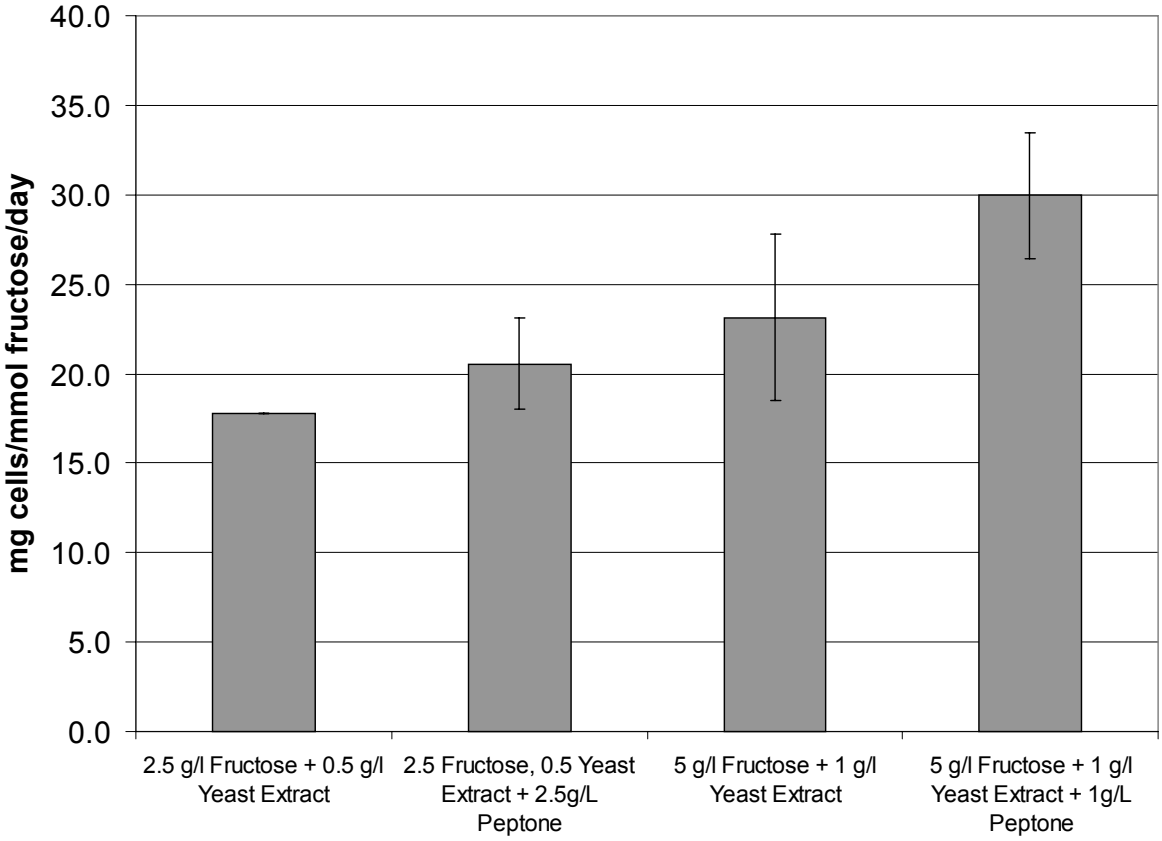


Figure 4.2-1. Effect of varying peptone and yeast extract concentrations in PETC 1754 growth medium on *C. ljungdahlii* growth

Original formula PETC 1754 contains 5 g/L fructose, 1 g/L yeast extract, and no peptone. Three trials were performed with the original formula (incubated 2, 2, and 3 days), two trials were performed in PETC 1754 with 2.5 g/l fructose and 0.5 g/l yeast extract (both incubated 3 days), two trials were performed in PETC 1754 with 2.5 g/l fructose, 0.5 g/l yeast extract, and 2.5 g/l peptone (incubated 3 and 4 days), two trials were performed in PETC 1754 with 5 g/l fructose, 1 g/l yeast extract, and 1 g/l peptone (incubated 1 and 2 days).

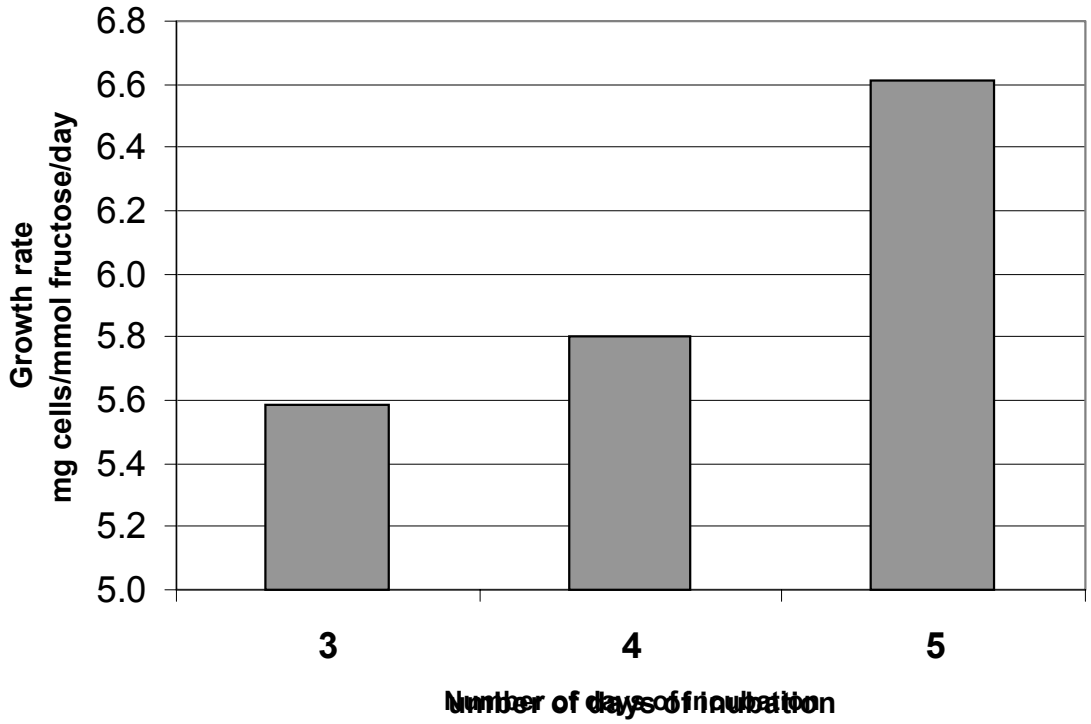


Figure 4.2-2. Growth rate of MSU1 in modified MSM at three, four, and five days

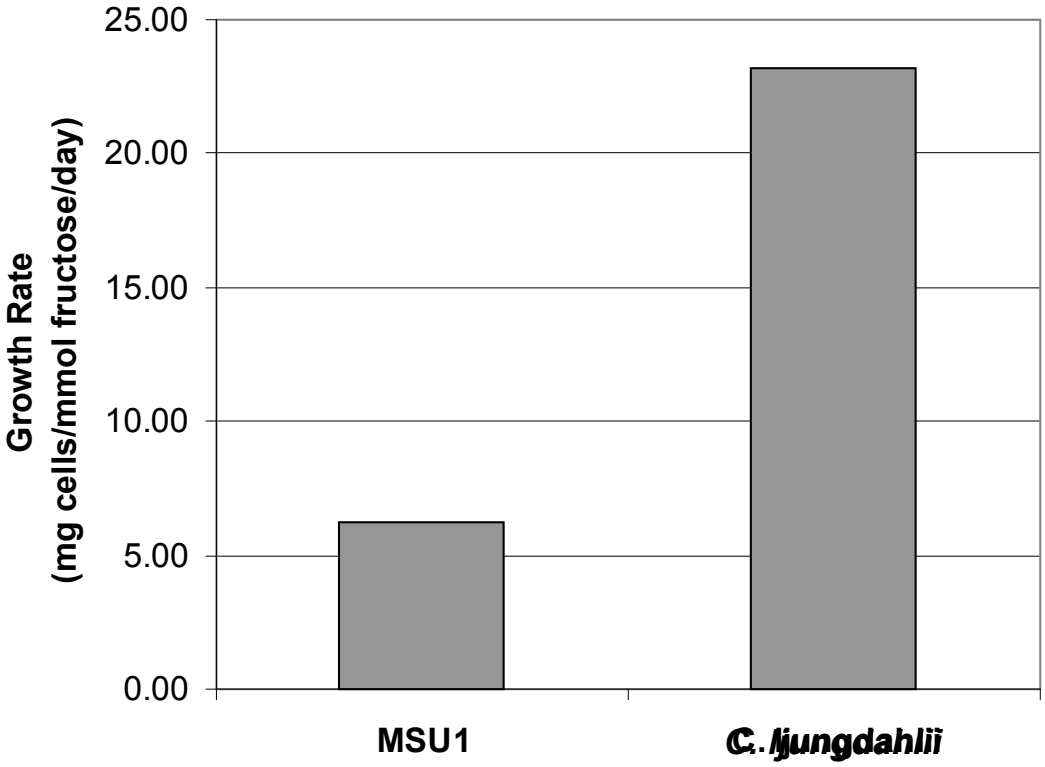


Figure 4.2-3. Comparison of growth rates of MSU1 grown in Modified MSM for 3-5 days and *C. ljungdahlii* grown in PETC 1754 for 2-3 days

Both media contained 5 g/l yeast extract and 1 g/l fructose. Each culture was incubated for three days.

4.3 Ethanol Production

Cultures like *Clostridium ljungdahlii* have been proven at the laboratory scale to convert synthesis gas to ethanol (Gaddy and Clausen, 1992). *C. ljungdahlii* was used in the current research to obtain baseline values for ethanol production and CO consumption by a known synthesis gas fermenting organism. This baseline production provided a basis of comparison for new organisms introduced in this work. Trials of *C. ljungdahlii* were conducted concurrently with trials of Mississippi State University's Mesophilic Culture 1 (MSU1), a novel ethanol producing culture isolated by Dr. Lewis Brown of Mississippi State University Biological Sciences. This section is divided into three phases. Each phase marks the implementation of new experimental methods and the desertion of failed techniques, that albeit traditional and widely acceptable protocol, were not of acceptable utility to this effort. In each phase, the discussion is divided into four sections: Method Development, Results, Data Presentation and Discussion, and Experimental and Application Significance. The Method Development section describes new methods that were employed during each phase. Results from experiments conducted using the new methods are presented in the Results Section. Comparisons between *C. ljungdahlii* and MSU1 as well as overall significance of the results are presented in the Data Presentation and Discussion Section. At the end of each phase, methods were re-evaluated based on observed results. Adjustments made to the methods at the end of each phase are outlined in Experimental and Application Significance.

Phase 1 Methods Development

Synthesis gas fermentation to ethanol has been reported to be limited by mass transfer of the gases to the targeted bacterial cells (Klasson et al., 1991). Concentrating large quantities of the desired ethanol-producing bacteria in production medium was suggested as a means of overcoming the mass transfer limitations. Therefore, each trial of *C. ljungdahlii* and MSU 1 was conducted in two steps. First, the bacteria were grown in media optimized for cell growth. Once the growth media was turbid with cell growth, 20 vials of turbid growth media were placed in the glove bag and the liquid from each vial was poured into centrifuge tubes and capped with airtight lids. The centrifuge tubes were removed from the glove bag and placed in the centrifuge where the cells and growth media were centrifuged for 20 minutes at 10ºC to 15ºC and 4000 rpm. The pelletized cells were then concentrated in EPM, and at that point, were ready for incubation to induce ethanol production (Step 2).

During Phase 1, the ethanol production vials had a 15 ml liquid phase and a 25 ml headspace. The gas within the headspace (which was the gas from the glove bag environment) was replaced with a 2:1 mixture of H_2 and CO as shown in Figure 4.3-1. Silicon septum caps were used to seal each vial.

During the course of experimentation with *C. ljungdahlii* and MSU1, oxygen was detected within the gas samples taken from supposed anaerobic production vials. According to initial headspace analyses, the production vials did begin as anaerobic systems. As testing time elapsed, a larger concentration of oxygen was measured in each serial gas sample. This oxygen was believed to have entered the sample via one of two

sources. The most obvious potential source of oxygen is that oxygen was actually in the vial when the sample was collected. For this conclusion to be valid, the sealed vials must be permeable to air or have consistent leaks. Jack Ford, a colleague at Mississippi State University, tested the vials and caps for leaks and concluded that the systems were sealed from ambient air at minimum over the first 72 hours of testing. The other potential source of oxygen in the gas samples was leakage during sampling. First consider that if the bacteria in a vial are utilizing CO and H_2, a drop in pressure will develop. If the pressure has fallen below atmospheric when samples are taken, the pressure in the sampling syringe will be lower than the surrounding atmosphere. It is then hypothesized that air enters the needle of the syringe once the needle is removed from the low-pressure environment of the vial and comes in contact with ambient air pressure (pressure gradient from outward in). Unfortunately, a good solution to this leakage was not found so it was decided that an air dilution correction would be applied to the gas analytical data. Therefore, the volume of CO loss observed in each vial was corrected for air to give a conservative estimate for CO uptake by the bacteria. The volume of air in each sample was calculated from the volume of oxygen. The calculated air volume was subtracted from the volume of CO lost by the system. The resulting volume calculated represents a conservative value for CO uptake by the cells.

Liquid samples were analyzed on an Agilent Technologies 6890N Network GC System with a Flame Ionization Detector (FID). During Phase 1, a 10ºC/minute ramp was used to analyze production media for ethanol.

To prevent bacterial contamination of the media in which MSU1 and *C. ljungdahlii* were cultured, the common microbiological practice of using an alcohol as a sterilizing agent was employed. During Phase 1, ethanol was used in the anaerobic glove bag to sterilize the silicon septa before each septum was punctured. Ethanol was also used to sterilize the workspace within the glove bag. The subsequent results presentation will highlight the detrimental impacts of following this widely used microbiological method on the intended ethanol fermentation experiments.

Phase 1 Results

During Phase 1, all testing with *C. ljungdahlii* failed to show evidence of CO consumption. The MSU1 culture did consume 0.49 ml ± 0.029 ml of CO over 73 hours. However, both cultures produced ethanol. Figure 4.3-2 shows that the test vials and the biotic controls of *C. ljungdahlii* produced equal concentrations of ethanol. Figure 4.3-3 shows that test vials and biotic controls of MSU1 also produced equal concentrations of ethanol. The ethanol observed in the MSU1 and *C. ljungdahlii* test vials and biotic controls was then attributed to the fermentation of sugars carried over from the growth media (see Table 3.1-2 for the composition of these media). It can be seen in both Figures 4.3-2 and 4.3-3 that ethanol was present in the abiotic controls at lower levels than in the test vials and the biotic controls for both *C. ljungdahlii* and MSU1 incubations. Ethanol in the abiotic controls and in medium blanks indicated that the medium was contaminated with ethanol. Since the only ethanol that either the vials or liquid samples were exposed to was the ethanol used for sterilization in the glove bag, the source of ethanol contamination was narrowed to ethanol absorbed into the medium from

the ethanol present in the glove bag, which comes into contact with the vials during attempts to establish anaerobic conditions. This is accomplished by allowing vented vials to remain the glove bag for long periods of time to allow equilibration of the media within the vials with the anaerobic glove bag atmosphere.

Phase 1 Data Presentation and Discussion

Ethanol was detected in all of the vials from the Phase 1 trials of both *C. ljungdahlii* and MSU1. It was concluded after a thorough review of test procedures that the source of the ethanol in the abiotic controls was ethanol contamination from the use of ethanol as a sterilizing agent in the glove bag. While the EPM was allowed to equilibrate with the nitrogen atmosphere within the glove bag and thus become anaerobic, the volatile ethanol within the glove bag atmosphere dissolved into the production media. Therefore, isopropanol was then used as a replacement to ethanol in the glove bag and on the bench top as a sterilizing agent in order to prevent further ethanol contamination as well as biotic contamination.

It was determined that MSU1 consumed enough CO to create an ethanol concentration of 24.0 ppm ± 1.3 ppm within each test vial. This determination was made when the baseline ethanol concentration in the abiotic controls of 42.3 ppm was subtracted from the ethanol concentration observed in the test vials yielding an estimated ethanol concentration of 21.6 ppm ± 4.6 ppm. Since the same concentrations of ethanol are seen in the MSU1 biotic controls, the ethanol observed in the MSU1 test vials was considered not to be a product of synthesis gas fermentation. Ethanol production seen in both the test vials and biotic controls was attributed to fructose fermentation of sugar

carried over from the growth medium. After the cells were centrifuged and the growth medium was decanted, it was visually observed that a small amount of growth medium still surrounded the cells. The sugars from the growth media were carried over into the production step when the cells were resuspended into the production media. Since ethanol production was observed and CO uptake was not, at this point, it is believed that the fructose was being fermented to ethanol.

Phase 1 Experimental and Application Significance

Ideally, all ethanol in the production vials of MSU1 and *C. ljungdahlii* would be a result of synthesis gas fermentation to ethanol. After ethanol was discovered in the production media, the ethanol was removed from the anaerobic glove bag and isopropanol used as a replacement.

It was preferable that all ethanol produced in the ethanol production media be derived from synthesis gas. For this reason, cell-washing techniques were adopted to keep fermentable sugars in the growth media out of the production media. After the culture in the growth media was centrifuged and the growth media decanted, the cell pellets were suspended and combined in physiological saline (0.85% NaCl) adjusted to a pH of 6.0. The saline solution containing cells was centrifuged, the saline decanted, and the resulting pellet suspended in ethanol production media.

One final new protocol was also adopted. This change was precipitated by opinion, not experimental evidence. Since *C. ljungdahlii* did not utilize CO during Phase 1, a concern was that the centrifugation temperature of 15ºC was so low that it could shock the ethanol-producing bacteria. After Phase 1, the centrifuge was operated at 20ºC.

After 20 minutes of centrifugation at 20ºC, only soft, watery pellets formed. Adding 5 minutes to the centrifuge time caused the cells to form tighter pellets. The effectiveness of the cell washing technique was tested in Phase 2.

Phase 2 Methods Development

Phase 2 began with the reevaluation of the synthesis gas composition employed (2:1 H_2:CO). The overall stoichiometric equation for ethanol production from synthesis gas required a higher concentration of CO than was provided during Phase 1 testing. For Phase 2, an 80% CO and 20% H_2 synthesis gas mixture was used to ensure that CO would never be limiting.

The headspace to liquid phase ratio was also reevaluated before Phase 2 experimentation began. The liquid volume in each vial was increased from 15 ml to 20 ml to slightly increase the area of interaction between media and synthesis gas. Since the 40 ml VOA vials were cylindrical and placed on their sides during culture incubation, filling each vial half full rather than less than half full provided a slight increase in liquid surface area. Maximizing the liquid surface area optimizes the interfacial area for mass transfer between the media and the synthesis gas.

After Phase 1, a color change was observed with the EPM over time. The original color source for this medium is neon-green colored B-vitamin solution that is added to the production medium. When combined with the other ingredients in EPM, the medium has a light green tint. This tint was still present after the medium was autoclaved but as the medium incubated in the glass-cover incubator or sat in the glove bag, the green tint faded until the medium turned clear. According to the label for each vitamin included in

the B-vitamin solution, every component is light sensitive. This meant that the B-vitamin solution and the production medium were light sensitive. Therefore, the decreases in color for the EPM were attributed to photodegradation of the B-vitamins in the medium. To remedy this problem, the glass covers of the shaker incubators were covered with aluminum foil to prevent degradation of production media during incubation. All production media left to equilibrate to anaerobic conditions in the glove bag were thereafter kept in shoeboxes to eliminate photodegredation.

Concurrently with the observations of EPM photodegradation, a potential secondary peak was observed at the ethanol peak in chromatographs of ethanol standards within EPM. This discovery of a second peak during liquid phase analysis coincided with the realization that the production media were degrading. This peak appeared on the chromatograph just before the ethanol peak. Figure 4.3-4 presents an example of the resulting chromatograph from a liquid medium sample from a biotic control that fermented fructose (from growth media carry-over) to ethanol. On the chromatograph, the ethanol peak appears at 4.148 minutes. Since photodegradation of the production media had been observed, the second peak was believed to be a degradation product in the production media and was then considered a "media peak". The so-called "media peak" appeared at 4 minutes. To get better separation of the ethanol peak and the "media peak," the GC/FID method was changed from a 10ºC/min ramp to a 5ºC/min ramp. Figure 4.3-5 shows that while lowering the ramp to 5ºC/minute did not necessarily promote a greater difference in elution time between the peaks, the peaks were more rigid and therefore more easily recognizable as two distinct, separate peaks.

After all the trials of MSU1 and *C. ljungdahlii* were complete, another peak was found to appear at 2.5 minutes in chromatographs from all enrichments. Since none of the enrichments were producing ethanol, the possibility existed that all synthesis gas fermentation was directed toward the product observed at 2.5 minutes. The possibility existed that this compound was a more valuable product than ethanol so efforts began to identify it. Since acetone and isopropanol were both readily available in the laboratory, and were both identified as potential synthesis gas fermentation products (Zeikus, 1980), calibration curves for isopropanol and acetone were created on the FID. All chromatographs were reintegrated to evaluate the peak areas of isopropanol and acetone.

Phase 2 Results

During Phase 2, both *C. ljungdahlii* and MSU1 were found to utilize CO. Figure 4.3-6 illustrates that equal volumes of CO were utilized per hour in both trials with *C. ljungdahlii*. Though the data reflect significant potential levels of CO uptake, the sizable deviation represented by the error bars was a disappointing indication that there was a high degree of variability between the individual vials in each trial. Liquid samples for these trials of *C. ljungdahlii* also displayed extreme variability between individual vials. For this reason, presenting data obtained from averaging the ethanol produced in each of the three test vials does not accurately reflect potential ethanol production in all of the test vials. Instead, ethanol and isopropanol concentrations in an individual test vial of *C. ljungdahlii* and an individual biotic control are presented in Figure 4.3-7 to illustrate that *C. ljungdahlii* did produce ethanol. Unfortunately, because of the aforementioned variability between vials, the ethanol in the test vial cannot be categorized definitively as

ethanol produced from synthesis gas fermentation or ethanol from fermentation of fructose carried over from the growth medium.

Both of the MSU1 trials conducted under Phase 2 utilized CO during their 73-hour test times. Figure 4.3-8 illustrates that the first trial of MSU1 utilized significantly higher volumes of CO than the second trial. Shortly after completing the second trial of MSU1 in Phase 2, Gram stains of the MSU1 culture indicated that MSU1 purity was lost to bacterial contamination. The fructose-rich growth medium provided ideal growth conditions not only for MSU1 but also for many other microorganisms. The undesired organisms out-competed MSU1 for nutrients in the growth medium and the MSU1 culture was lost. Less CO uptake was observed in the second trial of MSU1 because undesired organisms not capable of fermenting CO were effectively diluting MSU1. This experience with bacterial contamination highlights problems with the potential use of single isolates within industrial situations.

Figure 4.3-7 for *C. ljungdahlii* and Figure 4.3-9 for MSU1 both show ethanol production by the bacteria in less than 24 hours. With both cultures, the ethanol initially produced was no longer present later in the trials. The ethanol produced by MSU1 in the first 18 hours did not appear in samples of the vials taken only 6 hours later. The ethanol produced by *C. ljungdahlii* during the first 24 hours of testing was absent in samples taken at and beyond 96 hours of incubation.

Concerns developed that both of the cultures were metabolizing the ethanol that was produced from synthesis gas fermentation. This meant that there was a possibility that the cultures were producing large quantities of ethanol but the ethanol was degraded

and converted into cell mass before it could be detected in liquid samples. Since the cultures were degrading the ethanol produced from synthesis gas fermentation, a means of separating ethanol from EPM before it can be utilized by the cells must be developed before a scaled-up version of the synthesis gas fermentation process can be successfully employed. Experiments were then designed for *C. ljungdahlii* and MSU1 to test this theory. A concentration of 100 ppm ethanol was added to three vials of *C. ljungdahlii* in production media and the vials incubated along with three biotic control vials. Even though there was no observable CO utilization when cell washing techniques were employed, Figure 4.3-10 shows that *C. ljungdahlii* produced ethanol over the 96-hour trial. While this trial of *C. ljungdahlii* was intended to cast light on whether *C. ljungdahlii* was degrading the ethanol it produced from synthesis gas, instead it cast doubt on the effectiveness of cell washing techniques. MSU1 was incubated in ethanol production media with 500 ppm ethanol. Figure 4.3-11 shows little change in ethanol concentrations observed over 24 hours and clearly, no ethanol production occurred with this test system. The high levels of ethanol coupled with the unexpected presence of isopropanol rendered MSU1 inactive.

Phase 2 Data Presentation and Discussion

During Phase 2, the maximum observed ethanol concentration produced by *C. ljungdahlii* was 0.172 g/l ± 0.043 g/l. MSU1 produced a maximum of 0.471 g/l ± 0.077 g/l ethanol. The highest reported concentration of ethanol produced in batch fermentations with *C. ljungdahlii* was 7 g/l (Gaddy and Clausen, 1992). While MSU1 produced higher concentrations of ethanol than *C. ljungdahlii* in head-to-head tests,

neither culture approached ethanol concentrations rivaling the reported ethanol production values for *C. ljungdahlii*.

Phase 2 Experimental and Application Significance

At the time of experimentation, it was not known that isopropanol was present in the production media. The lower than expected performance by *C. ljungdahlii* was thought to be a result of the lack of available CO. As CO was utilized; lower pressures were created in the batch systems. Under lower pressures, CO was less likely to dissolve in the liquid phase where it was needed for ethanol production. Three new gassing methods were implemented to assure that sufficient CO was available to the organisms. First, the gassing time was increased from 1 minute per vial to 2 minutes per vial to provide time for nitrogen to be fully flushed from the headspace. Next, the method used to replace the headspace within the vial was altered so that a positive pressure would be created in each vial before incubation. Creating a positive pressure in the vials was believed to force a larger number of CO molecules into the liquid phase and control air leakage into the vials. With this new method of gas introduction, synthesis gas was added to each test vial and abiotic control for 15 seconds before the vial was vented. After simultaneously filling and venting each vial for 1½ minutes, the venting needle was removed and synthesis gas continued filling the unvented vial for 15 seconds. In this manor, each test vial and abiotic control was filled and slightly pressurized with CO.

Pressurizing the headspace only delayed eventual pressure loss caused by CO uptake. To assure that CO was available even during long trials, up to 192 hours, vials were regassed at least once every 72 hours.

Figure 4.3-9 shows that ethanol production was observed in the biotic controls of MSU1 even after cell washing techniques were implemented. Since no synthesis gas was present, the ethanol in the biotic controls was a result of the fermentation of fructose carried over from the growth medium. For this reason, cell washing was determined to be an ineffective technique. Cell washing techniques were abandoned after Phase 2.

Phase 3 Methods Development

Based on interactions with Department of Energy officials during the course of this study, the synthesis gas mixture was changed to a more accurate synthesis gas composition of 45% CO, 45% H_2, and 10% CO_2.

As time elapsed in each experiment, the oxygen concentrations in samples from each vial increased. At the time of experimentation, it was assumed that the increase in oxygen concentrations in the gas samples was a reflection of the failure of the silicon septum caps to seal the vial from ambient air. For this reason, the silicon septum caps were replaced by Mininert caps. Figure 3.1-1 shows the valve system associated with Mininert caps. Since only a single needle could be used with the Mininert caps, new methods for replacing the headspace were developed. Instead of using a venting needle, the Mininert caps were opened slightly to vent the vial. Each vial was gassed for 15 seconds before the cap was loosened. The vials were gassed for 1½ minutes before the cap was tightened. Each vial was gassed for an additional 15 seconds after the cap was tightened in order to create a positive pressure in the vial.

Phase 3 Results

C. ljungdahlii consumed CO throughout the 192 hour trial. Figure 4.3-12 shows that the cells utilized CO at the greatest rate from 47 to 70 hours. After 70 hours, the rate of CO utilization diminished. The most likely cause of the diminished CO uptake rate was nutrient depletion in the production media. As the culture utilized CO, it also consumed nutrients essential to cellular metabolism. Despite observed CO uptake, no ethanol was produced during the trial of *C. ljungdahlii* under Phase 3. Other possible sinks for the CO utilized by *C. ljungdahlii* in this trial are the conversion of CO to cell mass or to acetate. It was determined by HPLC that no acetate was formed and it was not optically discernable whether cell growth occurred during the ethanol production trials. The gas analysis technique used was unable to analyze for methane so it is possible that the CO was converted to methane or some other product by a bacterial contaminant.

Two other trials were conducted using *C. ljungdahlii* under the same conditions. Neither CO uptake nor ethanol production was observed during these trials. It was determined by Gram staining that the stock vials of *C. ljungdahlii* were indeed contaminated with undesired bacteria. Like the MSU1 culture, *C. ljungdahlii* was likely out-competed by the bacterial contaminant.

Phase 3 Experimental and Application Significance

After both MSU1 and *C. ljungdahlii* were lost to bacterial contamination, the search for a more robust organism capable of fermenting synthesis gas to ethanol was initiated. This work is described in detail in the Section 4.4 of the Results Chapter. While analyzing the liquid phase of these enrichments for ethanol, other peaks were

observed. In an effort to categorize the additional peaks, calibration curves for isopropanol and acetone were created. At this time, the so-called "media peak" was found to be the isopropanol peak. All chromatographs from Phases 2 and 3 were reintegrated for isopropanol, ethanol, and acetone. Although isopropanol was biologically available in all production vials in Phase 2 and 3 as a potential carbon and energy source, Figures 4.3-13 and 4.3-14 show that organisms in the presence of CO (test vials) did not consume isopropanol while organisms in the biotic control vials consumed isopropanol. Figure 4.3-12 illustrates that *C. ljungdahlii* in the presence of synthesis gas does not use isopropanol but rather consumes ethanol produced earlier in the fermentation. The cells in the biotic control consumed nearly 2 mg of isopropanol in each vial. Figure 4.3-14 shows that MSU1, in the absence of CO, utilized isopropanol while the same organism in a vial with a synthesis gas headspace consumed no isopropanol. While MSU1 and *C. ljungdahlii* in the biotic controls utilized isopropanol, the MSU1 and *C. ljungdahlii* in the test vials with synthesis gas did not. A larger quantity of ethanol was consumed in the test vials.

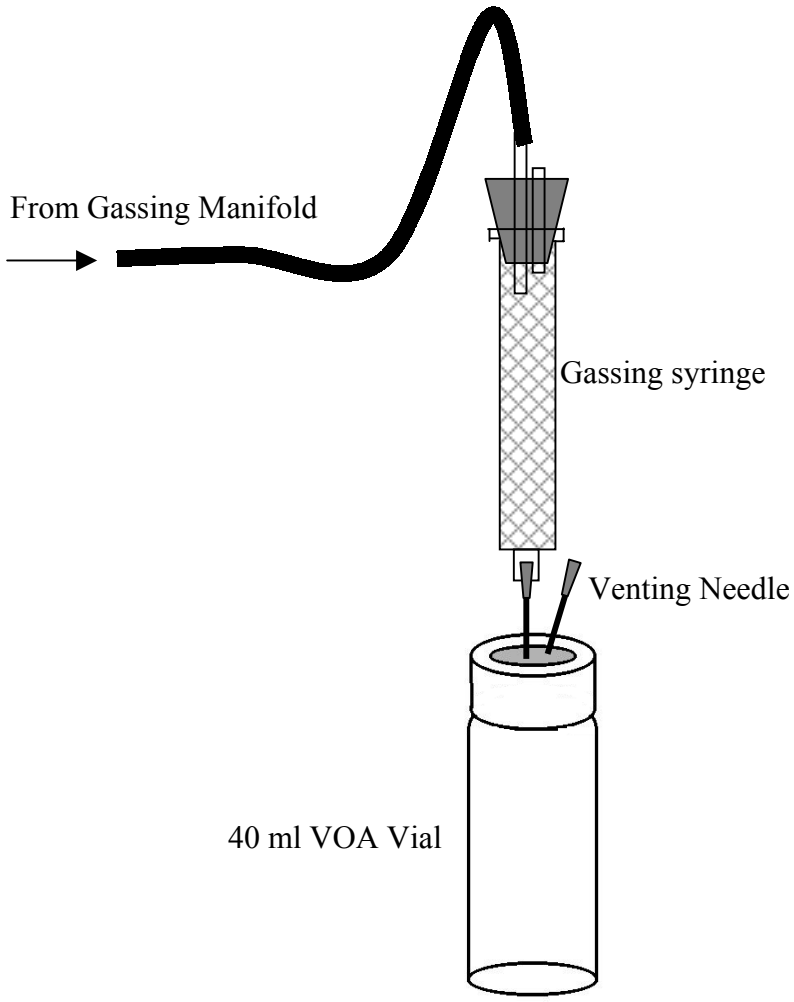


Figure 4.3-1. Headspace replacement

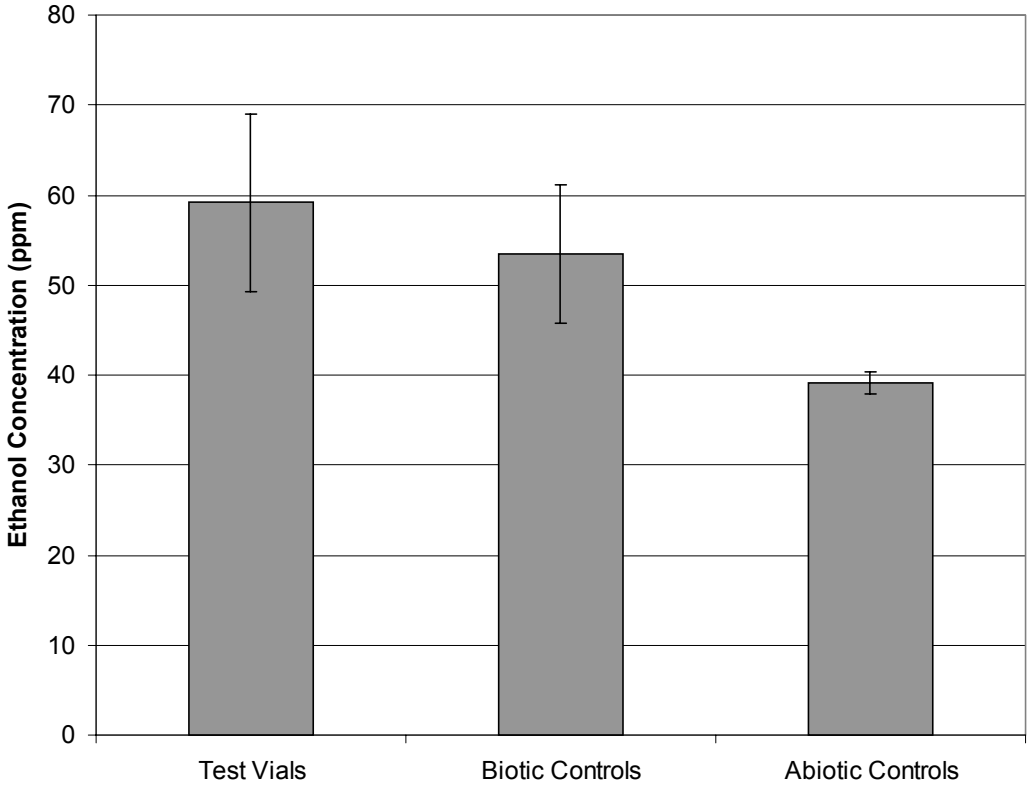


Figure 4.3-2. Ethanol production by *C. ljungdahlii* during Phase 1 after 24.5 hours

Test vials and abiotic controls were gassed with 2:1 H_2:CO. Test vials, biotic controls, and abiotic controls were run in triplicate. Two samples of each vial were analyzed.

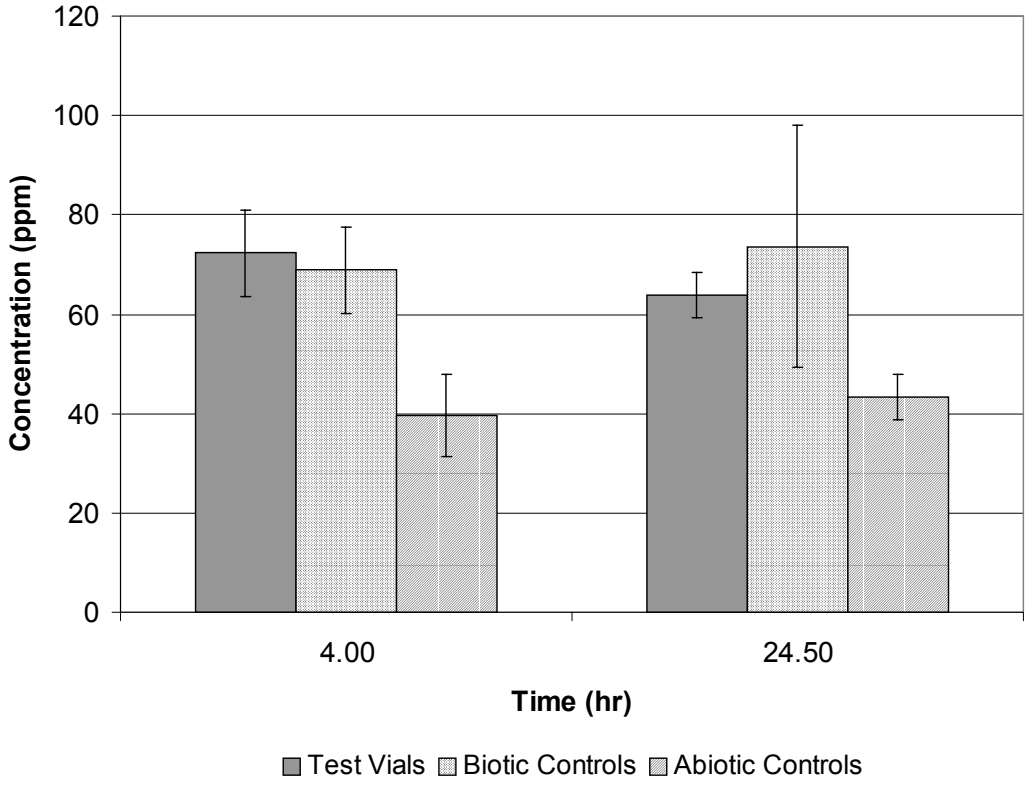


Figure 4.3-3. Ethanol production by MSU1 during Phase 1

Test vials and abiotic controls were gassed with 2:1 H_2:CO. Test vials, biotic controls, and abiotic controls were run in triplicate. Two samples of each vial were analyzed.

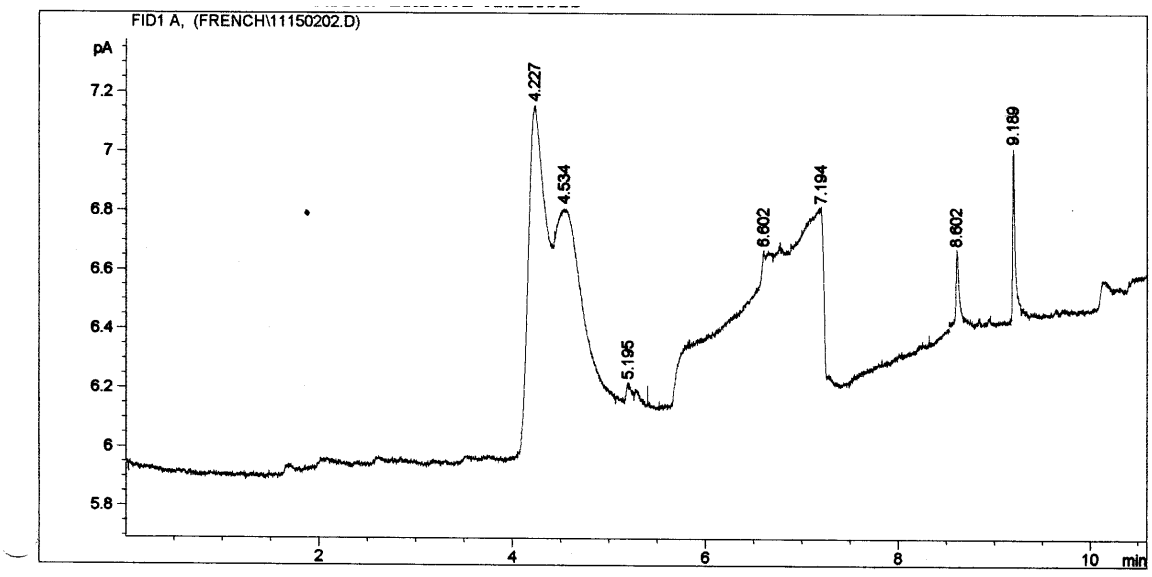


Figure 4.3-4. Chromatograph of biotic control that produced ethanol

The peak at 4.534 represents ethanol. The peak at 4.227 represents the "media peak" which was later discovered to be the isopropanol peak.

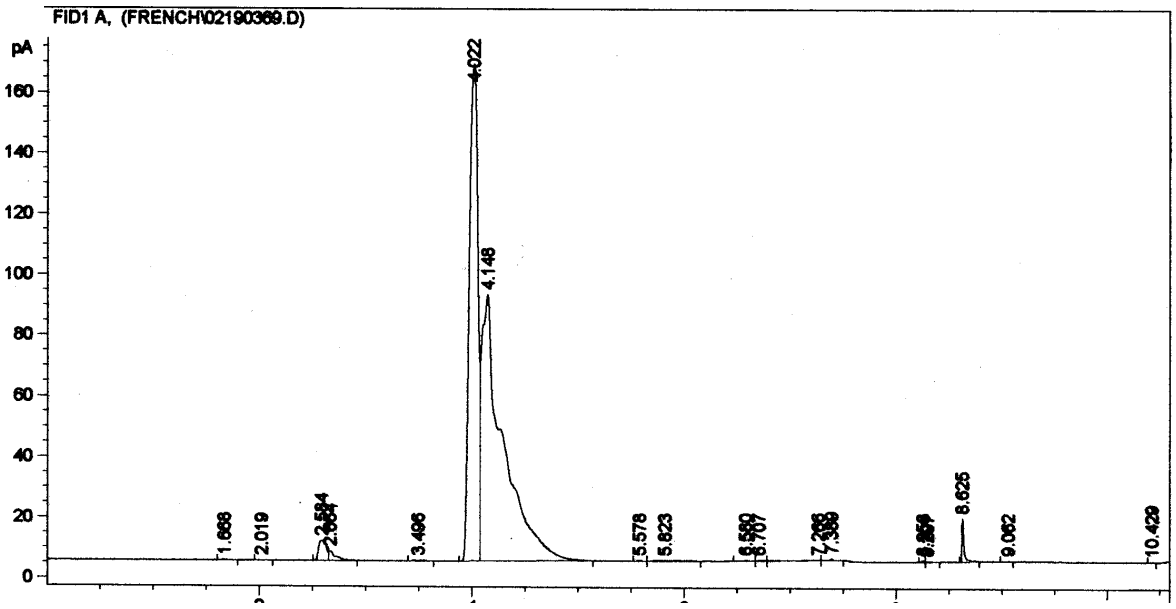


Figure 4.3-5. Chromatograph of EPM spiked with ethanol

The peak at 4.148 represents ethanol. The peak at 4.022 represents the "media peak" which was later discovered to be the isopropanol peak.

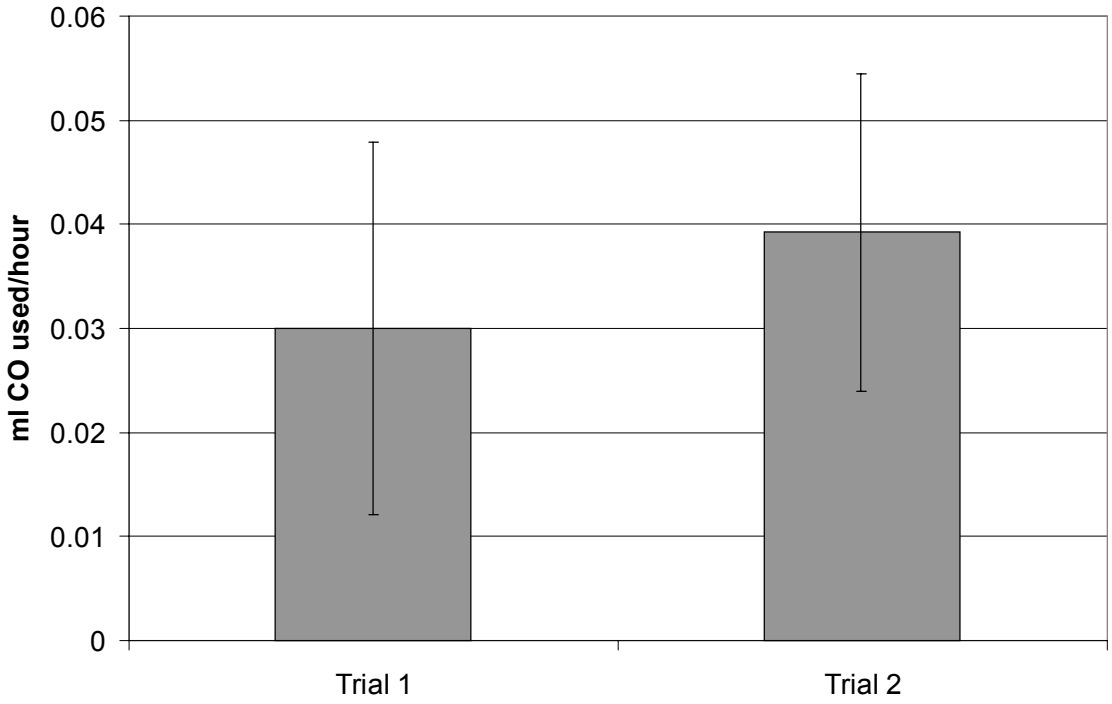


Figure 4.3-6. CO uptake per hour of incubation in trials of *C. ljungdahlii* during Phase 2

Test vials and abiotic controls were gassed with 20:80 H_2:CO. Test vials, biotic controls, and abiotic controls were tested in triplicate.

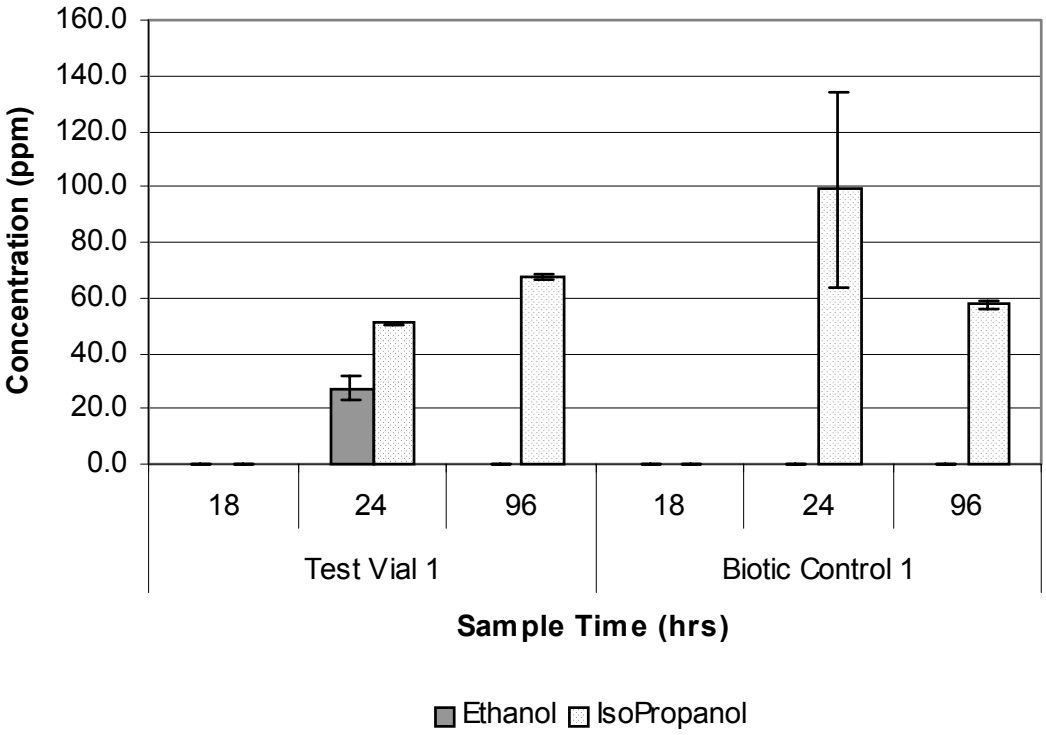


Figure 4.3-7. Ethanol production in two vials of *C. ljungdahlii* in EPM during Phase 2 Test vials were gassed with 20:80 H_2:CO. Two samples from each vial were analyzed.

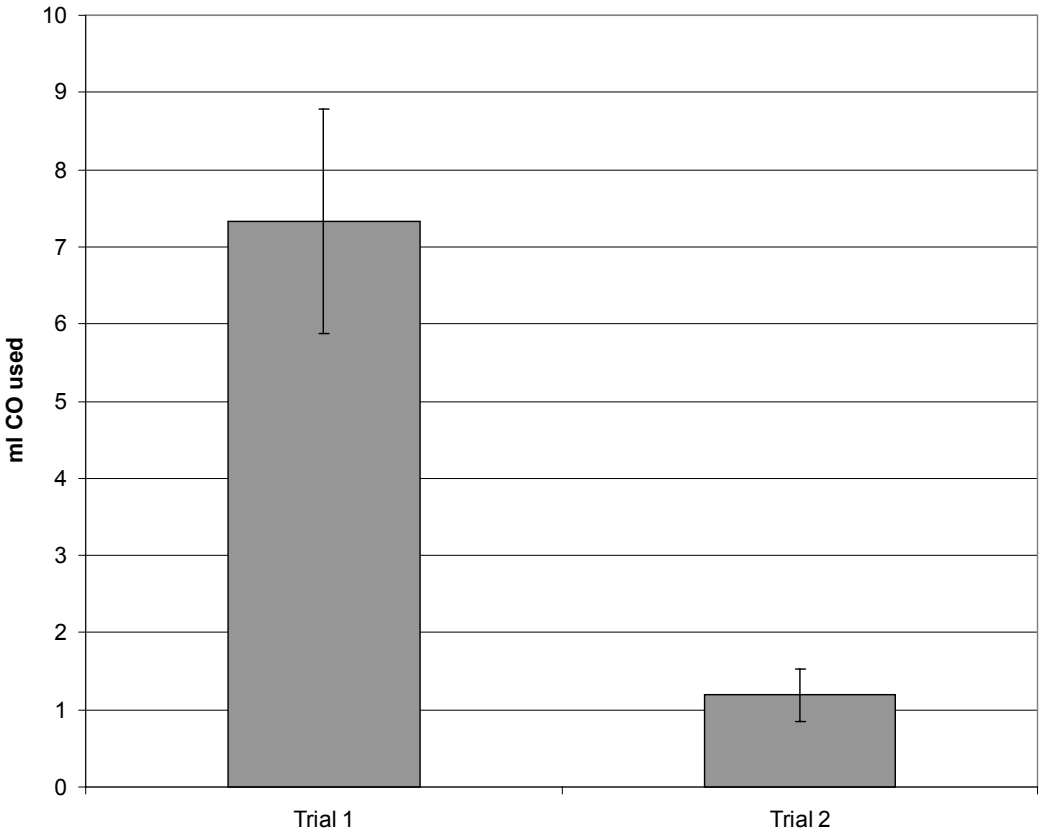


Figure 4.3-8. CO uptake in trials of MSU1 during Phase 2

Test vials and abiotic controls were gassed with 20:80 H_2:CO. Test vials, biotic controls, and abiotic controls were run in triplicate.

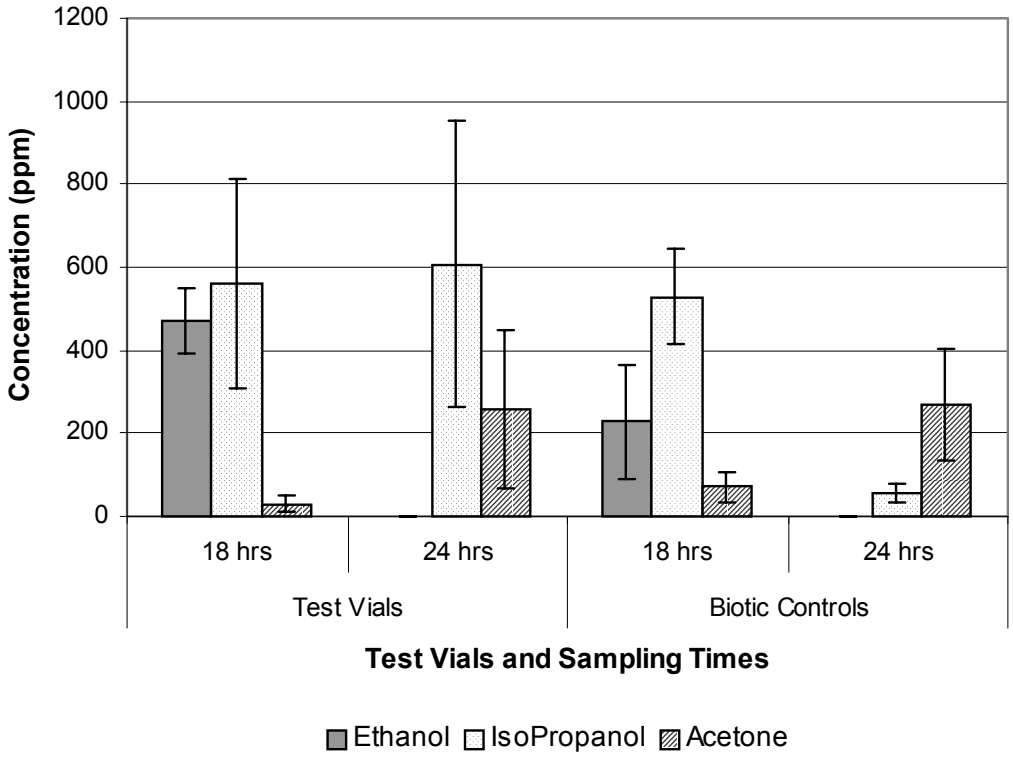


Figure 4.3-9. Ethanol production by MSU1 during Phase 2

Test vials and abiotic controls were gassed with 20:80 H_2:CO. Test vials and biotic controls were run in triplicate. Two samples of each vial were analyzed.

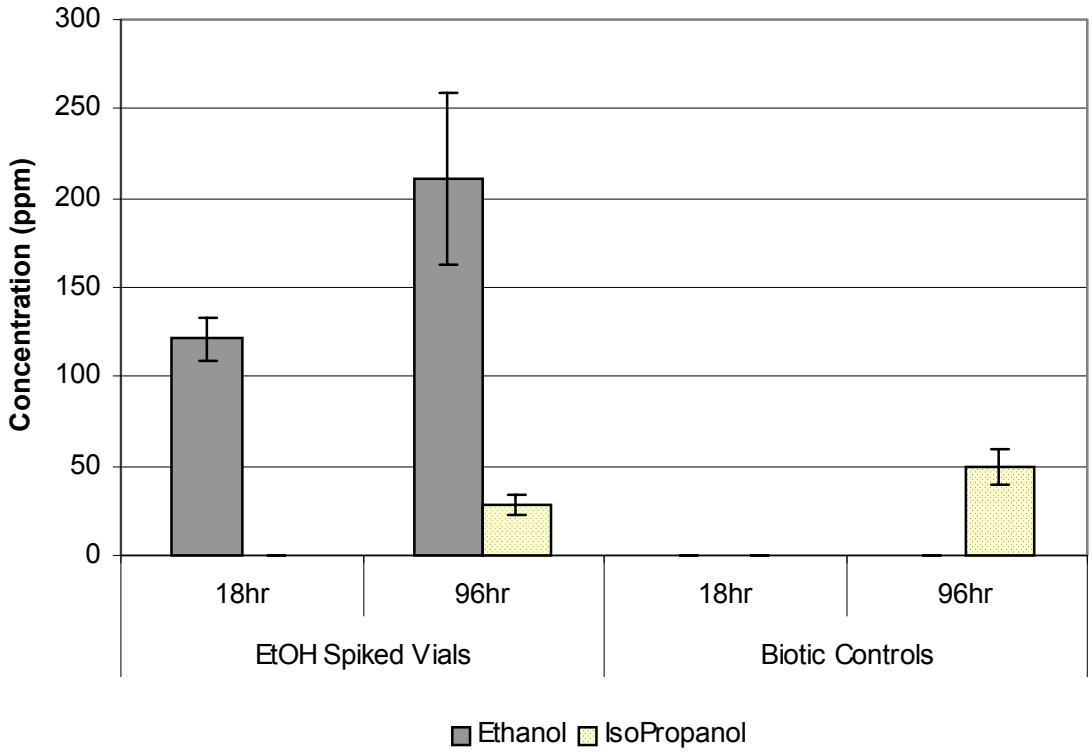


Figure 4.3-10. Effect of adding 100 ppm ethanol to *C. ljungdahlii* in EPM

The headspace of the ethanol spiked vials and the biotic controls was composed of 95% N_2 and 5% H_2. Two sub-samples from each liquid sample of the three ethanol-spiked vials and the three biotic controls vials were analyzed for ethanol.

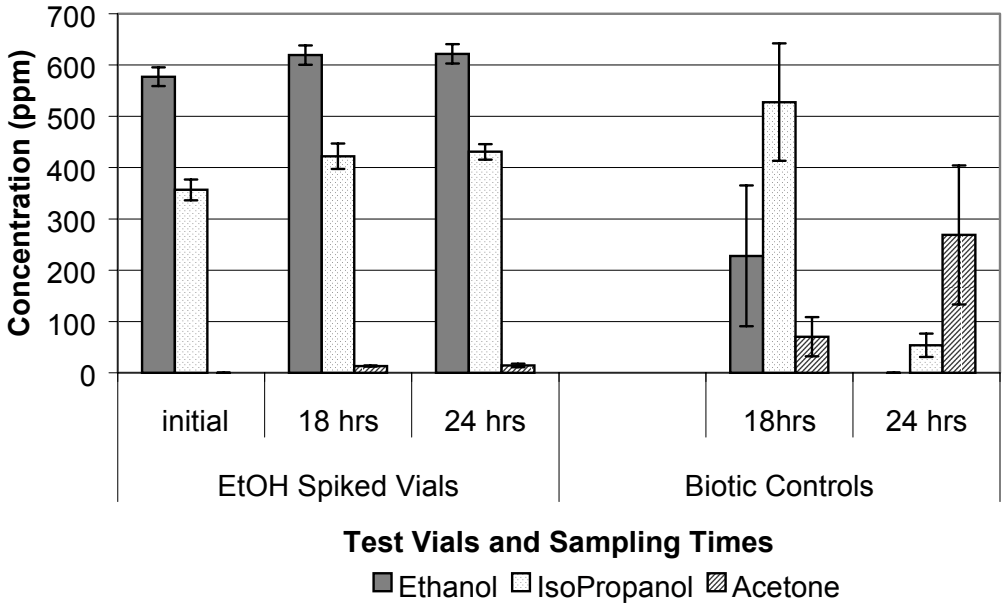


Figure 4.3-11. Effect of adding 500 ppm ethanol to MSU1 in EPM

The headspace of the ethanol spiked vials and the biotic controls were composed of 95% N_2 and 5% H_2. Two sub-samples from each liquid sample of the three ethanol-spiked vials and the three biotic controls vials were analyzed for ethanol.

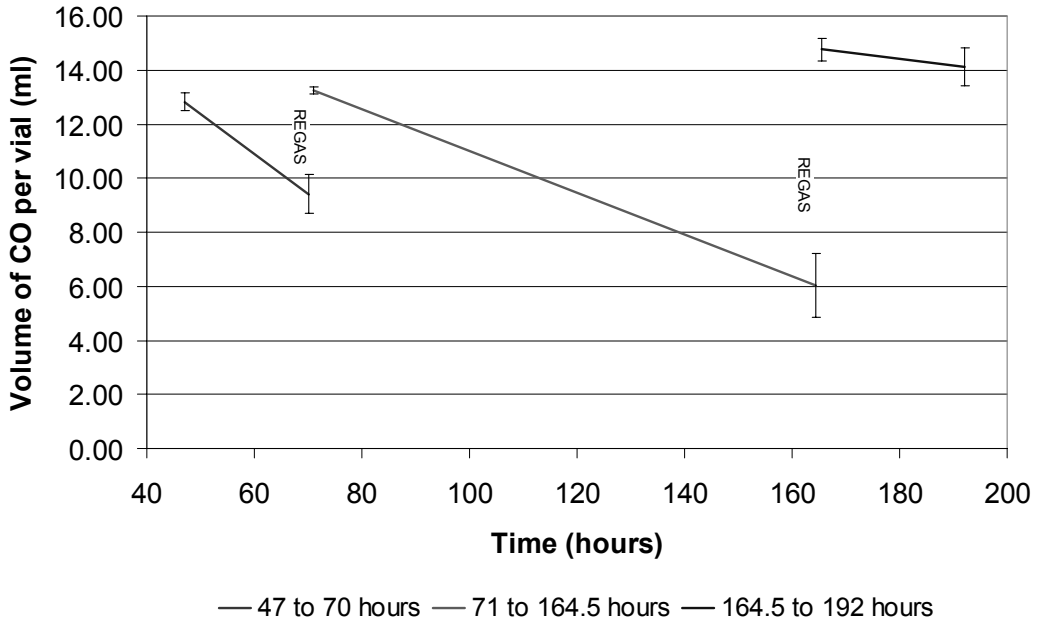


Figure 4.3-12. Carbon monoxide dissipation over a long incubation time

Test vials and abiotic controls were gassed with 45% CO, 45% H_2, and 10% CO_2. Test vials, biotic controls, and abiotic controls were run in triplicate. Two samples of each vial were analyzed.

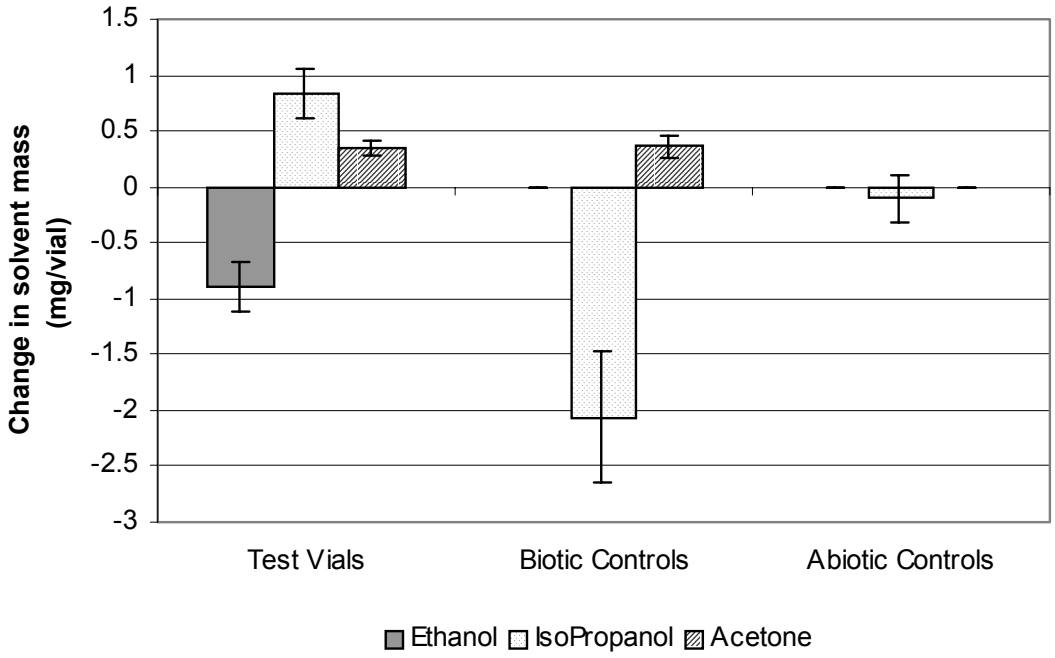


Figure 4.3-13. Ethanol, isopropanol, and acetone fluctuations with *C. ljungdahlii* between 24 and 192 hours

Bars above zero indicate solvent production while bars below zero indicate solvent consumption. Test vials, biotic controls, and abiotic controls were run in triplicate. Two samples were taken from each vial.

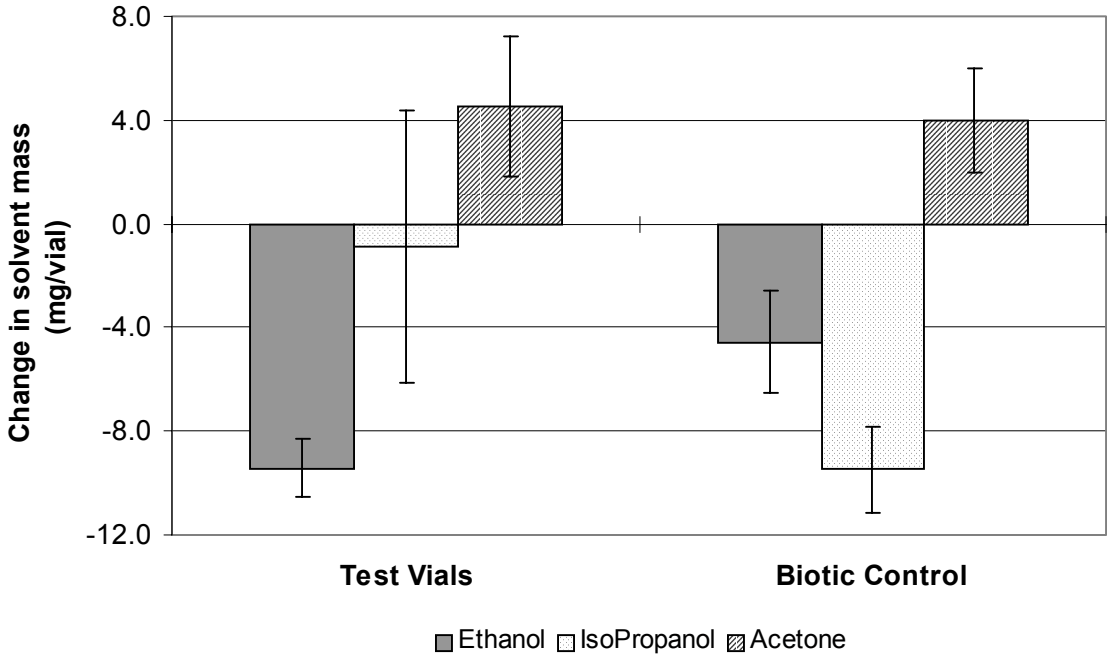


Figure 4.3-14. Ethanol, isopropanol, and acetone fluctuations with MSU1 from 18 to 24 hours

Test vials, biotic controls, and abiotic controls were run in triplicate. Two samples were taken from each vial. Bars above zero indicate solvent production while bars below zero indicate solvent consumption.

4.4 Enrichment Work

After both the MSU1 culture and *C. ljungdahlii* were lost as a result of bacterial contamination, the search for novel organisms capable of converting synthesis gas to ethanol began. Several candidate sources for bacterial isolates capable of fermenting synthesis gas were identified and used to create enrichments. These sources included horse manure, sludges from various sources, and enrichments begun by Dr. Lewis Brown of the Mississippi State University Department of Biological Sciences. These enrichments were continued in hopes of obtaining an isolate capable of fermenting synthesis gas to ethanol. A sample from each source was used to inoculate 20 ml of medium. The combination of the potential bacterial source and medium marked the creation of the first enrichment. APM and Brown media (see Tables 3.1-1 and 3.1-2) used in these enrichments did not contain a carbon and energy source. The headspace of each vial was replaced by CO, which provided the carbon and energy sources for any viable microorganisms within the enrichment. The conditions created in the first enrichment and all subsequent enrichments were intended to select for organisms capable of utilizing CO.

While enrichment techniques were used to enhance the bacterial composition of a potential source to only CO-utilizing organisms, streaking techniques were employed to isolate individual organisms. Cultures were streaked onto plate count agar (PCA) in petri plates or onto Acetate Production Media agar slants (APM agar) in an attempt to isolate organisms capable of utilizing CO. Each colony grown on APM agar or PCA was

thought to stem from one individual cell; therefore, a colony removed from the agar surface was considered at this stage of the experiment to be a pure isolate.

APM agar slants were prepared by combining APM with Difco Granulated Agar. Seven milliliters of APM agar was dispensed into a 40 ml VOA vial with a silicon septum cap and then autoclaved. The vials were removed from the autoclave, the caps tightened, and then the vials placed on their sides in the glove bag as shown in Figure 4.4-1. Once the media solidified, the caps were loosened to allow the media to become anaerobic by equilibrating with the oxygen deficient headspace within the glove bag. Enrichment cultures were streaked onto the APM agar surface. The atmosphere above the APM agar was replaced by CO and the agar was incubated in a stationary incubator at 37ºC.

Once a culture was isolated, ethanol production trials were set to begin; however, these ethanol production trials were delayed after isopropanol was discovered in the enrichment media. Instead, isopropanol was removed from the glove bag and the isolated cultures were transferred into isopropanol-free media. Neither growth nor CO uptake by isolated cultures was observed after isopropanol was removed from the media, indicating that the isolates were not CO-utilizing organisms; therefore, ethanol production trials were not conducted with these organisms.

Throughout the enrichment process, liquid samples were taken from the enrichment vials for ethanol analysis. While ethanol and acetate were not observed in any samples, a peak at 2.5 minutes appeared in most of the liquid samples. In an attempt to characterize this peak, samples with known concentrations of acetone and isopropanol

were analyzed on the FID, since acetone and isopropanol were known products from synthesis gas fermentations (Zeikus, 1980). The elution time for acetone was 2.5 minutes. Isopropanol appeared at the same elution time on the chromatograph as the so-called media peak discussed in the Section 4.3 under the heading "Phase 2 Method Development." It was concluded that the media peak observed in all previous testing was, in fact, the response generated from isopropanol that was absorbed into the media while they were left to gas-equilibrate within the anaerobic glove bag. A possible source of acetone is the anaerobic degradation of isopropanol, since the conversion of isopropanol to acetone has been observed under anaerobic conditions by methanogenic or sulfate reducing bacteria (Fox and Ketha, 1996). After the isopropanol was removed from the glove bag, all chromatographs generated from liquid samples of production media were re-integrated for isopropanol and acetone.

Manure and Sludge Enrichments

Initially, five candidate sources were screened concurrently for ethanol- and acetate-producing bacteria. The candidate sources included horse manure collected at Mississippi State University's School of Veterinary Medicine, secondary sewage sludge from the wastewater treatment facility (Tuscaloosa, Alabama), sludge from anaerobic lagoons at Bryan Foods (West Point, Mississippi) and Farbest Foods (Huntingburg Indiana), and sludge from a methane-producing bioreactor currently under development within the Chemical Engineering laboratories at Mississippi State University. For solid candidate sources, such as horse manure, one gram of the sample source was used to inoculate 20 ml of production media at pH 7.0 (APM). For liquid candidate sources, such

as sludge, three milliliters of the sample source were used to inoculate 20 ml of APM. The resulting first enrichments were incubated in the static incubator in the glove bag at 37ºC. After 10 days of incubation, CO uptake was observed and cell growth was visually observed on the vial walls for each of the first enrichments. The following steps were taken to produce the second enrichments:

1. Three milliliters of the liquid cell suspension were transferred into fresh APM and the enrichment was labeled "Suspended Growth."
2. The remaining cell suspension was poured into a sterile VOA vial.
3. Twenty milliliters of fresh APM were added to the vial with growth on the wall and the enrichment was labeled "Growth on Vial Walls".
4. The headspace of the "Suspended Growth" vials and the "Growth on Vial Walls" vials was replaced with CO and the vials were incubated on the shaker incubator at 37ºC.
5. The third vial was stored at room temperature as a precaution against permanently losing enrichments from each source. Specifically, the first enrichment vials were kept so that if any one of the second enrichment vials were broken, that enrichment could be re-created from the first enrichment.

CO uptake was observed no more than three times in enrichments of the sludge from Tuscaloosa, Alabama, sludge from Farbest Foods, and sludge from the methane producing laboratory bioreactor. Neither CO uptake nor cell growth was observed in the final enrichments from any of these sources. Since no cell growth occurred after the third serial transfer from these enrichments, no further enrichments could be performed. All

three of these sources enrichments were sited as failing to produce a single isolate. Work with these cultures is included in Appendix A.

The cultures derived from horse manure and Bryan Foods sludge were more successful. Figure 4.4-2 shows that six serial enrichments of horse manure were made based on CO uptake from a culture that exhibited cell growth on the vial walls. After six serial enrichments, the horse manure enrichment initiated from cells grown on vial walls was streaked onto APM agar and gassed with 100% CO. The APM agar was incubated upright in the stationary incubator in the glove bag at 37ºC. After 35 days of incubation, a colony was removed from the agar surface and was used to inoculate 20 ml of fresh APM. After the seventh serial enrichment, the horse manure enrichment initiated from cells grown on vial walls was streaked onto plate count agar (PCA). The PCA was incubated in the glove bag incubator. A colony was removed from the surface of the agar and used to inoculate 20 ml of fresh APM. Figure 4.4-2 also shows that only two serial enrichments were made from enrichments initiated with cells from the suspended growth of horse manure enrichments. After the fourth serial enrichment, the culture was streaked onto PCA, and a colony isolated and transferred into fresh APM. After three colonies were isolated as a result of horse manure enrichments, isopropanol was discovered in media that had equilibrated with the glove bag atmosphere.

The cultures were then transferred to isopropanol-free media and gassed with 100% CO. Neither growth nor CO uptake were observed after the transfer, and it was then concluded that the cultures isolated were isopropanol-utilizing cultures and were not capable of utilizing CO. Though CO was utilized by initial enrichments derived directly

from horse manure, Figure 4.4-2 shows that after colonies were isolated from APM agar and PCA, new enrichments were made on the basis of observed growth. Cell growth was discerned visually by an increase in media turbidity.

Since both PCA and APM agar were allowed equilibrate with the anaerobic atmosphere in the glove bag that contained isopropanol, isopropanol was present in both agars. Despite efforts to use media and agars that encouraged growth of CO-utilizing organisms over other organisms, the presence of isopropanol in the agars instead encouraged the isolation of isopropanol-utilizing colonies instead of the targeted CO utilizers.

Figure 4.4-3 illustrates that Bryan Foods sludge enrichments initiated with cells cultured in suspended growth were transferred six times based on measured CO uptake, while the Bryan Foods sludge initiated with cells grown on the vial walls consumed no CO. The sixth enrichment of Bryan Foods sludge was streaked onto APM agar and gassed with 100% CO. After 39 days of incubation in a stationary incubator at 37ºC, a tiny, clear colony was removed from the agar surface and transferred to a vial containing 20 ml of fresh APM. During the 71 days of incubation under a 100% CO headspace, an increase in turbidity was observed, but no CO uptake was measured. After isopropanol was determined to be in the media, the cell growth was determined to be a result of isopropanol utilization by the culture. Therefore, the colony isolated was in fact an isopropanol-degrading culture, not the desired CO-fermenting culture.

Figure 4.4-3 shows that the seventh enrichment of Bryan Foods sludge was streaked onto PCA. After incubation in the glove bag incubator, a colony was removed

from the agar surface and transferred into 20 ml of fresh APM. Despite the lack of CO uptake, an increase in turbidity indicating cell growth was observed. Both the culture isolated from PCA and the culture isolated from APM agar were transferred into isopropanol-free APM where neither growth nor CO uptake were observed. The cultures isolated from Bryan Foods sludge were determined to be isopropanol-utilizing organisms, not CO-utilizing organisms.

Summary of Manure and Sludge Enrichments

In efforts to isolate a CO-utilizing microorganism from potential manure and sludge sources, a total of 60 incubations were performed. Though an organism capable of fermenting CO into ethanol was not isolated from horse manure, Bryan Foods sludge, Farbest Foods sludge, Tuscaloosa sewage sludge, or the methane-producing bioreactor sludge, all sources were identified as containing organisms capable of utilizing CO. A total of 28 enrichments were made based on observed CO uptake. All five cultures isolated from manure or sludge enrichments were determined to be isopropanol-utilizing microorganisms, rather than CO-utilizing organisms.

Brown Enrichments

Concurrently with the performance of this study, efforts were underway in Dr. Lewis Brown's microbiology laboratory at Mississippi State University to find microorganisms capable of converting synthesis gas into ethanol. Twelve enrichments begun in Dr. Brown's laboratories were brought into the chemical engineering laboratories where further enrichments were performed in an effort to find an isolate capable of fermenting synthesis gas to ethanol. The twelve enrichments created by Dr.

Brown included six enrichment cultures from oil well cuttings (OWC1-6), four enrichment cultures from hog waste (HW1-4), and two enrichment cultures from sewage sludge (SS1-2). The enrichments from the same source differed only by the media formulation employed in their creation. Table 4.4-1 outlines the sources and media formulations used to create these enrichments.

All work with the enrichments begun by Dr. Brown performed in chemical engineering laboratories was conducted in a liquid medium at pH 7.0 called Brown Media, because it was formulated by Dr. Brown for use with these cultures. One milliliter of inoculum from each of the 12 enrichments was transferred into each of two vials of fresh Brown Media and each vial was gassed with 100% CO. Because some organisms prefer growth under static conditional and some prefer growth under shaking conditions, one vial of each enrichment culture was incubated in a stationary incubator at 37ºC while the other was incubated in the shaker incubator at 37ºC to provide agitation. Neither growth nor CO uptake was observed in the stationary incubations of vials containing HW3, OWC5, OWC3, or SS1. While CO uptake was observed in all shaking cultures, only the most prolific enrichments, which were OWC4, HW1, HW4, and SS2, are discussed in detail here. Work performed with all other enrichments started by Dr. Brown is included in Appendix B.

Of the enrichments started by Dr. Brown, OWC4 proved to be the most active culture incubated on the shaker incubator. Figure 4.4-4 illustrates that six serial enrichments were performed from the OWC4 enrichment, of which only 2 transfers were made based on measured CO uptake. The remaining enrichments outlined in Figure 4.4-

4 were made based on an observed increase in turbidity indicating cell growth. Five or fewer serial enrichments were made from each of the other enrichments initiated in Dr. Brown's laboratory; therefore, OWC4 was determined to be the culture best suited to growth on low concentrations of isopropanol. Since the goal of these enrichments was to find a CO-utilizing isolate, no further work was conducted with this culture.

CO uptake was observed in both the shaking and static incubations of HW1. Figure 4.4-5 illustrates that two serial transfers of HW1 incubated in the shaker incubator were made based on observed CO uptake while only one transfer was made based on CO uptake by HW1 incubated in the static incubator. While no CO uptake by the third enrichment of HW1 was observed after 26 days of incubation in the shaker incubator, an increase in turbidity indicating cell growth was observed. The enrichment was stained by Gram staining and the culture was viewed via microscopy. The enrichment was composed entirely of Gram-positive ovals, suggesting that the third enrichment of HW1 incubated on the shaker incubator was a pure culture. The third enrichment of HW1 incubated on the shaker incubator was streaked into PCA and incubated in the glove bag incubator for 8 days. After 8 days, a translucent-white colony that grew on the agar surface was transferred into fresh Brown Medium formulated for rapid cell growth by the addition of 5 g/l fructose. Though the cells grown in the fructose-rich Brown Medium were intended for use in ethanol production trials, the discovery that isopropanol was dissolving in media in the glove bag halted performance of any ethanol production experiments until it could be determined that the cultures were still capable of fermenting CO. After the HW1 incubated on the shaker incubator was transferred into isopropanol-

free media, neither growth nor CO uptake were observed. For this reason, it was concluded that the culture isolated from HW1 incubated on the shaker incubator was an isopropanol-utilizing organism and not a CO-utilizing organism.

After nearly 2 months of incubation, CO uptake by HW1 in the static incubator was observed and the following actions were taken:

1. The first enrichment was transferred into fresh Brown Medium creating a second enrichment. Neither growth nor CO uptake were observed after this transfer so further enrichments were not conducted.
2. The first enrichment was Gram stained. When viewed via microscopy, the cells of HW1 grown in the static incubator were thin, twisted cells that looked like whisps of hair, Gram-positive rods suggesting the possibility that HW1 was a pure culture. This hypothesis was proved incorrect when, as outlined below, two distinct colony morphologies were observed growing on PCA.
3. The enrichment was streaked onto PCA and incubated in the glove bag incubator. After 8 days of incubation, two distinct types of colonies were observed growing on the agar surface. Figure 4.4-6 provides an illustration of both colony morphologies. The clear, branching colony was labeled Colony One and the round, yellow colony was labeled Colony Two. Each colony was transferred into fresh Brown Medium with 5g/l fructose. Concerns surrounding isopropanol in the media prevented any further work with these isolated cultures.

Figure 4.4-7 shows that three serial transfers of HW4 incubated in the shaker incubator were made based on observed CO uptake. After CO uptake was observed in the third enrichment of HW4 incubated on the shaker incubator, the culture was streaked onto PCA and incubated for 7 days in the stationary incubator in the glove bag before colony growth was observed. A colony was removed from the agar surface and transferred into fresh Brown Medium that was formulated for rapid growth with the addition of 5 g/l fructose. Growth was observed shortly after the colony was transferred into the fructose-rich medium and the culture was stained using the Gram stain in an effort to determine culture purity and characterize the isolate. When viewed via microscopy, the culture was defined by short, squiggle-shaped, Gram-positive organisms. This culture was set aside pending the resolution of isopropanol issues. However, when it was determined that the isolate was not capable of utilizing CO, no further work was conducted with this culture.

Figure 4.4-7 further illustrates that HW4 incubated in the static incubator utilized CO after 72 days of incubation. The culture was transferred into fresh Brown Medium where neither growth nor CO uptake was observed in the second enrichment and as a result, no further work with this enrichment was performed.

Initially, each culture was streaked on to APM agar, gassed with 100% CO, and then incubated in an upright position in static vials within an incubator at 37ºC. Colony growth was observed only on the APM agar streaked with SS2. Figure 4.4-8 shows that after over 3 months of incubation, colony growth was observed on the surface of the APM agar and a colony was transferred into fresh APM. SS2 transferred from APM agar

was incubated in the shaker. Despite the lack of CO uptake by the isolate, an increase in turbidity indicating cell growth was observed after 71 days of incubation. The culture was then transferred into isopropanol-free, fresh APM where neither CO uptake nor cell growth was observed. Because of the lack of observable activity by the SS2 APM agar isolate, it was concluded that this culture was an isopropanol-utilizing organism, not a CO-fermenting bacteria.

Summary of Brown Enrichments

A total of 90 individual enrichments were created in efforts to isolate a novel microorganism capable of fermenting synthesis gas into ethanol from enrichments started by Dr. Lewis Brown at Mississippi State University. Though an organism capable of fermenting CO into ethanol was not isolated as a result of these efforts, all sources were identified as containing organisms capable of utilizing CO. A total of 30 enrichments were made based on observed CO uptake and five cultures were isolated from Brown enrichments. Unfortunately, all five cultures isolated were determined to be isopropanol-utilizing microorganisms rather than CO-utilizing organisms. Additional enrichments beginning with the original enrichments from Dr. Brown in isopropanol-free Brown Media are hypothesized to ultimately yield a CO-utilizing isolate.

Table 4.4-1. Sources and media formulations for enrichments obtained from Dr. Lewis Brown

Source	Enrichment Name	Media
Oil Well Cuttings	OWC1	Marine Broth with Fructose
	OWC2	Marine Broth
	OWC3	Ethanol Media with Brilliant Green and Fructose
	OWC4	Ethanol Media with Brilliant Green
	OWC5	Ethanol Media without Vitamins and Trace Minerals with Rila Salts and Fructose
	OWC6	Ethanol Media without Vitamins and Trace Minerals with Rila Salts
Hog Waste	HW1	Marine Broth with Fructose
	HW2	Marine Broth
	HW3	Ethanol Media without Vitamins and Trace Minerals with Rila Salts and Fructose
	HW4	Ethanol Media without Vitamins and Trace Minerals with Rila Salts
Sewage Sludge	SS1	Ethanol Media without Vitamins with Fructose
	SS2	Ethanol Media without Vitamins

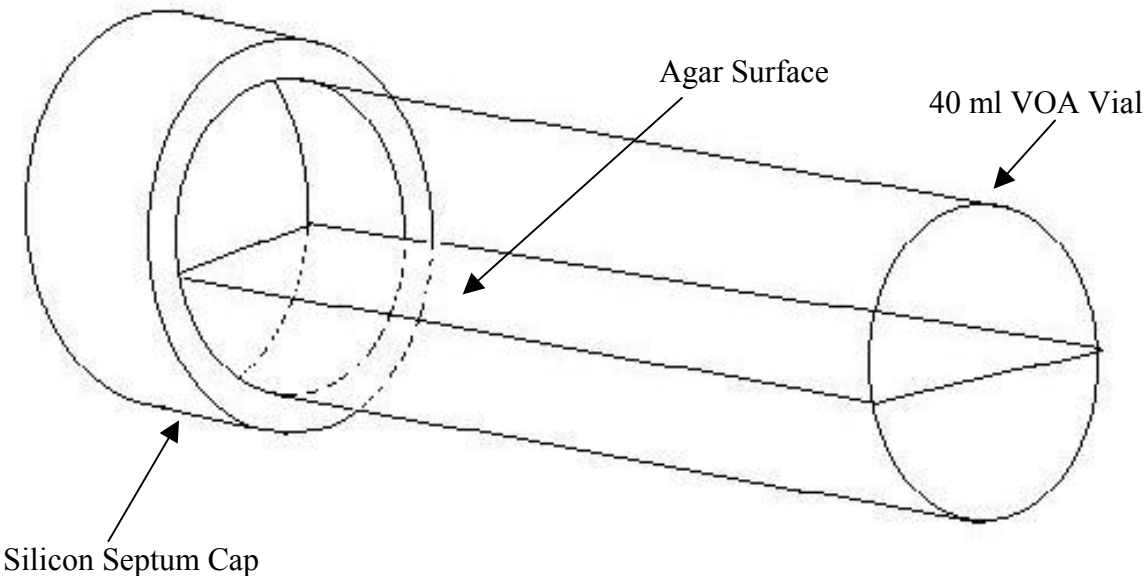


Figure 4.4-1. Acetate Production Medium Agar slant (APM agar) prepared in 40 ml VOA vial with a silicon septum cap

Culture was streaked onto the agar surface and gassed with 100% CO. The system was then incubated in the upright position.

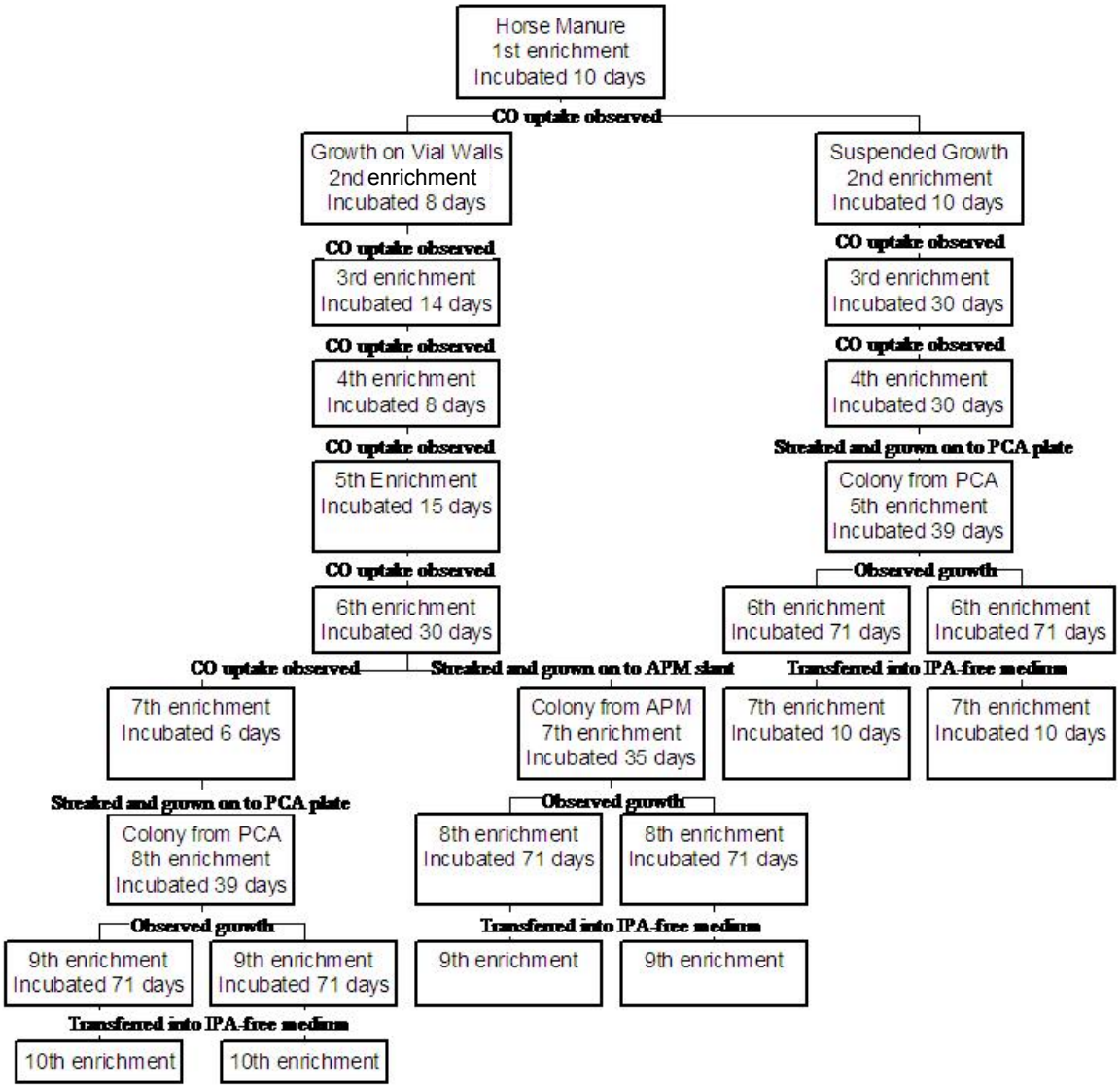


Figure 4.4-2. Enrichments of horse manure

All enrichments were gassed with 100% CO.

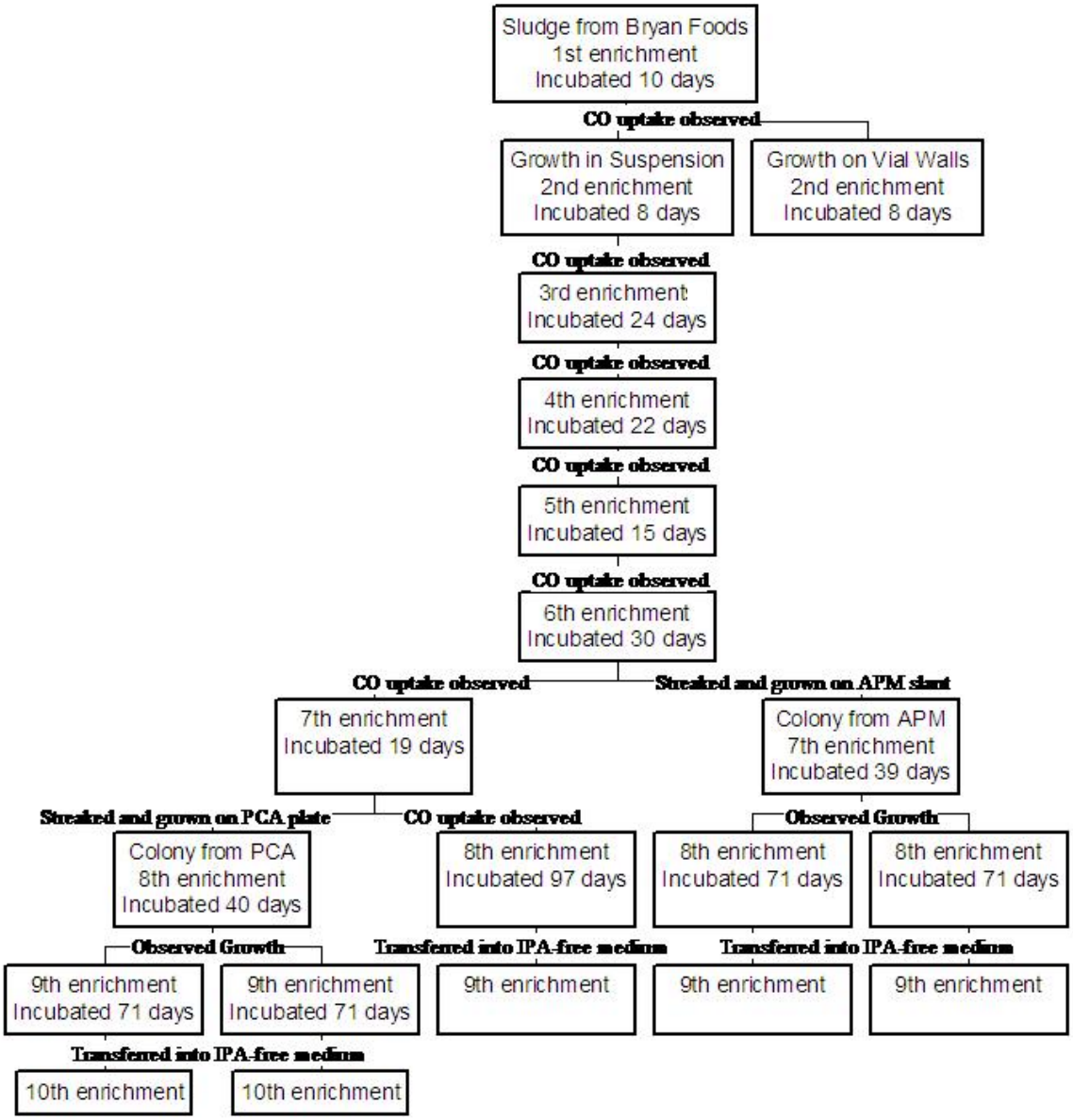


Figure 4.4-3. Enrichments of Bryan Foods sludge

All enrichments were gassed with 100% CO.

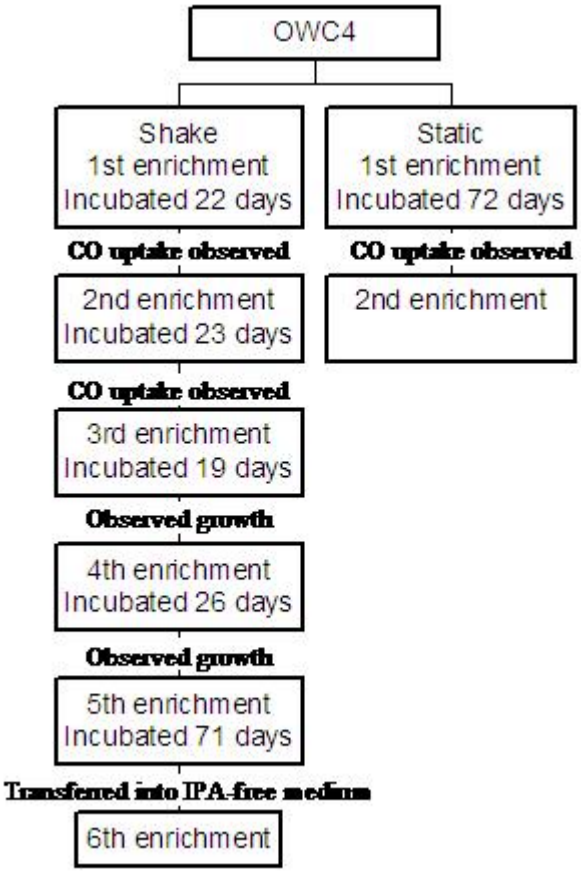


Figure 4.4-4. Enrichments of OWC4

All enrichments were gassed with 100% CO.

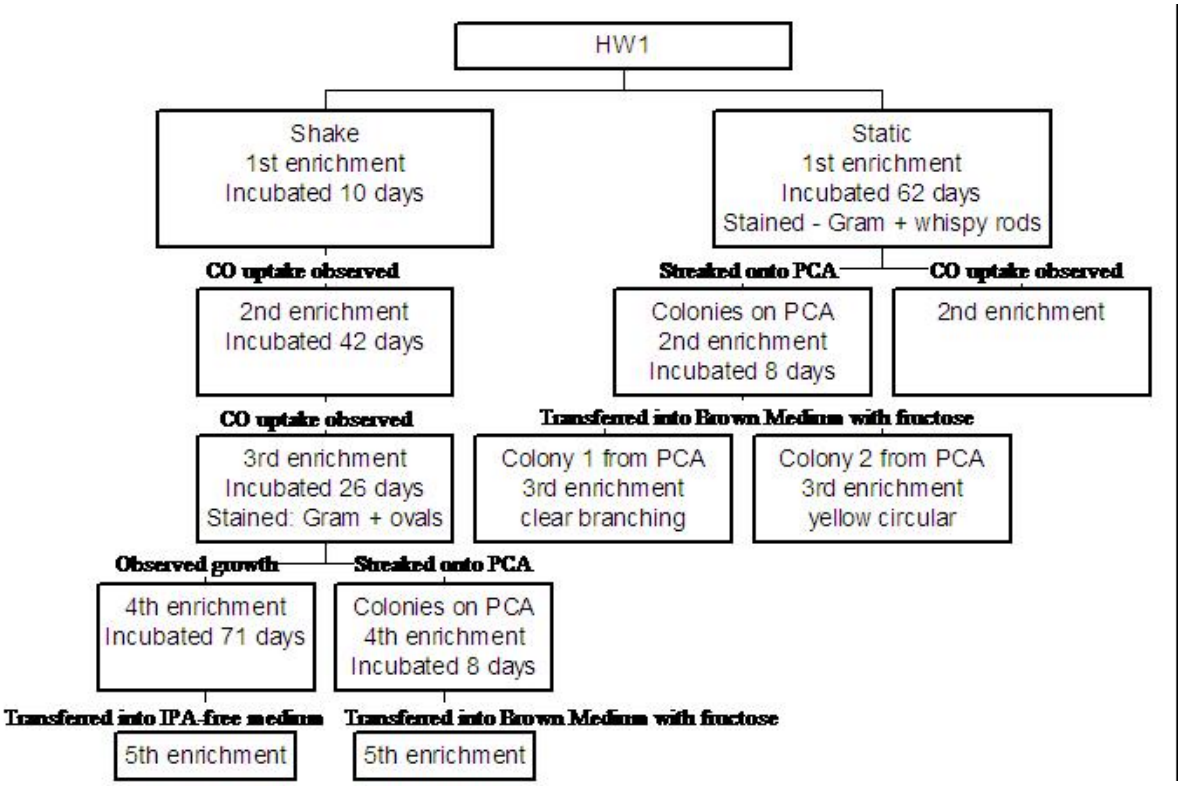
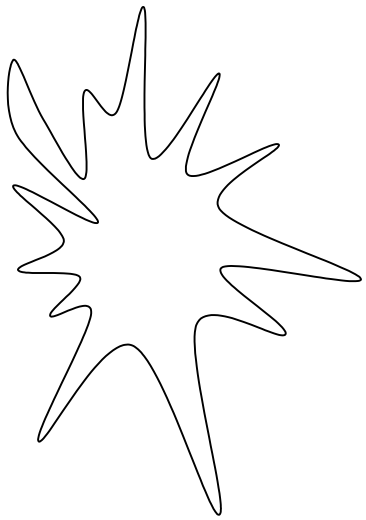
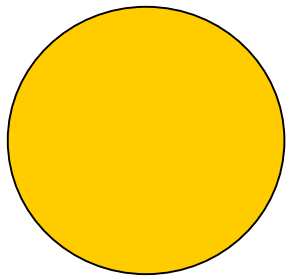


Figure 4.4-5. Enrichments of HW1

All enrichments were gassed with 100% CO.



Top View
Colony 1



Top View

Side View

Colony2

Figure 4.4-6. Colony morphologies observed when streaking HW1 incubated in the static incubator.

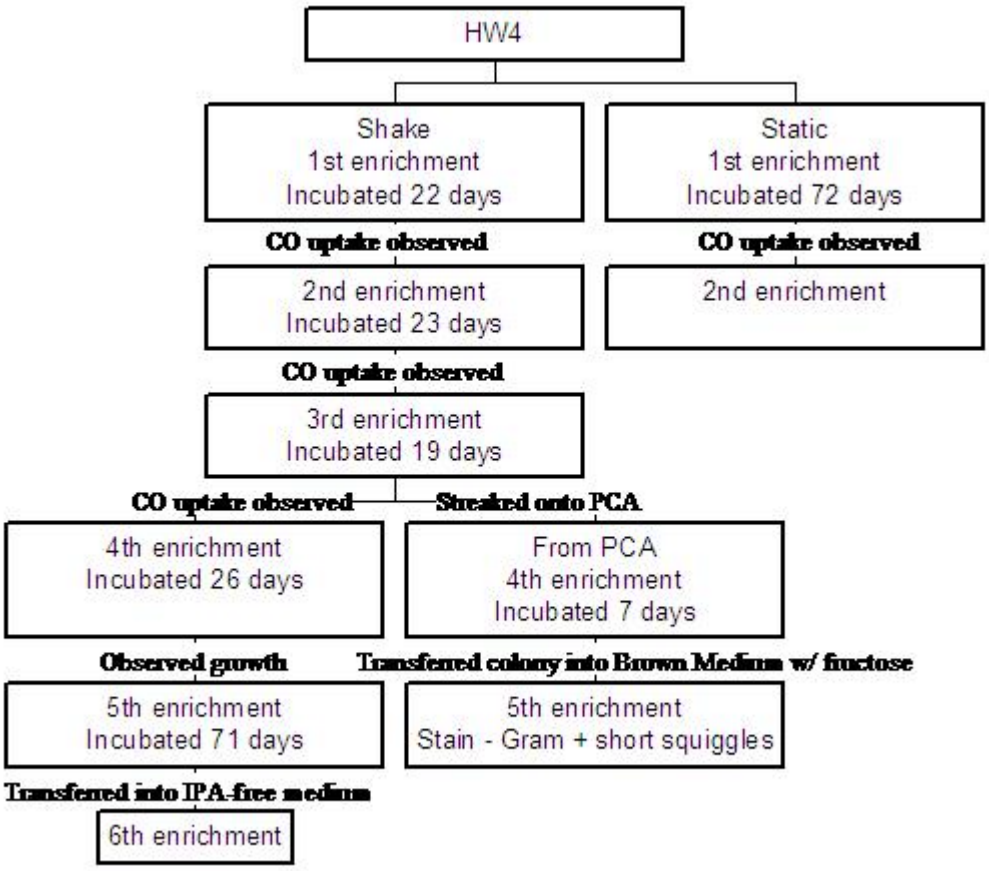


Figure 4.4-7. Enrichments of HW4

All enrichments were gassed with 100% CO.

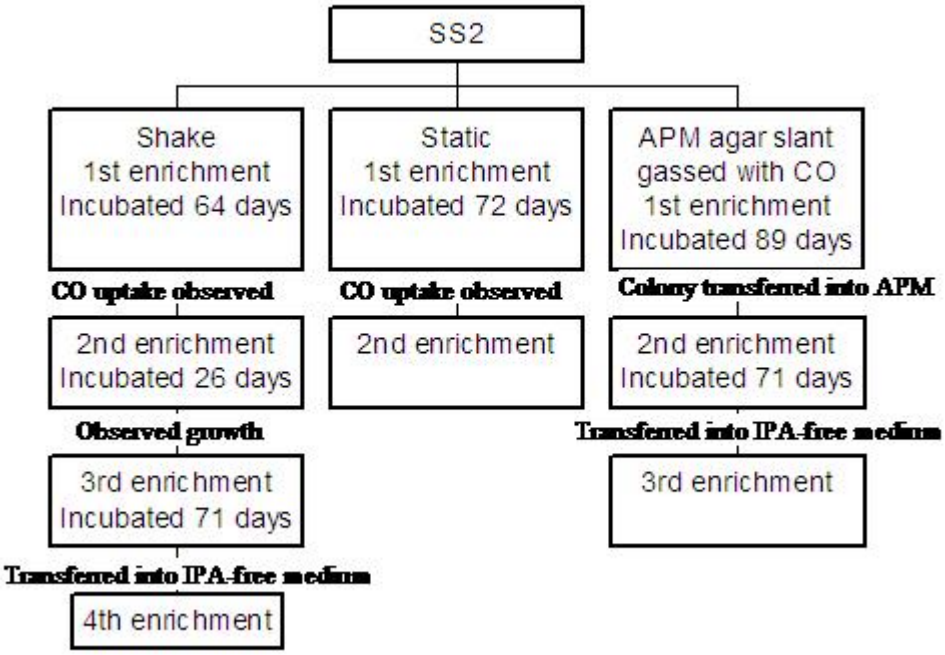


Figure 4.4-8. Enrichments of SS2

All enrichments were gassed with 100% CO

4.5 Media Optimization

After isopropanol was discovered in the media that had been equilibrated within the anaerobic glove bag, concerns arose regarding the use of Ethanol Production Medium (EPM) and Acetate Production Medium (APM) throughout the course of previous ethanol production trials and the enrichment work. EPM was the medium used in ethanol production trials while APM was used for enrichment studies. Although media pH was measured and adjusted prior to dispensing it into vials, neither media pH nor media redox potential were tested after the media was autoclaved and allowed to become anaerobic. Whether the EPM used in ethanol production trials maintained the correct pH or a low enough redox potential (less than -200 mV) during the experiments was not known. Additionally, a new formulation for production medium was found in US Patent # 5,807,722 (Gaddy, 1998). Before work with this new formulation began, both the stability of system pH and the media redox potential under a nitrogen atmosphere and a CO atmosphere needed to be tested. The new production medium formulation, outlined in Table 4.5-1, contained yeast extract, trypticase, and L-cysteine, and was referred to as Modified EPM or Modified APM depending on the production medium pH.

Three formulations each of EPM and APM and four formulations each of Modified EPM and Modified APM were tested for pH and redox potential stability. Klasson et al. (1992b), recommended including reducing agents in the production media formulation so 10 ml/l ATCC Reducing Agent (see Table 4.5-2) or 0.4 g/l L-cysteine were added to EPM and APM. The concentrations of ATCC Reducing Agent or L-

cysteine added to the production media were the same as those used in the ATCC recommended growth media for *C. ljungdahlii* (ATCC, 2004).

Four formulations each of Modified EPM and Modified APM were tested:

- Modified EPM and Modified APM with no additives
- Modified EPM and Modified APM with 10 ml/l ATCC reducing agent (resulting in media containing 0.9 g/l L-cysteine and 0.4 g/l Na_2S)
- Modified EPM and Modified APM plus 0.4 g/l Na_2S
- Modified EPM and Modified APM plus 0.1 g/l L-cysteine

Two vials of each medium formulation were adjusted to pH 4.5 and two vials of each medium were adjusted to pH 7.0. The vials were capped with silicon septum caps, autoclaved, and allowed to cool and become anaerobic in the anaerobic glove bag. One vial of each medium formulation at each pH was gassed with 100% CO while the other vial was left with the glove bag headspace composition of 95% N_2 and 5% H_2. After the vials were incubated in the shaker incubator at 37ºC for 24 hours, each vial was opened in the glove bag and the pH and redox potentials were measured.

Figure 4.5-1 shows that EPM plus ATCC Reducing Agent under the nitrogen headspace, and Modified EPM plus ATCC Reducing Agent under the nitrogen headspace, had the most stable pH after autoclaving and abiotic incubation. For the vials incubated under a CO headspace, modified EPM with 0.4 g/l Na_2S had the final pH nearest to 4.5 (pH 4.53) after autoclaving and incubation. The EPM formulation used in all ethanol production experiments (EPM with no additives) had a pH of 4.95 when incubated under a CO headspace, and a pH of 4.93 when incubated under a nitrogen

headspace. Therefore, the EPM used during ethanol production trials may not have been at the optimal pH for ethanol production.

While most formulations of EPM were closer to pH 5.0 than the intended pH 4.5, Figure 4.5-2 shows that the pH of APM formulations were much closer to the intended pH of 7.0, indicating that the pH of all formulations of APM remained relatively stable after autoclaving and incubating. Thus, any of these media formulations can be expected to maintain a stable pH.

The redox potential is an indication of whether or not a medium has the available electrons needed by anaerobic organisms to grow and produce ethanol. A highly negative redox potential, –200 mV or lower, is desired for work with anaerobic organisms (Vega et al., 1989b). Figure 4.5-3 shows that all media formulations initially at pH 4.5 used in this experiment have negative redox potentials of –45 mV or lower, with most between –150 and –250 mV. The EPM formulation used in all ethanol production trials (EPM with no additives) had a final redox potential of –45 mV after autoclaving and abiotic incubation under a 100% CO headspace, which is less than ideal for ethanol production from synthesis gas.

The range of observed redox potentials was much broader with production media formulations initially at pH 7.0 (See Figure 4.5-4). After autoclaving and incubation, the redox potential of the production media at pH 7.0 used for all enrichments was positive regardless of headspace composition. However, the inclusion of ATCC Reducing Agent in the APM formulation caused the redox potential to fall below –200 mV, while all other

tested formulations of APM and modified APM had redox potentials less than –300 mV regardless of headspace composition.

Data Presentation and Discussion

For synthesis gas fermentations, CO and CO_2 should be the only bioavailable carbon sources in ethanol production vials. Since the modified versions of the production medium contained yeast extract, a potential carbon source, Modified EPM was determined to be an inappropriate medium for ethanol production. The pH and the redox potentials of EPM with ATCC Reducing Agent under a CO headspace, and EPM with 0.4 g/l L-cysteine under a CO headspace, were nearly equal. Further ethanol production trials are needed to determine which EPM additive results in the most ethanol production.

Since all formulations of APM and modified APM except APM with no additives and APM with ATCC Reducing Agent had equally low redox potentials and equally stable pHs, any of these formulations will be ideal for use as enrichment media.

Table 4.5-1. Modified Production Medium

Medium Component	Amount (per 1.0 L)
Salt solution[1]	80 mL
Yeast extract	1.0 g
Trypticase	1.0 g
Pfenning trace metal solution[2]	3.0 mL
B-vitamins solution[3]	10.0 mL
Cysteine HCl	0.5 g
$CaCl_2 \cdot 2\ H_2O$	0.06 g
$NaHCO_3$	2.0 g
Distilled Water	920.0 mL

1. See Table 4.5-1A
2. See Table 4.5-1B
3. See Table 4.5-1C

Table 4.5-1A. Salt Solution

Medium Component	Amount
KH_2PO_4	3.00 g
K_2HPO_4	3.00 g
$(NH_4)_2SO_4$	6.00 g
NaCl	6.00 g
$MgSO_4 \cdot 7\ H_2O$	1.25 g
Distilled Water	1000 mL

Table 4.5-1B. Trace Metal Solution

Medium Component	Amount
$FeCl_2 \cdot 4\ H_2O$	1500 mg
$ZnSO_4 \cdot 7\ H_2O$	100 mg
$MnCl_2 \cdot 4\ H_2O$	30 mg
H_3BO_3	300 mg
$CoCl_2 \cdot 6\ H_2O$	200 mg
$CuCl_2 \cdot H_2O$	10 mg
$NiCl_2 \cdot 6\ H_2O$	20 mg
$NaMoO_4 \cdot 2\ H_2O$	30 mg
Na_2SeO_3	10 mg
Distilled Water	1000 mL

Table 4.5-1C. B-Vitamin Solution

Medium Component	Amount
Pyridoxal HCl	10 mg
Riboflavin	50 mg
Thiamine HCl	50 mg
Nicotinic acid	50 mg
Ca-D-Pantothenate	50 mg
Lipoic acid	60 mg
P-Aminobenzoic acid	50 mg
Folic acid	20 mg
Biotin	20 mg
Vitamin B_{12}	50 mg
Distilled Water	1000 mL

Table 4.5-2. ATCC Reducing Agent

NaOH	0.9 g
L-Cysteine HCl	4.0 g
$Na_2S \cdot 9\ H_2O$	4.0 g
Distilled water	0.1 L

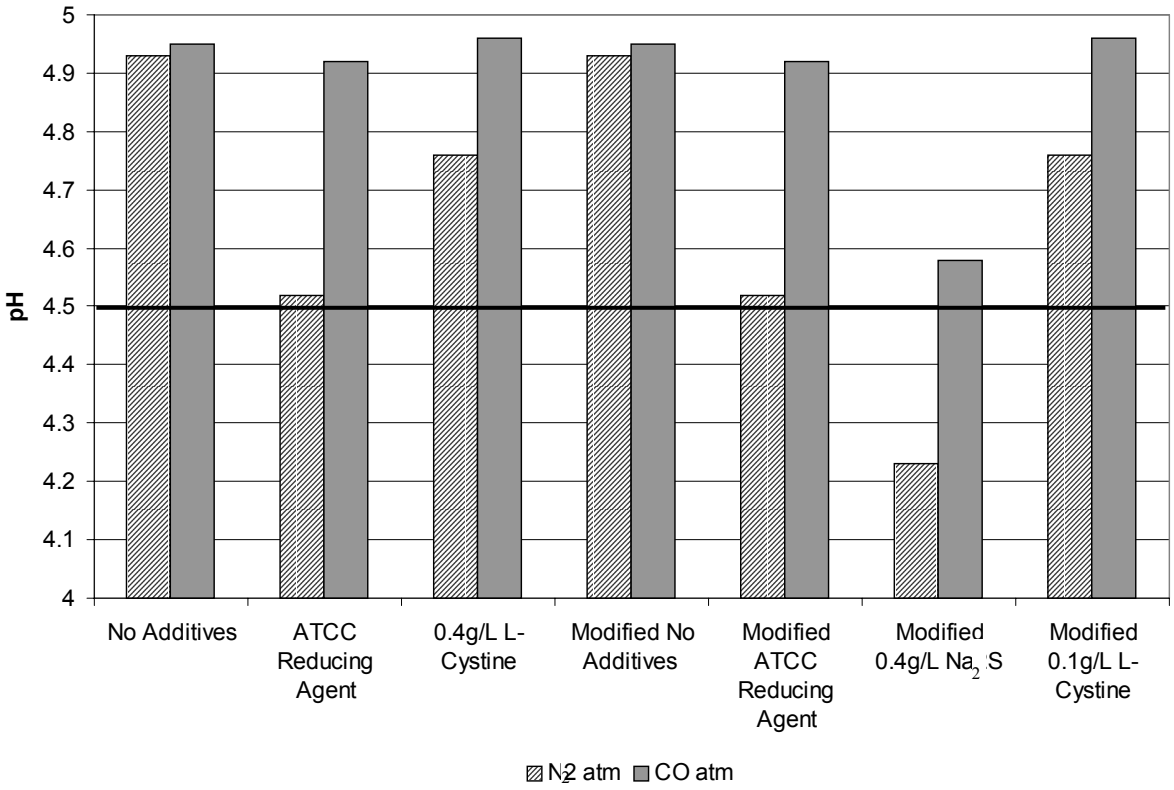


Figure 4.5-1. pH of EPM and Modified EPM formulations initially at pH 4.5 after autoclaving and incubating under either a 95% N_2 and 5% H_2 headspace or a 100% CO headspace for 24 hours in the shaker incubator.

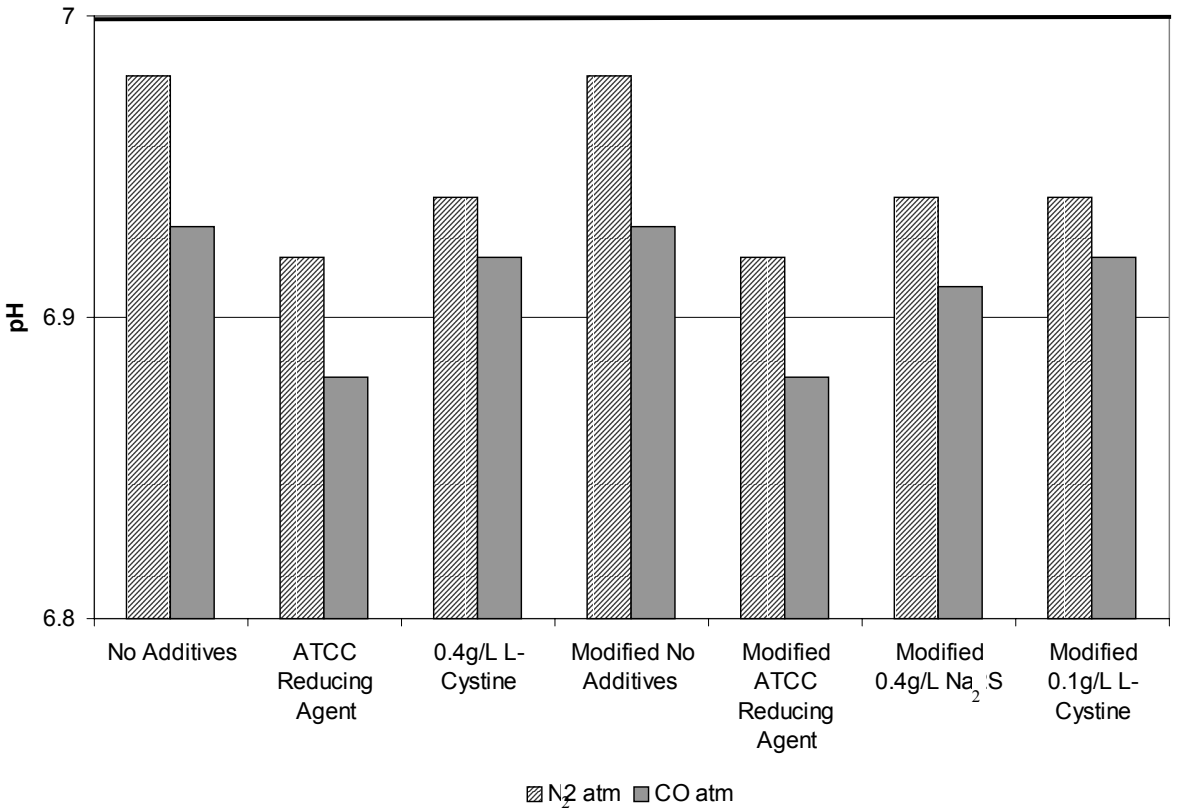


Figure 4.5-2. pH of APM and Modified APM formulations initially at pH 7.0 after autoclaving and incubating under either a 95% N_2 and 5% H_2 headspace or a 100% CO headspace for 24 hours in the shaker incubator.

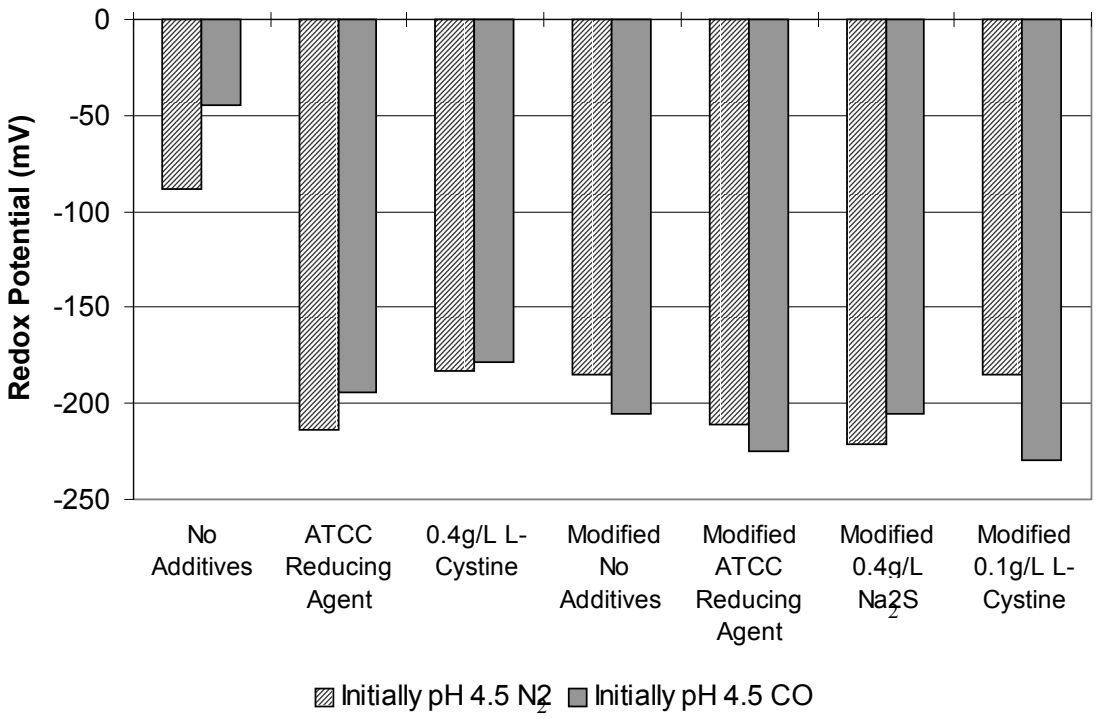


Figure 4.5-3. Redox Potential of EPM and Modified EPM formulations after autoclaving and incubating under either a 95% N_2 and 5% H_2 headspace or a 100% CO headspace for 24 hours in the shaker incubator.

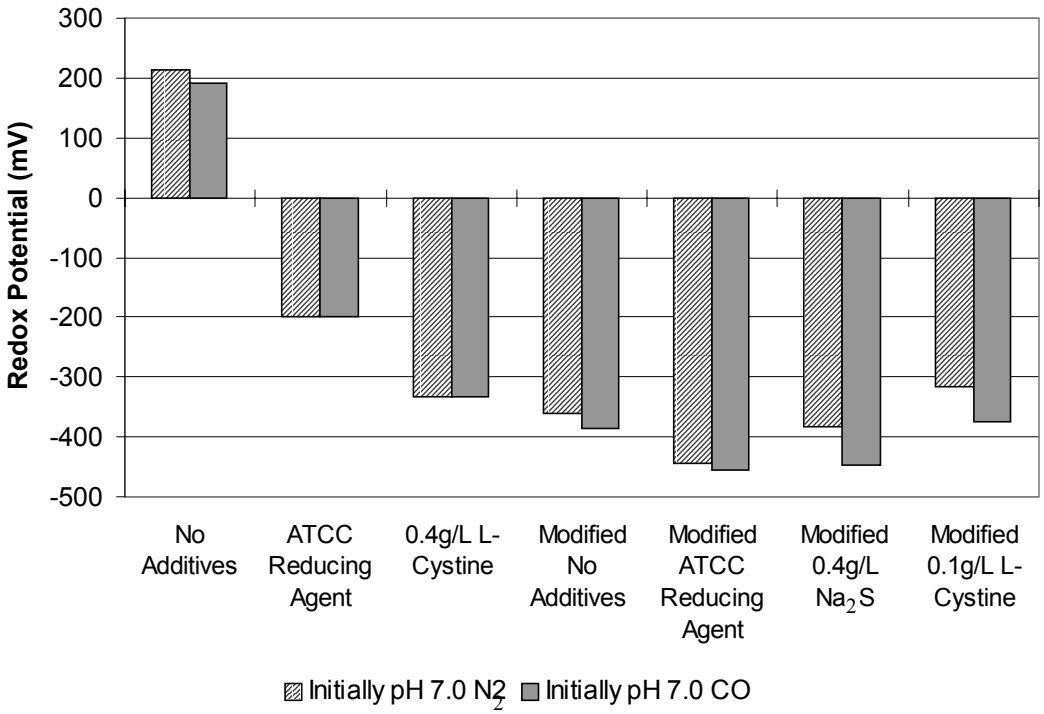


Figure 4.5-4. Redox Potential of APM and Modified APM formulations after autoclaving and incubating under either a 95% N_2 and 5% H_2 headspace or a 100% CO headspace for 24 hours in the shaker incubator.

CHAPTER V

CONCLUSIONS AND ENGINEERING SIGNIFICANCE

5.1 CONCLUSIONS

The following conclusions are drawn from the performance of this research effort:

- *Clostridium ljungdahlii* demonstrated its highest growth rate on PETC 1754 with 5 g/l fructose, 1 g/l yeast extract, and 1 g/l peptone. Since the growth rate per millimole of fructose increased with the nutrient concentration in the medium, PETC 1754 with 5 g/l fructose, 1 g/l yeast extract, and 1 g/l peptone is recommended for rapid growth of *C. ljungdahlii*.

- Mississippi State University Mesophilic Culture 1 (MSU1) grew at a much slower rate than *C. ljungdahlii* in medium with equivalent fructose and yeast extract concentrations. This result was expected since much work has been conducted to optimize PETC 1754 for *C. ljungdahlii* while a growth medium has not yet been optimized for MSU1.

- In ethanol production trials of MSU1 and *C. ljungdahlii* where equal concentrations of ethanol were produced in both test vials and biotic controls, ethanol production was a result of fermentation of fructose carried over from the growth medium.

- While MSU1 produced nearly three times more ethanol from synthesis gas than *C. ljungdahlii*, ethanol concentrations observed with both cultures were an order of magnitude lower than those reported in literature for *C. ljungdahlii*.

- Bacterial contamination of both MSU1 and *C. ljungdahlii* precipitated the loss of each culture despite the use of ethanol and isopropanol as sterilizing agents. Cultures less prone to succumbing to biological competition must be developed along with improved sterilization techniques.

- In the absence of synthesis gas, both MSU1 and *C. ljungdahlii* consumed isopropanol.

- In an effort to implement standard microbiological sterilization techniques, ethanol or isopropanol used in the glove bag as sterilizing agents contaminated all media used during ethanol production trials and nearly all media used to perform enrichments.

- All cultures isolated as a result of enrichment studies were isopropanol-utilizing cultures that were not capable of utilizing CO as a carbon and energy source.

- Ethanol Production Medium (EPM) with 10 ml/l ATCC reducing agent was equally optimized for ethanol production from synthesis gas as EPM with 0.4 g/l L-cysteine based on pH and redox potential after incubation under a CO

headspace. Due to simplicity, EPM with 0.4 ml of L-cysteine is recommended for future ethanol production trials. Any formulation of Acetate Production Medium (APM) containing levels of ATCC reducing agent, L-cysteine, or Na_2S tested during this research is recommended for use enrichment work.

5.2 Engineering Significance

Less than 1% of all microorganisms have been catalogued to date (Prescot, 2001). Microbiologists can isolate and identify every microbial culture on the planet but without the incorporation of these organisms into viable processes, all of this work would be for naught. Microorganism, both discovered and undiscovered, hold untapped catalytic abilities. A microbial catalyst holds an untold number of enzymatic pathways capable of conducting complicated chemical reactions at low temperatures and pressures. For this reason, microorganisms are an important catalytic resource.

This study clearly shows the ability of a microbial catalyst to resist chemical poisoning by isopropanol. Both *C. ljungdahlii* and MSU1 were even seen to adapt to isopropanol contamination in the media. Though both cultures preferentially utilized carbon monoxide, MSU1 and *C. ljungdahlii* also utilized the contaminating isopropanol as a carbon and energy source. Both cultures continued to produce low levels of ethanol despite the contamination.

The most powerful message this study sends is the need for further development of sterilization techniques for microbiological systems intended to utilize only single-carbon substrates. On the bench scale, a means of sterilizing caps before sampling must

be developed. The eventual loss of both MSU1 and *C. ljungdahlii* to bacterial contaminants further illustrates this need. Clearly, this process is not ready for scale up to the pilot scale.

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APPENDIX A

MANURE AND SLUDGE ENRICHMENTS

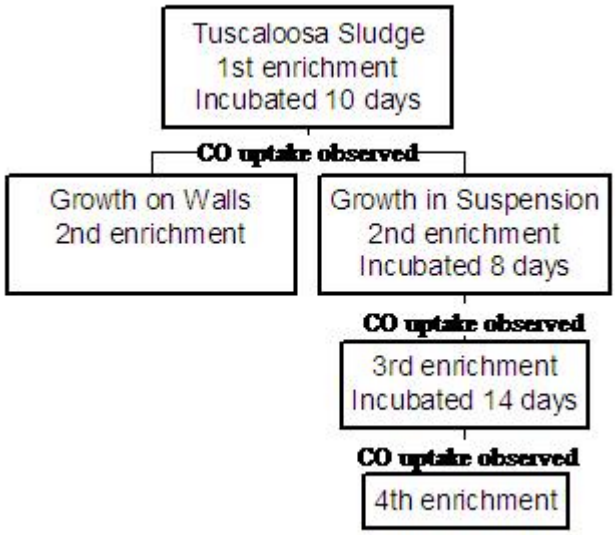

Figure A-1. Enrichments of sludge from the wastewater treatment facility in Tuscaloosa, Alabama

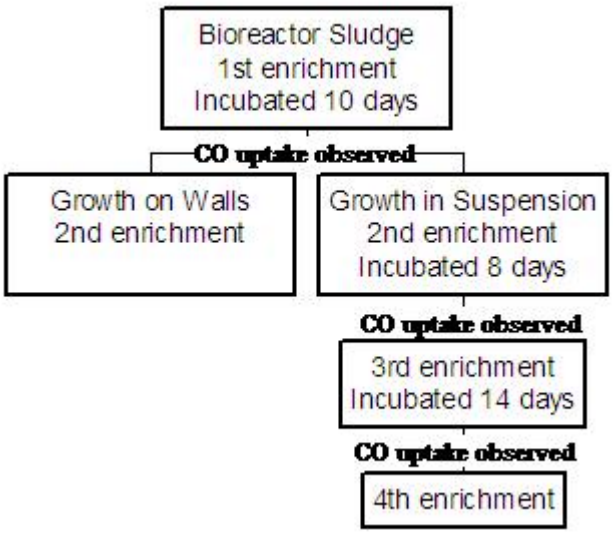

Figure A-2. Enrichments of sludge from a methane-producing bioreactor

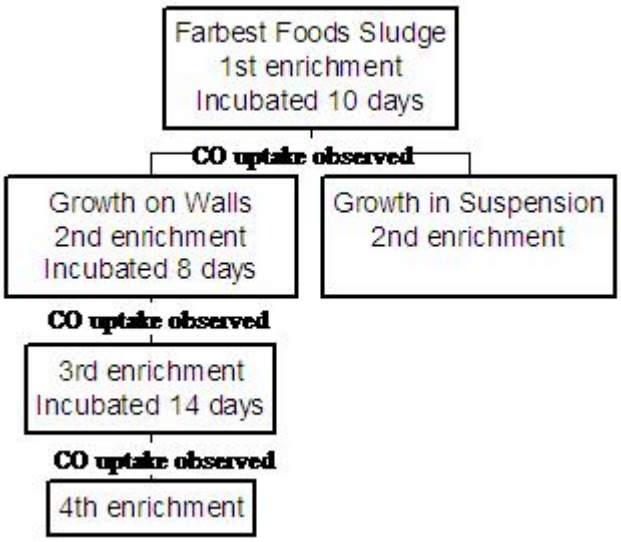

Figure A-3. Enrichments of sludge from Farbest Farms

FIGURE B

BROWN ENRICHMENTS

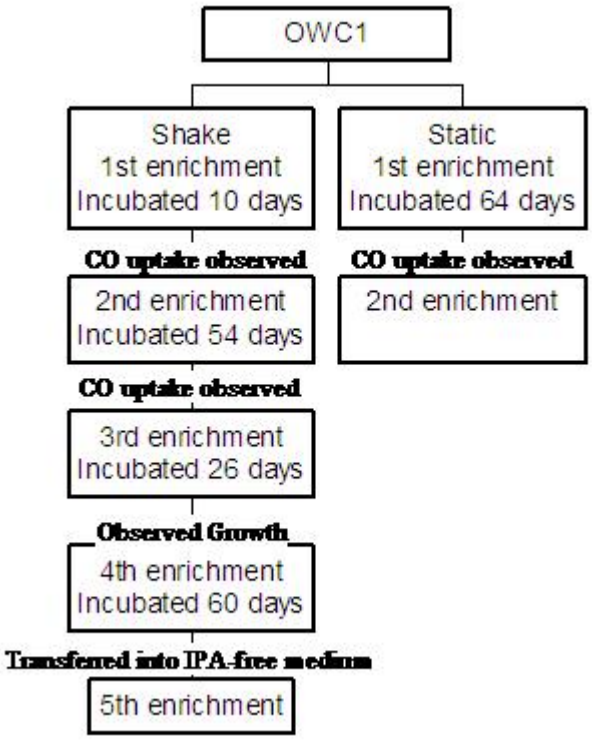

Figure B-1. Enrichments from OWC1

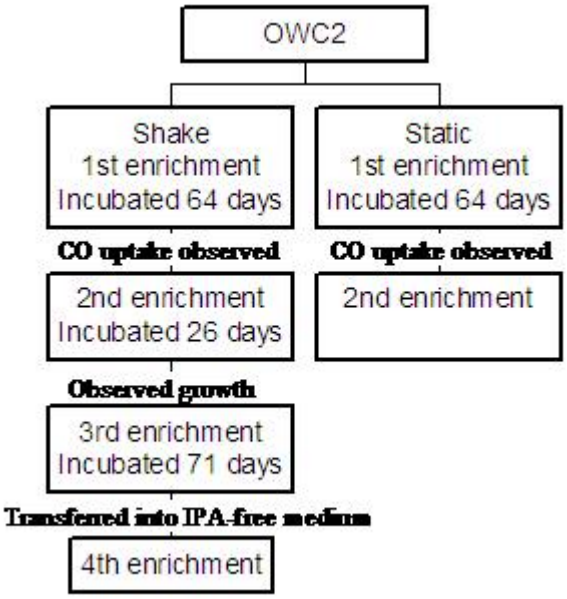

Figure B-2. Enrichments from OWC2

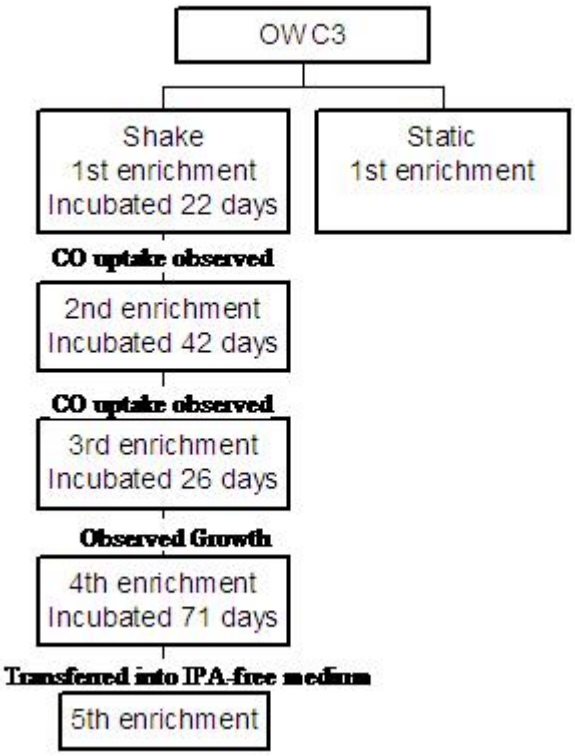

Figure B-3. Enrichments from OWC3

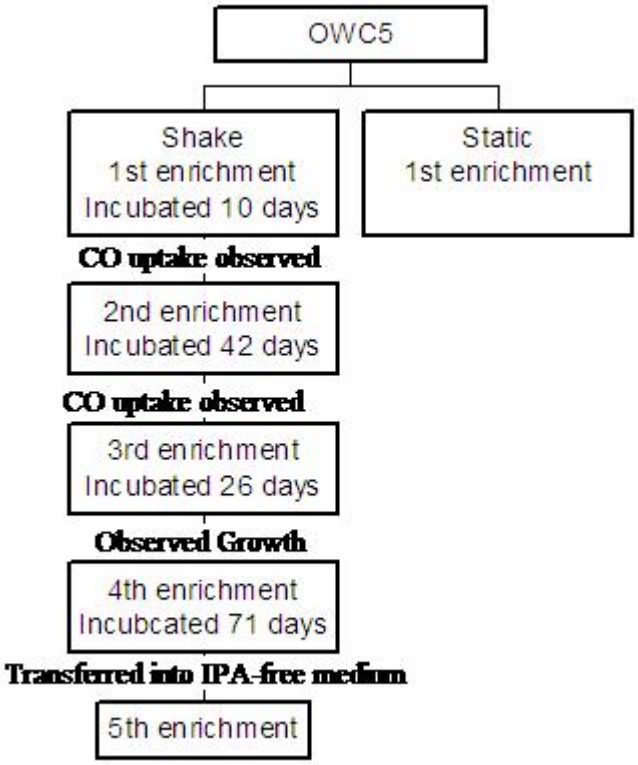

Figure B-4. Enrichments from OWC5

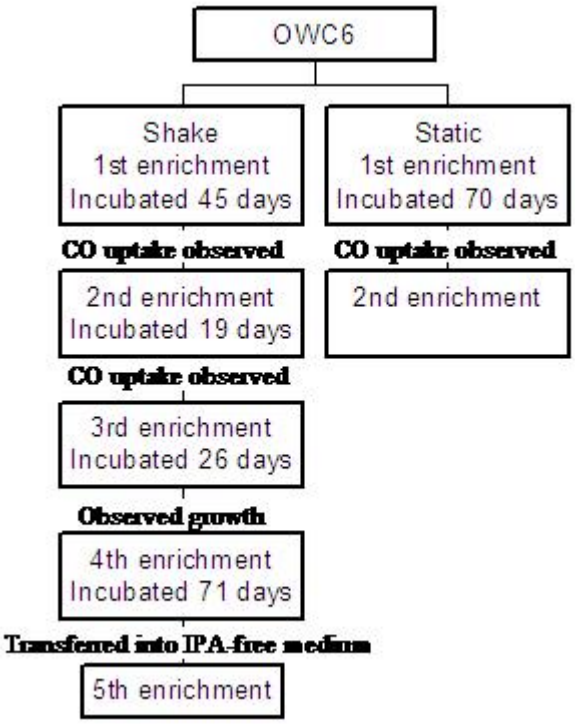

Figure B-5. Enrichments from OWC6

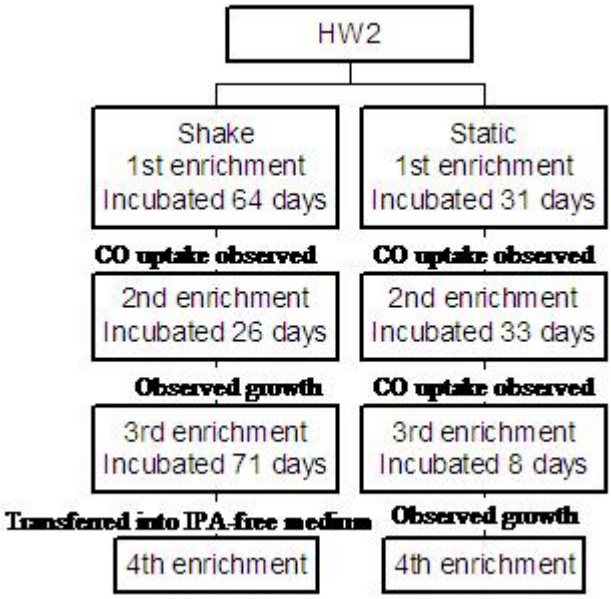

Figure B-6. Enrichments from HW2

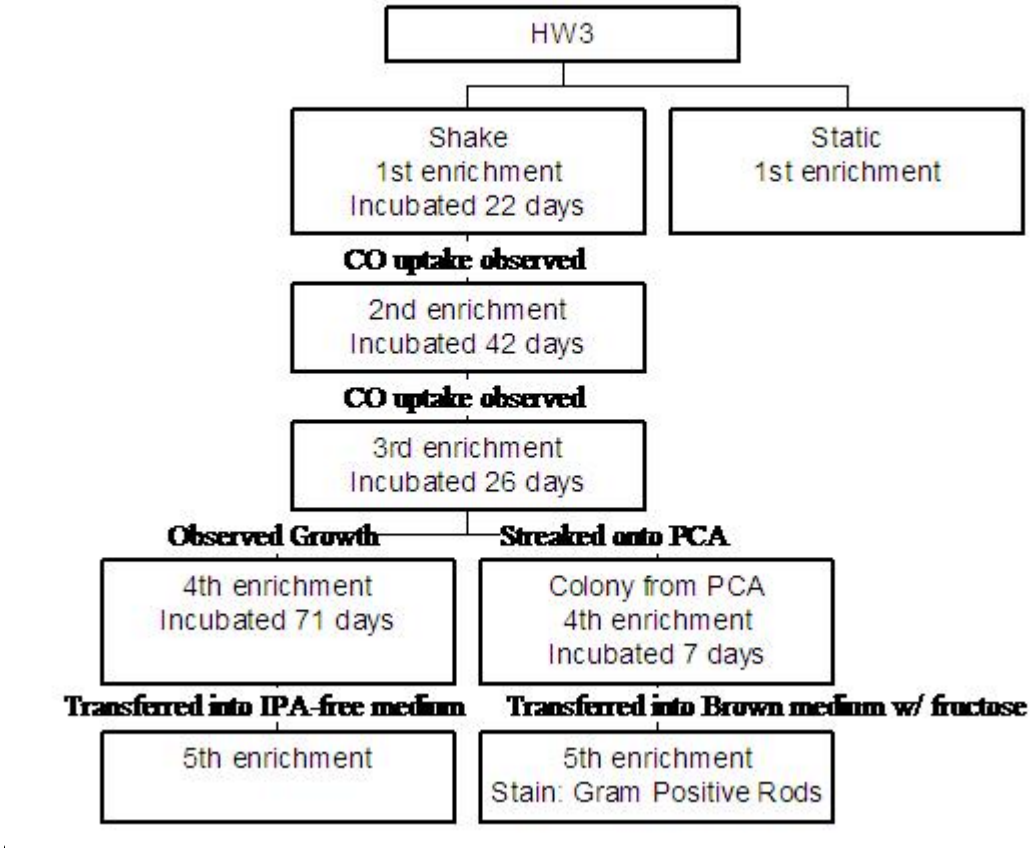

Figure B-7. Enrichments from HW3

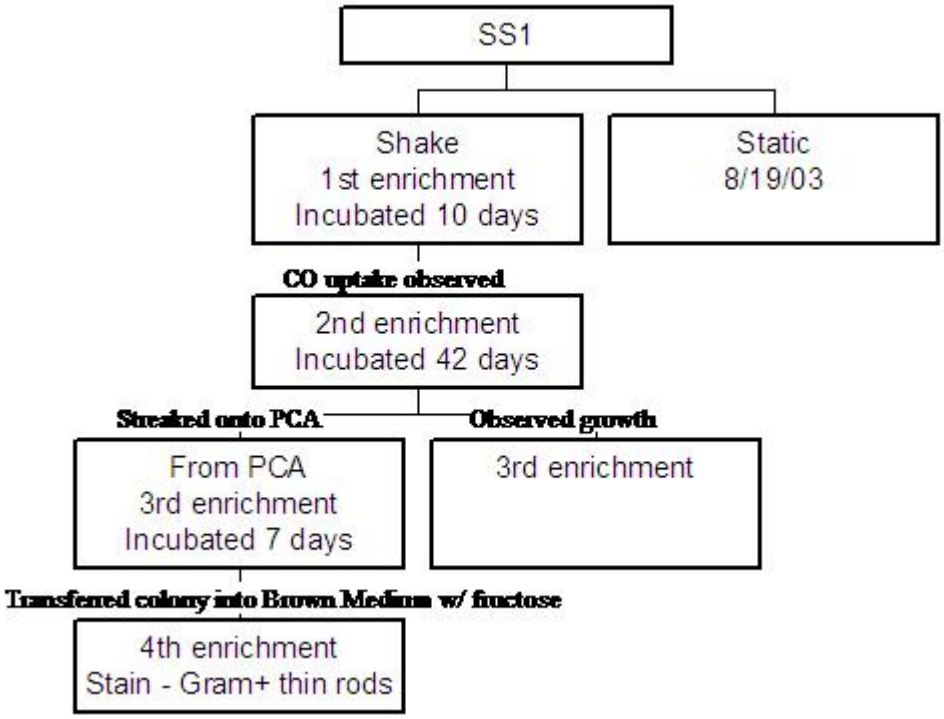

Figure B-8. Enrichments from SS1

TABLE C

RAW DATA

Table C-1. *C. ljungdahlii* Growth Experiments

***Clostridium ljungdahlii* Growth Data**

Date	Media	Fructose (g)	Peptone (g)	Yeast Extract (g)	Cell mass per 0.4L (mg)	Days of incubation
1/24/03	1754	2.5	2.5	0.5	296	3
1/25/03		2.5	2.5	0.5	296	3
1/27/03	1754	2.5	0	0.5	416	4
1/29/03		2.5	0	0.5	372	3
2/4/03	1754	5	0	0.9	507	2
2/7/03		5	0	1	621	2
2/11/03		5	0	1	620.7	3
2/18/03 [2]	1754	5	1	1	360	1
2/20/03		5	1	1	610	2

Table C-2. MSU1 Growth Experiments

MSU1 Growth Data

Date	Media	Fructose (g)	Yeast Extract (g)	Cell mass per 0.4L (mg)	Days of incubation
2/2/03	MSM	5	0.9	335	3
1/27/03	MSM	5	1	464	4
2/18/03	MSM	5	1	661	5

Table C-3. MSU1 Phase 1

LIQUID	GC fraction (ppm)					
sample time (hr	4 hour		24.5 hour		48.5 hour	
cells & gas 1	62.61331	81.81768	61.54518	70.67522	109.0399	100.4274
cells & gas 2	80.66286	66.6047	63.32528	61.85602	66.94919	70.52881
cells & gas 3	64.3028	77.8396	67.40703	58.31507	101.3211	100.1252
cells 1	69.19314	63.07512		33.78665		
cells 2	67.58528	85.07389	83.11847	99.34089		
cells 3	60.16765	68.34804	77.04492	75.06572		
gas 1	46.51802	50.55172	46.17392	46.90071		
gas 2	33.89057		48.85763	40.98148		
gas 3	33.90504	33.51497	38.65273	38.39978		

	GC fraction (%)								
GAS	Initial			24.5			48.5		
sample time (hr	CO	CO_2	O_2	CO	CO_2	O_2	CO	CO_2	O_2
cells & gas 1	14.03	0.64	0.00	13.22	0.30	1.94	11.50	0.92	5.19
cells & gas 2	13.79	0.00	0.00	12.74	0.37	2.52	11.53	1.96	6.31
cells & gas 3	14.15	0.00	0.00	13.49	0.37	1.93	11.66	0.88	4.54
cells 1	0.00	0.00	4.54						
cells 2	0.00	0.00	3.02						
cells 3	0.00	0.00	0.00						
gas 1	12.98	0.28	0.21						
gas 2	13.59	0.00	0.00						
gas 3	13.26	0.00	0.00						

Table C-4. *C. ljungdahlii* Phase 1

LIQUID	GC fraction (ppm)					
sample time (	4		24.5		48.5	
cells & gas 1			49.09	57.56		
cells & gas 2			53.67	52.02		
cells & gas 3			72.06	70.77		
cells 1	64.12	70.29	58.95	66.39		
cells 2			48.25	51.44		
cells 3			47.51	47.97		
gas 1	55.85	59.34	39.94	38.3		
gas 2	39.93	39.59	0	0		
gas 3	36.11	0	0	0		

	GC fraction (uL/100uL)								
GAS	Initial			24.5			48.5		
sample time (	CO	CO_2	O_2	CO	CO_2	O_2	CO	CO_2	O_2
cells & gas 1	13.30	0.48	0.00	13.79	10.57	1.24	2.22	4.35	4.12
cells & gas 2	13.50	0.00	0.00	13.59	0.50	1.01	12.26	17.73	10.51
cells & gas 3	13.97	0.00	0.00	13.31	0.74	2.06	10.54	0.00	7.94
cells 1	0.00	0.00	4.25						
cells 2	0.00	0.00	3.33						
cells 3									
gas 1	13.68	0.00	0.81						
gas 2	13.38	0.05	0.26						
gas 3	13.57	0.00	0.65						

Table C-5. MSU1 Phase 2, Trial 1

Trial 1

	GC fraction (uL/100uL)					
GAS	**Initial**			**73 hour**		
sample time (hr)	**CO**	**CO_2**	**O_2**	**CO**	**CO_2**	**O_2**
cells & gas 1	115.56	0.00	0.00	60.56	1.10	2.33
cells & gas 2	111.20	0.00	0.00	61.35	0.00	2.48
cells & gas 3	110.42	0.00	0.00	63.86	0.00	3.46
cells 1	0.00	0.00	0.00			
cells 2	0.00	0.00	0.00			
cells 3	0.00	0.00	0.00			
gas 1	117.29	0.00	0.00			
gas 2	110.29	0.00	0.00			

Table C-6. MSU1 Phase 2, Trial 2

Liquid Samples	Ethanol Concentration (ppm)					
Sample Time	Initial		18 hours		24 hours	
EtOH 1	566.9	599.0	645.3	601.2	629.2	600.1
EtOH 2	558.4	585.1	620.2	611.3	613.9	644.0
Cells 1			312.4	429.8	0.0	0.0
Cells 2			138.5	138.0	0.0	0.0
Cells 3			120.7		0.0	0.0

Liquid Samples	IsoPropanol Concentration (ppm)					
Sample Time	Initial		18 hours		24 hours	
EtOH 1	377.4	370.8	402.3	400.2	417.1	419.1
EtOH 2	339.5	339	449.7	436.4	448.7	438.3
Cells 1			703.4	572.5	68.22	68.72
Cells 2			442.9	419.5	24.5	24.5
Cells 3			500.9		68.7	68.2

Liquid Samples	Acetone Concentration (ppm)					
Sample Time	Initial		18 hours		24 hours	
EtOH 1	0	0	13.8	13.62	12.4	19.2
EtOH 2	0	0	13.2	11.7	12.6	12.8
Cells 1			36.2	37.2	489.9	
Cells 2			72.2	74.9	172.1	144.8
Cells 3			130.7		261	275.9

Liquid Samples	Ethanol Concentration (ppm)			
Sample Time	18 hours		24 hours	
Cells & Gas 1	446.3	365.7	0.0	0.0
Cells & Gas 2	603.5	487.4	0.0	0.0
Cells & Gas 3	446.3	476.3	0.0	0.0
Cells 1	312.4	429.8	0.0	0.0
Cells 2	138.5	138.0	0.0	0.0
Cells 3	120.7		0.0	0.0

Liquid Samples	IsoPropanol Concentration (ppm)			
Sample Time	18 hours		24 hours	
Cells & Gas 1		489.2	286.8	283.2
Cells & Gas 2	728.6	832.4	515.2	488.9
Cells & Gas 3	629.4	579.3	1037.6	1034.4
Cells 1	703.4	572.5	68.22	68.72
Cells 2	442.9	419.5	24.5	24.5
Cells 3	500.9		68.7	68.2

Liquid Samples	Acetone Concentration (ppm)			
Sample Time	18 hours		24 hours	
Cells & Gas 1	22.4	22.4	354.7	390.2
Cells & Gas 2	11.8	13.7	456.9	300.9
Cells & Gas 3	52.9	58.9	18.0	17.1
Cells 1	36.2	37.2	489.9	
Cells 2	72.2	74.9	172.1	144.8
Cells 3	130.7		261	275.9

GAS	GC Fraction (%)			
	Initial		72 hours	
Sample Time (hr)	CO	O_2	CO	O_2
Cells and Gas 1	78.38	0	69.73	0
Cells and Gas 2	78.88	0	73.38	0
Cells and Gas 3	77.71	0	73.98	0

Table C-7. *C. ljungdahlii* Phase 2 liquid samples

Liquid Samples	Ethanol Concentration (ppm)			
Sample Time	18 hours		96 hours	
EtOH 1	119.1	112.2	256.1	241.7
EtOH 2	119.1	144.7	243.1	226.4
EtOH 3	117.2	115.8	142.0	160.1
Cells 1	0.0	0.0		
Cells 2	0.0	0.0	0.0	0.0
Cells 3	0.0	0.0	0.0	0.0

Liquid Samples	IsoPropanol Concentration (ppm)			
Sample Time	18 hours		96 hours	
EtOH 1	0.0	0.0	20.6	22.7
EtOH 2	0.0	0.0	31.1	32.9
EtOH 3	0.0	0.0	32.7	33.7
Cells 1	0.0	0.0		
Cells 2	0.0	0.0	39.3	41.9
Cells 3	0.0	0.0	56.6	58.2

Liquid Samples	Acetone Concentration (ppm)			
Sample Time	18 hours		96 hours	
EtOH 1	0.0	0.0	0.0	0.0
EtOH 2	0.0	0.0	0.0	0.0
EtOH 3	0.0	0.0	0.0	0.0
Cells 1	0.0	0.0		
Cells 2	0.0	0.0	0.0	0.0
Cells 3	0.0	0.0	0.0	0.0

Liquid Samples	Ethanol Concentration (ppm)					
Sample Time	18 hours		24 hours		96 hours	
Cells & Gas 1	0.0	0.0	30.4	24.1	0.0	0.0
Cells & Gas 2	0.0	0.0	141.2	203.0		
Cells & Gas 3	0.0	0.0	102.6	0.0	0.0	
Cells 1	0.0	0.0				
Cells 2	0.0	0.0	0.0	51.3	0.0	0.0
Cells 3	0.0	0.0	133.1		0.0	0.0

Liquid Samples	IsoPropanol Concentration (ppm)					
Sample Time	18 hours		24 hours		96 hours	
Cells & Gas 1	0.0	0.0	51.3	50.7	68.0	67.0
Cells & Gas 2	0.0	0.0	252.3	207.2		
Cells & Gas 3	0.0	0.0	138.5	244.4	58.0	
Cells 1	0.0	0.0				
Cells 2	0.0	0.0	124.0	74.3	39.3	41.9
Cells 3	0.0	0.0	0.0		56.6	58.2

Liquid Samples	Acetone Concentration (ppm)					
Sample Time	18 hours		24 hours		96 hours	
Cells & Gas 1	0.0	0.0	0.0	0.0	0.0	0.0
Cells & Gas 2	0.0	0.0	0.0	0.0		
Cells & Gas 3	0.0	0.0	0.0	0.0	0.0	
Cells 1	0.0	0.0				
Cells 2	0.0	0.0	0.0	0.0	0.0	0.0
Cells 3	0.0	0.0	0.0		6.9	7.0

Table C-8. *C. ljungdahlii* Phase 2 gas samples

	GC fraction (uL/100uL)								
GAS	Initial			18 hour			36 hour[1]		
sample time (hr)	CO	CO_2	O_2	CO	CO_2	O_2	CO	CO_2	O_2
cells & gas 1	73.98	0.28	0.00	69.24	0.00	0.00	42.72	0.00	3.93
cells & gas 2	71.94	0.00	0.00	68.01	0.00	0.00	66.76	0.00	3.59
cells & gas 3	66.60	0.71	0.18	66.50	0.27	0.00	65.38	8.94	3.60
cells 1				0.00	0.00	6.36			
cells 2				0.00	0.00		0.00	0.00	5.45
cells 3				0.00	0.00		0.00	0.00	7.06
gas 1	68.84	0.54	0.00				57.09	0.00	5.67
gas 2	66.63	3.11	0.00	64.32	0.42	3.75			
gas 3	65.41	1.00	2.42	65.58	0.37	4.23	70.78	0.00	1.05
EtOH 1									
EtOH 2				0.00	0.00	0.00	0.00	0.00	2.73
EtOH 3				0.00	0.00	0.00	0.00	0.00	5.95

	66 hour			67 hour - regassed			167 hour		
sample time (hr)	CO	CO_2	O_2	CO	CO_2	O_2	CO	CO_2	O_2
cells & gas 1	40.43	0.00	3.21	78.45	0.00	3.15	46.10	0.00	4.47
cells & gas 2	57.52	0.00	3.11	84.40	1.00	0.26	79.95	0.14	1.56
cells & gas 3	30.74	0.00	3.17	84.66	0.00	0.77	36.87	0.00	2.97
cells 1									
cells 2							0.00	0.00	1.63
cells 3							0.00	0.00	8.54
gas 1							51.05	0.62	6.78
gas 2									
gas 3									
EtOH 1							0.00	0.00	1.94
EtOH 2							0.00	0.00	2.26
EtOH 3									

Table C-9. *C. ljungdahlii* Phase 3 liquid samples

LIQUID	Ethanol GC fraction (ppm)							
sample time (	24		48		70.5		165	
cells & gas 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells & gas 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells & gas 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
gas 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
gas 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
gas 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

LIQUID	IsoPropanol GC fraction (ppm)							
sample time (	24		48		70.5		165	
cells & gas 1		93.55	113.12	113.92	77.75	76.49	87.18	88.19
cells & gas 2	111.50	112.66	85.41	85.46	73.19	74.00	117.52	105.36
cells & gas 3	100.86	95.18	76.40	77.32	74.54	138.81	110.09	102.23
cells 1	82.35	84.70	66.68	69.15	218.21	181.37	300.22	206.75
cells 2	93.35	93.98	63.97	64.81	187.80	226.55	191.47	131.87
cells 3	102.63	101.60	64.79	67.19	204.65	233.55	161.79	263.69
gas 1	17.21		16.28	16.06	36.29	33.96	50.15	50.91
gas 2	25.89	26.75	20.86	21.73	24.97	26.55	72.98	60.42
gas 3	19.00	19.06	15.42	15.41	20.03	20.08		

LIQUID	Acetone GC fraction (ppm)							
sample time (	24		48		70.5		165	
cells & gas 1	0.00	0.00	0.00	0.00	0.00	0.00	6.92	7.37
cells & gas 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells & gas 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	4.32
cells 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
cells 3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
gas 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
gas 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
gas 3	0.00	0.00	0.00	0.00	0.00	0.00		

Table C-10. *C. ljungdahlii* Phase 3 gas samples

	GC fraction (%)								
GAS	47			70			71 regassed		
sample time (	CO	CO_2	O_2	CO	CO_2	O_2	CO	CO_2	O_2
cells & gas 1	62.94	0.96	0.96	49.55	0.67	0.67	66.63	3.26	0.00
cells & gas 2	65.36	0.28	1.29	44.46	0.52	3.81	65.69	2.64	0.00
cells 1	0.00	0.00	3.70						
cells 2	0.00	0.00	4.71						
cells 3	0.00	0.00	4.44						
gas 1	71.92	2.62	2.15						
gas 2	70.65	0.91	3.12						

GAS	164.5			166.5 regassed			192		
sample time (	CO	CO_2	O_2	CO	CO_2	O_2	CO	CO_2	O_2
cells & gas 1	26.06	0.44	0.00	75.27	2.11	0.00	68.20	1.80	0.00
cells & gas 2	34.28	1.06	0.00	72.40	2.43	0.00	73.10	2.53	0.00
cells 1									
cells 2									
cells 3									
gas 1							69.27	2.07	0.00
gas 2							65.16	1.49	4.94