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Production of Acetic Acid from the Fermentation of Synthesis Gas

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PRODUCTION OF ACETIC ACID FROM THE FERMENTATION OF SYNTHESIS GAS

By

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In recent years economic, environmental, and strategic concerns over the use of petroleum resources have led to increased interest in renewable alternatives. Biomass gasification produces a synthesis gas composed of primarily carbon monoxide, hydrogen, and carbon dioxide, which can be fermented into a variety of fuels and chemicals.

This study evaluated the performance of a two-stage approach to batch synthesis gas fermentations. The first stage employs a rich medium optimized for cell growth, while the second stage is designed to maximize production of acetic acid from synthesis gas. This two-stage approach is hypothesized to be more metabolically efficient than previous single-stage designs.

This study presents the evaluation of known acetic acid producing organisms described in the literature, and efforts to isolate a novel microbial catalyst for synthesis gas fermentations. Finally, new techniques were developed and implemented to develop a more effective system for batch synthesis gas fermentations.

DEDICATION

I dedicate this thesis to my grandparents, Mr. and Mrs. Thomas Jackson Ford, Jr., Mrs. Vance Walker Thompson and the late Mr. Vance Walker Thompson. Your courage, grace, and generosity have been a blessing in my life.

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CHAPTER I

INTRODUCTION

Traditional production of fuels and many chemicals has relied heavily on fossil fuel feedstocks. In recent years, the depletion of available petroleum reserves, the environmental harm caused by greenhouse gas emissions, and the strategic impacts of a dependency on imported petroleum have resulted in a revitalized interest in alternative methods of fuels and chemicals production (Dale, 2003). A major challenge in this effort is the development of processes to convert renewable resources, such as biomass, into usable chemicals and fuels (Zeikus, 1980).

Millions of tons of waste biomass are generated every year throughout the world. World biomass production is estimated to exceed 110 billion tons per year (Dale, 2003). Approximately 280 million tons of waste biomass are generated each year in the United States. This quantity is enough to support the production of all industrial chemicals readily made from biomass and to contribute significantly to the nation's fuel needs (NRC, 2003). Mississippi's struggling economy, which is based largely on agricultural and forest products, would receive a major boost from the utilization of this waste biomass as an industrial resource.

One of the most exciting proposed industrial concepts to utilize this material is the construction of "biorefineries" that convert complex biomass into a feedstock for

chemical production. This feedstock can then be converted into valuable commodity chemicals, such as ethanol, acetic acid, and methane (Zeikus, 1980). The concept of "cracking" the many chemicals found within biomass using an integrated industrial approach is commonly known as "biorefining." Biorefineries, like petroleum refineries, would produce a variety of products such as food, textiles, fuels, solvents, and plastics (NRC, 2003). Biorefineries offer many advantages over petroleum refineries, such as reducing the dependency on foreign feedstocks, benefiting farmers by opening new markets for agricultural products, and causing minimal adverse environmental impacts compared to petroleum refineries (Dale, 2003).

One biorefinery option under development at Mississippi State University involves the gasification of biomass to produce synthesis gas, which can be converted into a variety of useful products. Synthesis gas is composed primarily of hydrogen, carbon monoxide, carbon dioxide, and methane (Klasson et al., 1992), and is produced through the oxygen-starved combustion of biomass. Synthesis gas can be converted chemically or biologically into numerous valuable chemicals (Iranmahboob, 1999). Waste biomass, such as sawdust and poultry litter, are often seen as unwanted by-products posing disposal challenges. Using these products as feedstock for synthesis gas production followed by fermentation transforms waste biomass from a large disposal problem into a potentially profitable industry (Zeikus, 1980).

Much of the research on synthesis gas fermentation has been focused on conversion to ethanol because of its potential as a fuel additive, but acetic acid may be an attractive alternative product. Acetic acid prices are similar to the government-subsidized

prices of ethanol, making the proposed synthesis gas to acetic acid efforts particularly interesting from an economic standpoint. The price of ethanol in June 2004 was approximately $0.99/kg with government subsidies (CMR, 2004). The price of acetic acid during the same time period was $1.00/kg (CMR, 2004). World demand for acetic acid was estimated at over 15 billion pounds in 2003 (Johnson, 2000).

The following reactions, although greatly simplified, represent the overall intracellular chemical reactions used to convert synthesis gas to acetic acid (Gaddy, 1998):

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

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

A number of studies have been conducted on synthesis gas fermentation to acetic acid, but the lack of industrial deployment indicates the need for further research.

Commercial uses of industrial grade acetic acid include manufacturing of acetate esters, such as cellulose acetate (Sugaya et al., 1986), which is used in the production of photographic films and rayon (Johnson, 2000). Acetic anhydride, an acetic acid derivative, is used to produce cellulose acetate and pharmaceuticals (Johnson, 2000). Acetic acid can be used to make vinyl acetate monomer, the building block for polyvinyl acetate (Johnson, 2000). Polyvinyl acetate is used in latex paints and glues for paper and wood. Calcium-Magnesium Acetate (CMA) can be used as a de-icing agent for roads (Sugaya et al., 1986). Additionally, food grade acetic acid in a diluted form is vinegar. Acetic acid is also used as a solvent in the production of terephthalic acid, which is used in the manufacture of resins, fibers, and films (Johnson, 2000). Other uses for acetic acid

include the production of herbicides, pharmaceuticals, rubber chemicals, and explosives (Johnson, 2000).

Current acetic acid production methods include reaction of methanol and carbon monoxide (methanol carbonylation), catalytic liquid/vapor oxidation of petroleum gases, or oxidation of acetaldehyde (Johnson, 2000). This research explores the possibility of using microorganisms to convert synthesis gas to acetic acid. Biocatalysis has several advantages over traditional chemical catalysis, including high selectivity, mild operating temperatures and pressures, and high yields (Vega et al., 1989b).

Research Hypothesis and Objectives

The construction of biorefineries for biomass gasification and fermentation will result in a major economic boost for agricultural states such as Mississippi. In the proposed synthesis gas biorefinery, biomass goes through a series of size-reduction steps in preparation for gasification (Figure 1-1). The synthesis gas generated within the gasifier is fed to the fermentor as the carbon and energy source for anaerobic bacteria. These bacteria ferment the synthesis gas into the desired product or products, e.g. acetic acid or ethanol. Liquid medium (nutrient solution), containing bacteria and fermentation products, is continuously removed from the fermentor and replaced with fresh medium. Cells are removed from the product solution and recycled to the fermentor in order to maintain high cell densities. The cell-free product solution is transferred to the separations stage.

A two-stage batch fermentation approach is hypothesized to optimize acetic acid yield. In the first fermentation stage, the growth stage, bacteria will be grown in a fructose-rich environment in order to maximize cell yield. Cells will be harvested from the growth stage, and transferred to the production stage. In the production stage, synthesis gas components CO, CO_2, and H_2 will serve as the sole carbon and energy sources. This two-stage approach will yield higher cell mass, and consequently more bio-catalytic capacity, in the production stage. The hypothesized two-stage fermentor system could be easily employed in the biorefinery scheme described above, greatly increasing the production stage cell density and resulting product yields.

The original objective of this research was to determine the feasibility of producing acetic acid from synthesis gas. This objective was divided in two parts:

- To establish a benchmark for future acetic acid production using known homoacetogens described in the literature.
- To evaluate the capabilities of novel cultures isolated at MSU to ferment synthesis gas to acetate.

During the early stages of experimentation, deficiencies were noted in the existing experimental techniques. These deficiencies, combined with the sensitivity of the microorganisms to seemingly insignificant changes, required a shift in the overall experimental scope. Therefore, the following additional objectives were added:

- To evaluate and improve culture-handling techniques used in these experiments in order to develop a more effective system for the conversion of synthesis gas to acetic acid.
- To utilize the refined experimental techniques to evaluate the potential for synthesis gas fermentation to acetate by bacteria from two additional sources.

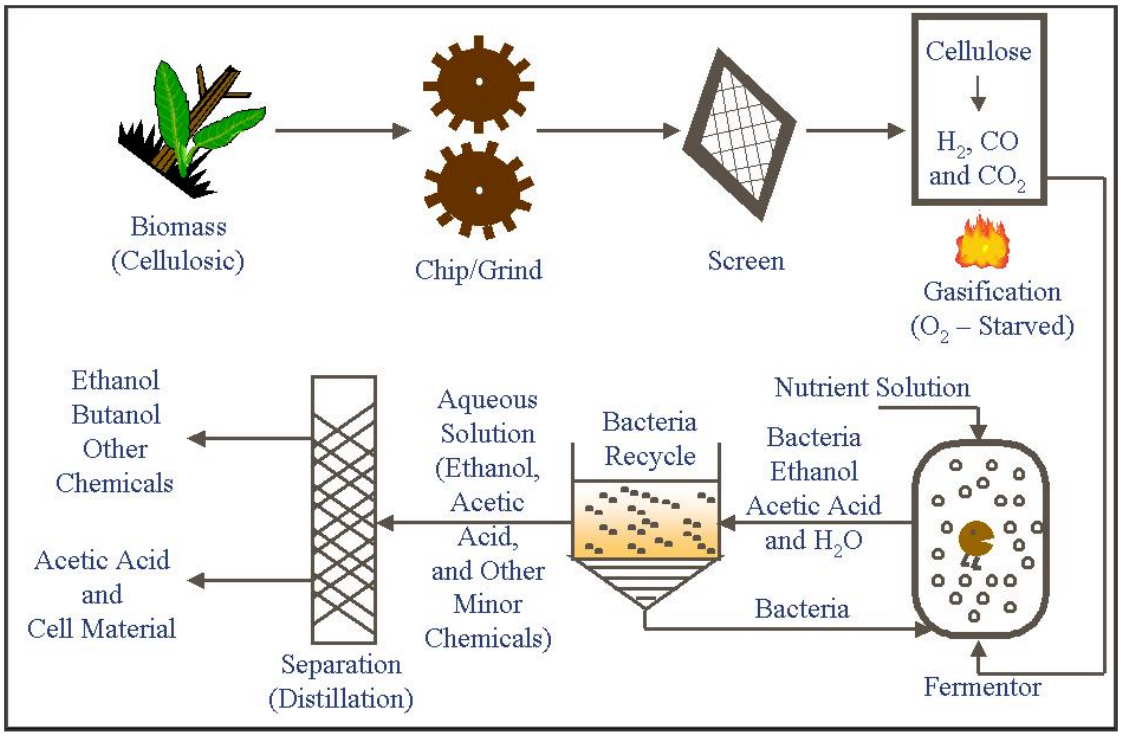

Figure 1-1. Synthesis Gas Fermentation Biorefinery

CHAPTER II

LITERATURE REVIEW

Synthesis gas fermentation has great potential for industrial deployment, but a major obstacle to commercialization is low fermentor productivity using existing techniques and organisms (Worden et al., 1997). Low cell density, low solubility of CO and H_2, inability to properly regulate pathways in order to obtain only the desired product, and biocatalyst inhibition due to reactants and products, contribute to this low productivity (Worden et al., 1997). In addition to productivity issues in the bioreactors, low product concentrations lead to separations issues downstream from both an economic and technical perspective (Worden et al., 1997).

Although strides have been made to address some of these concerns, the lack of commercialization of these processes indicates the necessity for further research. New organisms may be able to convert synthesis gas to acetic acid at higher rates than previously reported. Innovative fermentation techniques may also yield higher production rates. Clearly, further research is needed to help remove the barriers to commercialization of this technology.

Acetic Acid Production

Manufactured acetic acid is classified as either virgin or recovered (Johnson, 2000). Virgin acetic acid is produced by first intent; recovered acetic acid is recovered from processing of other products or chemicals, but is usually of similar quality to virgin acid when properly purified (Johnson, 2000). U.S. production of acetic acid exceeded 3.9 million tons in 2003, with virgin acetic acid accounting for roughly 75% of the total (Kirschner, 2003b). World capacity for acetic acid production was nearly 8 million tons in 1998, with BP-Amoco (19%) and Celanese (26%) accounting for almost half of this amount (Yoneda et al., 2001). Production methods for acetic acid are listed in Table 2-1 (Johnson, 2000).

Acetic acid was originally produced by the aerobic fermentation of ethanol, and this course remains the primary production route for vinegar (Yoneda et al., 2001):

$$CH_3CH_2OH + O_2 \rightarrow CH_3COOH \quad (1)$$

Acetic acid was first synthetically produced via oxidation of acetaldehyde, and oxidation of this and other hydrocarbons such as ethylene, *n*-butane, and naphtha still accounts for about a quarter of world acetic acid production (Johnson, 2000; Yoneda et al., 2001). Acetaldehyde oxidation, using a manganese acetate catalyst at 50-60ºC and atmospheric pressure, results in 95% conversion to acetic acid (Yoneda et al., 2001):

$$CH_3CHO + \tfrac{1}{2}\, O_2 \xrightarrow{\text{cat.}} CH_3COOH \quad (2)$$

Direct oxidation of ethylene over a palladium/heteropolyacid/metal catalyst at 150-160ºC and 80 atm yields 87% conversion to acetic acid (Yoneda et al., 2001):

$$CH_2{=}CH_2 + O_2 \xrightarrow{\text{cat.}} CH_3COOH \quad (3)$$

Oxidation of *n*-butane or naphtha utilizes a cobalt acetate or manganese acetate catalyst at 150-230ºC and 50-60 atm, resulting in yields of 50% for *n*-butane and 40% for naphtha (Yoneda et al., 2001). The oxidation of *n*-butane is presented below (Yoneda et al., 2001):

$$CH_3CH_2CH_2CH_3 + O_2 \xrightarrow{\text{cat.}} CH_3COOH + \text{by-products} \quad (4)$$

Acetic acid is most commonly produced by methanol carbonylation (Johnson, 2000). Methanol carbonylation routes to acetic acid were pioneered by BASF in the 1950s and 1960s, but in 1970, Monsanto dramatically improved this process using a methyl iodide-promoted rhodium catalyst (Yoneda et al., 2001). Under reaction conditions, the rhodium complexes are converted to the active catalyst $[RhI_2(CO)_2]^{-1}$, resulting in the overall reaction below (Yoneda et al., 2001):

$$CH_3OH + CO \xrightarrow{\text{cat.}} CH_3COOH \quad (5)$$

The "Monsanto Process" for methanol carbonylation boasts higher productivity and yields at much milder conditions than the BASF process (180-220ºC and 30-40 atm for Monsanto versus 230ºC and 600 atm for BASF) (Yoneda et al., 2001). The Monsanto process exhibits yields of 99% for methanol and 85% based on CO (Yoneda et al., 2001).

End uses for acetic acid are presented in Figure 2-1 (Kirschner, 2003b). Vinyl acetate monomer (VAM) is the largest end use for acetic acid (Johnson, 2000). VAM is used primarily in vinyl acetate homopolymer and vinyl acetate-n-butyl acrylate copolymer (Johnson, 2000). Acetic anhydride, an acetic acid derivative, is used in the

production of cellulose acetate and pharmaceuticals (Johnson, 2000). The fastest-growing market for acetic acid is the terephthalic acid (TPA) manufacturing process, in which acetic acid is a solvent. TPA is used in the production of polyethylene terephthalate (PET) resins; PET bottles are utilized by the carbonated beverage industry (Johnson, 2000; Kirschner, 2003b).

Biomass Resources

Lignocellulosic biomass (trees, grasses, etc.) is a largely untapped resource for chemical production. Biomass resources can be divided into silviculture crops, agricultural crops, and waste biomass. The biomass resources available in the United States are enough to satisfy our current demands for food, feed, and fiber, and still produce sufficient raw materials for biobased industrial products, with the exception of massive fuel production (NRC, 2003). The use of biomass resources as raw materials in bioprocessing is dependent on their regional availability and competing uses.

Agricultural products provide a valuable source of biomass for chemical production. Corn, wheat, soybeans, and hay currently comprise about 80 percent of the crops harvested in the U.S. (NRC, 2003). Although these products are used in food and animal feed, there is enough waste material to support additional biobased products, such as bulk chemical production. In addition to established crops, other high-yield crops may be produced as dedicated feedstocks for chemical production. Grasses such as Bermuda Grass and Switchgrass, and legumes such as alfalfa, may provide raw materials or feedstocks for future bioprocesses (NRC, 2003).

Forest productivity from silviculture has increased dramatically over the past 50 years, and the production capacity may still not be fully utilized (NRC, 2003). Although there is a high demand for softwood forest residues, hardwood residues have fewer competing uses, and may be a good choice as a raw material for chemical production (NRC, 2003).

Additional raw material sources for chemical production include waste biomass. It is estimated that enough carbon is present in waste biomass resources to generate the 100 million metric tons of organic chemicals consumed in the U.S. each year and supply an appreciable fraction of the nation's energy requirements (NRC, 2003). Waste biomass includes low-profit or negative-profit by-products of agricultural and industrial processes, such as corn stover, paper-industry waste streams, wood mill wastes such as sawdust, cotton gin wastes, mixed office paper, newsprint, and municipal solid waste (Claassen et al., 1999; NRC, 2003).

Current Biomass Processing Technologies

Several conversion options are available for biomass utilization. Wet milling and dry milling are used currently in the production of ethanol from corn. For complex biomass containing large amounts of hemicellulose and lignin, technologies such as acid hydrolysis, enzyme hydrolysis, and gasification are under development (Ladisch and Svarczkopf, 1991; Phillips et al., 1994).

The wet milling process (Figure 2-2) begins by steeping the corn kernels in water containing 0.1%-0.2% sulfur dioxide at 48-52˚C for 30-50 hours (Ladisch and

Svarczkopf, 1991). The steep water is removed and the softened grain is ground into four fractions: protein, fiber, germ, and starch (Ladisch and Svarczkopf, 1991). The protein fraction is sold as corn gluten meal and the fiber fraction as bran (Ladisch and Svarczkopf, 1991). The germ fraction is used to produce oil, and the starch is sent to further processing to make sweeteners, high fructose corn syrup, food starch, industrial starch, and ethanol (Ladisch and Svarczkopf, 1991).

Unlike wet willing, where water is added to corn at the beginning of the process, dry milling (Figure 2-3) begins by tempering the corn to about 20% moisture (Ladisch and Svarczkopf, 1991). The whole grain is cooked and fermented to ethanol (Ladisch and Svarczkopf, 1991). The remaining solids, as well as unfermented soluble components are dried and sold as distillers dried grain with solubles, or DDGS (Ladisch and Svarczkopf, 1991). DDGS contains mostly protein, oil, and fat, and is used as animal feed (Ladisch and Svarczkopf, 1991).

The advantages to wet and dry milling processes include technological maturity, readily available raw material sources in the Midwestern U.S., and established co-product markets. The major disadvantage of these processes is their dependency on corn feedstocks. While a great deal of capital and research has been devoted to corn starch-to-ethanol processing (Ladisch and Svarczkopf, 1991), complex biomass sources are not readily amenable to wet and dry milling technologies.

Enough lignocellulosic biomass is available to supply large amounts of liquid fuel without disrupting the food supply, but further research is needed to develop efficient and economical conversion technologies (Dale, 2003). These plant structures are composed

of 35-50% cellulose (a polymer of glucose), 20-40% hemicellulose (a polysaccharide containing xylans, mannans, and glucans), and 15-30% lignin (a polyphenolic structure) (Claassen et al., 1999; NRC, 2003). The cellulose fibers form a crystalline structure that is intermingled with hemicellulose, held together by lignin (Claassen et al., 1999). Traditional yeast fermentation by *Saccharomyces cerevisiae* is not effective for lignocellulose feedstocks (Claassen et al., 1999). This organism is limited to mono- or disaccharide substrates, and is unable to convert pentoses to ethanol (Claassen et al., 1999). As a result, hydrolysis is needed to break down the polymer structure of lignocellulose, break up the cellulose/hemicellulose polymers, and release the fermentable sugars (Claassen et al., 1999).

Acid hydrolysis has been used since World War II for the pretreatment of woody biomass (NRC, 2003). This process uses either concentrated or dilute sulfuric acid to hydrolyze the lignocellulose (NRC, 2003). The dilute acid process requires an acid concentration of 2-5%, a temperature of 160ºC, and a pressure of 10 atm (Iranmahboob et al., 2002). Concentrated acid hydrolysis uses a 10-30% acid concentration and a longer retention time than with dilute acid hydrolysis, but lower operating temperatures (100ºC) and pressures (Ladisch and Svarczkopf, 1991; Iranmahboob et al., 2002). The sugars liberated through the hydrolysis process can be fermented to ethanol (Ladisch and Svarczkopf, 1991). Although glucose yields as high as 90% can be obtained with acid hydrolysis (Ladisch and Svarczkopf, 1991), the need for corrosion resistant materials can result in high capital costs.

Enzyme hydrolysis is another pretreatment technology used for conversion of lignocellulosic biomass into ethanol. This process uses enzymes to convert cellulose to sugars (Iranmahboob et al., 2002). Enzymatic hydrolysis is economically infeasible at present due to the need for feedstock pretreatment, enzyme production, and enzyme recovery (Iranmahboob et al., 2002).

A final conversion technology, gasification followed by fermentation, will be discussed in-depth in the following section.

Synthesis Gas Production

Biomass gasification converts the complex structure of cellulose, hemicellulose, and lignin in biomass into a gas mixture known as synthesis gas (Phillips et al., 1994). This thermal treatment process yields large quantities of gaseous products along with small amounts of char and ash (Maschio et al., 1994). The resulting synthesis gas is typically a mixture of carbon monoxide (10-50%), hydrogen (10-40%), carbon dioxide (8-50%), nitrogen (if air is used in gasification, 0.5-60%), methane (0.01-10%), and sulfur compounds (up to 10% depending on the biomass type) (Gaddy and Chen, 1998). The feedstock composition, the type of gasification reactor, and the operating conditions all can affect the composition and quality of the synthesis gas (Maschio et al., 1994).

The first step of the gasification process involves the thermochemical degradation of lignocellulose to produce char and volatiles (Maschio et al., 1994). Next, the char is gasified, and further equilibrium reactions occur as shown in Equations (6)-(9) (Maschio et al., 1994):

$$C + H_2O \leftrightarrow CO + H_2 \quad (6)$$

$$C + CO_2 \leftrightarrow 2CO \quad (7)$$

$$CO + H_2O \leftrightarrow H_2 + CO_2 \quad (8)$$

$$CH_4 + H_2O \leftrightarrow CO + 3H_2 \quad (9)$$

These reactions along with side reactions, such as cracking and reforming of tar, reach equilibrium under the proper operating conditions to determine the final synthesis gas composition (Maschio et al., 1994). The high nitrogen content resulting from air gasification is considered unacceptable due to a complex and expensive separation; as a result, most gasification processes use steam or oxygen to produce a nitrogen-free synthesis gas (Maschio et al., 1994).

Internal or external heat generation is used to produce the energy required for gasification. For internal heating, air or oxygen is introduced within the reactor, and the biomass is partially oxidized to generate the energy for gasification (Maschio et al., 1994). Reactors using internal heat generation are either moving-bed gasifiers or fluidized-bed reactors (Maschio et al., 1994). For external heating, part of the biomass feedstock is burned outside the reactor, and the energy is transferred to the reactor (Maschio et al., 1994). External heating can be employed either by bringing the feedstock into contact with hot char or other solids heated in a separate vessel, or by entraining the feedstock in a carrier gas and passing it through an externally heated tubular reactor (Maschio et al., 1994).

Typically, the biomass is preheated to about 260ºC prior to entering the gasification reactor (Gaddy and Chen, 1998). Within the reactor, the biomass is gasified

at 500-1500ºC to produce synthesis gas (Gaddy and Chen, 1998). The gas is then cooled to around 40ºC in a heat recovery unit, regenerating steam for use in the gasification step (Gaddy and Chen, 1998). The cooled synthesis gas is then sent through a solids removal unit to remove ash, slag, tar, and other particulates (Gaddy and Chen, 1998).

Large energy input is one of the disadvantages of biomass gasification (Gaddy and Chen, 1998). Although gasification of coal is an established technology, poor economics have limited commercial development of biomass gasification (NRC, 2003). Gasification breaks down the complex form of biomass, destroying potentially useful polymer structures.

The major advantage of gasification over other biomass processing technologies is feedstock flexibility. The gasification system can be adapted for a variety of feedstocks, allowing the use of niche or seasonal resources (Phillips et al., 1994). Another advantage of gasification is the potential use of other hydrocarbon materials such as plastic and rubber as feedstocks (Phillips et al., 1994). Because of the flexibility of gasification, synthesis gas can serve as a major intermediate in the production of fuels and chemicals from a very wide variety of biomass raw materials (Gaddy and Chen, 1998).

Synthesis Gas Conversion

Chemical catalysis may be used to convert synthesis gas to fuels and chemicals such as methane, methanol, formaldehyde, and acetic acid (Klasson et al., 1992). The reaction of carbon monoxide and hydrogen via a chemical catalyst is known as the

Fischer-Tropsch reaction (Iranmahboob, 1999), which is described by the following equation (Keyser et al., 2000):

$$CO + (1 + m/2n)\ H_2 \rightarrow 1/n\ C_nH_m + H_2O \quad (10)$$

In this equation, *n* represents the average carbon chain length of the product, and *m* is the average number of hydrogen atoms per molecule (Keyser et al., 2000).

One proposed catalytic synthesis gas conversion technique uses methanol and dimethyl ether as intermediates in acetic acid production (Yoneda et al., 2001). The catalyst for this process is a mixture of the catalyst used in methanol synthesis (e.g. copper-zinc-aluminum oxide) and a dehydration catalyst, which combine to carry out the following reactions at 220ºC and 40 atm (Yoneda et al., 2001):

$$CO + 2\ H_2 \rightarrow CH_3OH \quad (11)$$

$$2\ CH_3OH \rightarrow CH_3OCH_3 + H_2O \quad (12)$$

$$H_2O + CO \rightarrow CO_2 + H_2 \quad (13)$$

The carbonylation of methanol and dimethyl ether over a rhodium carbonyl complex catalyst at 170-250ºC and 25-50 atm then yields acetic acid by the following reactions (Yoneda et al., 2001):

$$CH_3OH + CO \rightarrow CH_3COOH \quad (14)$$

$$CH_3OCH_3 + 2\ CO + H_2O \rightarrow 2\ CH_3COOH \quad (15)$$

These chemical catalytic routes from synthesis gas to acetic acid have high operating costs due to high temperatures and pressures (Chang et al., 1998). In addition, synthesis gas often contains sulfur, which can poison the catalysts (Chang et al., 1998).

Alternatively, microorganisms can be used to ferment synthesis gas into fuels and chemicals (Klasson et al., 1992). Microorganisms use similar reactions to conventional catalysts, but operate at ambient temperatures and pressures, resulting in significant energy and equipment savings (Vega et al., 1989b). Although biological processes are generally slower than chemical processes, they often have higher specificity, higher yields, lower energy costs, and greater resistance to poisoning (Klasson et al., 1992).

A number of organisms have shown promise in converting unicarbonic compounds (e.g. CO, CO_2) from synthesis gas into acetic acid. These organisms include *Acetobacterium woodii*, *Butyribacterium methylotrophicum*, *Clostridium aceticum*, *Clostridium thermoaceticum*, *Eubacterium limosum*, and *Acetogenium kivui* (Gaddy, 1998). A section entitled "Overview of Acetogens" later in this chapter describes these organisms in greater detail.

Mass transfer issues dominate much of the literature on synthesis gas fermentation. The reactants (synthesis gas components) must be transported across the gas-liquid interface, diffuse through the medium to the cell surface, and finally be consumed by the cell (Klasson et al., 1991). A general summary of the mass transfer resistances is as follows (Klasson et al., 1991):

1. Diffusion through the bulk gas to the gas-liquid interface.
2. Movement across the gas-liquid interface.
3. Diffusion of the gas through the liquid film adjacent to the bubble and into the bulk liquid.

4. Transport of the dissolved gas through the bulk liquid to the film surrounding the microorganism.
5. Transport across the cell envelope to the intracellular reaction site.

The Wood-Ljungdahl Pathway

As early as 1891, Russian microbiologist Winogradsky proved that some bacteria are capable of growth using CO_2 as the sole carbon and energy source (Wood, 1991). In 1942, researchers at the University of Wisconsin isolated an organism, *Clostridium thermoaceticum*, which was able to anaerobically ferment one mole of glucose into 3 moles of acetic acid (Wood, 1991). Metabolism of glucose to acetic acid in *C. thermoaceticum* involves the Embden-Meyerhof-Parnas glycolytic pathway, first converting glucose to pyruvate, then further metabolizing to acetic acid and CO_2 (Ljungdahl, 1986). The overall reaction is summarized in Equation 16 (Ljungdahl, 1986):

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 8H^+ + 8e^- \quad (16)$$

As shown in the above equation, only 2 moles of acetic acid are produced in these initial steps. Further research demonstrated that the third mole of acetic acid was produced by CO_2 fixation, representing a total synthesis of an organic compound from CO_2, shown below (Ljungdahl, 1986; Wood, 1991):

$$2CO_2 + 8H^+ + 8e^- \rightarrow CH_3COOH + 2H_2O \quad (17)$$

The CO_2 formed by glucose fermentation acts as an electron acceptor in the formation of the final mole of acetic acid (Ljungdahl, 1986). Researchers have shown

that *C. thermoaceticum* is capable of growth and acetic acid production on either H_2/CO_2 or CO (Ljungdahl, 1986). The pathway used to convert CO_2 into acetic acid has been named the Wood-Ljungdahl Pathway, and organisms using this pathway are termed homoacetogens (Müller, 2003).

The Wood-Ljungdahl pathway, shown in Figure 2-4, proceeds as follows: (1a) a series of enzyme-catalyzed reactions reduce one CO_2 molecule to a methyl group; (2a) the methyl group is bound to a corrinoid iron-sulfur protein (CFeSP in Figure 2-2); (3a) this protein transfers the methyl group to CO dehydrogenase (CODH), which plays an essential role in the pathway (Müller, 2003); (1b) in a parallel branch of the pathway, CODH converts another CO_2 molecule into CO; (3) the methyl group is condensed onto CODH along with CO, forming Acetyl-CoA; (4) phosphotransacetylase and (5) acetic acid kinase enzymes further convert Acetyl-CoA to acetic acid (Müller, 2003).

The Wood-Ljungdahl pathway enables growth on H_2/CO_2, which indicates it must be coupled to a net gain of ATP (Müller, 2003). One mole of ATP is consumed in the formyl-H_4F synthetase reaction, and another mole of ATP is produced by substrate-level phosphorylation in the acetate kinase reaction (Müller, 2003). Since the net ATP gain from substrate-level phosphorylation is zero, ion gradient-driven phosphorylation must occur as well (Müller, 2003). This ion gradient is used by either H^+ or Na^+ translocating ATP synthetases to generate the energy needed for chemolithotrophic growth (Müller, 2003).

Overview of Acetogens

Acetogens are diverse in origin, inhabiting soils and sediments, manures, and sewage (Ljungdahl, 1986), in addition to extremes of temperature, pH, and salinity (Müller, 2003). A common bond between the acetogens is the production of acetic acid as a primary or only product (Ljungdahl, 1986). Several of the most promising acetogens from an industrial perspective are discussed below. Although research is ongoing with respect to many of these organisms, there are currently no industrial-scale facilities using these organisms to convert synthesis gas components to acetic acid. Clearly, much research is needed to capitalize on the vast potential of synthesis gas fermentation to acetic acid.

Clostridium aceticum was the first pure culture obtained of an acetogenic bacterium that could produce acetic acid from H_2/CO_2 when it was isolated in 1936 (Ljungdahl, 1983). The culture was lost from 1948 until 1981, when it was recovered from a spore preparation of the original strain (Ljungdahl, 1983). *C. aceticum* is a spore-forming bacterium that grows chemolithotrophically on H_2/CO_2 at 30ºC at a pH of 8.3 (Ljungdahl, 1983).

Due to the loss of *C. aceticum*, *C. thermoaceticum* was the only acetogen available for study for nearly 40 years (Daniel et al., 1990). These studies led to the discovery of the Wood-Ljungdahl pathway, and our present understanding of acetic acid synthesis from one-carbon compounds (Ljungdahl, 1983). *C. thermoaceticum* was isolated from horse manure in 1942, and grows optimally at 60ºC and a pH of 6.8 (Ljungdahl, 1986). This organism grows on CO alone with an 18-hour doubling time,

theoretically producing 1 mole of acetic acid and 2 moles of CO_2 for every 4 moles of CO (Grethlein and Mahendra, 1992).

Acetobacterium woodii is an anaerobic, non-spore-forming rod isolated from pond sediment in 1977 (Ljungdahl, 1983). This culture is capable of utilizing a number of one-carbon substrates including H_2/CO_2, formate, and methanol/CO_2, to produce acetic acid by a mechanism similar to that of *C. thermoaceticum* (Ljungdahl, 1983). *A. woodii* grows at 30ºC at neutral pH (Ljungdahl, 1983).

Acetogenium kivui is a non-spore-forming, nonmotile, thermophilic, anaerobic rod (Ljungdahl, 1983). *A. kivui* is incapable of growth on CO alone (Daniel et al., 1990). This inability to grow on CO alone exists either because the acetyl-CoA pathway is altered in a way that prevents total acetic acid synthesis from CO, or because the utilization of CO is not effectively coupled to energy conservation or anabolic processes (Daniel et al., 1990). This species, isolated from lake sediment, grows optimally at 66ºC and a pH of 6.4 (Ljungdahl, 1986).

Butyribacterium methylotrophicum was isolated from sewage in 1980, and grows at 39ºC and pH 7.5 (Ljungdahl, 1986). *B. methylotrophicum* is perhaps the most metabolically versatile among organisms capable of anaerobic CO metabolism (Worden et al., 1997). This organism can grow on 100% CO, H_2/CO_2, methanol, formate, and glucose (Worden et al., 1997). *B. methylotrophicum* is capable of producing acetic acid, butyrate, and ethanol from CO, and contains a unique direct metabolic pathway from CO to butanol (Worden et al., 1997). At pH 6.8, this organism produces acetic acid and butyrate, with acetic acid production favored at a molar ratio of 32:1 (Grethlein and

Mahendra, 1992). Lowering the pH to 6.0 results in equimolar formation of acetic acid and butyrate (Grethlein and Mahendra, 1992).

Eubacterium limosum has been isolated in several habitats, including human intestine, sewage, rumen, and soil (Chang et al., 1999). *E. limosum* is capable of growth on CO_2/H_2, CO, and methanol/CO_2 at a temperature of 39ºC and pH 7.2 (Ljungdahl, 1986). Growth of this organism on H_2 and CO_2 results in a 14-hr doubling time, with acetic acid as the primary product, and small amounts of butyrate as a secondary product (Grethlein and Mahendra, 1992). When grown on 50% CO, *E. limosum* exhibited a seven-hour doubling time with acetic acid as the only product (Grethlein and Mahendra, 1992). When grown with 40% CO, *E. limosum* has been shown to produce CO_2 and acetic acid as fermentation products, favoring CO_2 at a molar ratio of approximately 2:1 (Genthner and Bryant, 1982).

Peptostreptococcus productus has shown the highest growth rate among CO-metabolizing bacteria (Grethlein and Mahendra, 1992). *P. productus* is a mesophilic, anaerobic coccus isolated from sewage sludge (Lorowitz and Bryant, 1984). *P. productus* has a doubling time of 1.5 hours when grown on 50% CO (Grethlein and Mahendra, 1992). At CO concentrations of 30-50%, approximately 1 mole of acetic acid is produced for every 4 moles of CO consumed (Lorowitz and Bryant, 1984).

Clostridium ljungdahlii, a rod-shaped, motile, gram-positive, strict anaerobe, was originally isolated from chicken waste (Gaddy and Clausen, 1992). This organism can produce ethanol and acetic acid from CO/H_2O, CO_2/H_2, or synthesis gas (Gaddy and

Clausen, 1992). The overall stoichiometry for the fermentation of synthesis gas to ethanol and acetic acid by *Clostridium ljungdahlii* is as follows (Klasson et al., 1992):

$$6CO + 3H_2O \rightarrow CH_3CH_2OH + 4CO_2 \quad (18)$$

$$2CO_2 + 6H_2 \rightarrow CH_3CH_2OH + 3H_2O \quad (19)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (20)$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (21)$$

Under optimal growth conditions with pH 5.0-7.0, *C. ljungdahlii* ferments synthesis gas to a mixture with acetic acid as the major product and ethanol as the minor product (Gaddy and Clausen, 1992). In batch fermentations at pH 5.0 with synthesis gas as a substrate, a product ratio of 0.05 moles of ethanol per mole of acetic acid was reported, with an ethanol concentration of less than 1 g/L (Gaddy and Clausen, 1992). At lower levels of yeast extract (0.005-0.05% versus 0.1-0.2%), the molar product ratio of ethanol to acetic acid was increased to 0.11 (Gaddy and Clausen, 1992). Under non-growth conditions with no yeast extract and pH 4.0, ethanol concentrations exceed acetic acid concentrations, with ethanol concentrations reaching 7 g/L compared to acetic acid concentrations of 1 g/L (Gaddy and Clausen, 1992). These results suggest that non-growth conditions favor ethanol production, and growth conditions favor acetic acid production (Gaddy and Clausen, 1992).

Synthesis Gas Fermentation Techniques

A number of approaches have been taken to optimize microbial conversion of synthesis gas to acetic acid and other products. The low solubility of synthesis gas

components under fermentation conditions is a problem in these processes. On a mass basis, carbon monoxide and hydrogen solubilities in water are 60% and 4% that of oxygen, respectively (Bredwell et al., 1999). Much of the work in this area has focused on innovative fermentor configurations designed to attack these mass transfer issues. Additional areas of research include media optimization and culture acclimation.

Batch Synthesis Gas Fermentations

Batch cultures are often the starting point for bacterial research. Gaddy et al. used 125 mL serum bottles with crimp tops in their batch studies for acetic acid production with *P. productus* (Klasson et al., 1991; Vega et al., 1989b). In these experiments, the bottles were half-filled with liquid medium and placed horizontally on a shaker incubator in the dark at 37ºC and 100 rpm (Vega et al., 1989b). The production medium was prepared by growing the organism in a rich medium containing vitamins, minerals, sodium bicarbonate, reducing agent, and 0.4% yeast extract, in the absence of CO (Vega et al., 1989b). When growth ceased, the medium was filtered to remove all microorganisms and aerated to remove dissolved gases (Vega et al., 1989b). This "spent" medium was added in place of the yeast extract in the production medium, so that the organisms had the benefit of the vitamins and minerals in yeast extract without an added carbon source (Vega et al., 1989b). Seed cultures were prepared by allowing a small inoculum of cells to grow overnight on an 80% CO, 20% CO_2 gas mix (Vega et al., 1989b). This acclimated seed resulted in reduced lag phase, providing cells in the exponential phase of growth for production experiments (Vega et al., 1989b). Prior to

inoculation, 1.5 mL of sodium sulfide was added to the bottles to ensure the low redox potential required by anaerobic bacteria (Vega et al., 1989b). The bottles were inoculated with a 5 mL seed culture (Vega et al., 1989b). The bottles were removed from the incubator for about 3 minutes during sampling (Klasson et al., 1991). Gaddy et al. employed similar techniques with other cultures, including *C. ljungdahlii* and *Methanosarcina barkeri* (Klasson et al., 1991).

Daniel et al. conducted experiments to determine acetic acid production rates from H_2 and CO using *C. thermoaceticum* and *A. kivui* (Daniel et al., 1990). These experiments used 27.2 mL culture tubes with butyl rubber-stoppered crimp seals (Daniel et al., 1990). Each tube was given a 0.5 mL inoculum and incubated at 55ºC without agitation under a gas headspace composition of 30% CO, 30% CO_2, and 40% N_2 (Daniel et al., 1990).

Advanced Fermentor Designs for Synthesis Gas Fermentations

Gaddy's group also conducted experiments using a Continuous Stirred Tank Reactor (CSTR). The CSTR experiments were conducted using a 750 mL Chemostat bioreactor with a 350 mL working volume (Klasson et al., 1991). This system allowed adjustment of a variety of parameters including agitation rate, temperature, pH, and gas and liquid flow rates (Klasson et al., 1991). Worden's group used a 0.3 μm cellulosic membrane in cross-flow mode for a CSTR with cell recycle, and Gaddy's group added a hollow fiber membrane unit for cell recycle, and extraction and distillation columns to this system (Worden et al., 1997; Gaddy, 1998). Another method of increasing CSTR

performance is to operate two CSTRs in series, with the first reactor used to promote cell growth, and the second to promote production of the desired chemicals (Klasson et al., 1991). Gaddy's group used this technique accompanied by shifts in pH, dilution rate, and medium components, to increase ethanol production (Klasson et al., 1991).

A CSTR bioreactor can operate under either mass transfer-limited or kinetically-controlled conditions (Vega et al., 1989a). Kinetically-controlled conditions exist when the rate of microbial uptake is lower than the maximum possible rate of transport (Vega et al., 1989a). These conditions are achieved using high liquid dilution rates, resulting in low cell concentrations and various steady states during reactor operation (Vega et al., 1989a). Mass transfer-limited operation of CSTRs occurs when the dissolved gas concentration is zero, i.e. cellular uptake is equal to gas input (Vega et al., 1989a). Systems operating under these conditions have maximum energy efficiency in that all power input for agitation is being utilized to promote gas-to-cell contact (Vega et al., 1989a). Mass transfer-limited operation in a CSTR provides several benefits over kinetically-controlled operation, including the elimination of inhibitory effects due to high concentrations of dissolved gases, efficient operation, and high production rates (Vega et al., 1989a).

Immobilized cell reactors (ICRs) can greatly increase the cell densities in the bioreactor while minimizing energy input for mixing (Klasson et al., 1991; Worden et al., 1997). Reactor configurations for ICRs include packed bubble columns and trickle-bed reactors (Klasson et al., 1991). These systems use countercurrent flow of gas and liquid and a gas pressure drop through the column rather than mechanical agitation (Klasson et

al., 1991). Cell immobilization techniques are classified as either entrapment, enclosure of a catalyst within a membrane or gel, or carrier binding, in which cells are directly bound to water-soluble carriers by physical adsorption or ionic or covalent bonds (Klasson et al., 1991). The immobilized cells are then packed in a column or fluidized bed (Worden et al., 1997). Advantages of ICRs include minimized diffusion resistance due to direct contact of the substrate (synthesis gas) with the catalyst (cells), nearly plug-flow performance resulting in kinetic advantages, higher cell densities, lower gas retention times, and lower energy costs (Klasson et al., 1991; Gaddy, 1998). For the same retention time and mass transfer rate, bubble column bioreactors have been shown to have 15% higher conversion than CSTRs (Vega et al., 1990). Disadvantages of ICRs include the fact that overgrowth of cells can lead to channeling in the reactor (Klasson et al., 1991).

High-pressure reactors have also been studied for synthesis gas fermentations. High-pressure fermentation increases CO uptake, and decreases the necessary reactor volume (Vega et al., 1990). The potential disadvantage to these reactors is higher levels of dissolved CO, which may be inhibitory to the organisms at the increased levels (Vega et al., 1990). Gaddy et al. experimented with a 600 mL bench top reactor with gas feed and release lines, gas and liquid sampling ports, a pressure gauge and a pressure release valve at 4.2 MPa, and mechanical agitation (Klasson et al., 1991). In experiments with *P. productus*, Gaddy et al. found that increasing the pressure in a stepwise fashion resulted in CO consumption at pressures as high as 14.6 atm (Vega et al., 1990). Gradually

increasing cell growth during each step maintained low dissolved CO concentrations and avoided cellular inhibition due to CO (Vega et al., 1990).

Another approach to reduce the mass transfer limitations of synthesis gas fermentation is the use of microbubble dispersions. Conventional bubbles in fermentors have a diameter of 3-5 mm, but surfactant-stabilized microbubbles can have diameters on the order of 50 μm (Bredwell and Worden, 1998). Microbubbles are formed by creating a localized high shear zone at the gas-liquid interface (Bredwell et al., 1999). Bredwell et al. used a high-speed spinning disk rotated at several thousand rpm within a few millimeters of a stationary baffle (Bredwell et al., 1999). Bubbles drawn into the high shear zone are broken into smaller sizes, and surfactants absorbed at the interface stabilize the bubbles (Bredwell et al., 1999). The surfactants Tween (polyoxyethylene sorbitans) and Brij (polyoxyethylene alcohols) were used to stabilize microbubbles in these experiments (Bredwell et al., 1997). These surfactants form a layer surrounding the microbubble that forms a diffuse electric double layer, creating ionic and/or stearic repulsion between bubbles, preventing coalescence (Bredwell et al., 1997; Bredwell et al., 1999). Dispersions of microbubbles exhibit colloidal properties and are stable enough to be pumped (Bredwell et al., 1997). In mass transfer-limited systems, the mass transfer rate is inversely proportional to the bubble diameter, suggesting the potential for order-of-magnitude increases in the mass transfer rate using microbubbles (Bredwell and Worden, 1998). In order to be useful in synthesis gas fermentations, however, the surfactant must be capable of microbubble stabilization at levels that are non-toxic to the cells and have no negative effects of product formation (Bredwell et al., 1997).

Table 2-1. World Capacity for Virgin Acetic Acid Production by Technology (Johnson, 2000)

Methanol Carbonylation	60%
Acetaldehyde Oxidation	18%
Ethyl Alcohol	10%
Butane/Naphtha Oxidation	8%
Other	4%
Total	100%

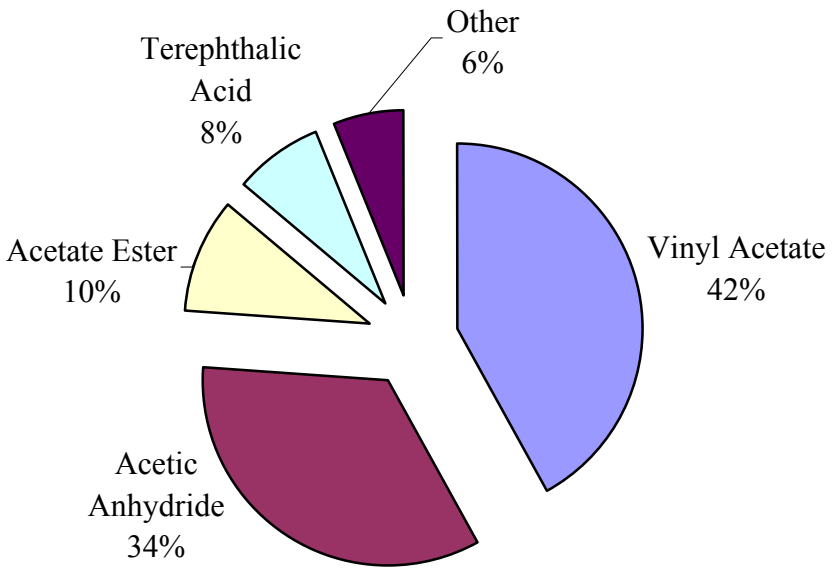

Figure 2-1. Acetic Acid Uses (Kirschner, 2003b)

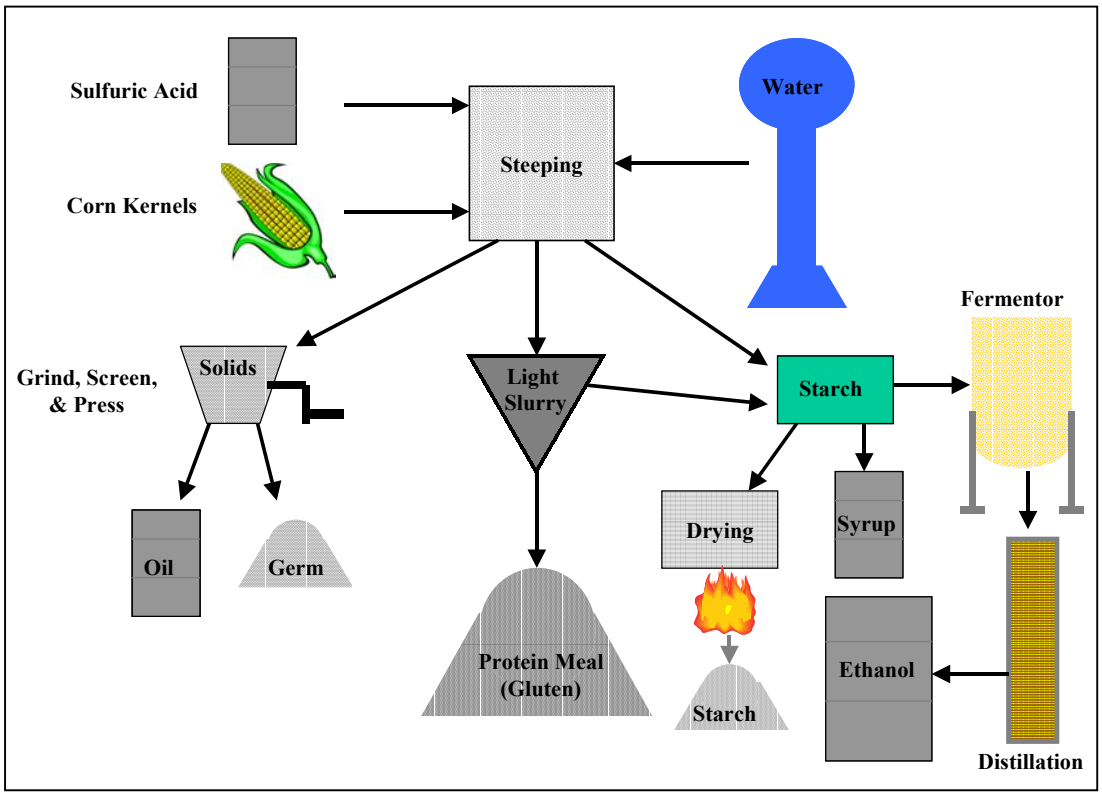

Figure 2-2. The Wet Milling Process

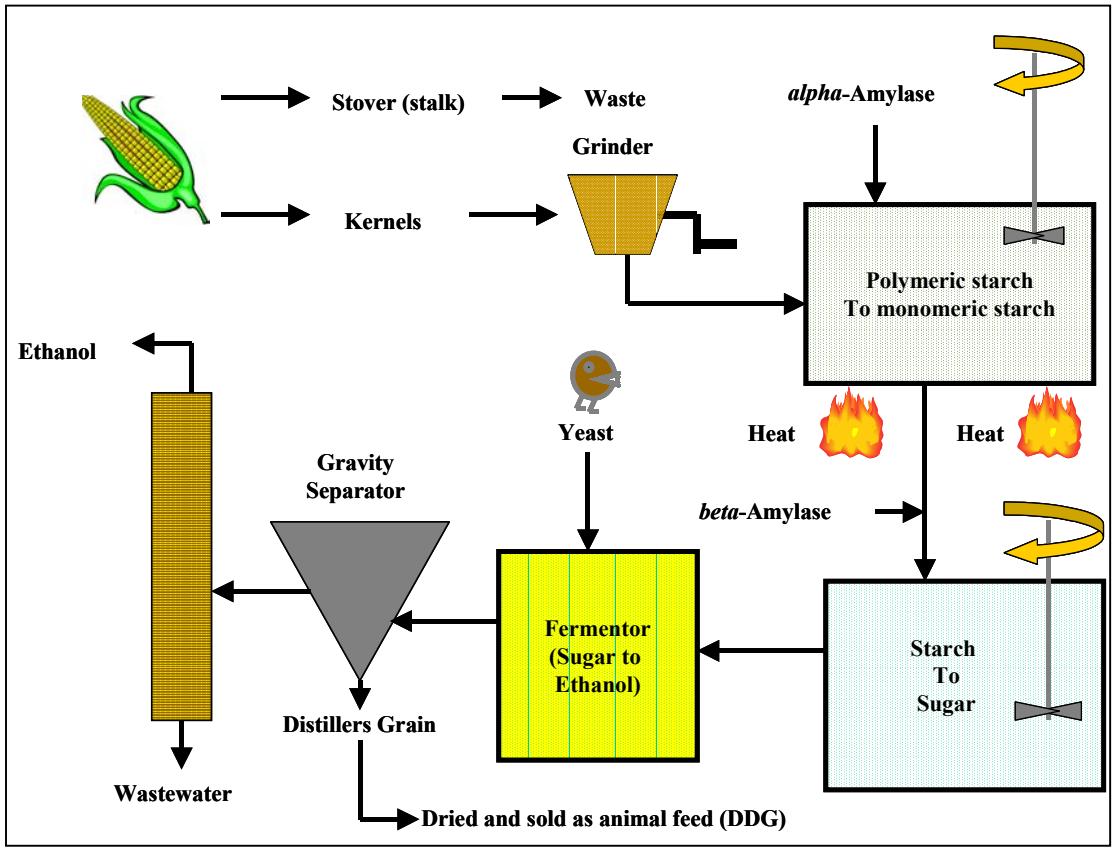

Figure 2-3. The Dry Milling Process

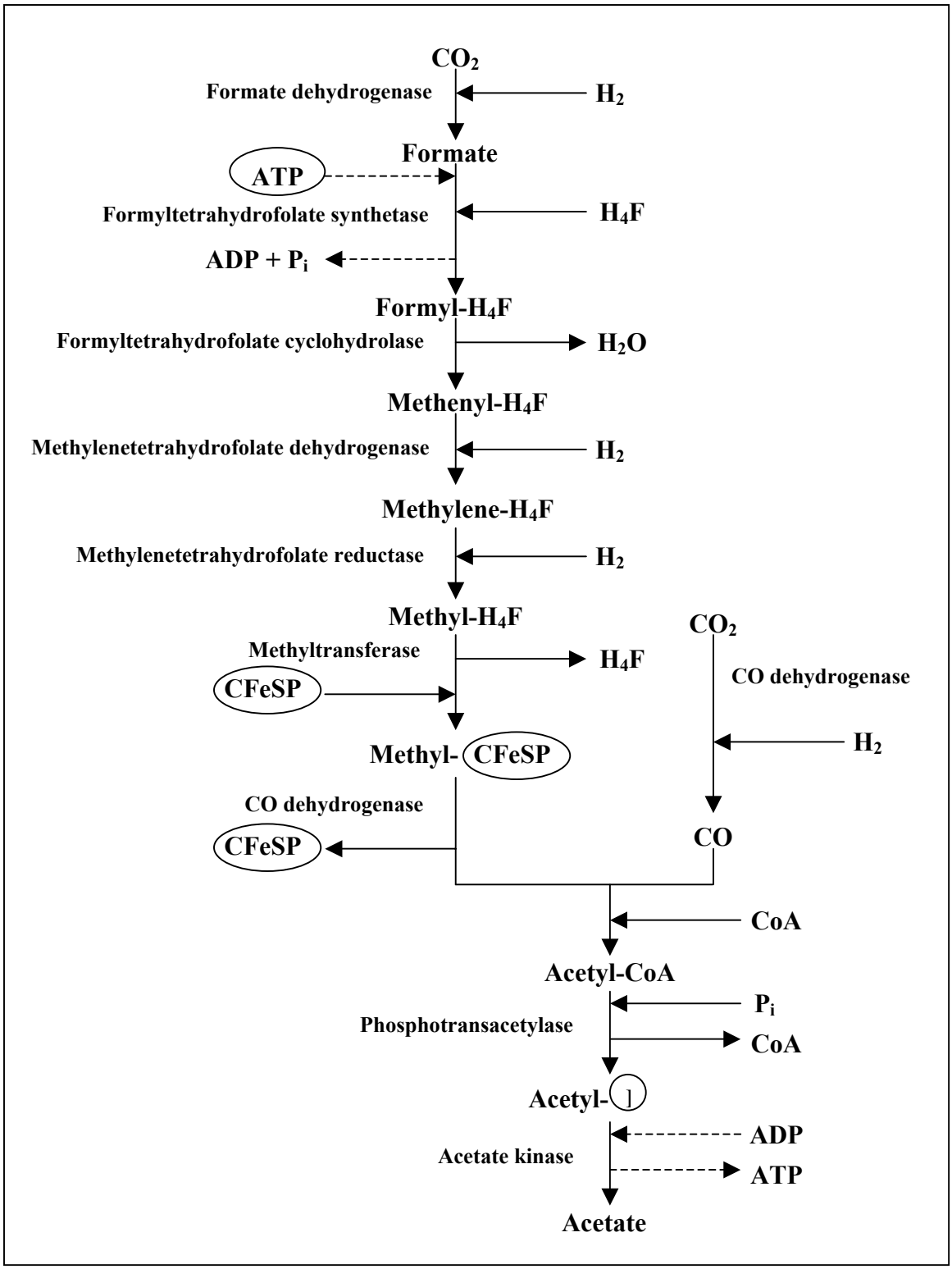

Figure 2-4. The Wood-Ljungdahl Pathway (Müller, 2003)

CHAPTER 3

MATERIALS AND METHODS

Materials

Bacterial Cultures

A number of different bacterial cultures were evaluated during the performance of this research. Two cultures, *Clostridium ljungdahlii* and *Clostridium thermoaceticum* were purchased from the American Type Culture Collection (ATCC). *C. ljungdahlii* (ATCC Number 55383) is an anaerobic bacterium originally isolated from chicken waste, and has been shown to produce acetate and ethanol from CO or CO_2 and H_2 at 37ºC (Gaddy and Clausen, 1992). *C. thermoaceticum* (ATCC Number 39073) is a thermophilic anaerobe that is capable of producing acetate from CO or CO_2 and H_2 at 55ºC (Daniel et al., 1990).

Additional cultures were developed from various sources, including anaerobic wastewater treatment lagoons, bovine stomachs, oil well drilling cuttings, and pond sediments. Samples from these sources were collected and evaluated for their ability to convert synthesis gas to acetate or ethanol. Details on attempts to isolate cultures are presented in the Methods Section of this thesis.

Fermentors

All bacteria were cultured in Fisherbrand 40 mL EPA vials. Acetate production experiments originally used these vials along with Si/PTFE caps. Si/PTFE caps consist of open-top polypropylene closures with 0.005" PTFE/0.120" silicone rubber septa. The PTFE side of the septum faces into the vial, while the silicone side faces out. Later experiments used mininert valve caps (Figure 3-1) from VICI Precision Sampling (Baton Rouge, Louisiana) to prevent leakage of headspace gases.

Vials for growth and production experiments were placed on a New Brunswick Scientific (Edison, New Jersey) C24 Incubator-Shaker for agitation at 400 rpm and incubation at 37ºC.

Media Preparation

Several different media formulations were used to promote cell growth or acetic acid production. ATCC recommends 1754 PETC Medium (hereafter referred to as 1754 Medium, or simply 1754) for growth of *C. ljungdahlii* and ATCC 1203 CM4 Medium (1203 Medium, or 1203) for growth of *C. thermoaceticum* (ATCC, 2004). Another medium also tried with both bacteria was Difco Reinforced Clostridial Medium (RCM) obtained from Fisher Scientific. RCM was purchased in powder form and prepared by adding 39 g of the powder per liter of distilled water. Mineral Salts Medium (MSM), a commonly used microbiological medium, was modified by adding 5 g/L fructose and 1 g/L yeast extract to support mesophilic growth. Detailed recipes for all growth media

formulations are presented in Tables 3-1 through 3-4. All chemicals used in growth and production media were obtained in technical or laboratory grades from Fisher Scientific (Hampton, New Hampshire) or Sigma-Aldrich (St. Louis, Missouri).

Acetate Production Medium (APM) (Table 3-5) was based on the formula defined in a patent by Gaddy and Clausen on the production of ethanol via synthesis gas fermentation (Gaddy and Clausen, 1992). The pH of the APM was adjusted to 7.0 rather than 4.5, which is recommended for ethanol production (Gaddy and Clausen, 1992). Acetate Production Medium #2 (APM2) (Tables 3-6 through 3-9) was based on the formula defined in a later patent by Gaddy involving the production of acetic acid from synthesis gas fermentation instead of ethanol (Gaddy, 1998). APM2 is a more complex media formulation than APM, containing a different mineral salts solution, yeast extract, trypticase, sodium bicarbonate, and reducing agents, in addition to the vitamins and trace minerals of APM.

Both growth and production media were prepared in a similar manner. Chemicals were measured using a Mettler Toledo (Columbus, Ohio) AG204 balance in Fisherbrand Disposable Polystyrene Weigh Dishes and added into a flask or beaker. When all components were added, the flask or beaker was placed on a Corning (Acton, Massachusetts) Stirrer/Hot Plate magnetic stir plate with a stir bar, and allowed to mix thoroughly. The pH of the medium was adjusted using a Fisher Accumet AP62 Portable pH Meter with an AccuTupH probe, and 1M KOH or 1M HCl. The medium was then placed into Fisherbrand 40 mL EPA vials using a Wheaton Adjustable-Volume Self-Refilling Repetitive Syringe (Millville, New Jersey). The vials were capped loosely and

placed in the autoclave for sterilization. A Steris Amsco Century SG-120 Scientific Gravity Sterilizer Autoclave (Mentor, Ohio) was used to sterilize the medium at 121ºC and 15 psi for 15 minutes.

Following sterilization, the caps of the vials were tightened and the vials were placed in an anaerobic glove bag. The glove bag used was a Coy Laboratory Products Flexible Anaerobic Chamber (Grass Lake, Michigan), equipped with catalyst boxes and an airlock chamber. Once the vials were placed in the glove bag, the caps were again loosened to allow oxygen to diffuse out of the headspace due to the oxygen gradient between the vial and glove bag atmosphere.

For some experiments, solid media were used in an attempt to isolate individual bacterial colonies. These experiments used either 1754 Agar or APM Agar. Both agar formulations were prepared by adding 17 g/L of Difco Laboratories Granulated Agar (Livonia, Michigan) to the media described above. The medium was stirred and heated to boiling on a Corning Stirrer/Hot Plate to dissolve the agar. The medium containing dissolved agar was placed in 250 mL Wheaton Media Bottles and autoclaved. After autoclaving, the agar was poured onto Fisherbrand Standard Sterile Polystyrene petri dishes and allowed to harden. The agar plates were placed in the glove bag for later use. Solid media were incubated in the glove bag in a Fisher Scientific Isotemp Incubator Model 625D.

Sampling Equipment

Liquid samples were taken using Becton Dickinson 3 mL luer-lok sterile syringes with 23 gauge needles (Franklin Lakes, New Jersey). All liquid samples were filtered using Millipore Isopore Membrane Filters (0.2 μm) (Bedford, Massachusetts) fitted with Millipore Swinnex filter holders prior to analysis. Samples of 1.5 mL were removed from the vials and filtered, yielding approximately 1 mL of liquid for analysis. Gas samples were taken with a Hamilton 100 μL Gastight Syringe (Reno, Nevada).

Chemical Analysis

Acetic acid in the liquid phase was quantified using a Waters HPLC System (Milford, Massachusetts). The Waters System consisted of a 515 Pump, a 717-Plus Autosampler, and a 2487 Dual λ Absorbance Detector. The HPLC used a Waters YMC ODS-AQ S-5 120 Å column (150 x 4.6 I.D., S-5 μm, 12nm), and the corresponding Waters YMC ODS-AQ S-5 120 Å guard column (4.0 x 23 mm threaded guard). The mobile phase for the HPLC was 20 mM NaH_2PO_4 with a pH of 3.5.

Ethanol in the liquid phase was analyzed using an Agilent 6890N Gas Chromatograph with a Flame Ionization Detector (Palo Alto, California). The GC was equipped with an Agilent 7683 Series Autosampler and Injector. The column used was an Agilent Innowax (30m x 0.250 mm, 0.25 μm).

Headspace gases were analyzed throughout the production experiments to determine uptake rates for the various synthesis gas components, and to assess potential

air leakage into the vials. Gas analysis was performed using an Agilent 6890N Network Gas Chromatograph System with a Thermal Conductivity Detector and a manual injection port. The system used a column selection method fitted with two Supelco columns (Bellefonte, Pennsylvania): 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). Calibration standards were prepared for carbon dioxide, oxygen, nitrogen, and carbon monoxide. Hydrogen could not be directly quantified using this gas chromatography setup. Although other components, such as water vapor, were likely present, it was assumed for the purposes of this research that the remaining volumetric balance of samples contained hydrogen.

Optical density was measured using a Hach Chemical (Loveland, Colorado) Pocket Colorimeter with a 580 nm Generic wavelength. This wavelength is typical for microbiological studies of this nature (Phillips et al., 1994).

Methods

Vial Cap Testing

This experiment was conducted to determine which of 3 vial cap types would perform best with respect to maintaining the headspace gas composition. A 1% resazurin in water solution was prepared for use as an oxygen indicator in this experiment. Resazurin solution reacts with oxygen, causing the solution color to change from colorless to bright pink when low levels of oxygen are present. Simultaneously boiling and sparging nitrogen into the solution prior to the experiment removed dissolved oxygen. Six vials were prepared using each of three types of vial cap: crimp tops, Si/PTFE screw caps, and mininert valve screw caps. Crimp tops were used with matching 20 mL vials, while the other caps were used with 40 mL vials. Vials were filled halfway with 1% resazurin solution (10 mL for crimp vials, 20 mL for others) in the anaerobic glove bag. All vials were capped with the appropriate tops and removed from the glove bag for gassing.

The vials were gassed with 100% CO using techniques outlined in the Gassing Techniques Section. Three vials with each cap type were placed on the shaker incubator at 37ºC. The remaining vials were placed in the 37ºC incubator in the anaerobic glove bag as controls. Gas samples of all vials were taken every 24 hours for the first 72 hours of the experiment. At the 168-hour mark, the caps were given 10 punctures with a gassing syringe needle prior to sampling. These punctures were given to simulate repeated sampling and determine whether oxygen was introduced into the vials during

sampling. Vials were observed throughout the experiment for color changes, which would indicate oxygen entry into the vials.

Cell Mass Quantification (Colorimeter Calibration)

The colorimeter was calibrated in order to correlate optical density readings with dry cell masses. As in the growth experiments, a rack of cells was grown in growth medium. The cells were concentrated as above, but resuspended into 35 mL of the same growth medium rather than APM. An optical density measurement was taken using the colorimeter and recorded. The cell concentrate was then diluted by a factor of 2. Another colorimeter measurement was taken, and the solution was again diluted by a factor of 2. This serial dilution continued until the colorimeter reading was below 0.05.

These colorimeter measurements were then compared to actual dry mass using the original cell concentrate. Ten milliliters (10 mL) of the cell concentrate were weighed on the balance in a pre-weighed Fisherbrand Aluminum crimped weighing dish with a 43-mL capacity. The dish was placed in a 90ºC oven overnight to evaporate the water. When dried, the dish was removed and weighed again to determine the mass of the solids. A calibration curve could then be prepared to correlate the optical density measurements to dry mass values. This curve was later used to quantify cell mass changes in growth experiments. Calibration curves for *Clostridium ljungdahlii*, *Clostridium thermoaceticum*, and the JAC-1 culture are presented in Figures 3-2 through 3-4.

Gassing Techniques

Synthesis gas was formulated on-site for use in the acetic acid production experiments. The apparatus used for gas mixing is shown in Figure 3-5. Compressed gas tanks filled with carbon monoxide, carbon dioxide, and hydrogen were obtained from Nexair (Memphis, Tennessee). The regulators for the tanks were configured such that the downstream pressure on each tank was 20 psi. The gas delivery apparatus was plumbed using 1/8" Stainless Steel Swagelok fittings. Relative pressures for the tanks were monitored using Gilmont Accucal Regulatory-Valved Rotameters (Barrington, Illinois). The rotameter flows were adjusted to volumetrically set the total synthesis gas composition. Synthesis gas composition was verified using a gas chromatograph.

The gases were introduced into the vials using a sterile gassing syringe. The gassing syringe (Figure 3-6) consisted of a pre-sterilized 10-cc glass syringe stuffed with glass wool with a rubber stopper on the plunger end. A glass tube inserted through a hole in the stopper connected the syringe to the tube leaving the gas mixing apparatus.

All gassing procedures were performed under a fume hood. The mininert valves on the vials were opened to insert the needle of the gassing syringe. The synthesis gas was allowed to flow into the sealed vial for 10-15 seconds to create a slightly positive pressure inside the vial and prevent oxygen from entering the headspace. At that time, the vial cap was loosened to purge the headspace of nitrogen. Synthesis gas flowed continuously into the vial for 2 minutes, and then the vial cap was tightened for the final 10-15 seconds to create a slight positive pressure within the vial headspace.

Bacterial Screening

Additional cultures from various bacterial sources were collected and evaluated for their ability to convert synthesis gas into acetate or ethanol. Although acetic acid production was of primary interest for this research, related research by Christine Morrison could benefit if an ethanol-producing organism was identified. For solid or sludge sample sources, 10 g of sample were placed anaerobically into 40 mL vials containing 10 mL of sterile APM. For liquid sample sources, 10 mL were placed into vials containing 10 mL of sterile APM. The vials were capped with mininert valves to limit the loss of headspace gases. The vials were then gassed with synthetically mixed synthesis gas as above, and initial headspace analysis was performed. The gassed vials were placed on the shaker incubator and allowed to incubate for one week. At that time, the headspace was again analyzed to determine the uptake of synthesis gas components. Liquid analyses were performed to measure acetate and ethanol production.

If after two weeks there were no substantial changes in headspace gas concentrations or there were no ethanol and acetate produced, studies were discontinued on that source. However, if the bacterial samples demonstrated CO or CO_2 uptake (greater than 20% reduction after 2 weeks) and/or ethanol or acetate production (greater than 10 ppm in the liquid phase after 2 weeks), further studies were performed to develop the cultures. At that point, the cultures were transferred (5 mL inoculums) into 15 mL of sterile APM, gassed, and incubated as before. Consortia that continued to uptake synthesis gas and produce ethanol or acetate at rates equal or greater than those presented

above were anaerobically streaked onto 1754 Agar in petri dishes in an effort to isolate the acetate and ethanol producers. The streaked plates were incubated anaerobically for several days or until colonies were visible.

Individual colonies were selected and transferred from the plates to vials containing 20 mL of 1754 Media. These vials were placed on the shaker incubator at 37ºC. Once a sufficient stock of cells was obtained, Gram stains were performed to determine the bacterial purity of the sample. Cultures determined to be pure based on these Gram stains were transferred (1-2 mL inoculums) into additional vials of 1754 for growth and production studies. Impure samples were again streaked onto 1754 Agar and treated as above.

Vial Slants for Culture Isolation

Another technique used for isolating cultures from bacterial consortia was a vial slant. A schematic of this technique is presented in Figure 3-7. Vial slants were prepared by allowing APM Agar to solidify in a 40-mL vial placed on its side, i.e. horizontally. The cultures were streaked onto the agar and the vial was gassed with 100% CO as the sole carbon and energy source. Mininert caps were used to maintain the headspace gas composition (i.e. prevent leakage). Single colonies that demonstrated growth on CO were isolated from the slants and tested to determine their ability to produce acetate and/or ethanol. Numerous cultures were isolated using this technique, with JAC-1 showing the most promise for acetate production.

Cell Growth Experiments

Vials of growth media were inoculated with 0.5-2.0 mL of a culture suspension, depending on the cell density of the inoculum source: the higher the cell density of the source, the smaller the inoculum. The vials were placed on the shaker incubator at 37ºC. Cell growth was monitored by changes in solution optical density.

Acetate Production Experiments

The acetate production experiments were run in triplicate using two types of controls. Gas controls contained 20 mL of APM with no cells. These controls were gassed with the same synthesis gas mixture used with the test vials. The purpose of the gas controls was to determine vial leakage rates and gas dissolution rates under sterile conditions. Cell controls contained 15 mL of APM with 5 mL of cell concentrate. These controls were given no synthesis gas; the headspace contained the same gas mix as the anaerobic glove bag: 95% nitrogen, 5% hydrogen. The cell controls were run to measure the rate of acetate production from residual growth medium components and dead cell organic matter.

When the cells in the growth medium reached a significant density, they were harvested for use in the acetate production experiments. A rack of 24 vials of growing cells was removed from the shaker incubator, with four vials set aside to inoculate the next batch of growth medium, and the remaining 20 vials taken into the anaerobic glove bag along with ten 40 mL centrifuge tubes. Two vials of growth medium, each

containing approximately 20 mL of cells and medium, were poured into each of the ten centrifuge tubes. The tubes were capped and removed from the glove bag. The tubes were weighed and counterbalanced on a Sorvall RT 6000 D Centrifuge (Asheville, North Carolina). The tubes were centrifuged for 20 minutes at 4000 rpm and then replaced in the glove bag. Once in the glove bag, the liquid decantate was removed from the tubes, leaving the cell pellet. The first pellet was resuspended in 35 mL of APM and vortexed using a Fisher Vortex Genie 2. This tube of suspended cells was then transferred into the next tube and vortexed, and so on until all the pellets had been concentrated into a single tube with 35 mL of APM. The 35 mL of cell concentrate was then distributed into the test vials (5 mL/vial in 3 vials) and the cell controls (5 mL/vial in 3 vials), with the remaining 5 mL used for dry cell mass analysis.

All 9 vials were capped with mininert caps in the anaerobic glove bag. These vials were removed from the glove bag for gassing. Synthesis gas was mixed on site and the vials were gassed as described above.

The vials were placed on the shaker incubator at 37ºC. At specified time intervals, the vials were removed from the incubator for liquid and gas sampling. Liquid sample collection was performed in the anaerobic glove bag. When samples had been taken, the vials were replaced on the shaker incubator for further incubation.

Table 3-1. Growth Media Formulations

ATCC 1754 PETC Medium is recommended for growth of *C. ljungdahlii*. Mineral Salts Medium (MSM) is a standard salts medium used for mesophilic cultures, and our modified version also contains fructose, yeast extract, and trace elements. Peptone-Yeast-Fructose (PYF) Medium was also used with mesophilic cultures. ATCC 1203 CM4 Medium recommended for growth of *C. thermoaceticum*.

	Amount (per 1.0 L)			
Medium Component	1754 PETC	Modified MSM	PYF	1203 CM4
NH_4Cl	1.0 g	1.0 g	---	---
$(NH_4)_2SO_4$	---	---	---	1.3 g
KCl	0.1 g	---	---	---
$MgSO_4 \cdot 7H_2O$	0.2 g	0.2 g	---	---
$MgCl_2$	---	---	---	0.75 g
NaCl	0.8 g	---	---	1.0 g
KH_2PO_4	0.1 g	0.38 g	---	1.5 g
K_2HPO_4	---	---	---	2.9 g
$CaCl_2 \cdot 2H_2O$	0.02 g	---	---	13.2 mg
$FeCl_3$	---	0.5 g	---	---
$FeSO_4$ (1.25% by weight)	---	---	---	0.1 mL
Resazurin (1.0% by weight)	---	---	---	0.2 mL
Sodium thioglycollate	---	---	---	0.5 g
Peptone	---	---	5.0 g	---
Yeast extract	1.0 g	1.0 g	10.0 g	5.0 g
Fructose	5.0 g	5.0 g	5.0 g	---
Cellobiose	---	---	---	6.0 g
Trace Elements[1]	10.0 mL	10 mL	---	---
Wolfe's Vitamin solution[2]	10.0 mL	---	---	---
Reducing agent[3]	10.0 mL	---	---	---
Distilled water	980 mL	990 mL	1000 mL	1000 mL
Solution final pH	5.9	7.0	6.0	---

1. See Table 3-2
2. See Table 3-3
3. See Table 3-4

Table 3-2. Trace Elements

Medium Component	Amount
Nitrilotriacetic acid	2.0 g
$MnSO_4 \cdot H_2O$	1.0 g
$Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$	0.8 g
$CoCl_2 \cdot 6H_2O$	0.2 g
$ZnSO_4 \cdot 7H_2O$	0.2 mg
$CuCl_2 \cdot 2H_2O$	20 mg
$NiCl_2 \cdot 6H_2O$	20 mg
$Na_2MoO_4 \cdot 2H_2O$	20 mg
Na_2SeO_4	20 mg
Na_2WO_4	20 mg
Distilled water	1.0 L

Table 3-3. Wolfe's Vitamin Solution

Medium Component	Amount
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 B12	0.1 mg
p-Aminobenzoic acid	5.0 mg
Thioctic acid	5.0 mg
Distilled water	1.0 L

Table 3-4. Reducing Agent

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

Table 3-5. Acetate Production Medium

Medium Component	mL/1L medium
Mineral solution	50
Trace minerals	5
B-vitamin solution	20
Distilled water	925

Mineral solution	g/L	Trace minerals	g/L
$(NH_4)_2SO_4$	10	Nitrilotriacetate	1.5
NH_4Cl	10	$MgSO_4 \cdot 7H_2O$	6.1
KH_2PO_4	136	NaCl	1.0
B-vitamins	mg/L	$FeSO_4 \cdot 7H_2O$	0.1
Biotin	20	$CoCl_2 \cdot 6H_2O$	0.1
Folic acid	20	$CaCl_2 \cdot 2H_2O$	0.1
Pyridoxal HCl	10	$ZnCl_2$	0.1
Thioctic acid	60	$CuCl_2 \cdot xH_2O$	0.01
Riboflavin	50	$AlK(SO_4)_2 \cdot 12H_2O$	0.01
Thiamine HCl	50	H_3BO_3	0.01
Calcium-D-Pantothenate	50	$Na_2MoO_4 \cdot 2H_2O$	0.01
Vitamin B12	50	$NiCl_2 \cdot 6H_2O$	0.05
P-Aminobenzoic acid	50	Na_2SeO_3	0.0005
Nicotinic acid	50	$MnSO_4 \cdot H_2O$	0.5

Table 3-6. Acetate Production Medium #2 (APM2)

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
$CaCl_2 \cdot 2H_2O$	0.06 g
$NaHCO_3$	2.0 g
Resazurin (0.01%)	1.0 mL
Distilled Water	920.0 mL

1. See Table 3-7
2. See Table 3-8
3. See Table 3-9

Table 3-7. Salt Solution for APM2

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
$MgSO_4 \cdot 2H_2O$	1.25 g
Distilled Water	1000 mL

Table 3-8. Trace Metal Solution for APM2

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

Table 3-9. B-Vitamin Solution for APM2

Medium Component	Amount
Pyridoxal HCl	10 mg
Riboflavin	50 mg
Thiamine HCl	50 mg
Nicotinic acid	50 mg
Calcium-D-Pantothenate	50 mg
Lipoic acid	60 mg
p-Aminobenzoic acid	50 mg
Folic acid	20 mg
Biotin	20 mg
Vitamin B12	50 mg
Distilled Water	1000 mL

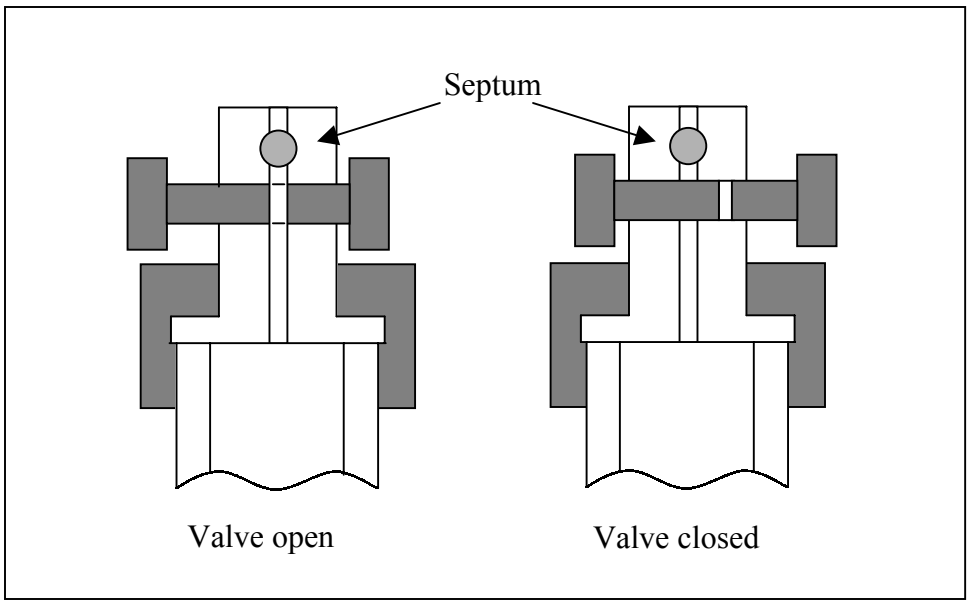

Figure 3-1. Mininert Valve Caps

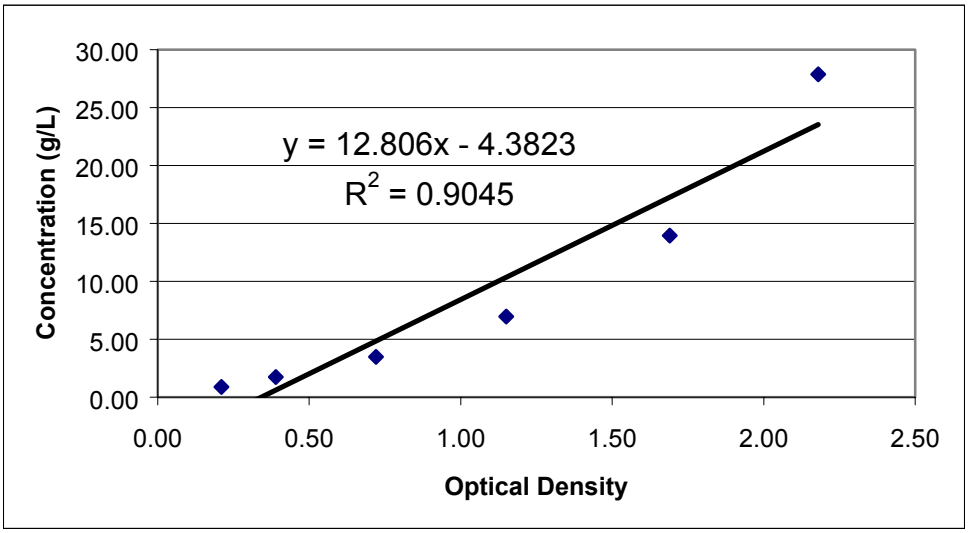

Figure 3-2. Cell Density vs. Optical Density for *Clostridium ljundahlii*

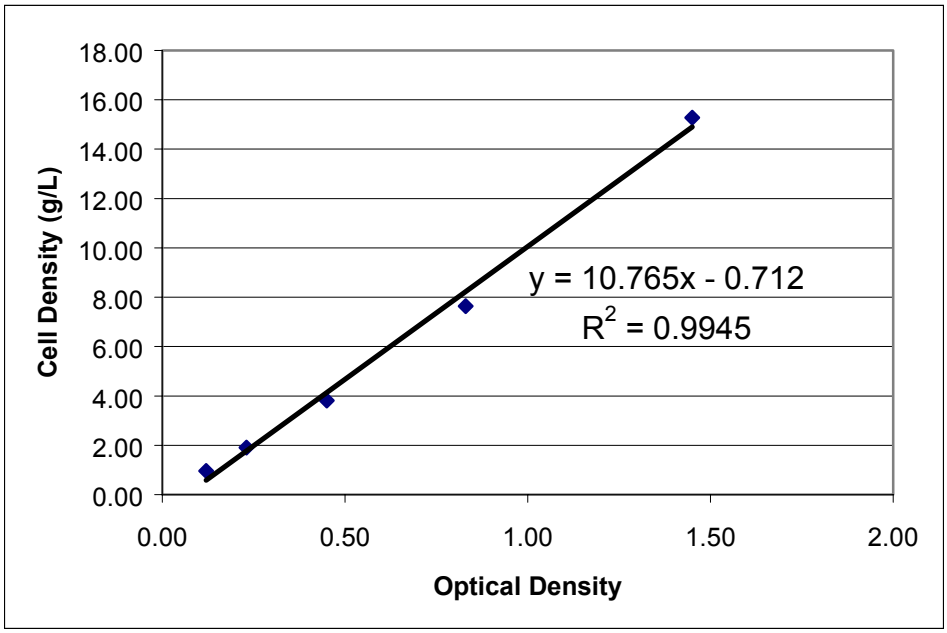

Figure 3-3. Cell Density vs. Optical Density for *Clostridium thermoaceticum*

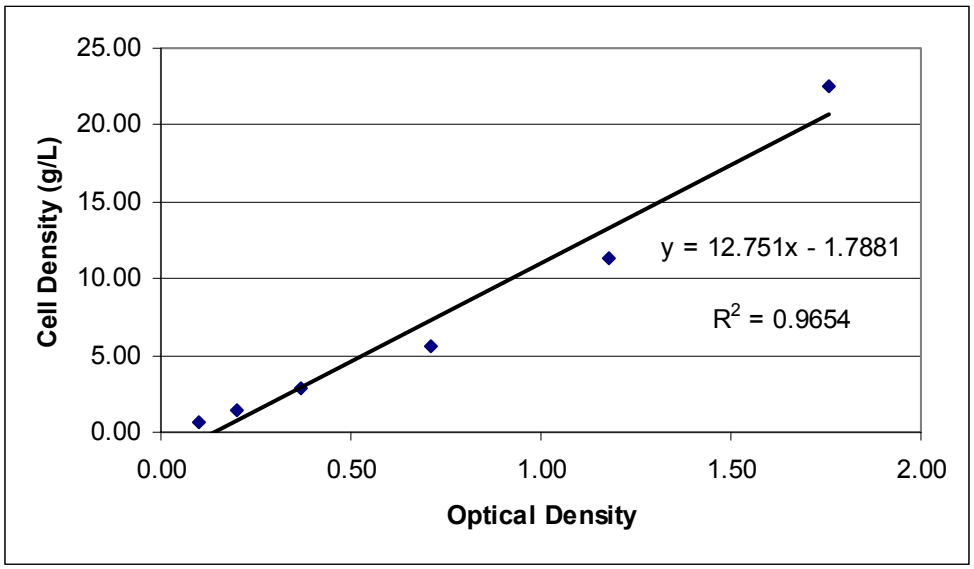

Figure 3-4. Cell Density vs. Optical Density for JAC-1 Culture

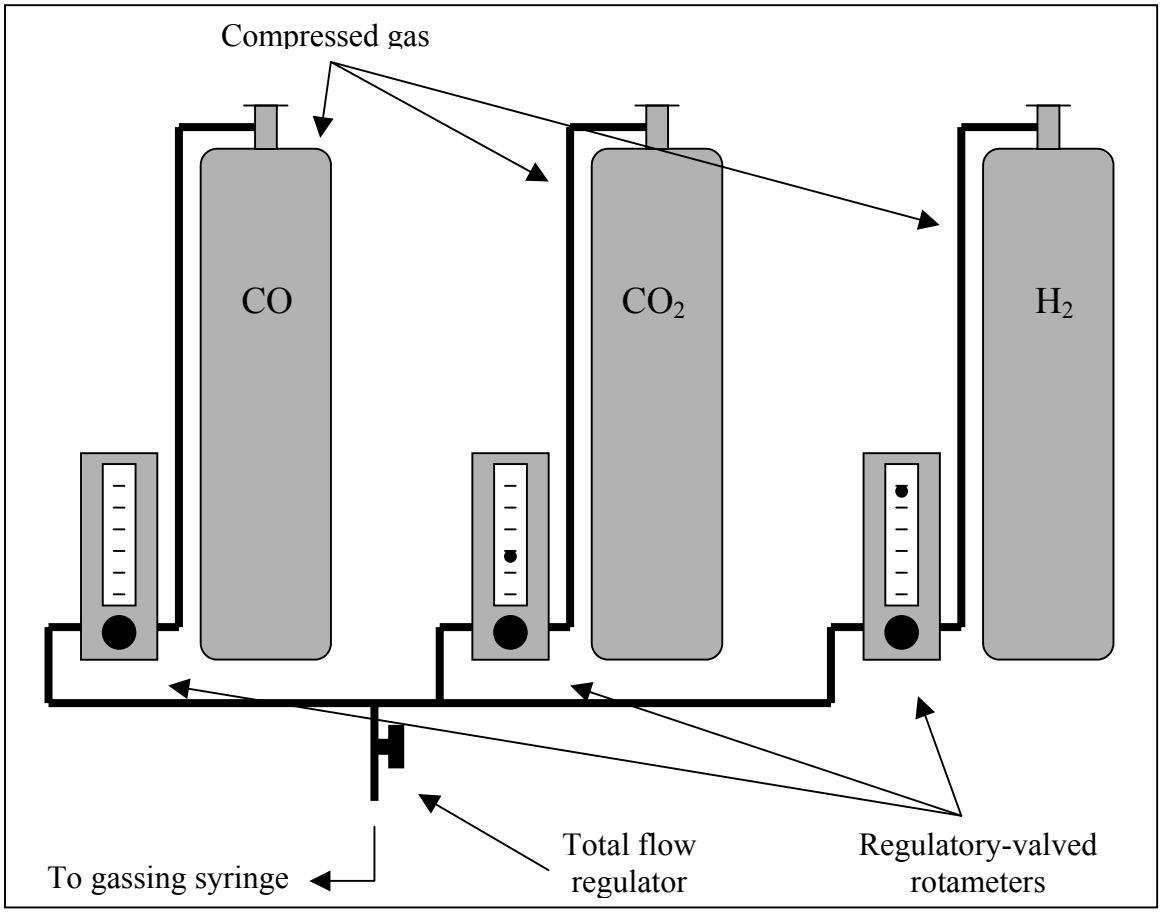

Figure 3-5. Gassing Apparatus

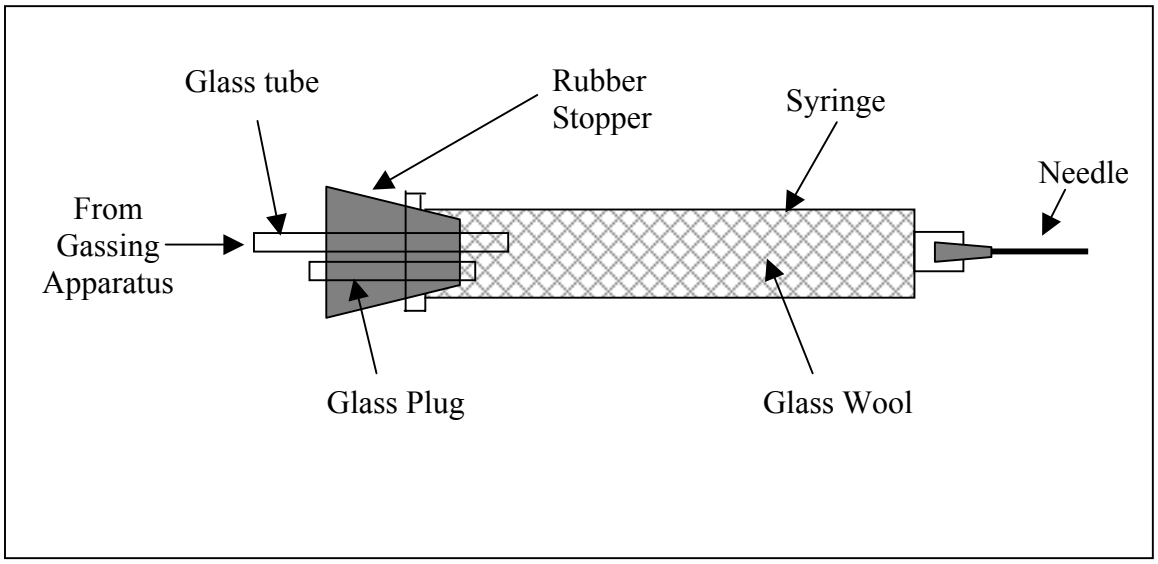

Figure 3-6. Gassing Syringe

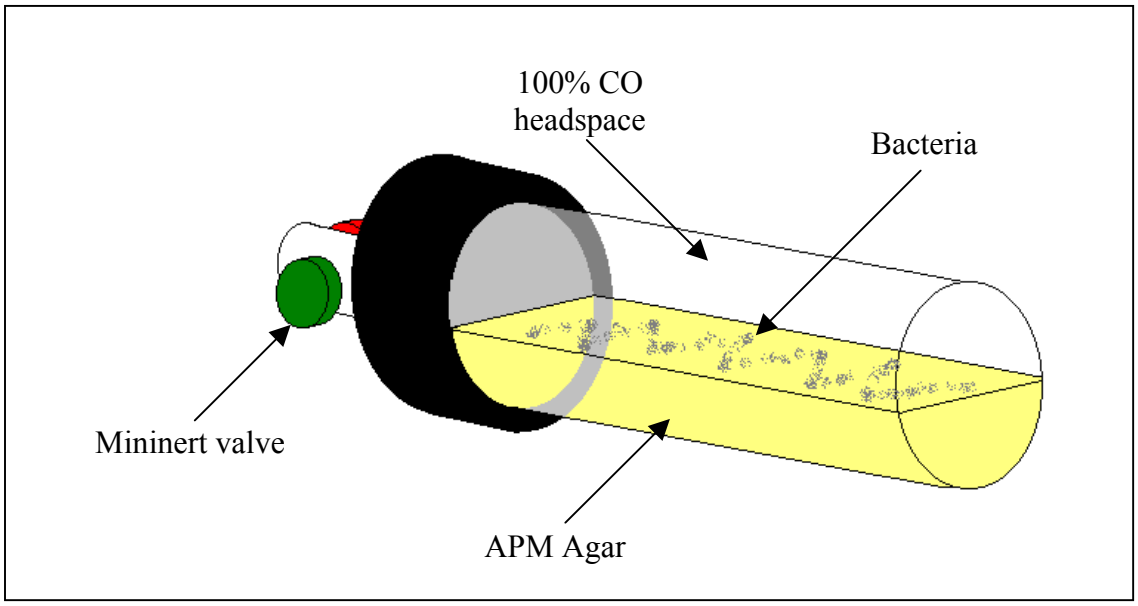

Figure 3-7. Vial Slant Schematic

CHAPTER 4

RESULTS

Overview

The original intent of this research project was to optimize a process in which synthesis gas was fermented to acetic acid via a microbial catalyst. As will be presented in the following pages, deficiencies with existing experimental techniques required a change in the overall experimental scope. The vast majority of the experiments presented in this chapter were hindered with unforeseen problems, making direct comparison of results with those found in the literature difficult. Experimental hurdles were identified throughout the research, and rectified whenever possible; however, the final, fully corrected system was employed in only the most recent experiments.

Although many problems were identified during early experiments, the results of Parts I and II are qualified by the fact that many of the setbacks were not anticipated. Commonly used microbiological techniques for culture handling and experimentation were employed in this research. *Clostridium ljungdahlii* and *Clostridium thermoaceticum* had been demonstrated many times in the literature to produce acetic acid from synthesis gas using similar techniques. Dr. Lewis Brown's research team had very promising results with the MSU-1 consortium, and good results were expected from JAC-1, an isolate from the MSU-1 consortium. The microorganisms used in this research

proved to be more sensitive than expected, and in many cases, well-established microbiological practices needed adjustments in order to be better suited to these experiments.

Isopropanol contamination, which resulted from commonly used sterilization techniques, hindered growth and acetate production for the bacteria in these experiments. Leaking vial caps prevented accurate evaluation of carbon monoxide uptake and allowed oxygen to inhibit and possibly kill strictly anaerobic microorganisms. Culture handling and sampling techniques for thermophilic microorganisms proved inadequate. These problems and their solutions are discussed in detail in Part III of this chapter.

While the results in Parts I and II were of little use toward the original goal, they demonstrated the need for a shift in the project's purpose, with that shift being oriented toward methods development and/or improvement. Part III presents a detailed description of the methods development resulting from the work presented in the earlier sections. Techniques and equipment were re-evaluated to identify and eliminate the problems of Parts I and II. Part IV employs these new techniques in order to determine their effectiveness toward technology development.

Part I. Proof of Concept with Known Homoacetogens

Preliminary experiments were conducted to reproduce the results presented in the literature related to acetate production from synthesis gas. These experiments were designed to use the same organisms and media from the literature in order to establish a benchmark to be used for evaluation of novel cultures. The organisms of primary interest for these experiments were *Clostridium ljungdahlii* and *Clostridium thermoaceticum*.

Clostridium ljungdahlii Growth Experiments

Several media formulations were evaluated to determine how best to grow *C. ljungdahlii*. ATCC 1754 PETC Medium, the growth medium recommended by the American Type Culture Collection for *C. ljungdahlii*, was used as the benchmark in these experiments. Peptone is a complex nutrient, containing carbon and amino acids, which can positively affect the growth of microorganisms. The 1754 PETC Medium, Acetate Production Medium, and Mineral Salts Medium (defined in the Materials and Methods chapter of this thesis) were augmented with varying levels of peptone to determine the best growth medium for *C. ljungdahlii*. The results of these experiments are presented in Figures 4.1-1 through 4.1-3, and the raw data for these experiments are presented in Appendix C.

Data points which seem to lie outside the trend, such as the sudden drop at 43 hours for 1754, can be explained by the fact that each data point in this experiment set represents a separate vial. As a result, some of the vials may have received a smaller number of cells initially despite having the same inoculum volume. Although the

medium for all the vials was prepared in large batches, some vials may have contained slightly different amounts of certain medium components due to imperfect mixing. Other possible causes of vial-to-vial variability include chemical or biological contaminants, worn caps with varying leakage rates that allowed oxygen into the vials, and human error associated with measuring the reagents. For these reasons, it is important to observe the overall trend in the data rather than to concentrate on individual data points.

Figure 4.1-1 shows the growth of *C. ljungdahlii* on 1754 with peptone. The same general growth trend is observed for all levels of peptone. The optical density is initially approximately 0.15, increases to nearly 0.35 after 24 hours, and plateaus to approximately 0.40 at 96 hours. These data suggest that adding peptone at 0.1, 0.5, and 1.0 g/L to 1754 does not significantly affect the growth of *C. ljungdahlii* within this medium. These data are not particularly surprising, since 1754 is already a very rich medium, containing 5 g/L fructose and 1 g/L yeast extract. Apparently, the additional nutrients are not necessary, and thus provide little benefit to the growth of *C. ljungdahlii*.

Growth of *C. ljungdahlii* on APM with peptone is shown in Figure 4.1-2. The goal of this experiment was to determine whether APM, which was already being used to promote acetate production in our experiments, could be augmented to promote growth as well. Using the same or a similar medium in both the growth and production phase of the process could potentially reduce scale-up costs by eliminating the time and energy requirements for transferring the cells from growth medium to production medium prior to fermentation. At 0.1 g/L peptone, the final optical density is 0.25, while the optical density for 1754 is 0.43. Increasing the peptone level to 0.5 and 1.0 g/L results in final

optical densities of 0.19 and 0.18, respectively. APM with peptone does not promote the rapid cell growth needed for this process; therefore, APM with peptone should not be used to grow *C. ljungdahlii*. Unlike 1754, APM contains no additional carbon source. The peptone added to the medium was the only source of carbon present for cellular growth. Gaddy et al. used a medium similar to APM containing 2 g/L yeast extract and 3.5 g/L sodium bicarbonate to promote growth of *P. productus* (Vega et al., 1989b), so adding additional nutrients to APM may indeed promote the necessary growth of *C. ljungdahlii*.

MSM with peptone showed mixed results for growth of *C. ljungdahlii* (Figure 4.1-3). Adding 0.1 g/L peptone and 5 g/L fructose resulted in very slow growth (0.16 initial optical density to 0.24 final optical density). Increasing the peptone to 1.0 g/L caused a decrease in the optical density over time. Adding 5.0 g/L peptone to MSM initially resulted in a high growth rate, but after 30 hours, the optical density began to decline. When using MSM and low levels of peptone (1.0 g/L), there are not enough nutrients to support rapid growth of *C. ljungdahlii*. Supplementing MSM with 0.1 g/L peptone and 5 g/L fructose caused slow growth, but still resulted in a much lower final optical density than 1754 (0.24 for MSM + 0.1 g/L peptone + 5.0 g/L fructose, versus 0.43 for 1754). MSM + 5.0 g/L peptone out-performed 1754 up to 30 hours of incubation, but after 96 hours, 1754 resulted in more sustained growth of *C. ljungdahlii*.

Adding 0.1 g/L peptone and 5 g/L fructose seems to have provided the necessary carbon, but perhaps lacked the vitamins and minerals needed for growth. Increasing the peptone level to 1 g/L and removing fructose provided the opposite effect, yielding more

amino acids, but less carbon. Using 5 g/L peptone supplied the carbon and nutrients needed for cell growth, resulting in the fastest growth seen in these experiments.

Comparing all the media over the 96-hour time frame of the experiments, 1754 performs as well or better than all the other formulations. If steady, sustained growth is desired for *C. ljungdahlii*, 1754 is the best medium studied in these experiments. However, for growing and harvesting *C. ljungdahlii* rapidly, MSM + 5.0 g/L peptone produced the highest observed optical density (0.47) after only 30 hours of incubation. A modified MSM may also be a simpler and more cost-effective medium than 1754, which contains small amounts of many different vitamins and minerals.

Production Experiments with *C. ljungdahlii* and *C. thermoaceticum*

A number of experiments were conducted to determine CO uptake and acetate production rates exhibited by *C. ljungdahlii*. The purpose of these experiments was to attempt to reproduce the results published by Gaddy (Gaddy, 1998). The experimental techniques are described in the section titled "Acetate Production Experiments" in the Materials and Methods chapter of this thesis. The results of two representative experiments are explained below. The results of all other experiments with *C. ljungdahlii* and an additional trial with *C. thermoaceticum* are presented in Appendix A, and raw data for all production experiments are presented in Appendix C.

As the data in Figures 4.1-4, -5, and -6 show, oxygen and nitrogen leaked into the vials during the experiment. Nitrogen was not an accurate indicator for vial leakage for two reasons: (1) nitrogen was present at small levels in the initial vial headspace due to

incomplete purging with the gas mixture, and (2) thorough mixing in the vial during incubation may lead to dissolved nitrogen leaving the liquid medium and entering the gas headspace. As a result, an "air concentration" was calculated by measuring the oxygen concentration and assuming stoichiometric composition of air entering the vial, i.e. approximately 1 mole of air for every 0.2 moles of oxygen.

Pressure or concentration gradients create driving forces that potentially cause air to enter the vials. One possibility is that as the microorganisms are utilizing one or more of the headspace gases, this generates a pressure gradient across the vial cap, causing a slight vacuum to be established within the vial. Air then enters the vial through a leaking cap or rushes in around the needle during sampling.

The headspace gas changes for *C. ljungdahlii* using Si/PTFE caps and an initial headspace composition of 2:1 ratio of H_2 to CO are shown in Figure 4.1-4. The figure shows a small, but not statistically significant, downward trend in the headspace CO concentration in abiotic controls and test vials. The air concentration in abiotic controls and test vials increased steadily to 15 to 20 uL/100uL after 48 h incubation.

Two possibilities exist for the results shown in Figure 4.1-4: (1) the organisms were able to fix hydrogen, creating a pressure gradient, or (2) leaking caps or sampling technique allowed air to enter the vials due to a concentration gradient. Since hydrogen was not analyzed directly using the GC method employed, hypothesis (1) cannot be ruled out; however, evidence presented later in this chapter suggests that (2) may be more likely.

Figure 4.1-5 shows the headspace gas changes for *C. ljungdahlii* using an 80% CO, 20% H_2 initial gas composition, and mininert caps. No statistically significant depletion of CO occurred in this experiment. In addition, the air concentration remains almost zero throughout the experiment. In this experiment, the pressure or concentration gradients discussed above were not created. The mininert caps seem to maintain the headspace composition, but the culture did not show CO uptake in this experiment. One possibility is that the culture was in the lag phase of growth, and was inactive during the experiment. Another possibility is that a more readily accessible carbon source was available to the cells, for example fructose inadvertently carried over from the growth medium.

Headspace changes for *C. thermoaceticum* are shown in Figure 4.1-6. The cells in this experiment were initially gassed with 60% CO, 20% H_2, and 10% CO_2, and incubated in a static 60ºC incubator. Mininert caps were used in all experiments with *C. thermoaceticum*. As in Figure 4.1-5 with *C. ljungdahlii*, the vials did not leak, but the cells did not uptake significant amounts of CO. A reason for this lack of activity may be a carryover of cellobiose (the carbon and energy source in the thermophilic growth medium 1203 CM4). A more likely cause, however, is the low solubility of CO at this temperature. The solubility of gases in liquids decreases as temperatures increase.

The solubility problem discussed above was perhaps compounded by the inability to effectively use the shaker incubator at 60ºC. The bacteria are very sensitive to small temperature changes, and the shaker incubator temperature was not as stable when set to 60ºC. As a result, a static incubator was used in these experiments. Without agitation,

gas dissolution can become a mass transfer limited step, causing the amount of dissolved gases within the bulk liquid to be greatly reduced. As described in the Literature Review chapter of this thesis, dissolution of gaseous substrates is one of the major mass transfer barriers for synthesis gas fermentations.

Figure 4.1-7 shows the acetate production by *C. ljungdahlii* in consecutive trials using a 2:1 gas mixture of H_2:CO. Other trials with this organism did not result in acetate production. In both trials, acetate production levels in biotic controls were similar to the production levels in test vials. As was explained in the Materials and Methods section, the purpose of biotic controls was to verify whether acetate observed in the test vials was produced from CO rather than other media carbon sources (e.g. fructose carryover from growth medium). The biotic controls contained cells, but no synthesis gas to use as a source of carbon and energy. The most likely cause of acetate production in the biotic controls was a carryover of fructose from the growth media. This carryover would also help to explain the low CO uptake rates in these experiments. Fructose dissolved in the medium is much more readily available to the cells than CO, which has a low solubility. Large standard deviations in these experiments are likely due to the complexity of the metabolic pathways used to produce acetic acid. Additionally, slight variations in the microenvironment can cause the cells to shift production from one compound to another.

Soon after the experiments presented were completed, a contaminant organism was identified in the vials thought to contain only *C. ljungdahlii*. This organism may have been present in earlier trials at lower levels. The contaminant organism was likely a competitor to *C. ljungdahlii* for carbon and energy, and could thus utilize fructose in the

growth phase and CO, acetate, or carryover fructose in the production phase. Bacterial contamination may also explain some of the variability in the results of these experiments. Neither significant CO uptake nor acetate production was observed in any experiments using *C. thermoaceticum*.

Part II: Development of Novel Cultures Capable of Converting CO to Acetic Acid

The data from Part I had not been fully analyzed when Part II was initiated. As a result, many of the same problems with experimental techniques became evident during these experiments. The purpose of these experiments was to evaluate two potential mesophilic homoacetogens identified at Mississippi State. Dr. Lewis Brown and his students in the MSU Department of Biological Sciences discovered a consortium called MSU-1, which could produce ethanol from synthesis gas. This consortium was also believed to have the potential for acetate production. A single organism from the MSU-1 consortium, JAC-1, was later isolated by the author using the vial slant technique described in the Materials and Methods section of this thesis. Both of these potential acetate producers were evaluated in order to determine their effectiveness relative to *C. ljungdahlii* and *C. thermoaceticum*. Results of a representative set of experiments with these cultures are presented below, with additional experiments presented in Appendix A. The raw data for these experiments are presented in Appendix C.

MSU-1 Production Experiments

Figure 4.2-1 shows the headspace gas changes for MSU-1 using Si/PTFE caps and an 80% CO, 20% H_2 initial headspace composition. The CO level in the abiotic controls and the test vials decreased at the same rate by approximately 20 uL/100 uL after 120 hours incubation. This decrease in CO is accompanied by an increase in air in the headspace. The potential mechanisms for vial cap leakage described in Part I of this chapter likely apply to this case.

Acetate production by MSU-1 in consecutive trials is shown in Figure 4.2-2. Both trials used Si/PTFE caps and a 2:1 initial headspace composition of H_2:CO. Acetate production in the test vials was not significantly higher than in the biotic controls, suggesting acetate production from carryover fructose within the growth medium. Net acetate production by this consortium was sporadic during these trials. Intermittent acetate production was likely due to scavenging organisms within the culture utilizing the products of the acetate-producers.

Unfortunately with the MSU-1 consortium, technical difficulties with the GC prevented gas sampling in early trials. Although gas samples were taken during later trials with this culture, no acetate production was observed in the liquid samples. Gram stains done during this period verified bacterial contamination of this consortium in the later trials.

JAC-1 Production Experiments

Bacterial consortia present a unique challenge for chemical production. Often these organisms work together to carry out a chain of chemical reactions, with one organism's product often used as another's reactant. The different organisms in a consortium are interdependent; therefore, a single isolate from the consortium may not show the same type of activity as all the organisms working in concert. Additionally, changes in the microenvironment may cause one organism to out-compete the others for nutrients, thus disrupting the entire system. For these reasons, the prospect of scaling-up a chemical production process based on a mixed culture is infeasible. Therefore, the

focus of this thesis was to eventually identify single isolates capable of acetate production from synthesis gas. In this vein, JAC-1 was isolated from the MSU-1 consortium to determine its ability to act alone as a homoacetogen.

Figure 4.2-3 shows the headspace gas changes for JAC-1 with mininert caps and an initial gas mix of 60% CO, 40% H_2. In this trial, the vials were sampled at 96 hours and re-gassed at 98 hours to determine the effect of repeated challenges to the culture. The CO level decreased significantly in the first 96 hours, with no increase in headspace air. This CO decrease was most likely due to cellular CO uptake, thus demonstrating the ability of JAC-1 to utilize CO. After re-gassing, air leaked into the vials, probably because of worn septa in the mininert caps.

Another test of JAC-1, showed almost no changes in the headspace gases (Figure 4.2-4). The trial used mininert caps and an initial headspace composition of 60% CO, 30% H_2, and 10% CO_2, which remained relatively constant during the experiment. This apparent lack of cellular activity was perhaps due to the loss of the JAC-1 culture, as Gram stains performed after this trial revealed bacterial contamination. Due to problems with the HPLC, liquid samples were not analyzed for JAC-1. The raw data for these experiments are presented in Appendix C.

Comparison of Cultures

Figure 4.2-5 compares CO uptake by MSU-1 with that of *C. ljungdahlii* when both cultures had the same initial headspace gas composition of 80% CO, 20% H_2. Both experiments were run with Si/PTFE caps. Under these conditions, *C. ljungdahlii* showed

no CO uptake, while MSU-1 showed a CO uptake of about 12%. Neither culture showed acetic acid production under these conditions.

A comparison of the CO uptake by all four cultures is shown in Figure 4.2-6. The initial headspace gas composition was identical (60% CO, 30% H2, and 10% CO_2) for these experiments, but Si/PTFE caps were used with *C. ljungdahlii* and MSU-1 while mininert caps were used with *C. thermoaceticum* and JAC-1. Under these conditions, *C. ljungdahlii* and JAC-1 showed the highest CO uptake, at 6-9%, with *C. thermoaceticum* showing some uptake, and MSU-1 showing none in this trial. Relatively low CO uptake by each of these cultures could be the result of the mass-transfer limitations of the batch system employed. Batch fermentations rely on simple agitation, e.g. stirring or shaking, to promote transfer of substrates from the gas phase to the aqueous phase. The mass transfer surface area for these systems is fixed based on the size of the vial and the volume of liquid medium. More advanced reactor designs often bubble the synthesis gas through the liquid, dramatically increasing the surface area for gas-to-liquid mass transfer. While batch fermentations can be useful for evaluating the ability of cultures to ferment synthesis gas to acetic acid, production rates are likely to increase if a fermentor design with greater mass-transfer efficiency is employed.

The fact that JAC-1 has a high CO uptake suggests the potential for high production rates of acetate or other valuable products. Although further experiments with JAC-1 might have confirmed this potential, the JAC-1 isolate was lost to bacterial contamination. This problem and others identified during the experiments of Parts I and II will be discussed in more detail in the following section.

Part III: Evaluation and Improvement of Techniques

The results of Part I and II demonstrated a necessity for improved techniques. A complete evaluation of equipment and procedures was performed in order to develop a more effective experimental system.

Si/PTFE caps were originally used in all experiments. Si/PTFE caps consist of open-top polypropylene closures with 0.005" PTFE/0.120" silicone rubber septa. The PTFE side of the septum faces into the vial, while the silicone side faces out. Mininert valves were used after concerns were developed over the gas leakage rates of Si/PTFE caps. Preliminary results showed lower gas leakage rates with mininert valves. Mininert valves consist of a PTFE cap with a butyl rubber septum. A diagram of a mininert valve cap is presented in the Materials and Methods chapter of this thesis.

Gaddy et al. used crimp-top bottles in batch synthesis gas fermentation experiments with positive results (Vega et al., 1989b). Crimp-top vials were not used in any production experiments presented in this thesis, but were studied as a potential replacement for mininert and Si/PTFE caps. These 20 mL borosilicate glass vials used a fitted cap consisting of a butyl rubber stopper and an aluminum crimp seal. Once the caps were attached, or crimped, to the vials, they could not be removed, which preventing reuse and increasing their cost relative to other vial cap configurations.

Initial results indicated high leakage rates from vials using Si/PTFE caps. Based on preliminary results, mininert caps were thought to reduce the leakage rates; so later experiments used that cap type. Figure 4.3-1 compared the results of earlier tests with *C. ljungdahlii* using a 2:1 gas mix of H_2:CO. The data represented in the graph were the

results of 72-hour samples from vials with Si/PTFE caps versus mininert caps. Clearly, the CO leakage was higher when Si/PTFE caps were used than with mininert caps. In addition, the vial-to-vial variation was higher with Si/PTFE caps. These results suggest that mininert caps reduce leakage rates and the variability between samples, i.e. mininert caps are more effective and more consistent. This hypothesis will be explored further in the following section. The raw data for these experiments are presented in Appendix C.

Evaluation of Crimp, Si/PTFE, and Mininert caps at 37ºC

Three cap types were investigated in the following experiment: crimp, Si/PTFE, and mininert caps. In this experiment, three vials with each cap type containing 20 mL APM were gassed with 100% CO and placed on the shaker incubator at 37ºC and 400 rpm agitation. Three more vials with each cap type were gassed and placed in an incubator at 37ºC in the anaerobic glove bag as controls (no agitation). The purpose of the controls was to determine whether CO leakage occurred through the caps during incubation or if CO leakage was due to sampling. Prior to the 168-hour samples, each cap was given 10 needle punctures to simulate the effect of additional sampling.

Figure 4.3-2 shows the changes in headspace CO for the cap test experiments. For crimp tops, the CO concentration decreased slightly throughout the experiment, showing approximately a 10% decrease after 72 hours for the glove bag experiments. On the shaker incubator, the decrease was on average about 15%, but statistically this change was not significantly higher than that in the glove bag. After the 10 needle punctures at 168 hours, the changes were on average larger for vials on the shaker incubator, but the

final headspace CO level for the vials on the shaker was within the standard deviation for the vials in the glove bag. Vials with Si/PTFE caps fared better than expected, showing no statistically significant change until the 10 punctures at 168 hours. It is interesting to note that with these caps, results in the shaker and glove bag were similar until the after repeated punctures. Results with mininert caps demonstrated almost no leakage until the 168-hour punctures. At that time, the decrease in headspace CO averaged approximately 10%.

Figure 4.3-3 compares the nitrogen level in the vials for this experiment. For crimp tops, the nitrogen in the headspace increases during the experiment to an average of 4 uL/100uL after 72 hours in the glove bag and on the shaker. At 168 hours, the repeated punctures result in a final average of nearly 8 uL/100 uL. Si/PTFE caps show a similar trend, reaching 4 uL/100uL by 72 hours. As with crimp tops, the Si/PTFE caps on average showed an increase at 168 hours. Mininert caps showed a less severe increase in nitrogen level during the first 72 hours, but a similar increase to the other caps after 168 hours.

Oxygen levels in the vials are compared in Figure 4.3-4. As with CO and N_2, the leakage rates and standard deviations for crimp tops are high. The Si/PTFE performed much better than expected, allowing no oxygen in the first 72 hours, and averaging 0.04 uL/100 uL O_2 after 10 needle punctures at 168 hours. Mininert caps also did well in the first 72 hours, showing an average of 0.08 uL/100 uL O_2. Continued needle punctures at 168 hours seemed to render the septa ineffective, causing an increase in oxygen to approximately 1 uL/100uL.

Overall, the Si/PTFE and mininert caps out-performed the crimp tops in these tests. Leakage for all vial caps seems to occur during sampling rather than incubation, since the leakage rates were similar for vials in the glove bag and in the shaker incubator. The reason for the poor performance of crimp tops was likely the slow recovery of the septa observed during and after sampling. The septa did not close quickly after being punctured by the needle, resulting in gas leakage during and shortly after sampling. Si/PTFE caps were more effective than preliminary tests indicated, suggesting the use of older, worn-out septa in some earlier experiments. Another possibility for the discrepancy between Si/PTFE cap results in these experiments and the results in preliminary tests is the continual refinement of gassing and sampling techniques by the students performing these experiments. Mininert caps performed reasonably well over a small number of injections. Repeated sampling, however, caused the butyl rubber septum to fail, allowing free exchange of headspace gases when the valve was opened.

Although the Si/PTFE caps performed well during this test, mininert caps may yield better long-term results. The advantage of using mininert caps is that the septa can be easily replaced during an experiment without disrupting the system, provided the valve is closed. The normal wear and tear can be neutralized by regularly replacing the septa. Based on the results, no significant leakage was observed until the 72-hour samples. Mininert caps should be used on condition that the septa are replaced after 10-12 injections, or approximately every 48-72 hours, to prevent leaking.

Evaluation of Mininert Caps at 60ºC

Thermophilic microorganisms that thrive at elevated temperatures, such as *C. thermoaceticum*, offer several advantages over mesophiles. Synthesis gas typically leaves the gasifier at temperatures over 1,300ºC (Gaddy and Chen, 1998), and cooling the gas to 60ºC rather than 37ºC may lead to significant energy savings over the operating life of the biorefinery. In addition, high temperatures increase reaction rates within thermophiles, resulting in increased production.

Mininert caps were used for all experiments incubated at 60ºC. The reasoning behind this was that preliminary data suggested high leakage rates for Si/PTFE caps relative to mininert caps at 37ºC. The results of the previous section suggest a different picture, with Si/PTFE caps performing as well or better than mininert caps under the same conditions. As stated above, mininert caps offer the advantage of replacing the septa during an experiment. In addition, since all previous experiments with thermophilic microorganisms used mininert caps, their performance needed to be evaluated under these conditions.

Figure 4.3-5 shows the headspace gas changes for vials with mininert caps containing 20 mL APM and a 100% CO initial headspace. On average, the trends suggest leakage of CO out of the vial and air into the vial. As the incubation time increases, however, the standard deviations in the data also increase, resulting in statistically negligible changes in the headspace gases during the experiment. The coefficient of variation (equal to the standard deviation divided by the mean) for CO and N_2 is approximately 20% at 48 h and 30% at 72 hours. For O_2, these values are even

higher, reaching 150% after only 24 hours. This high degree of variation among samples suggests that mininert caps may not be the best for use at this temperature.

One concern about operating at 60ºC is that the sampling procedure may produce unreliable results. Gases in the vials at 60ºC may contract rapidly as they enter the sampling syringe at room temperature. This contraction can cause a vacuum inside the syringe and lead to air entering the syringe to equalize the pressure gradient. This problem may occur at 37ºC also, but it has not been as noticeable in our experiments. A possible solution to this problem is preheating the syringe before sampling to the same temperature as the vials. Another option is to use locking sampling syringes.

Determination of Sampling Error

The purpose of this experiment was to determine the variation in the measured gas headspace concentration for vials with identical gas concentrations and the same researcher taking the sample. This sampling error was important in order to evaluate the precision of GC headspace concentration samples taken during the experiments presented in this thesis.

Empty vials without caps installed were placed in the anaerobic glove bag overnight and allowed to equilibrate to the glove bag atmosphere of 95% N_2 and 5% H_2. The vials were then capped with mininert caps and removed from the glove bag for sampling. A series of samples was taken and the standard deviation for all the analyses was calculated. This standard deviation, 1.82 uL/100uL or 1.82%, is the sampling error

for GC headspace concentration samples presented in this thesis. The data for this experiment are presented in Appendix C.

Determination of Optimum Gassing Time

Experiments were conducted to determine the effect of gassing time on headspace composition. The purpose of these experiments was to determine the gassing time required for the headspace of the vials to reach the desired headspace composition, i.e. the composition set using the regulatory-valved rotameters of the gassing apparatus (description and diagram provided in the Materials and Methods chapter of this thesis). Vials with mininert caps containing 20 mL APM were gassed with 100% CO for 30, 60, 90, 120, or 150 seconds, and sampled immediately to determine the headspace composition.

Figure 4.3-6 shows the headspace composition versus the gassing time. After 30 seconds the headspace composition was on average 34% N_2, 66% CO. As the gassing time increased, more of the nitrogen was purged from the system. After 60 seconds, the composition was approximately 90% CO, 10% N_2, and for 90 seconds the composition was 95% CO, 5%N_2.

As the gassing time increases, the nitrogen level continues to decrease. Figure 4.3-7 gives a closer look at the nitrogen content as the gassing time increases above 90 seconds. However, the principle of diminishing returns begins to take effect, with longer gassing times having a smaller effect. Ninety seconds gassing time yields approximately 5% N2, while 120 and 150 seconds reduce the nitrogen level to 2% and 1%, respectively.

Based on the sampling error determination of 1.82%, increasing the gassing time from 120 to 150 seconds results in a negligible 1% change. The optimum gassing time is 120 seconds.

Review of Culture Handling Techniques

Bacterial cultures can be extremely sensitive to variations in the microenvironment. Proper aseptic techniques must be used to avoid contamination. Throughout these experiments, culture-handling techniques were improved, but some traditionally used techniques for maintaining aseptic conditions in terms of bacterial contamination resulted in setbacks.

One common microbiological procedure is to use ethanol or isopropanol to sterilize vial caps prior to culture inoculation or sampling. Since most of the inoculation and sampling for these experiments occurred in the anaerobic glove bag, solvent rinse bottles of isopropanol were placed in the glove bag. Vials of prepared media were often left in the glove bag with loosened caps so that they could become completely anaerobic. Isopropanol is highly volatile and this volatility allowed it to contaminate the vials of media stored in the glove bag via gas phase diffusion. Low levels of isopropanol were detected in the production media based on liquid analyses performed by fellow researcher Christine Morrison. Observed growth rates were lower for the experiments conducted with isopropanol in the glove bag, signaling that isopropanol may have hindered the growth of the homoacetogenic bacteria. In some cases, Gram stains of these cultures indicated bacterial contaminants, suggesting that isopropanol-metabolizing organisms

may have contaminated the system. Once the problem was identified, isopropanol was removed from the glove bag and all potentially contaminated media vials discarded.

Although many aseptic techniques were properly designed to prevent contamination, a certain degree of practice and skill are required to perform them properly. As more experiments were performed, aseptic techniques generally improved, and contamination problems became less frequent. In future work, more practice using aseptic techniques prior to beginning experiments will likely prevent contamination of cultures.

Bacterial Contamination

Gram stains are commonly used to identify Gram-positive and Gram-negative microorganisms. This stain can also be used to verify culture purity for known cultures. For example, *C. ljungdahlii* is a Gram-positive rod. If a Gram stain of this culture showed non-rods or Gram-negative organisms, the culture was clearly contaminated.

Bacterial contamination was a significant problem in early experiments. *C. ljungdahlii* became contaminated several times, resulting in the purchase of replacement cultures from ATCC. The JAC-1 culture, a promising isolate that showed high CO uptake rates, was lost due to bacterial contamination. In later experiments, more frequent Gram stains were performed in order to identify and prevent bacterial contamination. In addition, a rigorous stock culture program was initiated in order to maintain a stock set of pure cultures for use in growth and production experiments.

Photolytic Degradation of Medium Components

Acetate Production Medium is pale yellow in color due to the presence of certain B-vitamins in the medium. During early production experiments, APM was observed to fade from yellow to clear in vials placed on the shaker incubator for several days. This phenomenon was also observed among vials placed in the anaerobic glove bag for extended periods. The loss of color was observed to be more dramatic among vials in direct light, e.g. on the top shelf in the glove bag or nearest to the windows of the shaker incubator.

Photolytic degradation of the B-vitamins in the medium was identified as a possible cause of the color change. Consequently, vials of prepared APM were thereafter placed in shoeboxes in the glove bag, and the windows of the shaker incubator were covered with aluminum foil to maintain dark conditions. These steps proved to prevent the color change, substantiating the hypothesis that this fading was due to photolytic degradation of the B-vitamins.

Part IV: Utilization of Refined Techniques with Additional Bacterial Sources

The techniques developed in Part III were utilized in experiments using two additional bacterial sources: cow and horse manure obtained from the MSU College of Veterinary Medicine. A series of enrichments were made using these sources in hopes of eventually isolating one or more organisms capable of producing acetic acid from synthesis gas.

Each bacterial source used the same enrichment scheme. In the anaerobic glove bag, three samples of manure, each weighing approximately 0.5 g, were placed in a vial containing APM. The vials, labeled CM1, CM2, and CM3 for cow manure cultures, and HM1, HM2, and HM3 for horse manure cultures, were capped with mininert caps and gassed with 100% CO. The vials were placed on the shaker incubator for 1 week, at which time, liquid and gas samples were taken. Two transfers from each vial were made into vials of fresh media. The two vials inoculated from CM1 of the originals were labeled CM1A and CM1B; those from CM2 were CM2A and CM2B, etc. Likewise, the vials inoculated from HM1 of the originals were labeled HM1A and HM1B; those from HM2 were HM2A and HM2B, etc. These vials, the 1st transfers, were gassed and incubated for 1 week. Liquid and gas samples were taken of these vials, and a second set of transfers was made. The 2nd transfer vials were gassed and incubated for two weeks, with liquid and gas samples taken each week. The results of these enrichments are presented in Figures 4.4-1 and 4.4-2, and the enrichment schemes are presented in Appendix B. The raw data for these experiments are presented in Appendix C.

Cow Manure Enrichments

Figure 4.4-1 shows the results of the cow manure enrichments. Abiotic controls showed less than 0.05% CO uptake in each week of the study. CO uptake by the original cultures was moderate in CM1 (14%), and low in CM2 (2%) and CM3 (4%). Because of the high solids content within these samples, they could not be filtered for liquid sampling.

The first transfers demonstrated much higher CO uptake rates than the originals. The highest CO uptake was recorded in CM1B, at 36%, while the lowest was in CM3B, at 29%. In addition, CM1B showed approximately 380 mg/L acetate production. None of the other cultures in the 1st transfer produced acetate.

Despite promising results in the first transfer samples, the second transfers had only limited success during the first week. Of these samples, only CM1B showed CO uptake or acetate production. The CO uptake level was a relatively low 3%, and the acetate production level was ~140 mg/L. The 1B sample showed positive results for CO uptake and acetate production in both the first and second transfers, suggesting the possible presence of one or more homoacetogens.

In Week 2, the 2nd transfers performed better than in Week 1. All six vials showed CO uptake of at least 20% and 3 vials demonstrated over 200 mg/L acetate production. CO uptake for the 2nd transfers in Week 2 ranged from 24% in CM2A to 31% in CM3A. CM1A and CM2A each produced approximately 250 mg/L acetate, and CM2B produced over 1,500 mg/L acetate.

Horse Manure Enrichments

The results of the horse manure enrichments are presented in Figure 4.4-2. CO uptake by the original cultures varied widely. As explained above, high solids content prevented liquid sampling for these original vials. CO uptakes by HM1, HM2, and HM3, were 18%, 1%, and 10%, respectively. Again, abiotic controls showed less than 0.05% CO uptake in each week of the study.

The 1st transfers of the horse manure consortium resulted in higher CO uptake rates, but still no acetate production. CO uptake ranged from a low of 25% by HM3A to a high of 47% by HM2B. CO appears to have been utilized for either cell growth or the production of chemicals other than acetate.

The first week of the 2nd transfers exhibited poor results for both CO uptake and acetate production. The CO uptake by HM1A and HM2B was approximately 10%, while no other transfers utilized a measurable amount of CO.

Allowing the 2nd transfers one more week of incubation led to more positive results in CO uptake and acetate production. All transfers showed approximately 25-30% CO uptake. Strangely, although HM3B had the highest CO uptake (32%), this transfer was the only one that produced no acetate. HM2B and HM3A produced nearly 250 mg/L acetate, while HM1A and HM2B produced 1,500 mg/L. The acetate production by HM1B exceeded the upper limit of the HPLC calibration curve of 2,000 mg/L (2 g/L). An acetate production rate of 1 g/L per week, although too low to be industrially viable, is high enough to suggest that isolation of a pure culture combined with further

optimization of fermentor configurations and media formulations may result in an organism capable of synthesis gas fermentation to acetate at commercially significant levels.

In summary, the techniques developed in Parts I-III of this chapter proved adequate in evaluating new bacterial sources. CO uptake was observed in the test vials, with minimal gas leak rates in abiotic controls. In addition, aseptic techniques were utilized to prevent contamination of the enrichments.

The bacterial consortia studied in this section demonstrated an ability to fix CO in the headspace of the vials. Some of the enrichments also displayed simultaneous CO uptake and acetate production, suggesting the conversion of CO into acetate. As explained in Part II, bacterial consortia present many challenges for chemical production and should not be considered for industrial scale-up. Although preliminary results indicate great potential for CO-to-acetate conversion by bacteria from both sources, the development of a pure culture should be the eventual goal.

Attempts were undertaken to isolate an organism or organisms from these sources. The vial slant technique presented in the Materials and Methods chapter of this thesis was employed in this effort. Isolates from these sources grew slowly or not at all on the vial slants. The cultures that did grow were transferred to 1754 in an attempt to induce more rapid growth, but no appreciable growth was observed in this medium. One possibility for the loss of these cultures is the interdependency of the individual cultures in a consortium. Often, one culture in a consortium depends on another to produce needed by-products. When separated from each other, neither culture is fully functional

on its own. Additional enrichments with cultures from cow and horse manure are hypothesized to yield CO-utilizing organisms. Future work with these cultures is needed to determine whether a homoacetogen can be isolated.

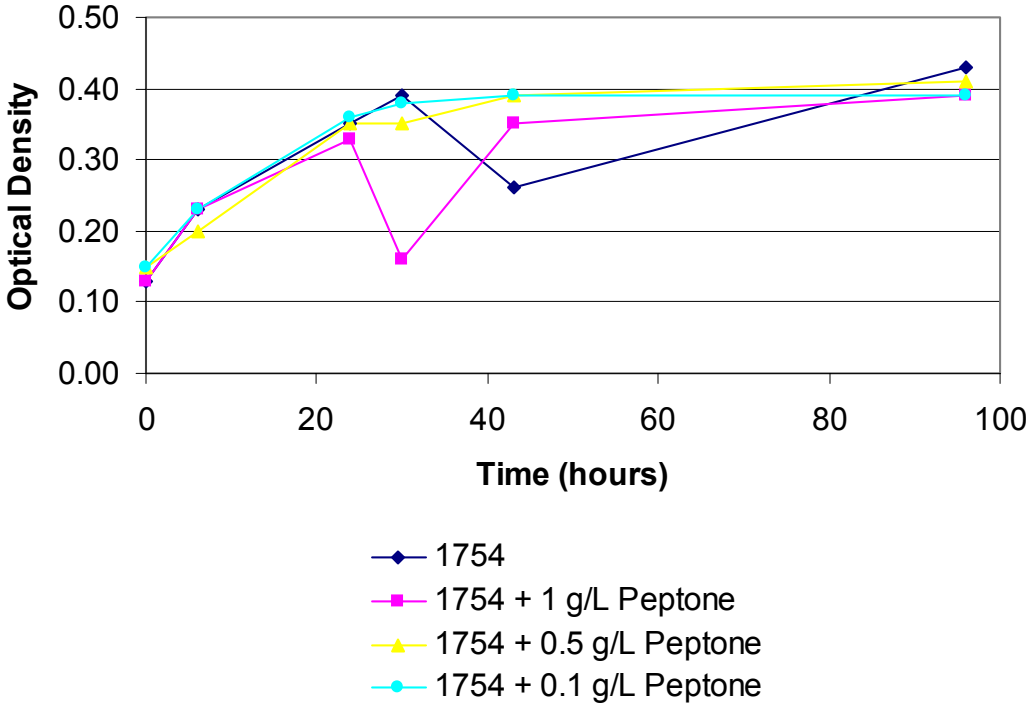

Figure 4.1-1. Growth of *C. ljungdahlii* on 1754 PETC Medium with varying amounts of peptone

0.2 mL inoculum in each vial. Agitation rate of 400 rpm at 37ºC. N=1.

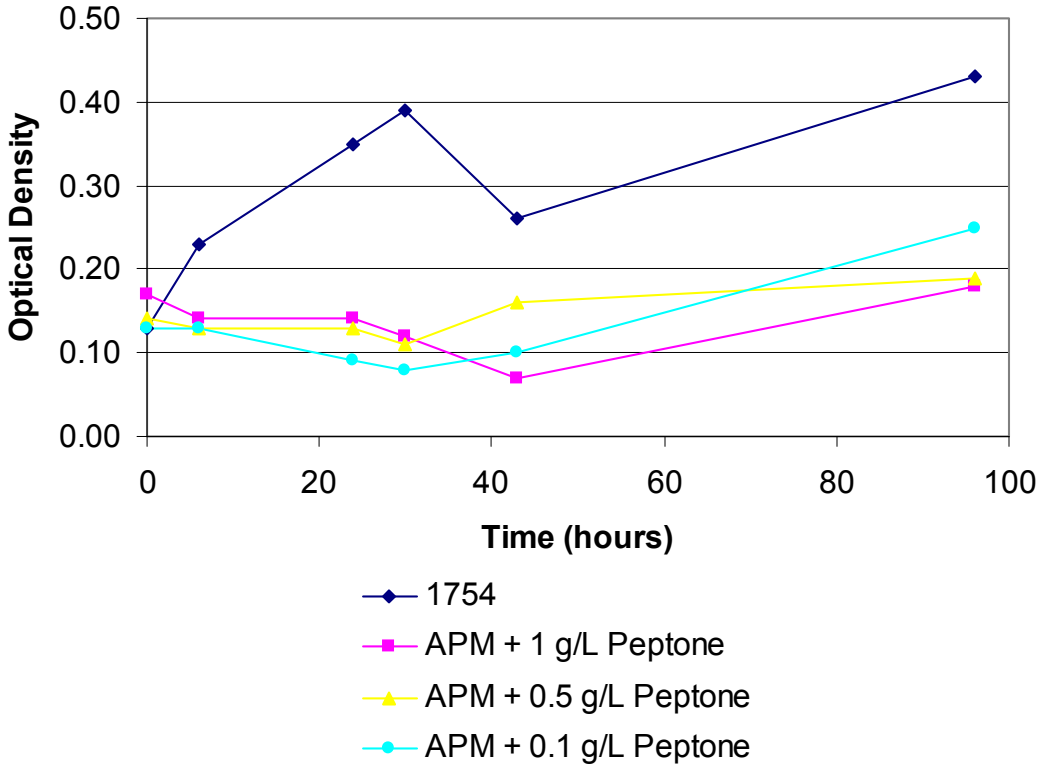

Figure 4.1-2. Growth of *C. ljungdahlii* on Acetate Production Medium with varying amounts of peptone

Growth on 1754 Medium is shown as the benchmark. 0.2 mL inoculum in each vial. Agitation rate of 400 rpm at 37ºC. N=1.

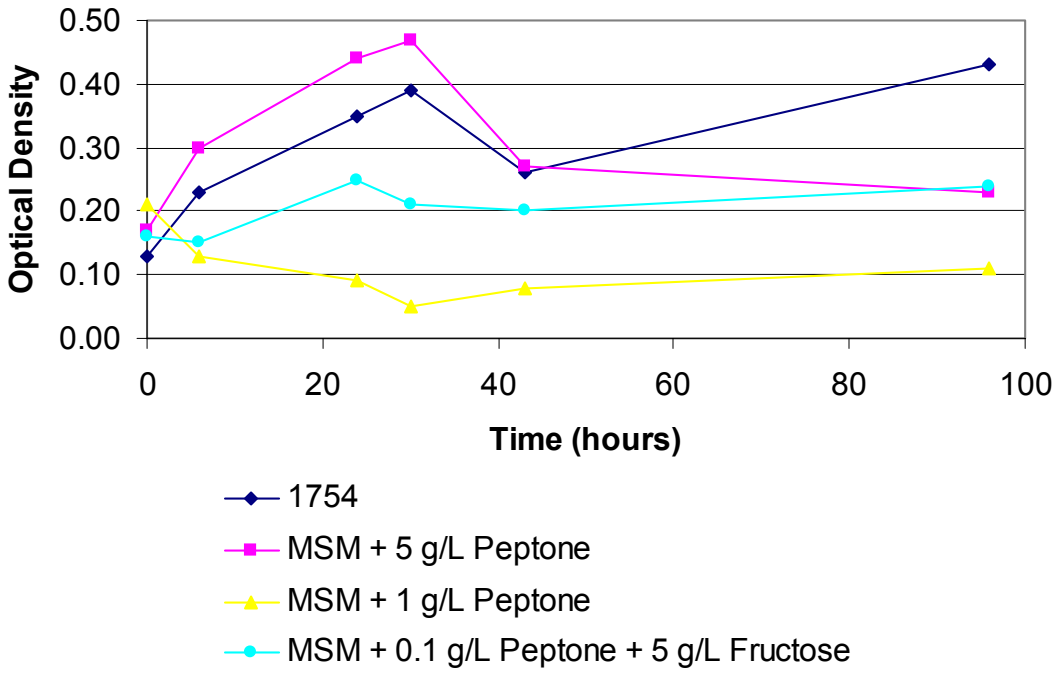

Figure 4.1-3. Growth of *C. ljungdahlii* on Mineral Salts Medium with varying amounts of peptone

Growth on 1754 Medium is shown as the benchmark. 0.2 mL inoculum in each vial. Agitation rate of 400 rpm at 37ºC. N=1.

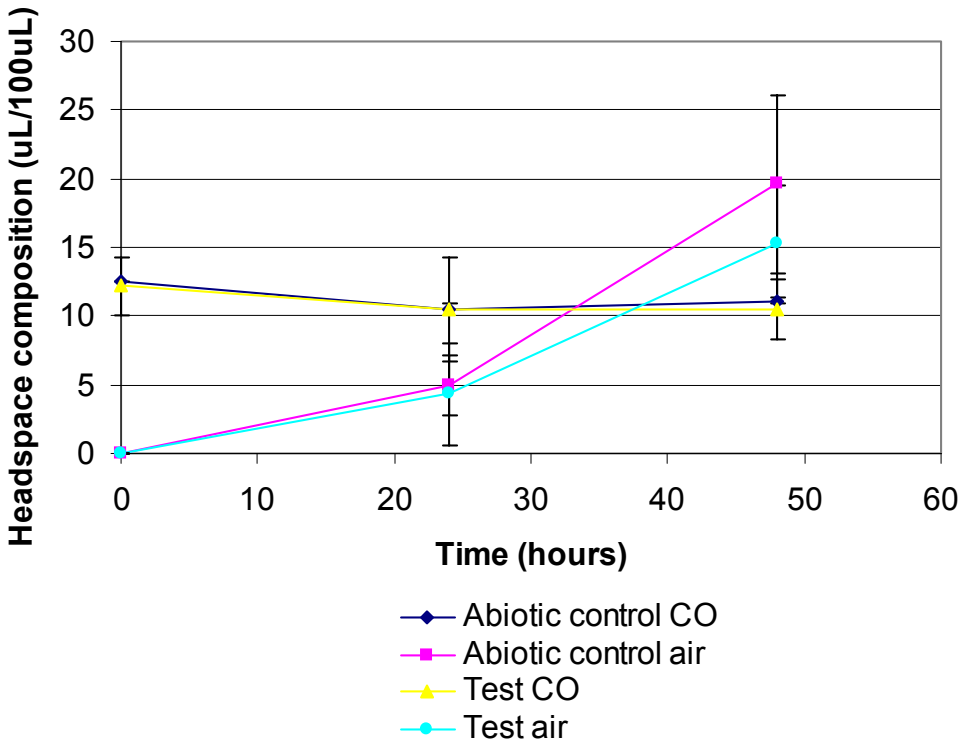

Figure 4.1-4. Headspace gas changes over time for *C. ljungdahlii* with an initial headspace composition of 2:1 of H_2:CO

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

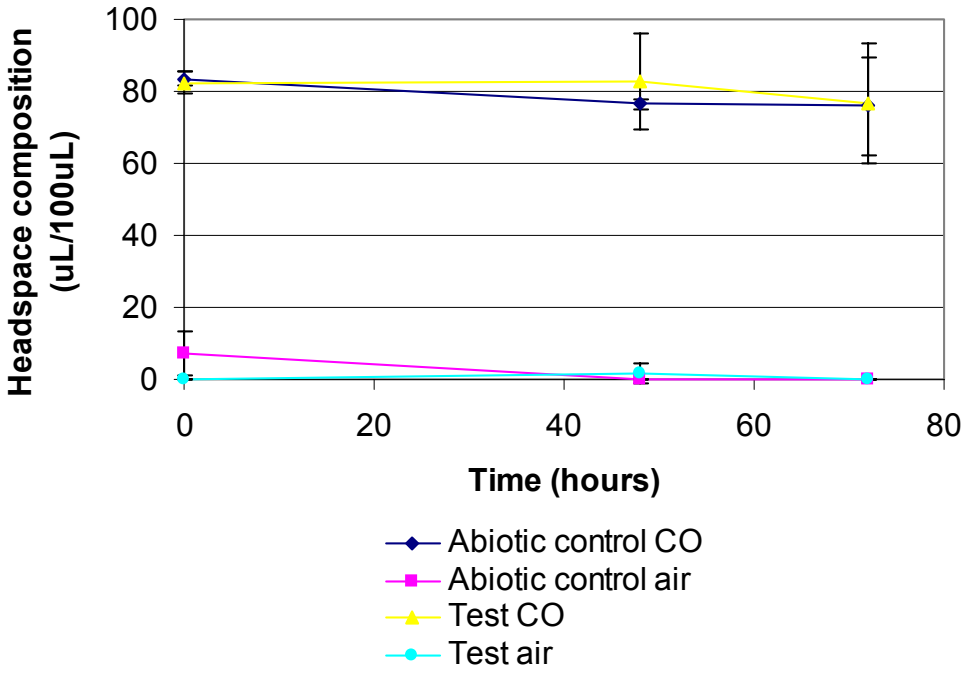

Figure 4.1-5. Headspace gas changes over time for *C. ljungdahlii* with an initial headspace composition of 80% CO, 20% H_2

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

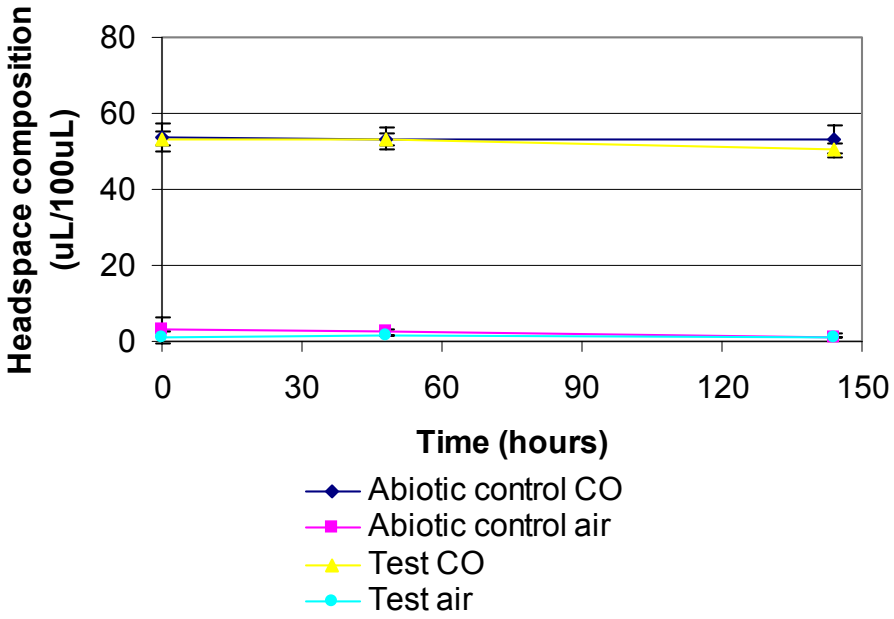

Figure 4.1-6. Headspace gas changes over time for *C. thermoaceticum* with an initial headspace composition of 60% CO, 30% H_2, 10% CO_2

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 60ºC and no agitation. N=3.

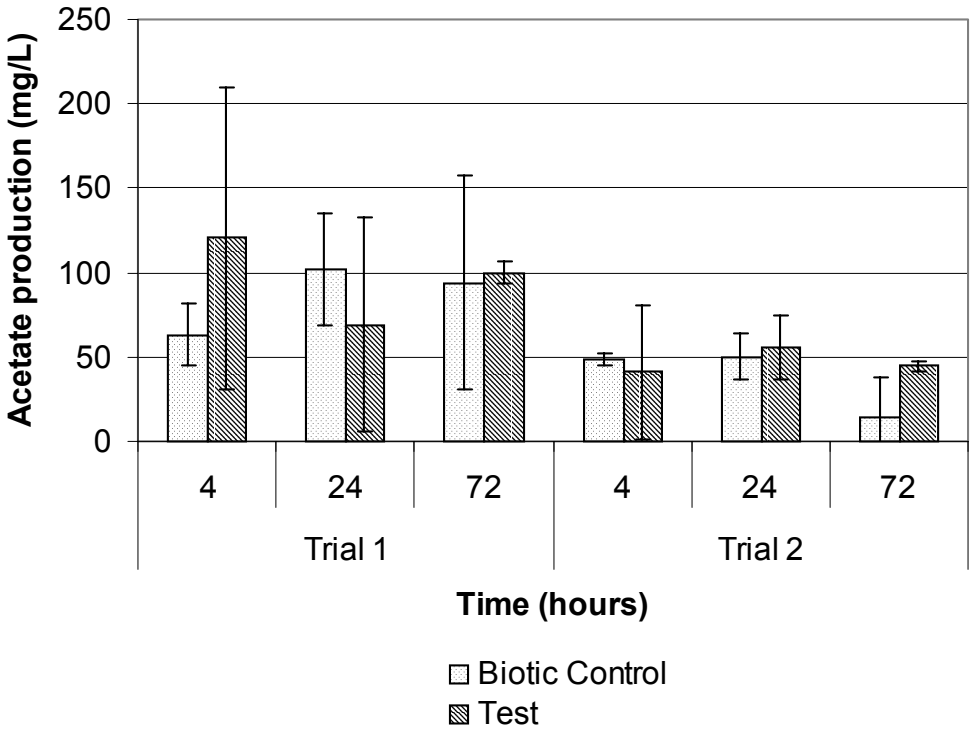

Figure 4.1-7. Acetic acid production by *C. ljungdahlii* over 72 hours of incubation in consecutive trials

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. Initial headspace composition was 2:1 of H_2:CO. Vials were incubated at 37ºC and 400 rpm agitation. N=3.

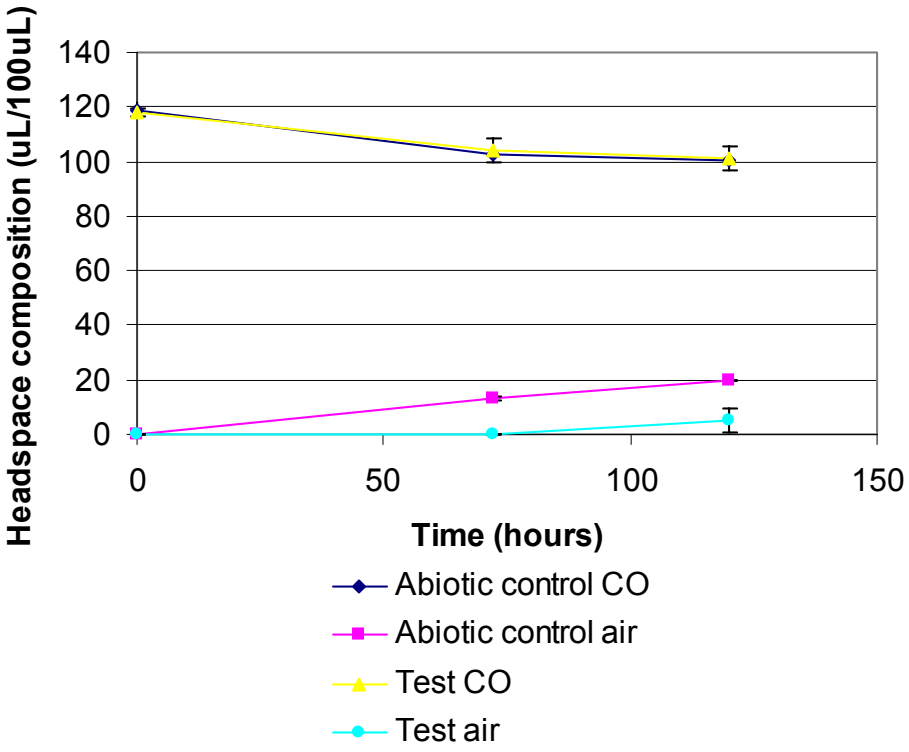

Figure 4.2-1. Headspace gas changes over time for MSU-1 with an initial headspace composition of 80% CO, 20% H_2

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

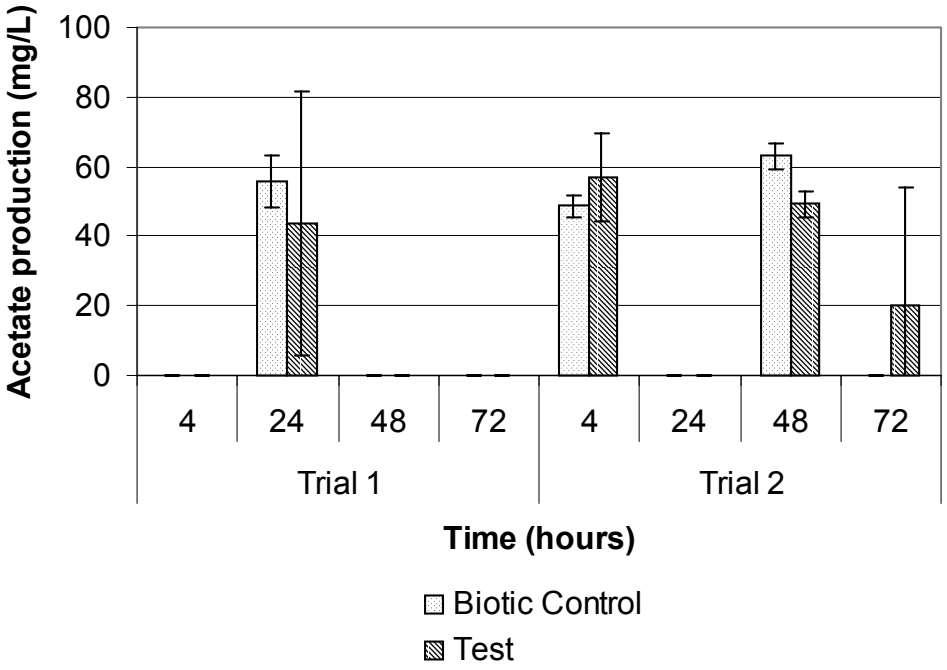

Figure 4.2-2. Acetic acid production by MSU-1 after 72 hours of incubation

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. Initial headspace composition was 2:1 of H_2:CO. Vials were incubated at 37ºC and 400 rpm agitation. N=3.

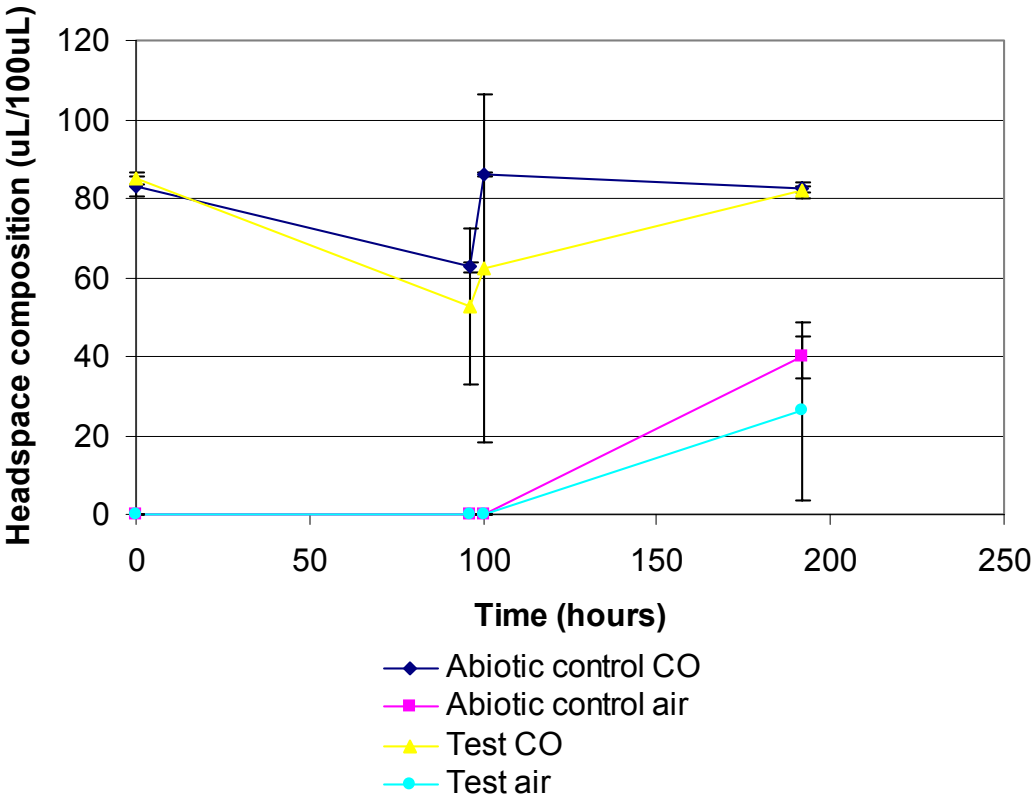

Figure 4.2-3. Headspace gas changes over time for JAC-1 with an initial headspace composition of 60% CO, 40% H_2, regassed at 98 hours

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

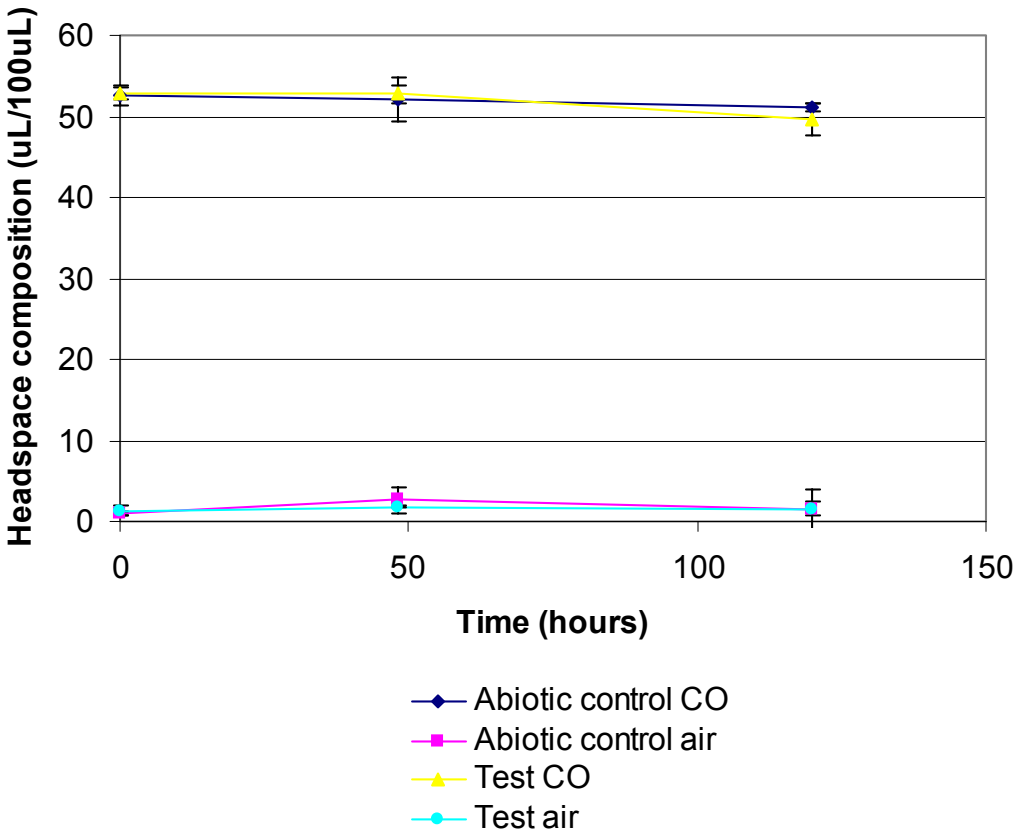

Figure 4.2-4. Headspace gas changes over time for JAC-1 with an initial headspace composition of 60% CO, 30% H_2, 10% CO_2

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

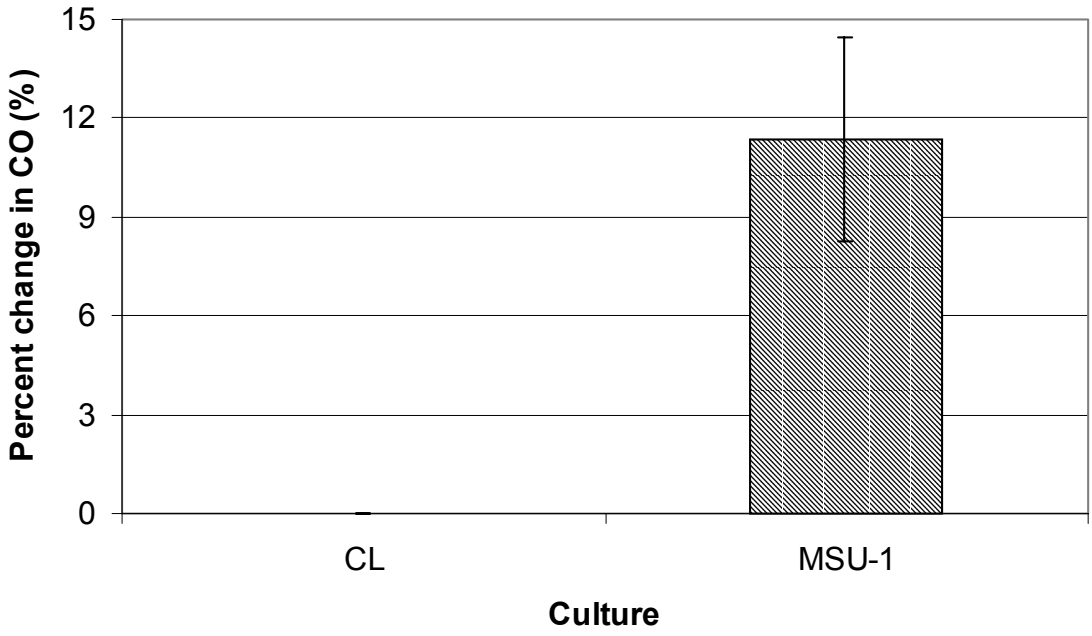

Figure 4.2-5. CO uptake by *C. ljungdahlii* and MSU-1 with initial headspace composition of 80% CO, 20% H_2, corrected for air leakage

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. CO uptake was calculated by subtracting the final CO concentration from the initial CO concentration. Uptake values were corrected for air leakage into the vial using the O_2 concentration, assuming stoichiometric composition of air entering the vial. Vials were incubated at 37ºC and 400 rpm agitation. N=3.

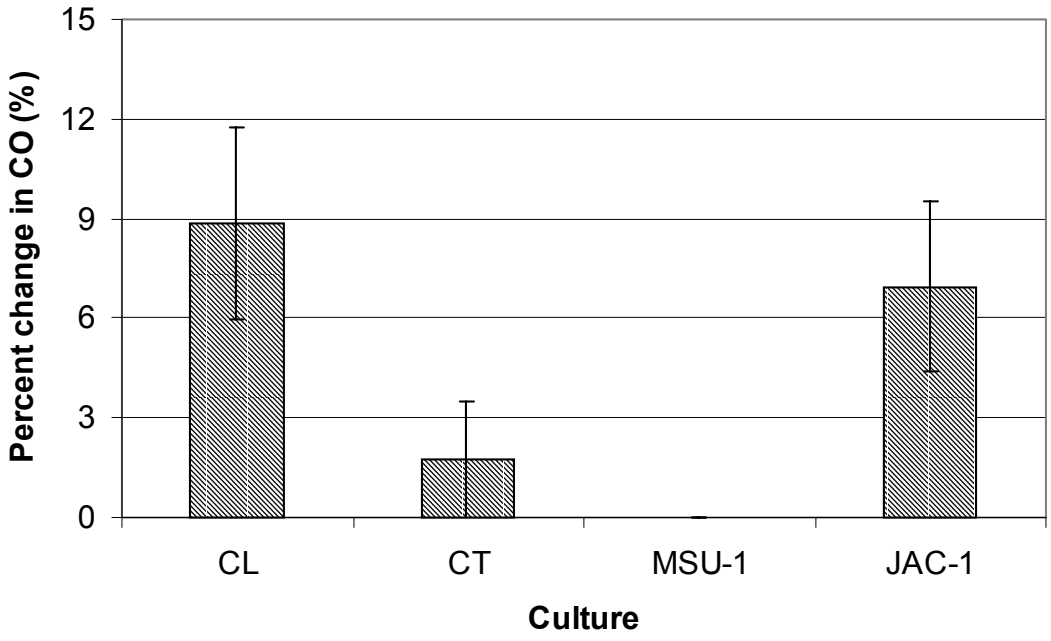

Figure 4.2-6. CO uptake by *C. ljungdahlii*, *C. thermoaceticum*, MSU-1, and JAC-1 with initial headspace composition of 60% CO, 30% H_2, 10% CO_2, corrected for air leakage

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. CO uptake was calculated by subtracting the final CO concentration from the initial CO concentration. Uptake values were corrected for air leakage into the vial using the O_2 concentration, assuming stoichiometric composition of air entering the vial. Vials were incubated at 37ºC and 400 rpm agitation. N=3.

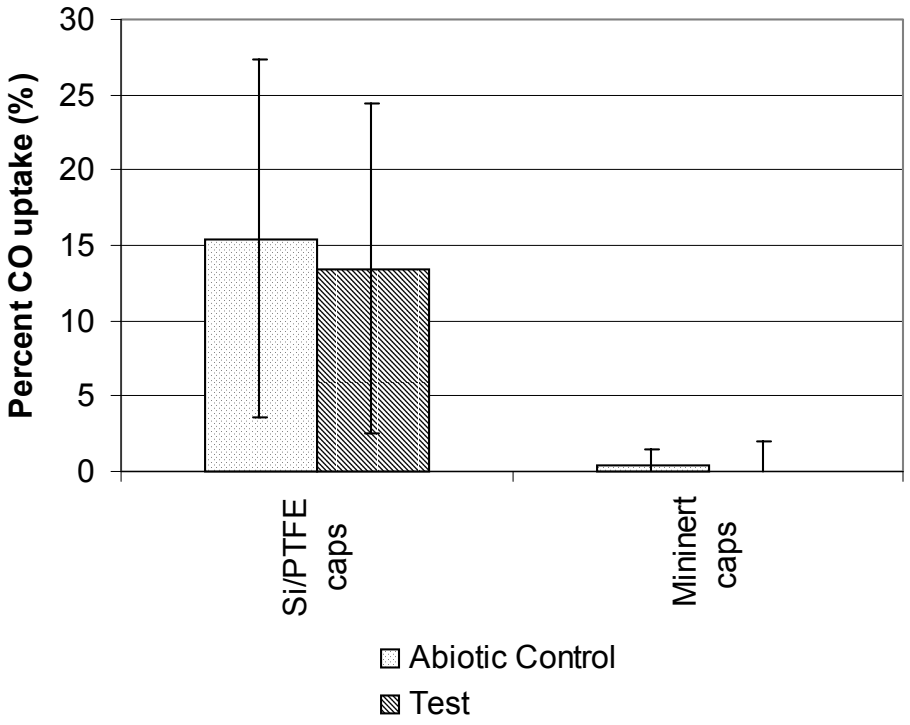

Figure 4.3-1. CO uptake by *C. ljungdahlii* after 72 hours of incubation with Si/PTFE caps and mininert caps

40 mL vials with 20 mL liquid volume containing APM and cells. Initial headspace composition was 2:1 of H_2:CO. Vials were incubated at 37ºC and 400 rpm agitation. N=9 for Si/PTFE caps, N=3 for mininert caps.

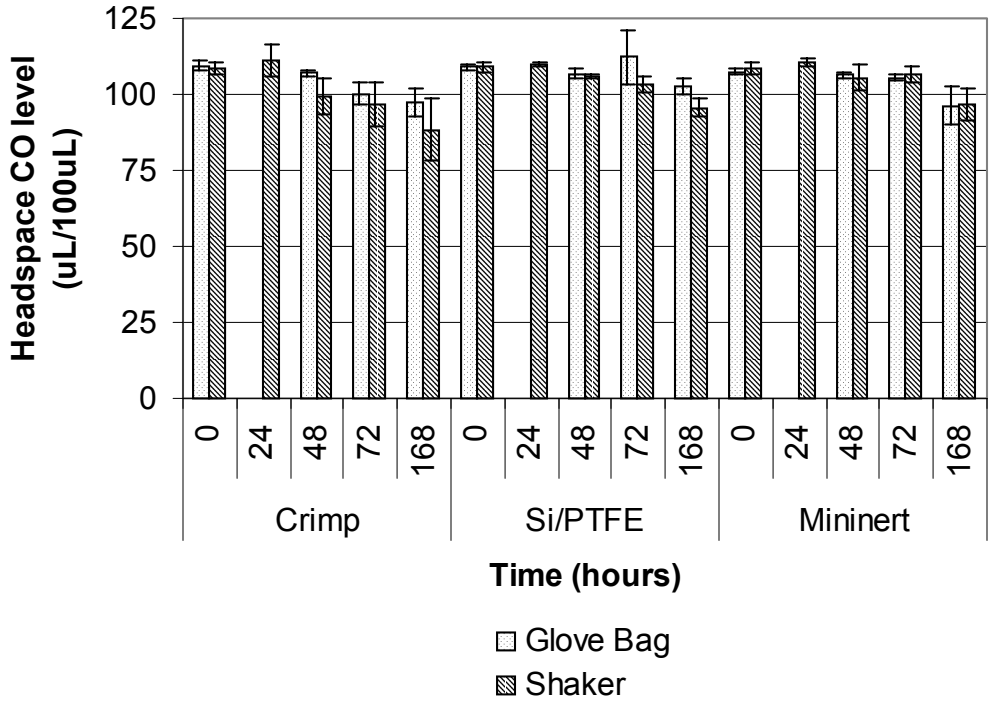

Figure 4.3-2. Cap test CO level

40 mL vials with 20 mL liquid volume containing APM with crimp, Si/PTFE, and mininert caps. 3 vials with each cap type were placed in the anaerobic glove bag (control) and the shaker incubator (test) to determine cap leakage rates. All Vials were incubated at 37ºC, with vials on the shaker incubator agitated at 400 rpm. Initial headspace composition was 100% CO. Cap septa given 10 sampling needle punctures prior to 168-hour samples to simulate additional samples. N=3.

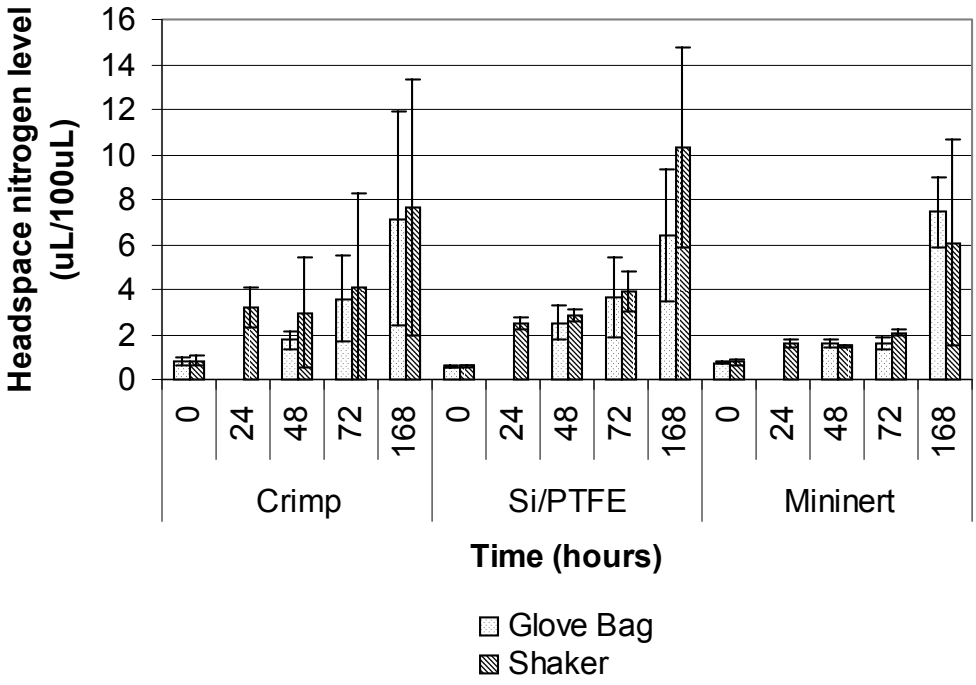

Figure 4.3-3. Cap test N_2 level

40 mL vials with 20 mL liquid volume containing APM with crimp, Si/PTFE, and mininert caps. 3 vials with each cap type were placed in the anaerobic glove bag (control) and the shaker incubator (test) to determine cap leakage rates. All Vials were incubated at 37ºC, with vials on the shaker incubator agitated at 400 rpm. Initial headspace composition was 100% CO. Cap septa given 10 sampling needle punctures prior to 168-hour samples to simulate additional samples. N=3.

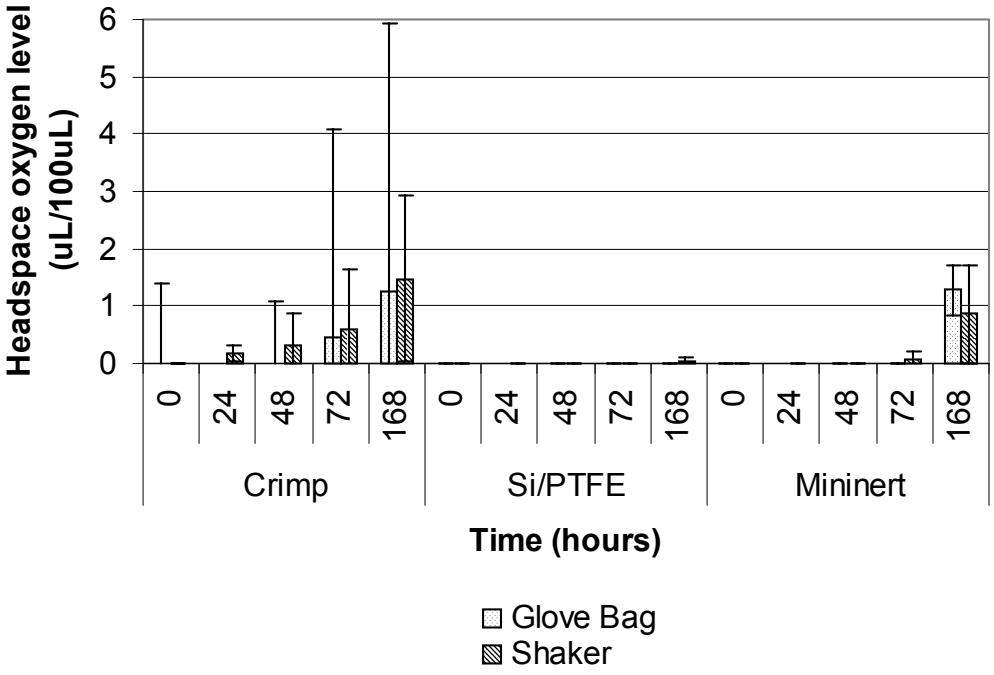

Figure 4.3-4. Cap test O_2 level

40 mL vials with 20 mL liquid volume containing APM with crimp, Si/PTFE, and mininert caps. 3 vials with each cap type were placed in the anaerobic glove bag (control) and the shaker incubator (test) to determine cap leakage rates. All Vials were incubated at 37ºC, with vials on the shaker incubator agitated at 400 rpm. Initial headspace composition was 100% CO. Cap septa given 10 sampling needle punctures prior to 168-hour samples to simulate additional samples. N=3.

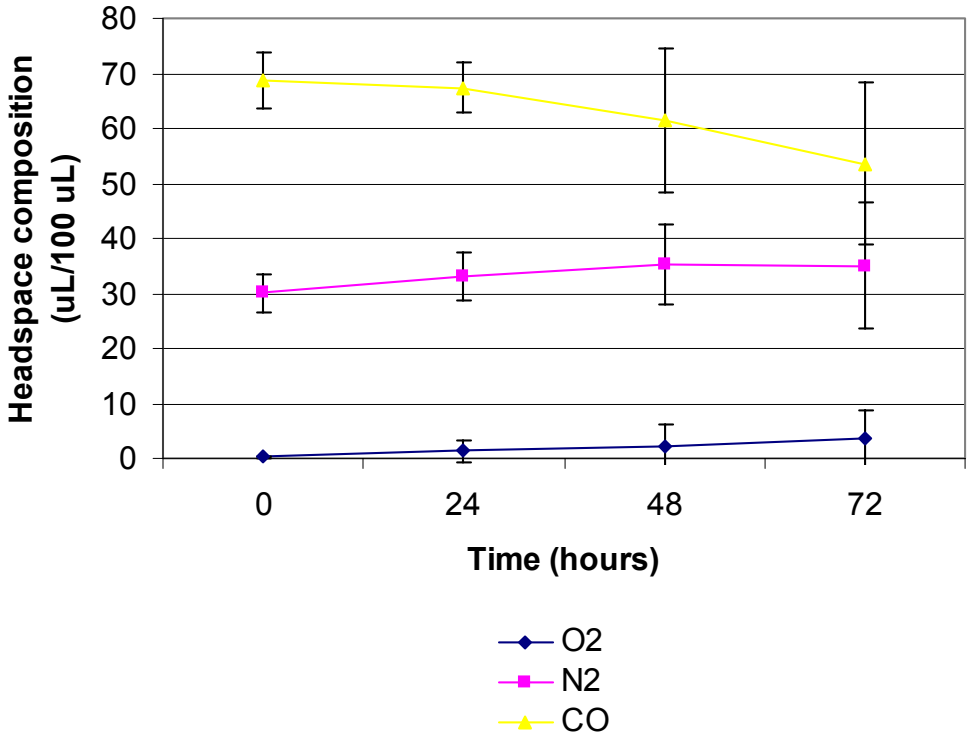

Figure 4.3-5. Headspace gas changes at 60ºC

40 mL vials with mininert caps and 20 mL liquid volume containing APM. Vials were incubated at 60ºC with no agitation. Initial headspace composition was 100% CO. N=3.

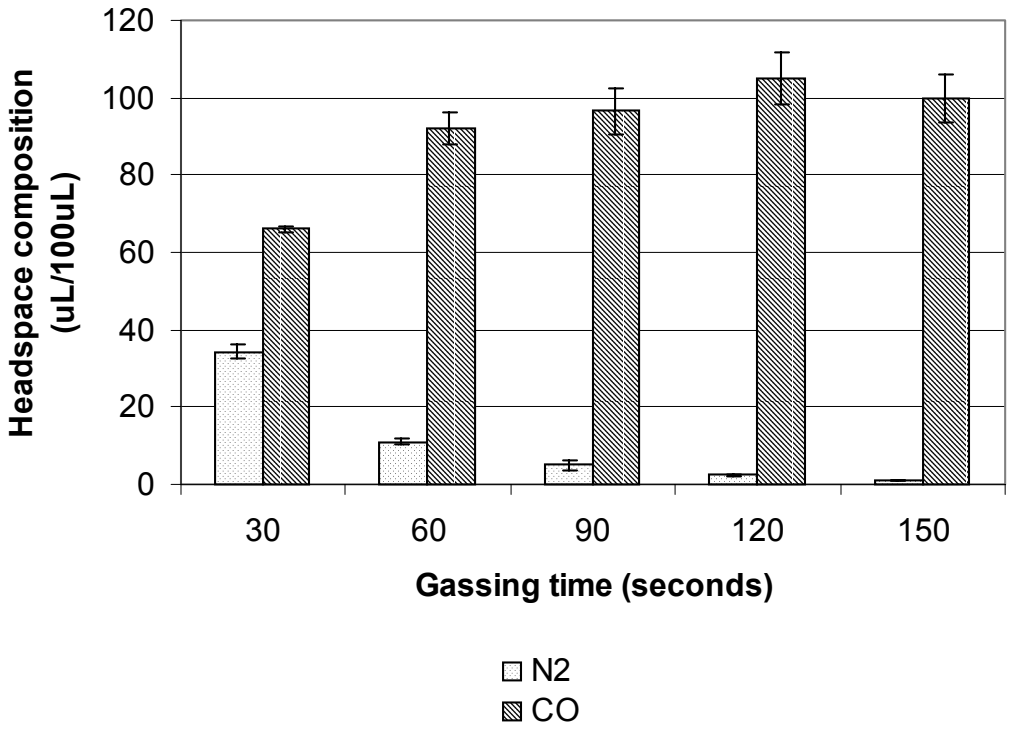

Figure 4.3-6. Effect of gassing time on headspace gas composition

40 mL vials with mininert caps and 20 mL liquid volume containing APM. Initial headspace composition was 100% CO. Vials sampled immediately after gassing. N=3.

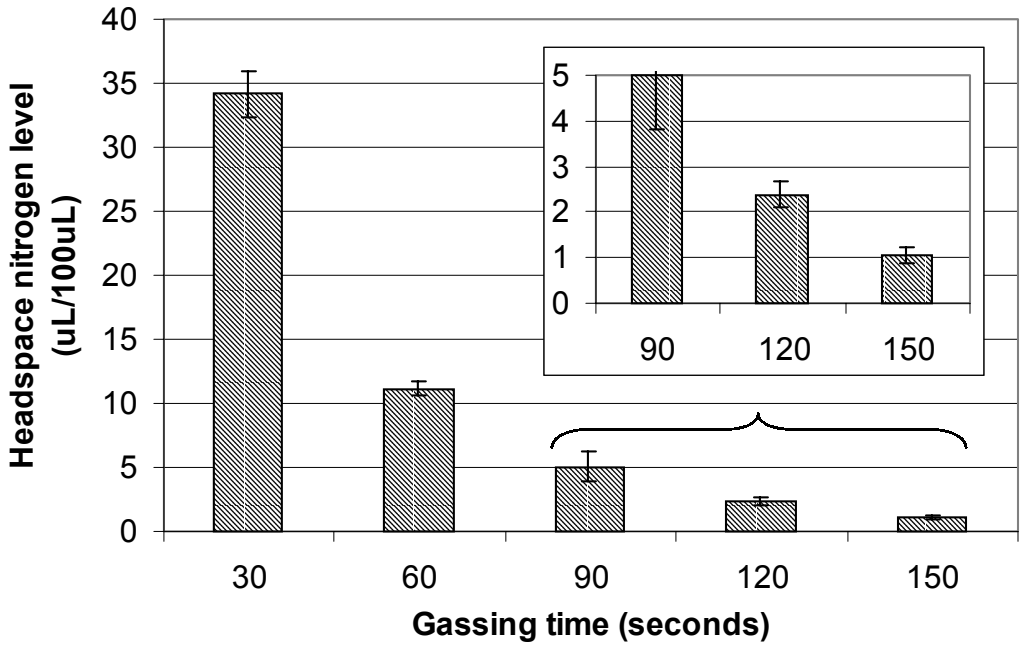

Figure 4.3-7. Effect of gassing time on headspace nitrogen content

40 mL vials with mininert caps and 20 mL liquid volume containing APM. Initial headspace composition was 100% CO. Vials sampled immediately after gassing. N=3.

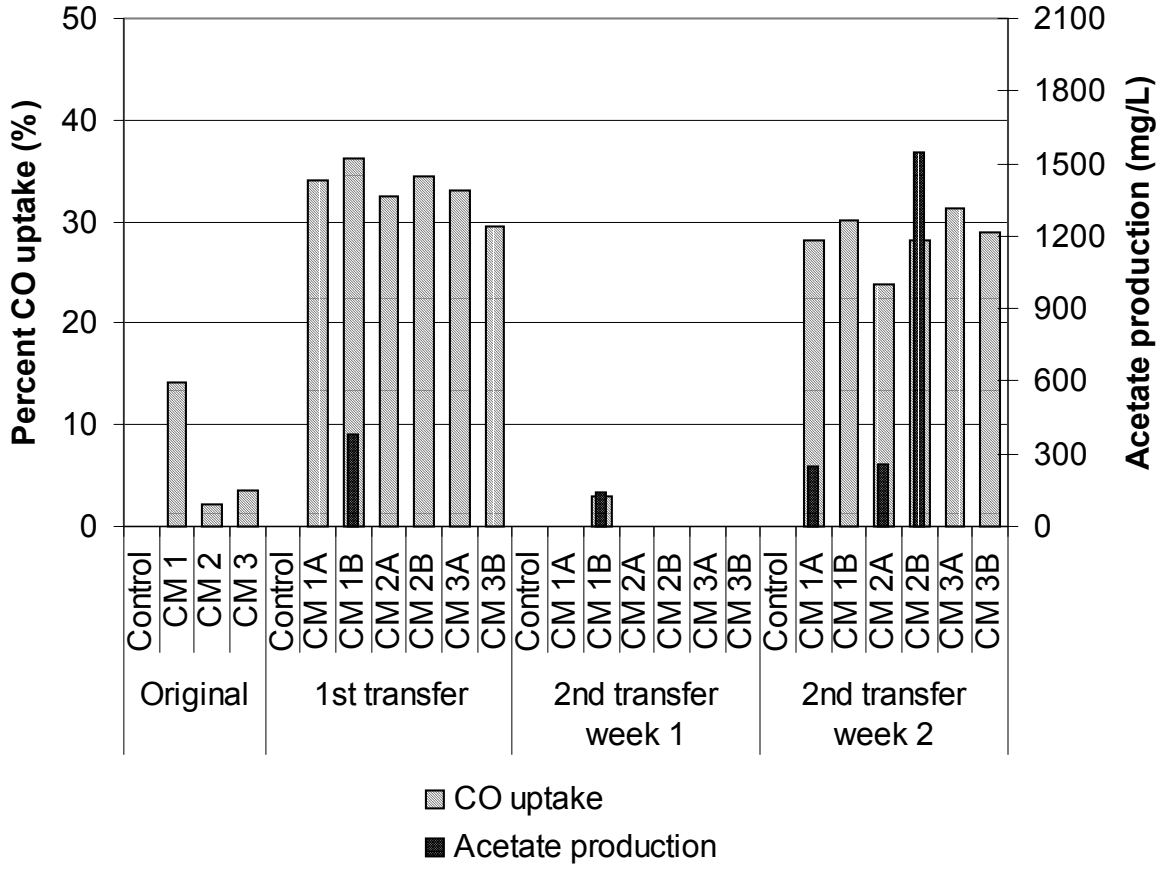

Figure 4.4-1. CO uptake and acetate production by cow manure consortium

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. Vials gassed with 100% CO and placed on the shaker incubator at 37ºC and 400 rpm agitation. See Figure B-1 for culture enrichment scheme. N=3 for abiotic controls, N=1 for all other samples.

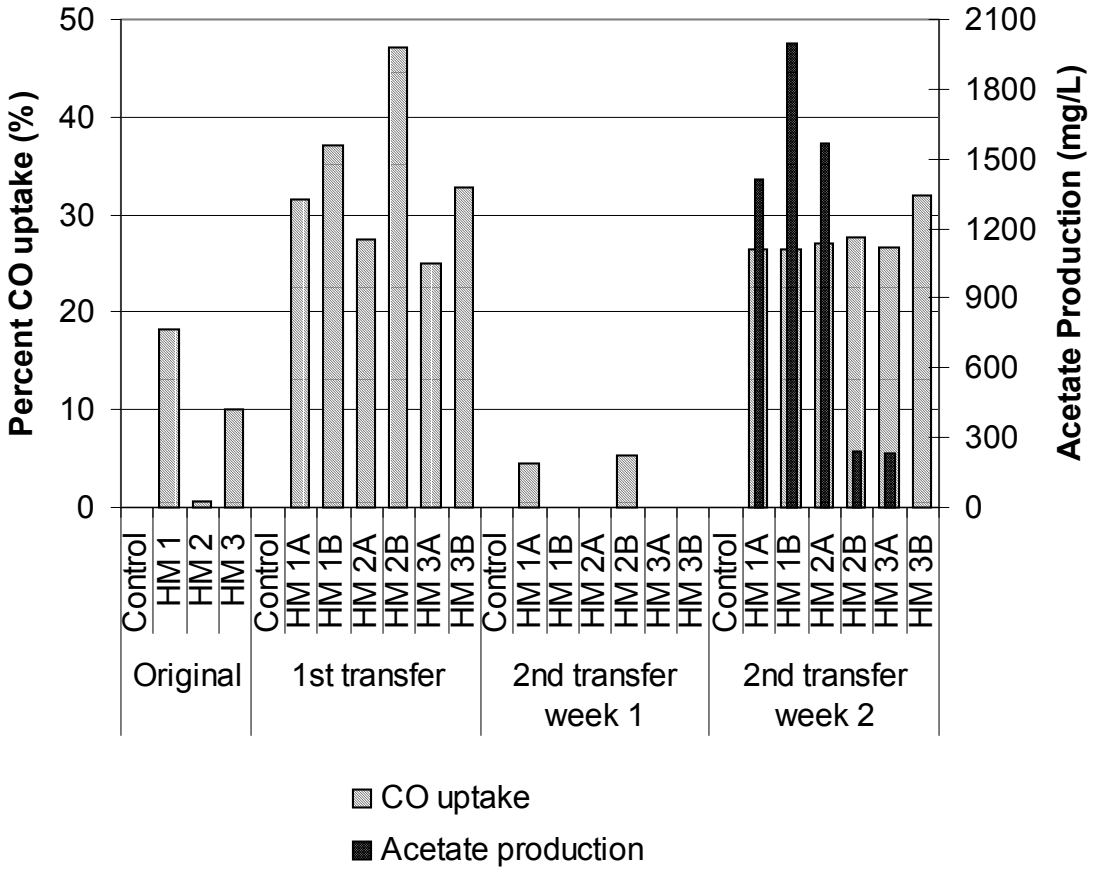

Figure 4.4-2. CO uptake and acetate production by horse manure consortium

40 mL vials with mininert caps and 20 mL liquid volume containing APM and cells. Vials gassed with 100% CO and placed on the shaker incubator at 37ºC and 400 rpm agitation. See Figure B-2 for culture enrichment scheme. N=3 for abiotic controls, N=1 for all other samples.

CHAPTER 5

CONCLUSIONS AND ENGINEERING SIGNIFICANCE

Conclusions

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

- *Clostridium ljungdahlii* exhibited the fastest growth rate on Mineral Salts Medium with 5.0 g/L peptone, but the most sustained growth on 1754 Medium. Due to the relative simplicity of the MSM + 5.0 g/L peptone medium, this medium is recommended to promote growth for *C. ljungdahlii*.

- Synthesis gas fermentation capabilities of *C. ljungdahlii* could not be evaluated due to problems in the experimental setup. Leaking vial caps and bacterial contamination issues, which prevented proper evaluation of this culture, have been addressed in this thesis and changes will be recommended for future experiments.

- The current fermentation scheme used for thermophilic microorganism such as *C. thermoaceticum* is ineffective for evaluating the performance of these organisms. Mass transfer limitations are exaggerated under static conditions, and the sampling system will require further development to ensure accuracy of headspace analyses.

- MSU-1 and other bacterial consortia present many challenges for a full-scale synthesis gas fermentation facility. The results presented in this thesis suggest that the use of mixed cultures for synthesis gas fermentations may be infeasible due to the unpredictability of bacterial consortia.

- The vial slant technique for culture isolation proved to be very effective for identifying and removing single cultures for study.

- JAC-1 demonstrated the highest CO uptake of any of the cultures studied in this research. Although problems with analytical equipment prevented the determination of products formed by this organism, its ability to utilize CO makes it a strong candidate for further research.

- Si/PTFE and mininert vial caps leaked headspace gases at similar rates at 37ºC. Since mininert caps can be regularly refitted with new septa, they are recommended for use in future synthesis gas fermentation experiments.

- Crimp tops, although commonly used for batch synthesis gas fermentation experiments (Vega et al., 1989b; Daniel et al., 1990; Klasson et al., 1991), performed poorly under the conditions used in these experiments. This poor performance was likely due to a slow recovery time for the butyl rubber septa employed for this cap type, which allowed gases to leak during and shortly after sampling.

- The use of mininert caps at 60ºC resulted in high variability among samples treated alike. In addition to concerns about mininert caps, the sampling procedure at this temperature may cause this variability. Rapid cooling of headspace gases from the vial temperature of 60ºC to the sampling syringe temperature (approximately 25ºC) may result in contraction of the gases, causing air to rush into the syringe to alleviate the resulting vacuum.

- The use of sterilizing agents in close proximity to culture media (e.g. isopropanol stored in the anaerobic glove bag with vials of media with loosened caps) resulted in chemical contamination of the media used in many experiments, causing decreased growth and production rates by the organisms.

- Frequent culture purity analyses (e.g. Gram stains) are necessary to identify and prevent bacterial contamination during synthesis gas fermentation experiments.

- Initial bacterial screening with cultures from cow and horse manure showed promising results for both CO uptake and acetate production. Although pure cultures had not been isolated from these sources at the publication of this thesis, future work should be done in order to identify and isolate potential homoacetogens.

Engineering Significance

Synthesis gas fermentation presents a unique opportunity for researchers to utilize waste biomass as a feedstock for chemical production. Fermentation of synthesis gas to acetic acid would generate a valuable commodity chemical from material that would otherwise be discarded. Commercialization of this process would result in a major new industrial market for states whose economies are traditionally agriculture-based.

Chemical engineers possess many of the tools necessary to commercialize bio-catalytic processes: process design and integration skills, understanding of process economics, and a wealth of related experience with chemical catalysis. Microbiological processes present a challenge for engineers who often lack a deeper understanding of the complexity of bacterial life. As organic chemistry has been the scientific backbone of the petrochemical industry, microbiology will provide the foundation for the bioprocessing industry.

Bioprocessing is an emerging field in chemical engineering, and as with any new technologies, growing pains can be expected. Although this research did not result in a refined bioprocess ready for scale-up, it does represent an important step for chemical engineers moving into bio-catalysis research. The significance of this work lies in a greater appreciation and understanding of microbial processes among the engineers involved in this research. In addition, meaningful contributions were made in technique development for synthesis gas fermentations. Most importantly, this research demonstrates the need for greater collaboration between chemical engineers and microbiologists in the bioprocessing arena.

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APPENDIX A

ADDITIONAL ACETIC ACID PRODUCTION TRIALS

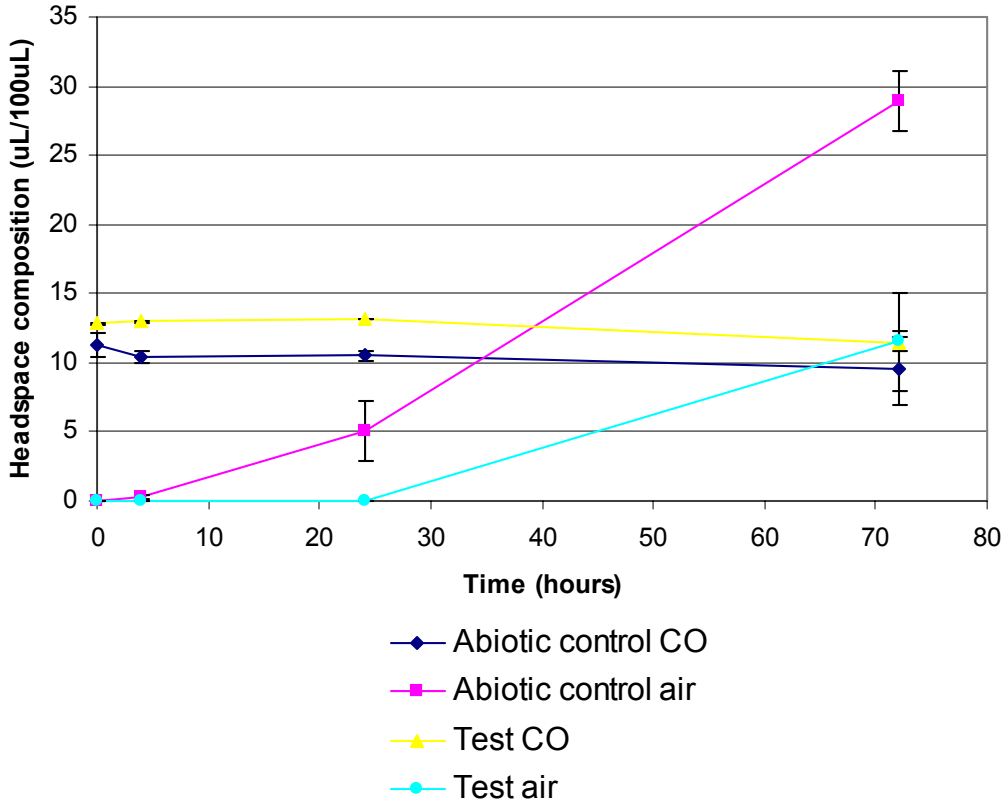

Figure A.1-1. Headspace gas changes over time for Trial A-1 of *C. ljungdahlii* with an initial headspace composition of 2:1 of H_2:CO

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

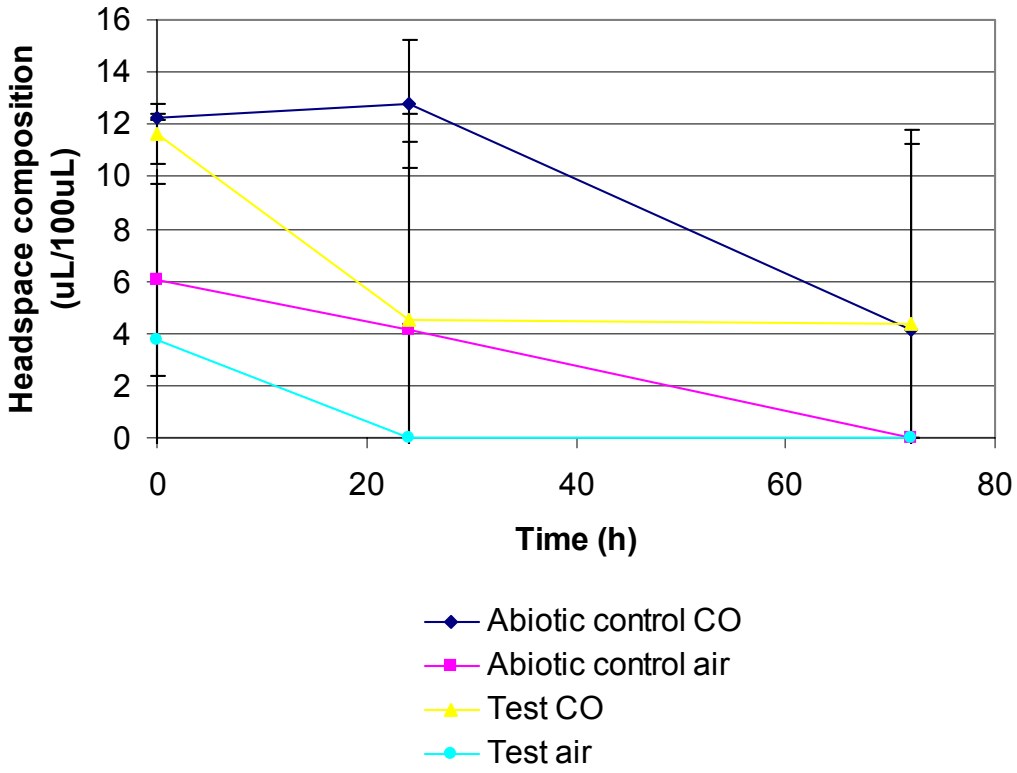

Figure A.1-2. Headspace gas changes over time for Trial A-2 of *C. ljungdahlii* with an initial headspace composition of 2:1 of H_2:CO

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

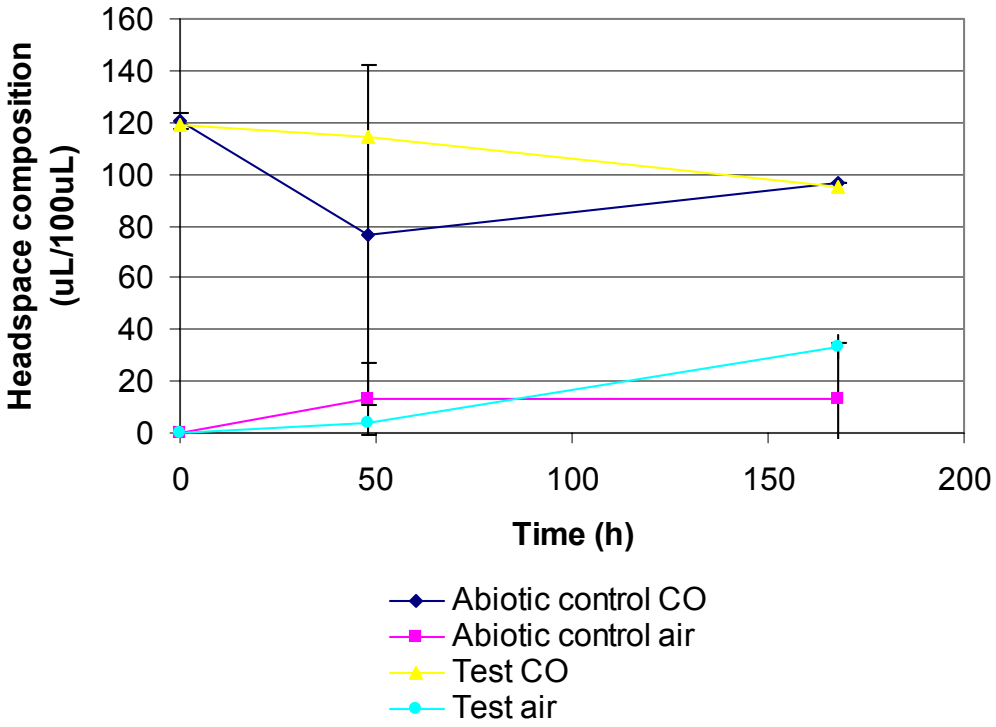

Figure A.1-3. Headspace gas changes over time for Trial A-3 of *C. ljungdahlii* with an initial headspace composition of 80% CO, 20% H_2

Cells washed three times with physiological saline prior to transferring to APM. 40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

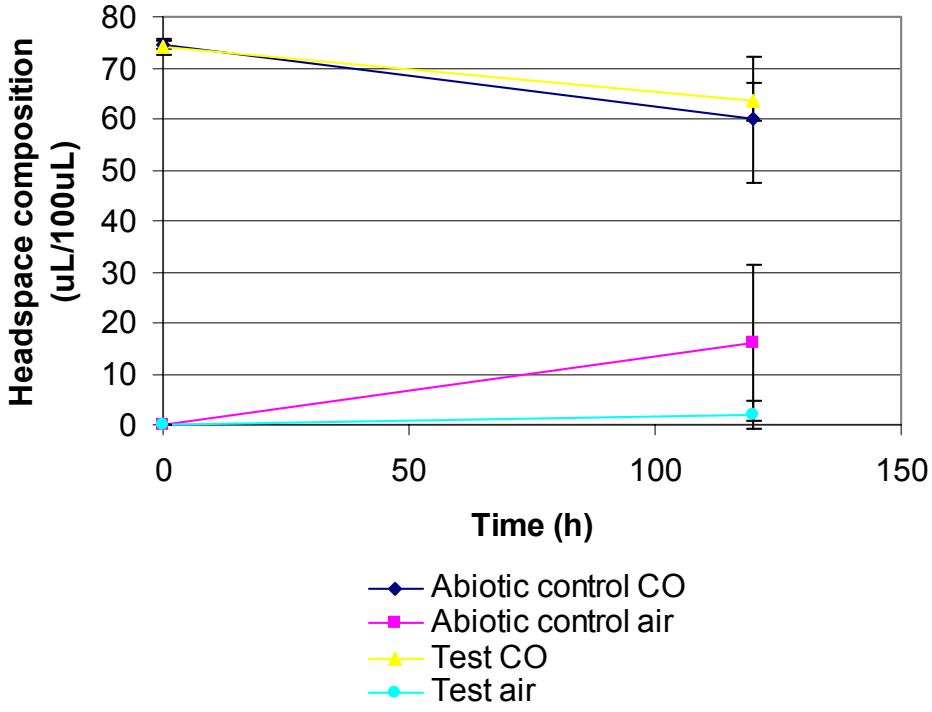

Figure A.1-4. Headspace gas changes over time for Trial A-4 of *C. ljungdahlii* with an initial headspace composition of 60% CO, 30% H_2, 10% CO_2

Cells washed once with physiological saline and allowed to sit overnight in the glove bag prior to transferring to APM. 40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

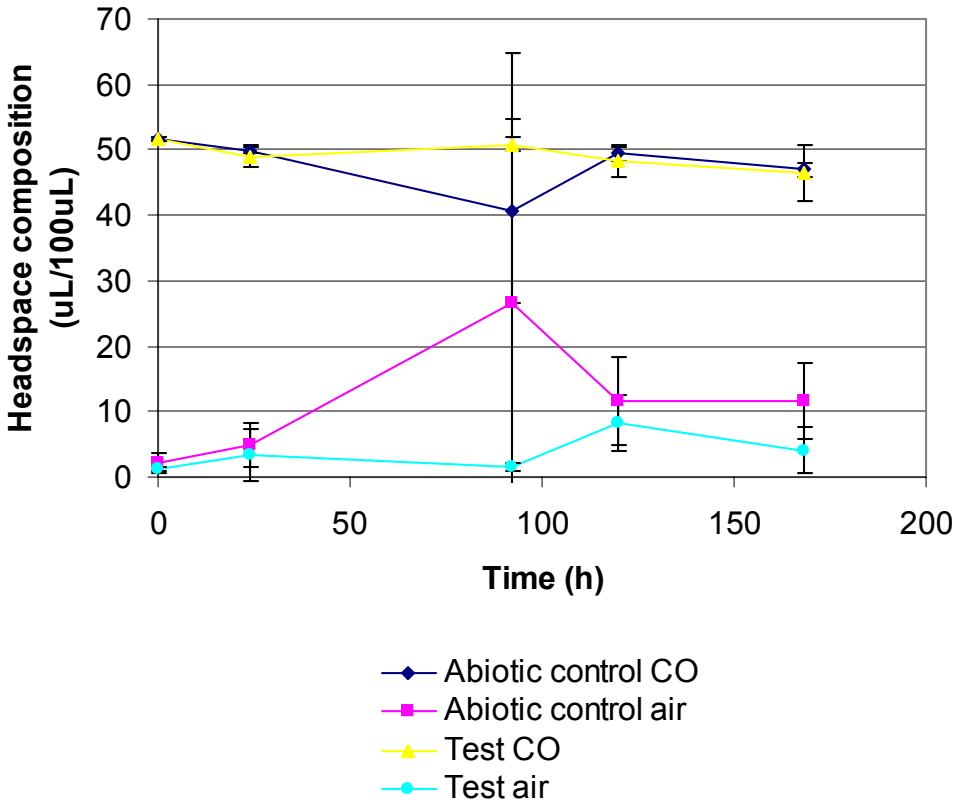

Figure A.1-5. Headspace gas changes over time for Trial A-5 of *C. ljungdahlii* with an initial headspace composition of 60% CO, 30% H_2, 10% CO_2

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

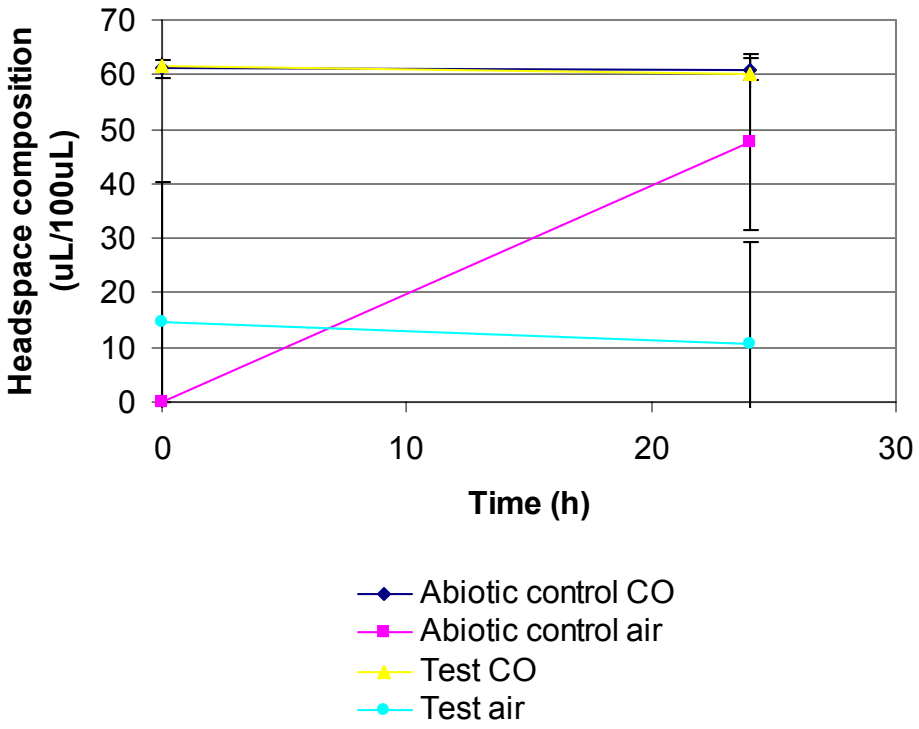

Figure A.2-1. Headspace gas changes over time for Trial A-1 of *C. thermoaceticum* with an initial headspace composition of 60% CO, 40% H_2

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 60ºC and no agitation. N=3.

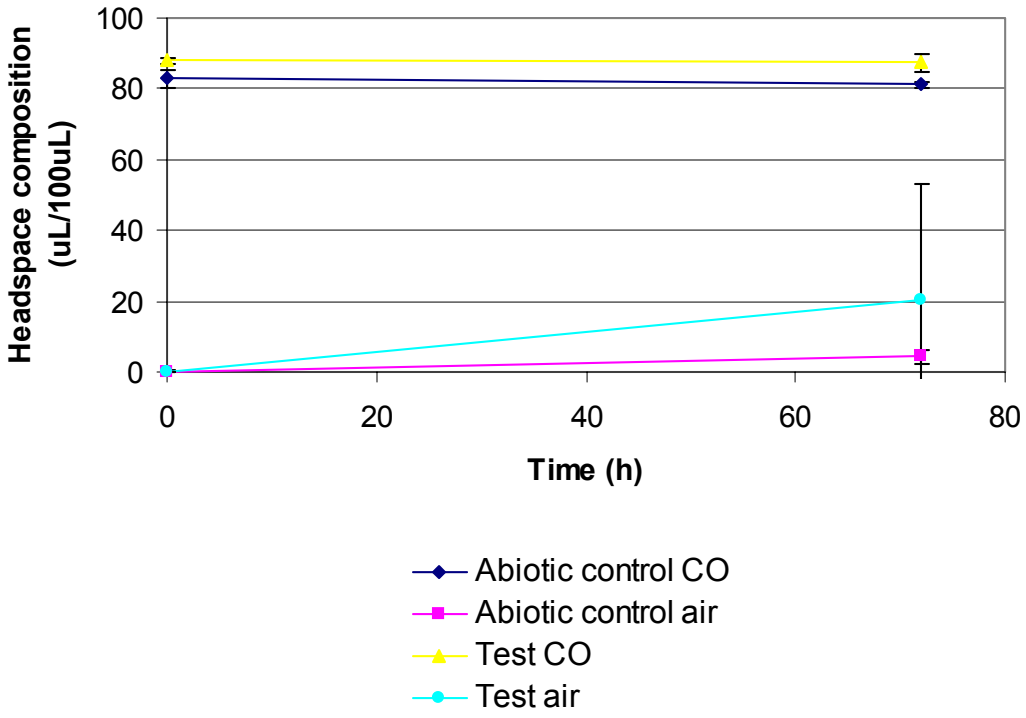

Figure A.3-1. Headspace gas changes over time for Trial A-1 of MSU-1 with an initial headspace composition of 60% CO, 20% H_2, 20% CO_2

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and 400 rpm agitation. N=3.

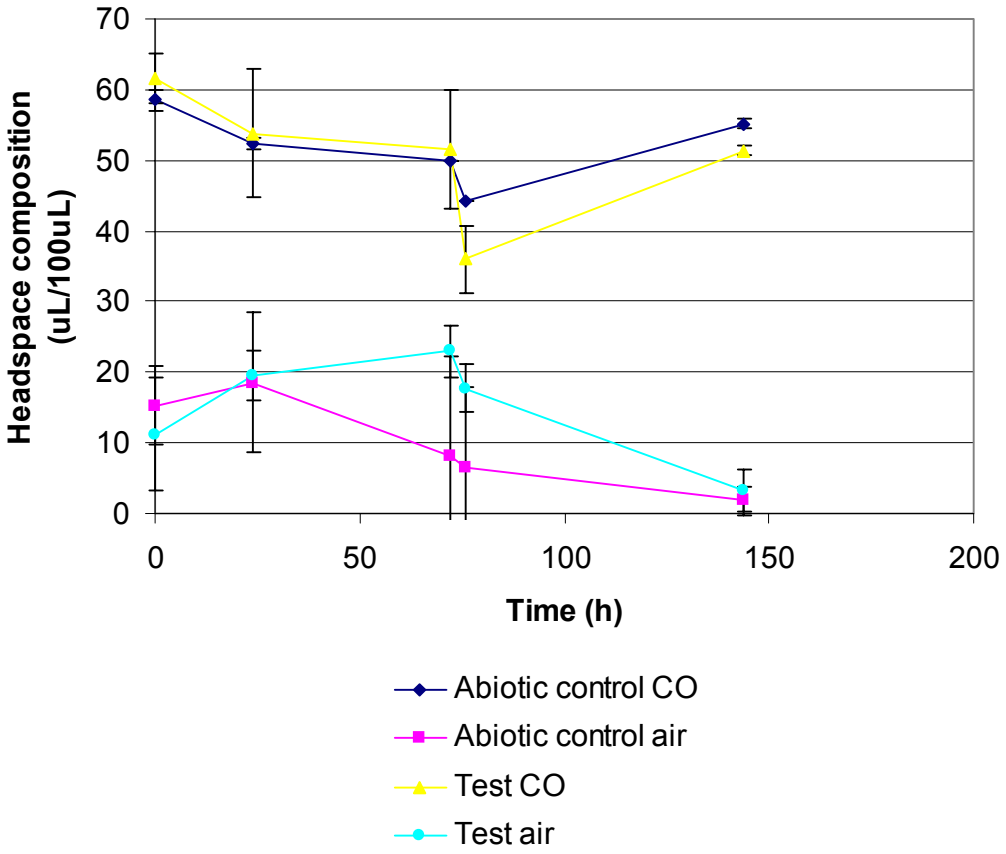

Figure A.4-1. Headspace gas changes over time for Trial A-1 of JAC-1 with an initial headspace composition of 60% CO, 30% H_2, 10% CO_2, regassed at 76 hours

40 mL vials with Si/PTFE caps and 20 mL liquid volume containing APM and cells. The air concentration was calculated by measuring the O_2 concentration and assuming stoichiometric composition of air entering the vial. Incubated at 37ºC and no agitation. N=3.

APPENDIX B

ENRICHMENT SCHEMES FOR MANURE CONSORTIA

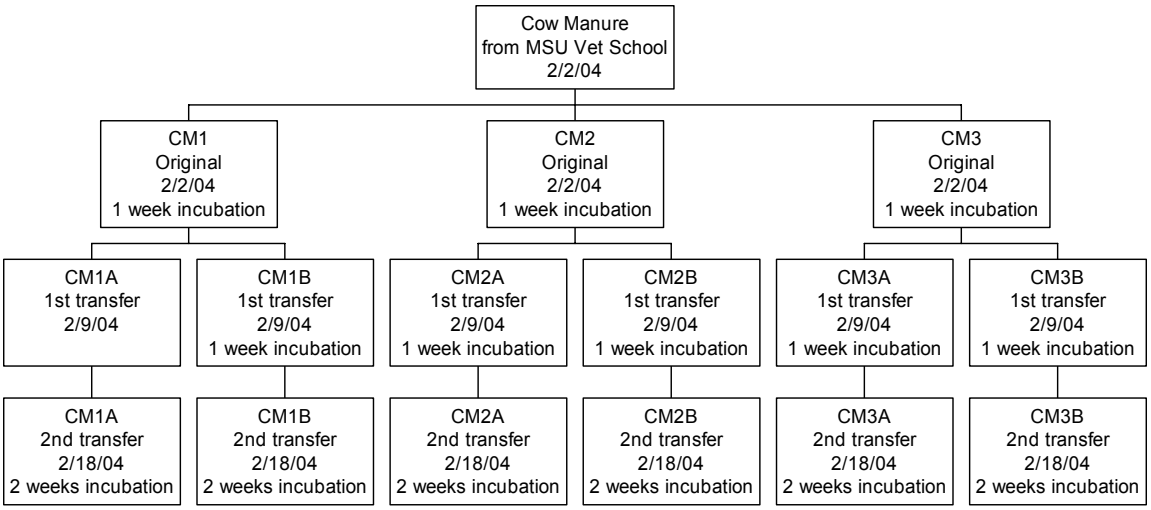

Figure B-1. Enrichment scheme for cow manure consortium

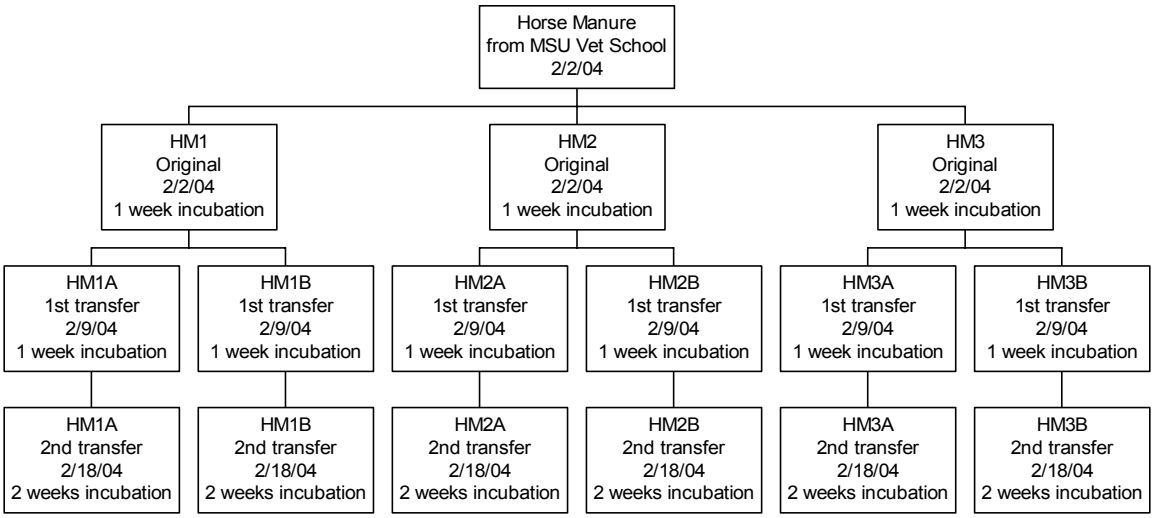

Figure B-2. Enrichment scheme for horse manure consortium

APPENDIX C

RAW DATA FOR GROWTH AND PRODUCTION EXPERIMENTS

Table C.1-1. Optical density for *C. ljungdahlii* over time with different media formulations

	Time (hours)					
Media	**0**	**6**	**24**	**30**	**43**	**96**
1754	0.13	0.23	0.35	0.39	0.26	0.43
1754 + 1 g/L Peptone	0.13	0.23	0.33	0.16	0.35	0.39
1754 + 0.5 g/L Peptone	0.15	0.20	0.35	0.35	0.39	0.41
1754 + 0.1 g/L Peptone	0.15	0.23	0.36	0.38	0.39	0.39
APM + 1 g/L Peptone	0.17	0.14	0.14	0.12	0.07	0.18
APM + 0.5 g/L Peptone	0.14	0.13	0.13	0.11	0.16	0.19
APM + 0.1 g/L Peptone	0.13	0.13	0.09	0.08	0.10	0.25
MSM + 5 g/L Peptone	0.17	0.30	0.44	0.47	0.27	0.23
MSM + 1 g/L Peptone	0.21	0.13	0.09	0.05	0.08	0.11
MSM + 0.1 g/L Peptone + 5 g/L Fructose	0.16	0.15	0.25	0.21	0.20	0.24

Table C.1-2. Data for *C. ljungdahlii* gassed with 2:1 mix of H_2:CO, a

Experiment Date 10/11/02

Description Clostridium ljungdahlii grown in PYF. Centrifuged at 4000 rpm for 25 min. Resuspended in APM. 5 mL conc. cells inoculated into 5 mL APM. Gassed with 2:1 mix of H2:CO. Placed on the shaker incubator at 35 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	72
cells 1	385.794	0.000	0.000
cells 2	0.000	455.850	0.000
cells 3	0.000	0.000	0.000
gas 1	0.000	0.000	37.759
gas 2	0.000	0.000	62.607
gas 3	0.000	40.729	55.086
cells & gas 1	0.000	0.000	0.000
cells & gas 2	0.000	0.000	453.677
cells & gas 3	0.000	0.000	0.000

GAS	GC fraction (uL/100uL)							
sample time (hr)	Initial				4			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	123.58974	0.00000				
cells 2	0.00000	0.00000	124.02176	0.00000				
cells 3	0.00000	0.00000	125.35437	0.00000				
gas 1	0.00000	0.00000	1.80523	10.30234	0.00000	0.07944	2.56081	9.97431
gas 2	0.00000	0.00000	2.27965	11.43830	0.00000	0.08565	3.07608	10.81747
gas 3	0.00000	0.00000	2.47057	12.08827	0.00000	0.00959	2.90797	10.30780
cells & gas 1	4.37948	0.00000	1.70272	12.76745	3.12146	0.00000	2.61253	13.05297
cells & gas 2	0.00000	0.00000	1.71036	12.90497	2.73603	0.00000	2.17233	12.90647
cells & gas 3	0.43895	0.00000	1.50357	12.79909	0.41077	0.00000	2.32919	12.94914

	24				72			
sample time (hr)	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1					0.00000	1.22481	110.47616	0.00000
cells 2					0.00000	1.33660	112.28148	0.00000
cells 3					0.00000	1.31834	111.74292	0.00000
gas 1	0.00000	1.46674	6.35721	10.16600	0.00000	6.25544	24.91782	6.49172
gas 2	0.00000	0.94673	5.04866	10.47227	1.79689	5.72000	20.33548	11.12919
gas 3	0.00000	0.59664	4.39932	10.94076	2.01436	5.39679	20.56895	11.14624
cells & gas 1	1.45631	0.00000	5.52177	13.23010	2.53760	2.57263	16.88868	11.04561
cells & gas 2					0.18318	2.84434	17.69163	11.08356
cells & gas 3					2.15994	1.49378	11.85847	12.01074

Table C.1-3. Data for *C. ljungdahlii* gassed with 2:1 mix of H_2:CO, b

Experiment Date	10/16/02
Description	Replication of 10/11/02 experiment. Clostridium ljungdahlii grown in PYF. Centrifuged at 4000 rpm for 25 min. Resuspended in APM. 5 mL conc. cells inoculated into 5 mL APM. Gassed with 2:1 mix of H2:CO. Placed on the shaker incubator at 35 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	72
cells 1	299.086	398.856	551.551
cells 2	0.000	359.649	0.000
cells 3	335.183	459.227	479.231
gas 1	0.000	0.000	32.415
gas 2	0.000	0.000	48.656
gas 3	0.000	66.862	54.669
cells & gas 1	300.612	0.000	632.455
cells & gas 2	323.590	494.363	423.348
cells & gas 3	335.379	446.275	0.000

GAS	GC fraction (uL/100uL)							
sample time (hr)	Initial				24			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	1.26224	116.43431	0.00000				
cells 2	0.00000	1.77737	114.83267	0.00000				
cells 3	0.00000	0.23356	117.54656	0.00000				
gas 1	3.83388	0.00000	1.33747	12.57560	0.00000	1.46674	6.35721	10.16600
gas 2	3.56547	0.00000	1.43552	12.33239	0.00000	0.94673	5.04866	10.47227
gas 3	4.00167	0.00000	1.49895	12.48361	0.00000	0.59664	4.39932	10.94076
cells & gas 1	0.18243	0.00000	2.00476	9.80664	0.32063	1.20306	11.29058	6.18829
cells & gas 2	0.00000	0.00000	2.10892	13.61450	4.32064	0.00000	1.08350	12.99433
cells & gas 3	0.06451	0.00000	1.88631	13.16770	4.19272	1.38546	11.07999	12.38601

	48			
	CO_2	O_2	N_2	CO
cells 1	0.00000	2.97259	122.04686	0.00000
cells 2	0.00000	1.32202	120.39006	0.00000
cells 3	0.00000	2.22353	119.76139	0.00000
gas 1	0.46423	4.98743	23.58977	10.78787
gas 2	0.44286	4.31101	17.41434	11.23974
gas 3	0.00000	2.47123	17.52451	11.14054
cells & gas 1	0.00000	2.56274	21.41571	7.97672
cells & gas 2	0.37288	2.55900	16.99979	11.83057
cells & gas 3	15.14507	4.04841	18.31454	11.67317

Table C.1-4. Data for *C. ljungdahlii* gassed with 2:1 mix of H_2:CO, c

Experiment Date	10/25/02
Description	Clostridium ljungdahlii grown in PYF. Centrifuged at 4000 rpm for 25 min. Resuspended in physiological saline. Allowed to sit overnight, then centrifuged and resuspended in APM. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 2:1 mix of H2:CO. Placed on the shaker incubator at 35 C.

LIQUID	[Hac] (mg/L)	[Hac] (mg/L)	[Hac] (mg/L)
sample time (hr)	4	24	72
cells 1	78.927	139.749	86.365
cells 2	42.901	77.126	90.195
cells 3	66.895	88.559	105.178
gas 1	0.000	44.893	0.000
gas 2	39.212	66.611	73.416
gas 3	102.003	53.513	54.903
cells & gas 1	90.229	0.000	107.231
cells & gas 2	221.352	83.848	96.848
cells & gas 3	49.596	123.899	96.104

GAS	GC fraction (uL/100uL)							
sample time (hr)	4				24			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	2.52030	123.76832	0.00000	0.00000	0.00000	2.65839	0.00000
cells 2	0.00000	0.00000	125.70868	0.00000	0.00000	0.00000	120.18310	0.00000
cells 3	0.00000	0.03892	125.96838	0.00000	0.00000	0.00000	0.38199	0.00000
gas 1	10.72666	1.17396	2.69623	12.16615	0.00000	2.48141	10.35305	11.03557
gas 2	19.00627	1.95421	3.33545	12.35301	1.50209	0.00000	2.96440	14.51601
gas 3	0.91686	0.49643	2.98060	12.33117				
cells & gas 1	0.50114	0.00000	4.52298	12.31042	0.00000	0.00000	8.31883	0.00000
cells & gas 2	0.38653	0.00000	5.74714	12.23351	6.97546	0.00000	2.32951	0.00000
cells & gas 3	0.51675	2.24537	13.18978	10.29500	0.30723	0.00000	9.81723	13.62431

	72			
sample time (hr)	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	3.94740	0.00000
cells 2	0.00000	0.00000	183.48213	0.00000
cells 3	0.00000	0.00000	0.00000	1.03725
gas 1	4.53138	0.00000	23.64562	12.37200
gas 2	0.00000	0.00000	22.79767	0.00000
gas 3	0.34070	0.00000	7.70327	0.00000
cells & gas 1	7.44668	0.00000	27.34449	12.97880
cells & gas 2	2.38182	0.00000	4.89186	0.00000
cells & gas 3	3.51726	0.00000	3.52672	0.00000

Table C.1-5. Data for *C. ljungdahlii* gassed with 80% CO, 20% H_2

Experiment Date 2/1/03 4:00 PM
Description Clostridium ljungdahlii grown in 1754 Medium. Centrifuged at 4000 rpm for 20 min. Washed 3 times in physiological saline. Resuspended in APM and allowed to sit overnight. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 80%CO, 20%H2. Placed on the shaker incubator at 35 C.

CELL MASS (g/L) 1.2964

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	17	25	48	70	120	168
cells 1	0.000	0.000	0.000	0.000	0.000	0.000
cells 2	0.000	0.000	0.000	0.000	0.000	0.000
cells 3	0.000	0.000	0.000	0.000	0.000	0.000
gas 1	0.000	0.000	0.000	0.000	0.000	0.000
gas 2	0.000	0.000	0.000	0.000	0.000	0.000
gas 3	0.000	0.000	0.000	0.000	0.000	0.000
cells & gas 1	0.000	0.000	0.000	0.000	0.000	0.000
cells & gas 2	0.000	0.000	0.000	0.000	0.000	0.000
cells & gas 3	0.000	0.000	0.000	0.000	0.000	0.000

GAS	GC fraction (uL/100uL)							
sample time (hr)	0				46			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	114.42235	0.00000	0.00000	0.80781	115.14308	0.00000
cells 2	0.00000	0.00000	114.27766	0.00000	0.00000	0.71216	114.70937	0.00000
cells 3	0.00000	0.00000	115.03219	0.00000	0.00000	0.63502	113.42682	0.00000
gas 1	0.06895	0.00647	5.31967	118.06324	0.00000	5.90389	107.15499	0.69584
gas 2	0.30929	0.00000	5.12316	123.82364	0.00000	0.88358	8.22218	114.27270
gas 3	0.36034	0.00000	4.10903	119.85410	0.27086	1.09072	7.83340	114.74629
cells & gas 1	0.08320	0.00000	3.79487	121.22272	1.36031	0.57788	7.16198	114.40129
cells & gas 2	0.21052	0.00000	4.88023	117.04728	0.45054	0.81746	8.30584	114.23110
cells & gas 3	0.00000	0.00000	4.25188	118.59123	0.00000	0.88552	8.79544	114.21651

	167			
sample time (hr)	CO_2	O_2	N_2	CO
cells 1				
cells 2	0.00000	6.04574	122.78575	0.00000
cells 3				
gas 1				
gas 2	0.00000	7.65921	27.23468	96.46567
gas 3				
cells & gas 1	0.00000	6.37389	27.19175	94.04739
cells & gas 2	0.00000	5.97173	28.28167	94.29548
cells & gas 3	0.00000	7.67360	30.87283	97.23176

Table C.1-6. Data for *C. ljungdahlii* gassed with 60% CO, 30% H_2, 10% CO_2, a

Experiment Date 2/15/03 12:00 PM
Description Clostridium ljungdahlii grown in 1754 Medium. Centrifuged at 4000 rpm for 20 min. Washed 1 time in physiological saline. Resuspended in APM and allowed to sit overnight. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60%CO, 30%H2, 10%CO2. Placed on the shaker incubator at 35 C.

CELL MASS (g/L) 1.5242

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1	0.000	0.000	0.000
cells 2	0.000	0.000	0.000
cells 3	0.000	0.000	0.000
gas 1	0.000	0.000	0.000
gas 2	0.000	0.000	0.000
gas 3	0.000	0.000	0.000
cells & gas 1	0.000	0.000	0.000
cells & gas 2	0.000	0.000	0.000
cells & gas 3	0.000	0.000	0.000

GAS	GC fraction (uL/100uL)							
sample time (hr)	0				122			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	126.78815	0.00000	0.00000	0.82470	127.35220	0.00000
cells 2	0.00000	0.00000	125.00033	0.00000	0.01230	0.49154	122.62322	0.00000
cells 3	0.00000	0.00000	126.96528	0.00000	0.00000	0.00000	121.87372	0.00000
gas 1	0.00000	0.00000	8.06489	73.76552	0.00000	3.47768	37.22273	59.27842
gas 2	0.26662	0.00000	8.47425	74.78253	0.10944	6.08245	43.59079	47.81147
gas 3	0.80690	0.00000	8.94620	75.24093	0.00000	0.00000	44.16583	72.61816
cells & gas 1	0.00000	0.00000	8.00728	74.86402	0.00000	1.04765	37.80120	61.19493
cells & gas 2	1.93871	0.00000	7.64856	75.49157	0.00000	0.00000	44.25392	67.81418
cells & gas 3	1.20962	0.00000	7.99106	72.43955	0.00000	0.11642	35.45710	61.38924

Table C.1-7. Data for *C. ljungdahlii* gassed with 60% CO, 30% H_2, 10% CO_2, b

Experiment Date 4/8/03

Description Clostridium ljungdahlii grown in 1754 Medium. Centrifuged at 4000 rpm for 20 min. Washed 1 time in physiological saline. Resuspended in APM and allowed to sit overnight. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60%CO, 40% H2/CO2 mix. Placed on the

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1	NO SAMPLES TAKEN		
gas 2			
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0				42			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	121.93467	0.00000	0.00000	0.00000	0.50391	0.00000
cells 2	0.00000	0.01319	126.95016	0.00000	0.11979	0.00000	0.35178	0.00000
cells 3	0.00000	0.00000	131.25681	0.00000	0.00000	0.00000	135.43288	0.00000
gas 1	0.00000	2.75146	18.11788	83.82756	1.78386	0.00000	10.33874	75.00471
gas 2	1.71326	0.23930	11.76373	81.30909	1.02533	0.00000	16.44386	77.44905
gas 3	0.75409	1.30003	9.64390	85.05116	1.02533	0.00000	16.44386	77.44905
cells & gas 1	0.00000	0.00000	7.38550	85.38605	3.98048	0.00000	8.68010	88.34968
cells & gas 2	0.05652	0.00000	9.19726	82.20738	4.53568	0.00000	8.99838	92.13582
cells & gas 3	0.10746	0.00000	10.15472	79.32678	1.80533	1.00112	2.91537	67.42097

	GC fraction (uL/100uL)			
sample time (hr)	70			
component	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	169.51979	0.00000
cells 2	0.00000	0.00000	149.14951	0.00000
cells 3	0.00000	0.00000	157.39085	0.00000
gas 1	0.00000	0.00000	12.44125	60.39199
gas 2	0.00000	0.00000	9.22464	84.30098
gas 3	1.61747	0.00000	8.85851	82.98148
cells & gas 1	0.00000	0.00000	3.38080	86.03455
cells & gas 2	0.00000	0.00000	7.60438	86.41772
cells & gas 3	2.18226	0.00000	10.52042	57.42595

Table C.1-8. Data for *C. ljungdahlii* gassed with 60% CO, 30% H_2, 10% CO_2, c

Experiment Date 5/29/03

Description Clostridium ljungdahlii grown in 1754 Medium. Centrifuged at 4000 rpm for 20 min. Resuspended in APM and allowed to sit overnight. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60%CO, 30% H2 10% CO2. Placed on the shaker incubator at 35 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1	NO SAMPLES TAKEN		
gas 2			
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0				24			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	10.85543	0.49355	98.99957	0.00000	5.69099	0.27832	95.23991	0.00000
cells 2	4.10936	0.45515	97.05756	0.00000	5.33180	0.31802	95.18442	0.00000
cells 3	4.27553	0.50424	98.13260	0.00000	35.69208	0.30237	95.84938	0.00000
gas 1	4.44190	0.22079	4.05364	51.61830	5.99608	1.11991	7.26528	49.58443
gas 2	3.20109	0.25373	4.87337	51.27188	39.49280	0.27092	5.39650	50.58400
gas 3	3.52356	0.76227	3.65063	52.08330	39.13712	1.56734	6.13428	49.75200
cells & gas 1	25.81616	0.19355	3.98319	51.93197	0.00000	1.57027	9.46662	47.65266
cells & gas 2	16.21747	0.30156	5.11147	51.37773	14.35091	0.28426	5.83815	48.37261
cells & gas 3	5.85055	0.21479	4.71746	51.41497	12.76391	0.11520	5.51673	50.80237

	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	92				120			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	29.62974	0.42842	93.01194	0.00000	5.05642	1.44227	94.53733	0.00000
cells 2	10.07062	0.77132	95.56677	0.00000	0.00000	1.52899	94.24534	0.00000
cells 3	20.41258	0.42534	90.76333	0.00000	18.68624	1.58324	95.02427	0.00000
gas 1	107.19557	1.14570	7.16805	49.40243	4.63647	2.50409	11.28105	48.15111
gas 2	93.48599	0.61423	5.78163	48.20654	27.22445	0.86014	6.61959	50.38951
gas 3	66.83917	14.13541	43.34953	24.32325	90.15315	3.54178	11.73361	49.97294
cells & gas 1	27.71373	0.37808	9.20036	49.73208	22.35996	2.51828	15.97360	45.49397
cells & gas 2	16.29491	0.16116	6.33115	50.81292	38.37557	1.57129	10.35189	49.24805
cells & gas 3	33.59158	0.37904	6.61383	52.05588	26.70453	0.83312	8.94353	49.81104

	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	164				192R			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	2.30450	95.40249	0.00000	0.00000	3.86369	117.96813	1.42837
cells 2	5.33923	2.53224	95.72345	0.00000	0.00000	4.11545	122.13064	1.43015
cells 3	16.06029	2.81059	95.06504	0.00000	0.00000	3.30431	114.77881	0.00000
gas 1	93.31572	1.50188	12.81365	46.04256	1.19243	5.71452	8.28790	49.13475
gas 2	51.26832	1.77635	12.40014	48.02780	1.28935	6.79839	7.96717	51.90640
gas 3	17.30096	3.67128	14.37195	46.87606	1.23744	5.50914	6.11381	52.25042
cells & gas 1	64.33673	0.42410	26.35740	41.48602	2.20875	5.44494	6.81797	54.66029
cells & gas 2	20.25281	1.61351	12.46542	48.98308	2.08462	6.64479	4.37985	55.27919
cells & gas 3	13.95289	0.38708	13.06607	48.77610	2.07353	7.12577	7.21986	51.39818

	GC fraction (uL/100uL)			
sample time (hr)	260			
component	CO_2	O_2	N_2	CO
cells 1	0.00000	0.98456	91.92885	0.00000
cells 2	0.00000	1.40996	93.53367	0.00000
cells 3	4.66265	4.30596	92.45262	0.00000
gas 1	4.66880	1.20249	7.96213	50.52224
gas 2	27.15227	0.38248	5.69869	52.26892
gas 3	77.06835	0.81564	5.41630	51.16989
cells & gas 1	6.94314	0.53899	6.81292	54.98589
cells & gas 2	8.55795	0.80411	5.22686	57.01187
cells & gas 3	18.39342	0.31246	5.80618	53.18001

Table C.1-9. Data for *C. thermoaceticum* gassed with 60% CO, 40% H_2

Experiment Date 5/2/03
Description Clostridium thermoaceticum grown in 1203 Medium. Centrifuged at 4000 rpm for 20 min. Resuspended in APM. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60%CO, 40% H2. Placed on the shaker incubator at 60 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1	NO SAMPLES TAKEN		
gas 2			
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0				19			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	129.19378	0.00000	0.00000	0.44649	131.68350	0.00000
cells 2	0.00000	0.29755	127.04461	0.00000	0.00000	0.00000	143.05633	0.00000
cells 3	0.00000	0.00000	133.44034	0.00000	0.00000	0.00000	131.23716	0.00000
gas 1	0.00000	0.00000	11.50846	63.06531	0.00000	13.21614	13.54751	63.17617
gas 2	0.00000	0.00000	7.12993	60.02117	0.00000	7.49361	10.20148	59.89242
gas 3	0.00000	0.00000	8.10503	60.04533	0.00000	7.80976	10.02545	59.65118
cells & gas 1	0.00000	8.86875	6.53741	61.17307	0.00000	0.00000	10.23360	60.52994
cells & gas 2	0.00000	0.00000	7.23853	60.17365	0.00000	0.00000	10.73141	62.72483
cells & gas 3	0.00000	0.00000	11.06467	63.19406	0.00000	6.43392	8.87446	57.40351

Table C.1-10. Data for *C. thermoaceticum* gassed with 60% CO, 30% H_2, 10% CO_2

Experiment Date 5/21/03

Description Clostridium thermoaceticum grown in 1203 Medium. Centrifuged at 4000 rpm for 20 min. Resuspended in APM. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60% CO, 30% H2, 10% CO2. Placed on the shaker incubator at 60 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1	NO SAMPLES TAKEN		
gas 2			
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	22				45			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	5.98672	4.71132	98.73009	0.00000	12.22519	0.36518	97.30613	0.00000
cells 2	16.67652	2.04629	97.50361	0.00000	11.38282	0.34671	96.15288	0.00000
cells 3	9.82178	5.05611	99.88698	0.00000	34.16880	1.29387	96.52106	0.00000
gas 1	11.14104	0.00000	5.64070	57.53031	11.57931	0.39730	6.47918	56.78560
gas 2	50.55845	1.14403	7.09697	53.00650	51.59901	0.52012	6.35135	52.06850
gas 3	12.81700	0.77350	7.07913	50.57013	66.96934	0.60595	7.26136	51.31587
cells & gas 1	58.21490	0.00000	6.39971	55.38833	67.11306	0.35035	7.36685	55.21327
cells & gas 2	51.57651	0.00000	7.73693	52.09501	12.63354	0.32991	7.61510	52.53726
cells & gas 3	56.36707	0.62370	8.22146	52.65385	20.56254	0.28880	7.26361	51.90645

	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	142				144R			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	9.95731	0.36991	95.16189	0.00000	8.66793	0.25619	95.63321	0.00000
cells 2	24.10662	0.36269	94.79139	0.00000	4.92264	0.76179	94.40510	0.00000
cells 3	59.89397	2.13493	94.07971	0.00000	9.46638	0.84531	97.64665	0.00000
gas 1	29.87118	0.22814	6.95456	57.23986	3.43400	0.00201	2.53341	56.79168
gas 2	49.23685	0.11202	6.57644	51.27452	8.83783	0.74917	3.20721	55.25313
gas 3	14.71706	0.41572	8.32406	50.53622	6.06601	0.21463	3.37408	55.46567
cells & gas 1	7.66041	0.23865	7.73019	52.09051	8.99891	0.31468	2.15917	56.31215
cells & gas 2	19.27163	0.24054	9.94954	50.68804	47.09014	0.76218	3.02471	54.78958
cells & gas 3	23.96967	0.25402	13.98784	48.49027	38.32681	0.19493	3.06553	55.85946

	GC fraction (uL/100uL)			
sample time (hr)	288			
component	CO_2	O_2	N_2	CO
cells 1	10.57110	0.27544	92.32207	0.00000
cells 2	6.32410	0.38777	93.25717	0.00000
cells 3	66.74244	0.33054	95.05739	0.51323
gas 1	21.43949	0.50719	2.90335	54.31542
gas 2	11.08260	0.62585	4.01638	55.91304
gas 3	87.44324	0.53311	3.77083	56.03234
cells & gas 1	24.47012	5.13436	18.49347	32.67737
cells & gas 2	26.57413	0.09433	4.42939	43.13067
cells & gas 3	12.03336	2.52864	12.24818	52.99306

Table C.2-1. Data for MSU-1 gassed with 80% CO, 20% H_2, a

Experiment Date 2/7/03 12:30 PM

Description MSU1 culture grown in New Medium. Centrifuged at 4000 rpm for 20 min. Washed 3 times in physiological saline and allowed to sit overnight. Resuspended in APM. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 80%CO, 20%H2. Placed on the shaker incubator at 35 C.

CELL MASS (g/L) 3.8114

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	25	48
cells 1	0.000	0.000	0.000
cells 2	0.000	0.000	0.000
cells 3	0.000	0.000	0.000
gas 1	0.000	0.000	0.000
gas 2	0.000	0.000	0.000
gas 3	0.000	0.000	0.000
cells & gas 1	0.000	0.000	0.000
cells & gas 2	0.000	0.000	0.000
cells & gas 3	0.000	0.000	0.000

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0				74			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.22516	0.00000	128.93955	0.00000	0.00000	0.00000	122.80118	0.00000
cells 2	0.00000	0.00000	126.22591	0.00000	0.00000	0.00000	129.77648	0.00000
cells 3	0.00000	0.00000	125.50797	0.00000	0.00000	0.00000	122.60465	0.00000
gas 1	0.00000	0.00000	6.38298	119.18284	0.00000	2.53213	18.05416	101.85777
gas 2	0.00000	0.00000	8.17470	115.47889	0.00000	2.52714	19.62922	102.73875
gas 3	0.00000	0.00000	6.66298	121.54123	0.00000	2.89023	18.36417	102.96734
cells & gas 1	0.00000	0.00000	7.26922	116.43788	0.00000	0.00000	17.71907	98.79448
cells & gas 2	0.00000	0.00000	5.48023	117.93051	0.00000	0.00000	14.20812	105.44459
cells & gas 3	0.00000	0.00000	4.29498	119.23868	0.00000	0.00000	14.48042	107.52933

sample time (hr)	123			
component	CO_2	O_2	N_2	CO
cells 1	0.00000	0.18250	127.12163	0.00000
cells 2	0.00000	0.48715	126.18585	0.00000
cells 3	0.00000	0.22516	128.18325	0.00000
gas 1	0.00000	4.00240	25.22457	100.75480
gas 2	0.00000	3.90734	26.35142	100.58955
gas 3	0.00000	3.95066	24.48338	99.46505
cells & gas 1	0.00000	2.01986	28.26614	96.46904
cells & gas 2	0.00000	0.37527	22.49258	104.26775
cells & gas 3	0.00000	0.77958	22.37234	102.85077

Table C.2-2. Data for MSU-1 gassed with 80% CO, 20% H_2, b

Experiment Date 2/23/03

Description MSU1 culture grown in New Medium. Centrifuged at 4000 rpm for 20 min. Washed 1 time in physiological saline and allowed to sit overnight. Resuspended in APM. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60% CO, 20% H2, 20% CO2. Placed on the shaker incubator at 35 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1			
gas 2	NO SAMPLES TAKEN		
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0				68			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.01834	124.10015	0.00000	0.00000	0.00000	118.51350	0.00000
cells 2	0.00000	0.05607	120.81862	0.00000	0.00000	0.77036	126.23276	0.00000
cells 3	0.00000	0.11797	122.67281	0.00000	0.03293	10.56194	130.34086	0.00000
gas 1	0.63771	0.08984	6.91966	83.74467	0.42990	0.66949	14.69979	81.87320
gas 2	0.63272	0.00000	9.39819	80.13556	0.34735	0.58383	16.71647	80.54311
gas 3	0.87250	0.00000	8.22625	84.46878	0.54744	1.31830	16.10180	80.84946
cells & gas 1	1.39178	0.00000	5.38995	88.37764	0.00000	11.59389	8.74517	87.76380
cells & gas 2	0.13741	0.00000	5.06522	88.05028	0.16489	0.00000	6.51035	89.83872
cells & gas 3	0.12000	0.00000	5.25272	87.19231	0.03744	0.68680	11.13510	84.64739

Table C.2-3. Data for JAC-1 gassed with 60% CO, 30% H_2, 10% CO_2, a

Experiment Date 4/25/03
Description JAC-1 grown in 1754 Medium. Centrifuged at 4000 rpm for 20 min. Resuspended in APM and allowed to sit overnight. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60%CO, 40% H2/CO2 mix. Placed on the shaker incubator at 35 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1	NO SAMPLES TAKEN		
gas 2			
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	25				94			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	115.25463	0.00000	0.00000		31.15849	0.00000
cells 2	0.00000	0.00000	116.18296	0.00000	0.00000	0.00000	121.37437	0.00000
cells 3	0.00000	0.00000	117.90520	0.00000	0.90877	0.00000	119.09787	0.00000
gas 1	0.82759	0.00000	1.33773	80.19778	3.04841	0.00000	8.92387	62.91774
gas 2	0.49481	0.00000	1.18776	83.40490	1.40725	0.00000	5.29652	63.78341
gas 3	1.73562	0.00000	1.32421	85.03018	3.93374	0.00000	7.25612	61.27085
cells & gas 1	0.00000	0.00000	1.17222	86.09190	1.44675	0.00000	3.13890	29.97712
cells & gas 2	1.82723	0.00000	1.09208	85.38326	3.11867	0.00000	4.91963	64.84088
cells & gas 3	1.30911	0.00000	1.19925	83.42899	7.30300	0.00000	8.45449	63.05791

	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	98R				192			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	119.58174	0.00000	0.00000	0.00000	131.41702	0.00000
cells 2	0.00000	0.00000	120.58947	0.00000	0.00000	0.00000	132.13537	0.00000
cells 3	0.00000	0.00000	120.10719	0.00000	0.00000	0.00127	133.17642	1.93371
gas 1	1.72883	0.00000	1.12856	86.74719	0.00000	6.75799	13.64807	81.44640
gas 2	0.89860	0.00000	0.90786	85.57251	0.00000	8.35459	11.60935	83.22212
gas 3	1.16828	0.00000	1.05851	85.86995	0.00000	8.77094	16.24881	82.18722
cells & gas 1	0.00000	0.00000	0.25931	11.61370	0.00000	0.00000	0.00000	80.72451
cells & gas 2	0.32924	0.04993	1.32374	88.69343	0.00000	7.54054	10.30768	84.51602
cells & gas 3	1.67218	0.00000	1.04283	86.87335	0.00000	8.15192	16.49607	80.75162

Table C.2-4. Data for JAC-1 gassed with 60% CO, 30% H_2, 10% CO_2, b

Experiment Date 5/16/03

Description JAC-1 grown in 1754 Medium. Centrifuged at 4000 rpm for 20 min. Resuspended in APM and allowed to sit overnight. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60%CO, 40% H2/CO2 mix. Placed on the shaker incubator at 35 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1	NO SAMPLES TAKEN		
gas 2			
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0				27			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	1.33880	128.65028	0.00000	0.00000	2.18980	115.74480	0.00000
cells 2	0.00000	1.45434	127.04272	0.00000	0.00000	2.33961	112.51001	0.00000
cells 3	0.00000	6.95851	122.17141	0.00000	0.03597	3.72314	110.53541	0.00000
gas 1	1.55693	3.78722	5.83257	57.80635	0.00000	1.72389	6.44869	52.73384
gas 2	1.53560	3.58758	5.95397	60.28196	0.00000	3.68025	7.03193	51.45708
gas 3	1.30932	1.78535	2.65156	57.34524	0.00000	5.69092	4.97209	52.89210
cells & gas 1	6.93153	4.09351	11.71118	65.62031	1.48411	4.04973	14.70603	61.69816
cells & gas 2	2.28801	1.10250	4.75145	60.32847	0.28258	4.50320	8.21068	55.95663
cells & gas 3	1.65938	1.53543	5.06240	58.93708	0.00000	3.12182	6.10767	43.90466

	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	70				76R			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	0.00000	2.72957	96.41931	0.00000	0.00000	1.35458	65.17616	0.00000
cells 2								
cells 3								
gas 1	0.00000	4.88980	6.72247	49.90509	1.47265	3.93165	5.50460	44.26888
gas 2								
gas 3								
cells & gas 1	1.50319	4.38195	18.21682	58.81261	1.93902	4.31243	6.35082	41.18945
cells & gas 2	0.11889	3.97807	5.66034	42.50398	1.28790	3.11567	4.22778	35.16903
cells & gas 3	0.22620	5.40558	8.14861	53.36229	1.18074	3.18184	4.76431	31.70638

	GC fraction (uL/100uL)			
sample time (hr)	141			
component	CO_2	O_2	N_2	CO
cells 1	3.37632	1.35695	98.27213	1.21508
cells 2	0.00000	5.05486	97.32159	0.00000
cells 3	4.28034	2.16586	96.59722	0.00000
gas 1	45.25792	0.79956	5.99116	54.56908
gas 2	26.02220	0.31571	5.91638	55.85876
gas 3	32.98747	0.00000	7.86201	54.96808
cells & gas 1	54.48401	0.00000	8.17773	50.53104
cells & gas 2	7.59502	1.14060	7.12469	51.55710
cells & gas 3	10.52153	0.85447	8.17336	51.96128

Table C.2-5. Data for JAC-1 gassed with 60% CO, 30% H_2, 10% CO_2, c

Experiment Date 6/4/03

Description JAC-1 grown in 1754 Medium. Centrifuged at 4000 rpm for 20 min. Resuspended in APM and allowed to sit overnight. 5 mL conc. cells inoculated into 15 mL APM. Gassed with 60%CO, 30% H2, 10% CO2 mix. Placed on the shaker incubator at 35 C.

LIQUID	[HAc] (mg/L)	[HAc] (mg/L)	[HAc] (mg/L)
sample time (hr)	4	24	48
cells 1			
cells 2			
cells 3			
gas 1	NO SAMPLES TAKEN		
gas 2			
gas 3			
cells & gas 1			
cells & gas 2			
cells & gas 3			

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0				46			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
cells 1	4.48646	1.09249	96.27721	0.00000	9.20127	1.30989	95.29538	0.00000
cells 2	9.22787	11.39344	89.16893	0.00000	6.48149	6.25117	91.19405	0.00000
cells 3	0.00000	1.28405	96.21960	0.00000	22.51319	0.49358	92.68222	0.00000
gas 1	27.20155	0.27636	6.72205	52.55830	94.66548	0.29800	7.67484	54.94493
gas 2	5.64178	0.16331	6.92284	51.38720	40.50970	0.38266	7.91878	51.75032
gas 3	4.08116	0.20101	7.34814	53.95739	25.68159	0.89921	9.47183	49.45699
cells & gas 1	3.66244	0.20031	5.96782	52.78714	37.80213	0.35975	7.30043	53.52507
cells & gas 2	8.40109	0.40289	6.45934	52.14112	39.97031	0.35136	10.75263	51.47615
cells & gas 3	5.68539	0.18671	6.12840	53.65168	107.42416	0.37940	7.71971	53.23994

	GC fraction (uL/100uL)			
sample time (hr)	119			
component	CO_2	O_2	N_2	CO
cells 1	0.00000	0.00000	94.11545	0.00000
cells 2	0.00000	6.44739	89.29178	0.00000
cells 3	0.00000	0.00000	92.74022	0.00000
gas 1	5.48599	0.00000	10.32135	50.66302
gas 2	7.90268	0.83975	9.23447	51.23429
gas 3	0.00000	0.02330	13.25640	51.61287
cells & gas 1	58.66852	0.20393	12.38828	51.12503
cells & gas 2	34.25484	0.21667	17.68868	47.49457
cells & gas 3	6.17451	0.53568	11.88704	50.26465

Table C.3-1. Data for 37ºC cap test

Experiment Description Vials were filled to 1/2 total volume with 1% Resuzurin in H2O in the glove bag. Resazurin turns blue in the presence of oxygen. 6 vials used crimp tops, 6 used white caps, 6 used mininert tops. Vials were removed from the glove bag, and gassed with 100% CO. 3 vials with each top type were placed in the 37C incubator in the anaerobic glove bag (control). The remaining vials were placed on the shaker incubator at 37C (test). The purpose of the experiment was to determine which tops leak the fastest, and how fast they leak.

			Initial samples			
Cap type	Location	Number	CO2	O2	N2	CO
crimp	glove bag	1	0.00000	0.00000	0.61874	110.78613
crimp	glove bag	2	0.00000	0.00000	0.70037	109.76181
crimp	glove bag	3	0.00000	0.00000	0.98655	108.03735
crimp	shaker	1	0.00000	0.00000	0.65556	106.57636
crimp	shaker	2	0.00000	0.00000	0.76717	109.93232
crimp	shaker	3	0.00000	0.00000	1.06595	109.94631
silicone/PTFE	glove bag	1	0.00000	0.00000	0.55721	110.28373
silicone/PTFE	glove bag	2	0.00000	0.00000	0.53649	108.19963
silicone/PTFE	glove bag	3	0.00000	0.00000	0.57874	108.28309
silicone/PTFE	shaker	1	0.00000	0.00000	0.54251	107.01262
silicone/PTFE	shaker	2	0.00000	0.00000	0.59902	109.93045
silicone/PTFE	shaker	3	0.00000	0.00000	0.63703	109.84999
mininert	glove bag	1	0.00000	0.00000	0.73780	106.65972
mininert	glove bag	2	0.00000	0.00000	0.76388	108.04241
mininert	glove bag	3	N/A	N/A	N/A	N/A
mininert	shaker	1	0.00000	0.00000	0.68730	110.24009
mininert	shaker	2	0.00000	0.00000	0.90262	106.32494
mininert	shaker	3	0.00000	0.00000	0.69359	108.61318

			24 hr samples			
Cap type	Location	Number	CO2	O2	N2	CO
crimp	glove bag	1	N/A	N/A	N/A	N/A
crimp	glove bag	2	N/A	N/A	N/A	N/A
crimp	glove bag	3	N/A	N/A	N/A	N/A
crimp	shaker	1	0.00000	0.00000	2.14058	105.69291
crimp	shaker	2	0.00000	0.21606	3.60979	115.63349
crimp	shaker	3	0.00000	0.26256	3.76404	112.35887
silicone/PTFE	glove bag	1	N/A	N/A	N/A	N/A
silicone/PTFE	glove bag	2	N/A	N/A	N/A	N/A
silicone/PTFE	glove bag	3	N/A	N/A	N/A	N/A
silicone/PTFE	shaker	1	0.00000	0.00000	2.36912	110.09462
silicone/PTFE	shaker	2	0.00000	0.00000	2.79754	110.07114
silicone/PTFE	shaker	3	0.00000	0.00000	2.31389	108.78524
mininert	glove bag	1	N/A	N/A	N/A	N/A
mininert	glove bag	2	N/A	N/A	N/A	N/A
mininert	glove bag	3	N/A	N/A	N/A	N/A
mininert	shaker	1	0.00000	0.00000	1.51697	111.92746
mininert	shaker	2	0.00000	0.00000	1.76115	110.23797
mininert	shaker	3	0.00000	0.00000	1.49059	109.26372

Table C.3-1. Data for 37ºC cap test, continued from last page

			48 hr samples			
Cap type	Location	Number	CO2	O2	N2	CO
crimp	glove bag	1	0.00000	0.00000	1.36576	107.89097
crimp	glove bag	2	0.00000	0.00000	2.15509	105.81194
crimp	glove bag	3	0.00000	0.00000	1.70144	107.29894
crimp	shaker	1	0.00000	0.00000	1.44553	95.92790
crimp	shaker	2	0.00000	0.00000	1.60807	106.09510
crimp	shaker	3	0.00000	0.97027	5.75204	95.37940
silicone/PTFE	glove bag	1	0.00000	0.00000	2.02613	106.93878
silicone/PTFE	glove bag	2	0.00000	0.00000	2.14474	108.13822
silicone/PTFE	glove bag	3	0.00000	0.00000	3.37211	105.00804
silicone/PTFE	shaker	1	0.00000	0.00000	2.81140	106.39123
silicone/PTFE	shaker	2	0.00000	0.00000	2.61048	105.52606
silicone/PTFE	shaker	3	0.00000	0.00000	3.16525	105.37072
mininert	glove bag	1	0.00000	0.00000	1.70759	105.55019
mininert	glove bag	2	0.00000	0.00000	1.47099	107.15690
mininert	glove bag	3	N/A	N/A	N/A	N/A
mininert	shaker	1	0.00000	0.00000	1.46601	108.57709
mininert	shaker	2	0.00000	0.00000	1.51452	100.64110
mininert	shaker	3	0.00000	0.00000	1.46409	107.42735

			72 hr samples			
Cap type	Location	Number	CO2	O2	N2	CO
crimp	glove bag	1	0.00000	0.00000	1.48588	103.44299
crimp	glove bag	2	0.00000	0.57440	4.25260	101.12446
crimp	glove bag	3	0.00000	0.79568	5.05973	96.36128
crimp	shaker	1	0.00000	0.00000	1.80807	104.63161
crimp	shaker	2	0.00000	0.00000	1.57882	93.49566
crimp	shaker	3	0.00000	1.79768	8.88301	91.29475
silicone/PTFE	glove bag	1	0.00000	0.00000	2.52195	108.75828
silicone/PTFE	glove bag	2	0.00000	0.00000	2.71728	105.59159
silicone/PTFE	glove bag	3	0.00000	0.00000	5.64010	122.57009
silicone/PTFE	shaker	1	0.00000	0.00000	3.78910	104.25472
silicone/PTFE	shaker	2	0.00000	0.00000	3.14781	100.66092
silicone/PTFE	shaker	3	0.00000	0.00000	4.90729	105.04954
mininert	glove bag	1	0.00000	0.00000	1.76968	106.36012
mininert	glove bag	2	0.00000	0.00000	1.39637	104.78118
mininert	glove bag	3	N/A	N/A	N/A	N/A
mininert	shaker	1	0.00000	0.00000	1.42520	109.59584
mininert	shaker	2	0.00000	0.23653	3.34520	105.07727
mininert	shaker	3	0.00000	0.00000	1.46272	105.13228

			168 hr samples			
Cap type	Location	Number	CO2	O2	N2	CO
crimp	glove bag	1	0.00000	0.00000	1.72266	102.73021
crimp	glove bag	2	0.00000	1.82865	9.14521	95.52868
crimp	glove bag	3	0.00000	1.97174	10.58716	94.02225
crimp	shaker	1	0.00000	0.49809	3.75511	78.76309
crimp	shaker	2	0.00000	0.78335	5.03626	99.04501
crimp	shaker	3	0.00000	3.16146	14.14239	86.91486
silicone/PTFE	glove bag	1	0.00000	0.00000	4.45185	103.53610
silicone/PTFE	glove bag	2	0.00000	0.00000	5.02535	104.60129
silicone/PTFE	glove bag	3	0.00000	0.00000	9.72900	99.76523
silicone/PTFE	shaker	1	0.00000	0.00791	9.82293	98.32094
silicone/PTFE	shaker	2	0.00000	0.00000	6.18150	96.64882
silicone/PTFE	shaker	3	0.00000	0.11332	14.99824	92.18784
mininert	glove bag	1	0.00000	0.97110	6.30267	100.81359
mininert	glove bag	2	0.00000	1.59562	8.55652	91.95148
mininert	glove bag	3	N/A	N/A	N/A	N/A
mininert	shaker	1	0.00000	0.00000	1.88525	101.53514
mininert	shaker	2	0.00000	1.71897	10.92310	90.98227
mininert	shaker	3	0.00000	0.85639	5.42325	98.19370

Table C.3-2. Data for 60ºC cap test

Experiment Description Empty vials were gassed with 100% CO and placed in the 60C incubator to determine leakage rate.

Date 4/5/04

	Initial samples			
Trial	CO2	O2	N2	CO
1	0.00000	0.31299	30.33615	74.44625
2	0.00000	0.16471	26.42985	65.29855
3	0.00000	0.29321	33.32457	66.56064

	24 hr samples			
Trial	CO2	O2	N2	CO
1	0.00000	3.75827	37.04163	62.53775
2	0.00000	0.20409	28.67152	71.60150
3	0.00000	0.19790	33.52221	68.17453

	48 hr samples			
Trial	CO2	O2	N2	CO
1	0.00000	6.76535	43.41649	46.58979
2	0.00000	0.08312	29.67088	71.64687
3	0.00000	0.16851	32.78526	65.87328

	72 hr samples			
Trial	CO2	O2	N2	CO
1	0.00000	9.69673	47.51904	36.85036
2	0.00000	0.55686	25.08550	59.24568
3	0.00000	0.34861	32.39889	64.78183

Table C.3-3. Gassing time data

Time	N2	CO
30	32.11437	66.84741
30	35.58738	65.40963
30	34.81453	66.02865
60	10.50111	95.34679
60	11.44604	87.39727
60	11.40235	93.70589
90	6.35671	99.75489
90	4.38917	100.33948
90	4.26547	89.40537
120	2.29929	102.38724
120	2.13161	112.87828
120	2.68475	100.37975
150	1.05744	101.73830
150	1.21211	104.82283
150	0.89468	92.88896

Table C.3-4. Sampling error data

Experiment Description Sampling error test. 3 vials were placed in the anaerobic glove bag overnight. The next day, the caps were tightened and they were removed from the glove bag for sampling.

Date 4/7/04

O2	Sample number									
Vial number	1	2	3	4	5	6	7	8	9	std. dev.
1	0.01776	0.06907	0.14603	0.04291	0.26055	0.26433	0.33469	0.33401	0.43699	0.15
2	0.04984	0.89986	1.09442	0.54131						0.46
3	0.00000	0.24253	0.29186							0.16
std. dev.	0.02526	0.43825	0.51069	0.35242						0.31

N2	Sample number									
Vial number	1	2	3	4	5	6	7	8	9	std. dev.
1	84.79739	85.19098	85.65832	85.65371	86.87938	85.68373	84.14452	85.57793	86.09636	0.77
2	86.62958	81.04268	84.93263	81.16972						2.79
3	86.84550	83.51691	82.80353							2.16
std. dev.	1.12534	2.08697	1.48377	3.17066						1.82

For O2:
0.31 uL/100uL
For air:
0.31 uL/0.20 = 1.55 uL/100 uL

1.55% sampling error

Table C.4-1. Data for Cow Manure Consortium

Experiment Date 2/9/04

Description Cow dung sampled from MSU CVM. Approximately 5g dung placed in each of 3 vials of APM and gassed with 100% CO on 2/9/04. After 1 week, 1st transfer was made, and gassed with 100% CO. At that time, a transfer of 2 mL was made to 2 vials from each of the 3 primary vials. After 2 weeks, 2nd transfer was made: 2mL from each vial into 1 vial each, and gassed with 100% CO.

	Acetate produced			
sample time	Original	1st transfer	2nd transfer(1)	2nd transfer(2)
Cow 1A	0.00	0.00	N/A	244.06
Cow 1B	379.76	137.25	N/A	0.00
Cow 2A	0.00	0.00	N/A	255.55
Cow 2B	0.00	0.00	N/A	1547.43
Cow 3A	0.00	0.00	N/A	0.00
Cow 2B	0.00	0.00	N/A	0.00

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0 (2/2/04)				1 week			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Cow 1	0.00000	0.00000	0.00000	95.85498	0.00000	0.62265	22.60258	81.69457
Cow 2	0.00000	0.00000	0.74440	97.75117	0.00000	0.00000	6.02430	95.58162
Cow 3	0.00000	0.00000	0.00000	101.83307	0.00000	0.00000	5.00059	98.27604

1st transfer	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	1st transfer (2/9/04)				48 hrs			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Cow 1A	0.00000	0.00000	0.00000	109.21360	0.00000	0.00000	3.05853	93.30390
Cow 1B	0.00000	0.00000	0.00000	108.58457	0.00000	0.00000	3.52281	89.47152
Cow 2A	0.00000	0.00000	0.67103	112.91966	0.00000	0.00000	1.96981	93.99309
Cow 2B	0.00000	0.00000	0.00000	108.87215	0.00000	0.00000	3.30733	94.97700
Cow 3A	0.00000	0.00000	0.00000	110.38233	0.00000	0.00000	2.39019	91.35132
Cow 3B	0.00000	0.00000	0.48062	106.65101	0.00000	0.00000	2.60420	98.17774
sample time (hr)	1 week				1 week			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Cow 1A	6.96511	0.00000	8.33422	92.41515	4.71588	1.37097	13.03058	75.16133
Cow 1B	6.87141	0.25924	10.26735	85.15500	4.83941	1.39406	14.99898	72.36183
Cow 2A	4.09043	0.00000	3.71712	76.44816	3.94966	1.21651	7.16118	80.45537
Cow 2B	7.03671	0.38759	8.03742	92.63713	5.06847	1.31649	12.27253	74.48476
Cow 3A	5.98046	0.00000	5.20694	86.49204	4.63385	1.22821	8.89805	77.28671
Cow 3B	6.56395	0.00000	5.67794	92.98747	4.77407	1.20578	8.66096	77.14264

2nd transfer	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	2nd transfer (2/18/04)				1 week			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Cow 1A	0.00000	0.00000	1.39854	92.00645	0.00000	0.00000	6.54762	100.43392
Cow 1B	0.00000	0.00000	1.46809	103.14225	0.00000	2.52085	7.21461	100.11675
Cow 2A	0.00000	0.00000	1.16943	92.91125	0.04724	0.00000	7.06134	101.17116
Cow 2B	0.00000	0.00000	1.36935	96.04425	0.00000	0.34917	8.42773	98.66742
Cow 3A	0.00000	0.00000	1.80235	94.01705	0.35797	0.23272	8.31172	100.13438
Cow 3B	0.00000	0.00000	1.45180	99.60146	0.00000	0.00000	6.93094	101.83567
sample time (hr)	1 week regassing				1 week regassing, 48 hr			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Cow 1A	0.00000	0.00000	1.42591	105.05910	0.37592	0.00000	3.72076	99.88973
Cow 1B	0.00000	0.00000	1.53650	107.76883	0.00000	1.40224	4.39110	102.53492
Cow 2A	0.00000	0.00000	1.34307	102.24189	0.52915	0.00000	3.27858	100.25200
Cow 2B	0.00000	0.00000	2.05304	108.37968	0.00000	0.00000	3.83217	103.37484
Cow 3A	0.00000	0.00000	1.63378	106.51231	0.46087	0.00000	3.66037	100.66876
Cow 3B	0.00000	0.00000	1.67352	108.02679	0.00000	0.00000	3.10206	104.30674
sample time (hr)	2 weeks							
component	CO_2	O_2	N_2	CO				
Cow 1A	1.93296	1.28262	9.82711	76.86683				
Cow 1B	0.00000	6.65755	10.33619	77.67023				
Cow 2A	0.91128	1.34810	10.84359	78.33266				
Cow 2B	0.79985	1.28356	9.95201	80.28732				
Cow 3A	0.37473	2.87374	16.50347	75.21868				
Cow 3B	2.10824	1.73784	10.74074	79.14404				

Table C.4-2 Data for Horse Manure Consortium

Experiment Date 2/9/04

Description Horse dung sampled from MSU CVM. Approximately 5g dung placed in each of 3 vials of APM and gassed with 100% CO on 2/9/04. After 1 week, 1st transfer was made, and gassed with 100% CO. At that time, a transfer of 2 mL was made to 2 vials from each of the 3 primary vials. After 2 weeks, 2nd

	Acetate produced			
sample time	Original	1st transfer	2nd transfer (1)	2nd transfer (2)
Cow 1A	0.00	1410.48	N/A	N/A
Cow 1B	0.00	2000.00	N/A	N/A
Cow 2A	0.00	1565.36	N/A	N/A
Cow 2B	0.00	243.37	N/A	N/A
Cow 3A	0.00	228.97	N/A	N/A
Cow 2B	0.00	0.00	N/A	N/A

GAS	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	0 (2/2/04)				1 week			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Horse 1	0.00000	0.00000	0.68816	94.37208	0.00000	1.17858	19.52562	76.11050
Horse 2	0.00000	0.00000	0.52904	95.89055	0.00000	0.00000	7.52086	95.28429
Horse 3	0.00000	0.00000	0.76692	104.86405	0.00000	0.00000	7.14410	94.86097

1st transfer	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	1st transfer (2/9/04)				48 hrs			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Horse 1A	0.00000	0.00000	0.00000	109.08074	0.00000	0.00000	2.72166	98.57296
Horse 1B	0.00000	0.00000	0.00000	107.98293	0.00000	0.00000	3.94622	94.89126
Horse 2A	0.00000	0.00000	0.00000	104.24828	0.00000	0.00000	3.31384	95.39576
Horse 2B	0.00000	0.00000	0.00000	104.60605	0.00000	0.00000	3.19613	92.66661
Horse 3A	0.00000	0.00000	0.00000	103.76261	0.00000	0.00000	2.61793	96.87053
Horse 3B	0.00000	0.00000	0.00000	107.36464	0.00000	0.00000	2.75706	96.97085
sample time (hr)	1 week				2 week			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Horse 1A	5.22015	0.00000	5.49289	85.11790	4.52805	1.22528	9.15143	77.51872
Horse 1B	7.16187	0.08683	8.60077	88.55882	5.45112	1.27023	12.25412	70.85124
Horse 2A	4.77806	0.00000	6.64056	91.98380	3.90880	1.23971	10.26703	76.83076
Horse 2B	5.27415	0.01925	7.36221	87.57159	3.50850	5.47209	26.87789	57.49387
Horse 3A	5.61136	0.00000	6.41701	95.96303	5.66414	1.21096	9.58844	78.66259
Horse 3B	4.68948	0.00000	5.88517	85.29853	4.32338	1.23198	10.06082	74.49366

2nd transfer	GC fraction (uL/100uL)				GC fraction (uL/100uL)			
sample time (hr)	2nd transfer (2/18/04)				1 week			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Horse 1A	0.00000	0.00000	1.35094	106.40647	0.00000	0.00000	6.71665	101.81878
Horse 1B	0.00000	0.00000	0.71055	89.59642	0.00000	0.00000	6.52145	102.15582
Horse 2A	0.00000	0.00000	1.51557	100.38179	0.00000	0.00000	6.17513	102.15068
Horse 2B	0.00000	0.00000	1.46737	106.12990	0.00000	0.00000	6.25262	100.85497
Horse 3A	0.00000	0.00000	1.43503	87.40959	0.00000	0.00000	5.13920	102.58129
Horse 3B	0.00000	0.00000	1.65975	92.68537	0.00000	0.00000	8.46742	99.40429
sample time (hr)	1 week regassing				1 week regassing, 48 hr			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Horse 1A	0.00000	0.00000	1.65966	107.54448	0.00000	0.00000	3.52573	102.19398
Horse 1B	0.00000	0.00000	1.27409	106.79060	0.00000	0.00000	3.03759	100.97173
Horse 2A	0.00000	0.00000	1.29280	108.06329	0.02031	0.00000	3.15765	105.20614
Horse 2B	0.00000	0.00000	1.62999	108.38856	0.00000	0.00000	3.18838	101.19790
Horse 3A	0.00000	0.00000	1.33318	108.72121	0.00000	0.00000	2.33259	99.58712
Horse 3B	0.00000	0.00000	1.42451	108.17287	0.00586	0.00000	3.62477	102.12328
sample time (hr)	2 week				2 week regassing			
component	CO_2	O_2	N_2	CO	CO_2	O_2	N_2	CO
Horse 1A	0.00000	1.52919	11.83120	81.10058	0.00000	0.00000	0.75291	108.33845
Horse 1B	0.00000	1.30732	10.27285	80.41289	0.00000	0.00000	0.67903	108.86830
Horse 2A	0.04090	1.38246	9.43210	80.93136	0.00000	0.00000	0.76305	107.64904
Horse 2B	0.00000	1.45573	10.46120	80.79304	0.00000	0.00000	0.57982	103.41611
Horse 3A	0.00000	1.45432	7.98732	81.98774	0.00000	0.00000	0.39639	105.77591
Horse 3B	0.00704	2.27951	15.12217	76.30210	0.00000	0.00000	0.77995	99.54986