ABSTRACT

COTTER, JACQUELINE LOUISE. Ethanol and Acetate Production from Synthesis Gas using Microbial Catalysts. (Under direction of Mari S. Chinn.)

The hybrid technology of gasification and fermentation has the potential to serve as a viable approach for ethanol production from plant biomass. Autotrophic bacteria can use gaseous product streams from a gasifier, CO and CO_2 and H_2, to produce ethanol and acetate. The work presented investigates the potential of resting cells to enhance ethanol production by *Clostridium ljungdahlii* and *Clostridium autoethanogenum* as well as the processing parameters significant to synthesis gas fermentation. Resting cells can offer improved cell stability under harsh environmental conditions and enhance the production of secondary metabolites (i.e. ethanol). The objectives of the resting cell culture experiments were to develop methods to induce the resting state in key autotrophic bacteria and evaluate performance based on culture stability over time, ethanol and acetate production, and culture viability. In effort to increase ethanol formation by resting cultures, the effects of benzyl viologen and medium pH were also examined. Studies describing microbial performance of growing *C. ljungdahlii* and *C. autoethanogenum* cultures supplied continuous synthesis gas streams have been limited. The objectives assessing synthesis gas processing conditions included: examining the overall culture metabolism of *C. autoethanogenum* grown on bottled synthesis gas supplied at different flow rate; and the effects of pH and bottled synthesis gas flow rate on substrate utilization and metabolic end product formation by *C. ljungdahlii* in liquid-batch, continuous-gas fermentation.

Variations of nitrogen limited media were tested for function in creating non-growing cells, while maintaining cell viability and density. *C. ljungdahlii* was able to maintain a

stable cell density when transferred to basal medium supplemented with vitamins and trace elements and all major nitrogen removed. Despite the culture's viability, these resting cells did not produce ethanol and acetate in large quantities. Cultures at pH 4.5 and 6.8 produce maximum ethanol concentrations of 0.52 mM and 1 mM over 144 hours, respectively. Cultures at pH 5.5 did not produce any ethanol. Poor performance at the lower pH levels may also be related to viability, where less than 50% viability was observed. The addition of benzyl viologen negatively affected culture viability and resulted in little ethanol and acetate production. *C. autoethanogenum* was sensitive to the amount and source of nitrogen in the different media formulations. Ammonia chloride was necessary for minimizing culture density loss, however non-growing cells did not produce significant quantities of ethanol and acetate. Considering viability of the cells, ethanol seems to be a primary metabolite for these autotrophic bacteria on sugar substrates.

Growth of *C. ljungdahlii* and *C. autoethanogenum* on continuous synthesis gas substrates was slower with longer lag phases than what was observed for growth on sugars. For *C. ljungdahlii* higher cell densities were achieved at a pH of 6.8 (579 mg/L) compared to pH 5.5 (378 mg/L) after 48 hours. In addition, the ethanol concentration at pH 6.8 (3.8 mM) was 110% greater than that at pH 5.5. Acetate concentrations were not statistically affected by pH level. For ethanol formation and ethanol to acetate ratios, flow rate was not a significant factor. Unlike *C. ljungdahlii, C. autoethanogenum* was significantly influenced by gaseous flow rates. More dense cultures were achieved at 10 ml/min compared to 5 ml/min gas flow, 136 mg/L and 109 mg/L, respectively. Although ethanol concentrations were less than 0.5 mM after 60 hours, the 10 ml/min flow rate resulted in a 38% increase in ethanol compared top the 5ml /min treatment.

Overall, ethanol was not observed as a secondary metabolite on sugar and synthesis gas substrates. More acidic initial medium pH levels do not promote ethanol production over acetate and reduced microbial growth potential. Synthesis gas flow rate will have a greater impact on cell culture densities and growth than regulation on end product formation.

ETHANOL AND ACETATE PRODUCTION FROM SYNTHESIS GAS USING MICROBIAL CATALYSTS

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

BIOLOGICAL AND AGRICULTURAL ENGINEERING

Raleigh

2006

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Biography

Jackie Cotter was born the 6th and final member of the 'Cotter Clan' on Canada Day in 1982. Growing up her family moved every few years to a new town that always seemed more exciting than the last. They started in Florida with weekly trips to Disney World, then off to California to hit up the beaches, how they ended up in the hustle-and-bustle of New York they still cannot figure out, and they finally ended up in good ole' Denver, North Carolina. So, it was here and there that Jackie grew up from a little girl who loved to play in the dirt, beat up the boys, and come home to Mama's cookin' to a grown woman who ... still loves the exact same things!

Albeit young, Jackie has had countless *interesting* life experiences of which only a few are worth writing down on this permanent record. In high school she performed extremely well in academics and consistently placed 2nd or 3rd, and sometimes 4th, in almost all of her track and cross country races (not-so-noteworthy). Her freshman year in college, Jackie moved away from home, 35 minutes away to be exact, and discovered that while she *could* make it on her own she'd rather not. Then Jackie moved to Raleigh, NC where she became one with the 'Pack' and earned a degree in biomedical engineering.

Since Jackie began her career as a graduate student she has spent her days dreaming of turtles in a Mexican lagoon and of her next opportunity to kick up dirt and play rugby. All the while she remained dedicated to the billions of little bacteria under her care that might not be visible, but certainly know how to put up a stink! Now that she has completed her Master's degree, Jackie is bound for more adventures and big times, hopefully in the form of a world-saving job that allows for tropical vacations at least 3 times a year.

Acknowledgements

First and foremost, I would like to thank Dr. Mari Chinn. As an advisor she has taught me more than any graduate student could ever hope for. She was there to guide me in the beginning when I wasn't quite sure what 'background' research was all about and she stayed with me till midnight when I just couldn't manage one more late night. Without her by my side to encourage me when there seemed to be no light at the end of the tunnel by wiping away my tears and helping me to laugh it off, I would not have made it through. I cannot say it enough, thank you Dr. Chinn for everything.

An icon in my life, Dr. Boyette: as an undergrad he dealt with my silly questions and gave me a few riddles of his own. His never-ending support is what brought me to graduate school in the first place. Over the years he has spent many hours entertaining my naivety around the shop showing me how to put things together and also how to take them apart, all the while teaching me how to be an engineer. I know I share my expression of gratitude for Dr. Boyette with countless students who have passed through Weaver, the home of Biological and Agricultural Engineering and the heart of many young engineers. Thank you Dr. Boyette for your constant dedication to a truly genuine form of education.

Dr. Amy Grunden is a wonderfully patient advisor whom I called upon throughout my research. Thank you for your understanding when as an engineer I could not figure out just how much was going on inside these crazy bacteria. Your willingness to help me has inspired me to continue research; I hope to one day be the person that people come to decipher their most difficult questions.

To all of my fellow grad students, thank you for the times we have spent together. The laughter we've shared, the glassware we've broken, and the nights roller skating around downtown have made being in grad school worthwhile.

I would like to thank my father for his hard work to support our family over all these years; he has been the perfect example for me. My mother is the rock of our family and everything I've become I owe to her for her selfless love. To my brothers, Chris and Michael, thank you for your comedic support over the years... although there has been considerable physical distance between us, knowing that you are there for me has meant the world. Last but not least, I need to thank my doting sister Jenny for being my first teacher and my number one fan. My sister is the most talented person I know and has taught me some of life's greatest lessons. When I was two, she taught me how to find and follow a good leader. When I was twelve she told me that the only way to cure the blues is to buck-up and laugh it off. And now that I'm twenty-five she's teaching me that if she can grow up, I can too.

Over the last two years my home away from home has been on a green field on the outskirts of Raleigh with my second family, the Raleigh Rugby Football Club. The friends I've made while kickin' tale on the field have all been more than memorable. Being part of a team where unconditional love is the standard has helped the unbearable parts of life just float on by, of course winning a national championship or two doesn't hurt either.

Scott, my sweet crazy baby, thanks for every little, "you can do it, babe," because I needed every one. Because of you, I have been able to grow stronger and dream bigger than I ever imagined. Thank you for bringing me the kindest of friendships.

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Chapter 1: Background and Literature Review

The U.S. currently imports over one-half of its crude oil and will need to import more than two-thirds by 2020 (EIA, 2004). A significant amount of valuable national resources go into securing consistent oil imports from the Middle East. Additionally, CO released from the partial combustion of the low oxygenated fuels used today is a major factor in global warming and ozone depletion. Therefore, the U.S. is looking for more environmentally, socially, and economically sound energy resources.

Ethanol is a renewable high-oxygen content fuel that can be used as a fuel alternative or additive. Traditionally ethanol is made from corn hydrolysis and fermentation: the breakdown of starch into simple sugars by amylase enzymes and conversion of the sugars into ethanol using yeast (RFA, 2005c). However, current U.S. corn production rates for food and feed will not be sufficient enough to handle the increasing demand for ethanol (Baker and Zahniser, 2006).

Biomass is a highly available resource that can be converted to ethanol. Plant biomass consists of industrial and agricultural residues as well as low-cost dedicated energy crops such as corn stover, wood chips, pulp and paper wastes, cotton stalks, and switchgrass (EERE 2005).

Current research involves the conversion of biomass to ethanol through enzyme hydrolysis and fermentation (Sun and Cheng, 2002). However, unlike corn conversion, the process is neither simple nor direct. Plant biomass is made up of lignocellulosic materials which make up the majority of plant cell walls (Pandey et al., 2000). This lignocellulose is difficult for microorganisms to break down through direct fermentation (Kamm et al., 2006). There are costly and time-consuming steps such as size reductions, chemical pretreatments

and enzymatic conversion that must take place before a microorganism can ferment the carbon available in biomass materials.

Biomass can also be combusted in a gasifier to produce synthesis gas containing H_2, CO, and CO_2 (Demirbaş et al., 2002). These simple carbon gases can be converted to fuels through various methods. One method involves utilizing the Fischer-Tropsch cycle in which the synthesis gas is converted to long and short hydrocarbons with multi-stage converters that require chemical catalysts and activators. However, the conversion of syngas to ethanol via chemical catalysts is not an efficient process (Stiles et al., 1991). Therefore biological conversion of synthesis gas to ethanol has become a promising area of research.

Several research groups have explored the use of anaerobic bacteria to convert syngas to ethanol (Gaddy et al., 1992; Datar et al., 2004; and Younesiet al., 2005). However, these studies have yet to define a methodology for generating high ethanol production levels with a stable culture. Additionally, the effects of environmental factors such as syngas flow rate, metabolic stage, and extracellular pH on culture stability and ethanol production have not been well defined. This research further explores how these environmental factors affect ethanol production and culture stability enhancement.

1.1 Energy Trends

Energy consumption rates have steadily risen, and are expected to continue to increase, at a steep rate internationally among both industrialized and developing nations. The International Energy Outlook of 2004 reports that in 1970 297 Quadrillion Btu (QBtu) of energy was consumed throughout the world, including the 97.8 QBtu of energy in oil consumption alone. By 2002 the total world energy consumption increased to 412 QBtu (159.4 QBtu from crude oil) and is projected to rise to almost 600 QBtu by 2020 (EERE,

2006a). The United States energy consumption levels have also steadily been rising with no indication of slowing down. The U.S. consumed 98.8 QBtu in 2003 with over 25% of the energy consumption being dedicated towards transportation. Crude oil provides 97% of the energy used for transportation in the U.S. (MacLean et al., 2004; National Energy Policy Development Group, 2001). Therefore, the U.S. currently depends on foreign oil for over one half of its crude oil needs and it is projected that it will depend on foreign oil for two-thirds of its crude oil needs by 2020 (EIA 2004) .

1.2 Renewable Resources

The limited energy provided by traditional non-renewable sources such as oil, natural gas, coal, and nuclear energies compared to the increasing oil consumption has led the U.S. towards a growing dependency on foreign oil. Therefore, the U.S. has implemented energy policies that aim to focus resources on energy conservation and increased production. An increase in domestic energy production would include exploring renewable energy sources such as hydro, geothermal, wind, and solar power as well as biomass-derived energy (Demirbaş, 2004a). In addition to traditional energy sources being non-renewable, the burning of natural gas or coal for heat or transportation needs generate environmental pollution in the form of excess CO emissions. On the other hand, renewable energy resources provide clean, sustainable energy. Renewable energy sources provide electricity and heat from natural elements, thereby encouraging a balanced environment and economy dependent on domestic energy sources. However, energy production from the main renewable energy sources, excluding biomass, has only provided 3.2% of the energy consumed in the U.S. from 2000 to 2004 (EIA, 2005). The primary limitation on energy production from these major renewable resources is the cost-effectiveness of the overall processes. Biomass

provides a readily available renewable resource that could potentially serve as a viable source for producing energy.

The potential for biomass to serve as an alternative source to meet growing energy needs stems from its high availability and carbon density. Biomass consists of any plant or crop material, whether from a dedicated crop, plant residue, or agricultural byproduct (EERE, 2005). Biomass can be generated from an extensive range of sources. Industrial processing residues from organic sources such as pulp and paper processing plants provide readily available carbon-dense biomass. There are numerous agricultural residues such as corn stover and cotton stalk that are produced as a byproduct from harvesting processes. Forest residues that result from seasonal upkeep to prevent forest fires lead to excess woody masses. Additionally, crops that are produced for industrial purposes that require only a plant's products (enzymes and chemicals) could potentially serve as biomass sources after the desired product is extracted and purified (EERE, 2005). Dedicated crops of both perennial herbaceous plants and rapidly growing woody plants such as switchgrass and willow trees, respectively, can provide biomass for energy production (Lemus and Lal, 2005).

The urgent need for a shift from an economy highly dependent on foreign oil, to a more sustainable economy based on renewable energy sources has led to a change in government policies. Recent legislation including the comprehensive energy bill promotes the use of renewable fuels by providing increased tax credits for the use of renewable energy sources including wind, biomass, solar, and landfill gases. The H.R.6 bill also raises the U.S. Renewable Fuel Standard (RFS) by requiring at least 4 billion gallons of renewable fuel consumption in 2006 with a steady increase to 7.5 billion gallons a year by 2012 (RFA, 2005a). Elevating the renewable fuels standard facilitates the transition towards energy

independence by forcing industries to think innovatively outside of using oil to meet their energy demands.

Ethanol and biodiesel are two primary renewable fuels used in the U.S. as fuel blends and possible fuel alternatives. In 2005, 75 million gallons of biodiesel were produced in the U.S. (NBB, 2006a). With new biodiesel production plants being built, by 2008 the U.S. production capacity for biodiesel is expected to reach over 700 million gallons per year. Each gallon of biodiesel creates 128,000 Btu of energy. Therefore, this increase in biodiesel production could potentially provide up to 8 % of the U.S. energy needs per year (NBB, 2006b). Ethanol production levels have been increasing over the past decade from 1.4 billion gallons of ethanol produced in the U.S. in 1995 compared to 3.9 billion gallons produced in 2005. The total capacity for ethanol production continues to rise as over 33 more ethanol production plants are under construction which will add 1.9 billion gallons of production capacity in 2006 (RFA, 2005b).

1.3 Trends in Ethanol Consumption and Uses

The use of reformulated gasoline has been mandated by the Clean Air Act to reduce CO emission in highly polluted areas around the country (EPA, 1990). Both ethanol and methyl tertiary-butyl ether (MTBE) are used as fuel oxygenates in gasoline mixes ($\leq$ 10 %) to reduce CO emissions of transportation vehicles (ACE, 2005). Midwestern states use ethanol readily available from corn hydrolysis and fermentation as a main fuel oxygenate source. Still, MTBE was the major oxygenate used nationwide due to transportation costs of ethanol from production sites. However, MTBE has been found to contaminate ground water and reach drinking water from “releases” in underground gasoline storage tanks (Stephenson,

2002). “Releases” from underground tanks are primarily due to abandoned tanks, inactive tanks, and tanks with poor leak detection and prevention systems (Stephenson, 2002).

In 1999, 11% of California’s gasoline supply consisted of MTBE. Due to the threat of contamination Governor Davis issued the final MTBE phase-out, Executive OrderD-52-02, planning the complete elimination of MTBE from California gas by 2004. By May of 2003 all of the major refineries supplying gas to California replaced MTBE with ethanol. Because of increasing numbers of local laws around the nation implementing the use of ethanol over MTBE for reformulated gasoline, the demand for ethanol is steadily increasing.

In addition to its uses as a fuel additive, ethanol can be used as the main energy source for automobile engines, acting as a fuel alternative. Flexible Fuel Vehicles, FFVs, can use gasohol blends of 15 – 85% ethanol and are often used in government and private fleet vehicles (NEVC, 2006). More than 5 million vehicles, including at least 20 car models ranging from sports cars to utility trucks, have been sold in the U.S. that can use gasohol blends with up to 85% ethanol (EERE, 2006b). Using ethanol as a primary fuel source for transportation vehicles would greatly reduce the U.S. growing dependency on foreign oil supplies. Although record high levels of ethanol were produced in 2004, 161 million gallons of ethanol were imported to the U.S to supply ethanol for the increasing gasohol market (RFA, 2005b).

1.4 Ethanol Production Methods

The prominent current ethanol production method used in the U.S. is conversion of corn starch to ethanol at a rate of 2.7 gallons of ethanol per bushel of corn (Baker and Zahniser, 2006). This method of ethanol production is a three-step process involving a wet or dry milling procedure in series with a hydrolysis and fermentation step. The milling process,

wet or dry, of ethanol production is the cost consuming step. The main difference between wet and dry milling is the treatment of the grain prior to milling. The U.S. ethanol industry consists of over two-thirds dry milling plants. Dry milling ethanol production grinds the entire corn kernel into a 'meal' which is then treated with enzymes to form the usable simple sugars (dextrose) that are subsequently fermented by *Saccharomyces cerevisiae* to produce ethanol. Wet milling uses a water and acid pretreatment or 'steeping' process before the milling step. This pretreatment allows for extraction of co-products prior to the fermentation step. Both wet and dry milling ethanol production methods can yield valuable co-products such as 17.5 pounds of Distiller's Dried Grains with Solubles (DDGS), used as an animal feedstock, and 17 pounds of CO_2 per bushel of corn (RFA, 2005c).

The basic steps in these methods have remained relatively constant over the last decade due to the necessary processing and treatment steps such as milling and enzyme conversion. The few changes in the overall corn-ethanol conversion process have led to a minimal increase in overall yield (from 2.5 gal per bushel to 2.7 gal per bushel from 1995 to 2005) (Baker and Zahniser, 2006; Simrad et al., 1995).

The U.S. Energy Policy Act of 2005 specifies an increase to 7.5 billion gallons of renewable fuel to be blended into gasoline per year by 2012 (EERE, 2006c). Due to this policy, a higher supply of ethanol is needed to meet the demand. There is an expected increase U.S. ethanol production capacity from 4.8 to 6.8 billion gallons from 2005 to 2006 (RFA, 2006). The corn needed to produce this ethanol will most likely be diverted from U.S. corn exports (Baker and Zahniser, 2006). The U.S. corn market can handle this initial increase in corn demand because of the current large reserve stocks. However, if ethanol production continues to rise to 7.5 billion gallons per year by 2012 the corn reserves will

diminish. Additionally, 23% of the U.S. annual corn crop will need to be used to maintain a 7.5 billion gallon a year production rate. This shift in corn usage will have a significant impact on the corn market (food and feed) considering that in 2005 ethanol production reached its highest use of the total U.S. corn crop at 12% (Baker and Zahniser, 2006).

The prices of ethanol per gallon in the U.S., over the last 10 years, ranged between 1.19 and 2.91dollars per gallon with current subsidies of $ 0.51 (EIA, 2006). Ethanol prices are dependent on geographic location and processing cost which include the current price of fossil fuel used for production, planting, harvest, conversion and transport. The U.S. cost of gasoline has steadily risen (with spiked exceptions following Hurricanes Katrina and Rita) from 1.50 to over 3.00 dollars per gallon from November 2003 to September 2005 (EIA, 2006). Assuming a low-heat value, a gallon of ethanol provides 76,330 Btu while a gallon of gasoline provides 116,090 Btu (Ewing and Jaffoni, 2004). Based on these energy values, it would take approximately 1.5 gallons of ethanol to produce as much energy as one gallon of gasoline.

According to the USDA Agricultural Baseline Projections of 2005 the increase in ethanol demand over the next 10 years will create a need for U.S. farmers to increase their corn production (Baker and Zahniser, 2006). This needed increase in corn production will have several detrimental effects including an increase on the cost of corn production, a decrease in land available for other crops, and loss of soil fertility (Baker and Zahniser, 2006). Even though the price of ethanol with current corn hydrolysis and fermentation techniques yield competitive prices per gallon compared to gasoline, the need for a more sustainable ethanol production method is still present.

1.5 Lignocellulosic Conversion

The focus of ethanol production research has involved the development of general lignocellulosic biomass to ethanol conversion processes. The most recently developing technique being studied is the enzymatic hydrolysis and fermentation of more readily available biomass feedstocks. The following process schematic displays the multi-stage process (Figure 1.1). The biomass is first hydrolyzed to produce fermentable sugars and then these reducing sugars are fermented into ethanol by yeast or bacteria (Sun and Cheng, 2002). The development of lignocellulosic-derived ethanol stems from the need for a sustainable ethanol production method. The main advantage to this method of ethanol production is the high availability of many lignocellulosic-biomass sources such as corn stover, sugar cane bagasse, cotton stalks or perennial grasses.

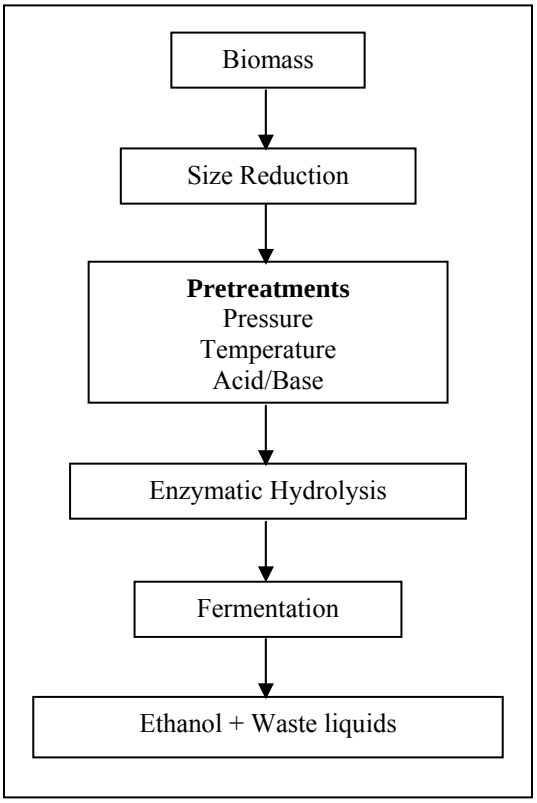

Figure 1.1. Lignocellulosic conversion to ethanol by hydrolysis and fermentation approach

However, there are limiting factors to the maximum possible efficiency of the hydrolysis and fermentation of lignocellulosic biomass. Lignocellulose is composed of cellulose, hemicellulose, and lignin. Lignin is a complex compound composed of aromatic rings (coniferyl, sinapyl, and coumaryl alchohols) that strengthens the cell walls. It is what makes up the 'woody' part of biomass materials such as cotton stalks. The hemicellulose is

also found in cell walls. It is made up of pentose sugars and is bound to cellulose, chains of β-1,4 linked glucose (Pandey et al., 2000). The lignin and hemicellulose in plant material prevents enzymes from being able to access the cellulose for saccharification to glucose and subsequent direct fermentation by yeast.

In order to access and breakdown the cellulose, size reductions, physical and chemical pretreatments, enzymatic treatments or combinations of these procedures must be performed to reduce interference with lignin and hemicellulose. The pretreatment methods are costly and add complications to waste management for the developing process (Palmarola-Adrados et al., 2005; Thorsell et al., 2004). Another limiting factor for lignocellulosic conversion is the general inability of yeast to use other sugars besides the base sugar, glucose, in the fermentation process. The 5-carbon sugars, such as xylose, cannot be metabolized by the common *Saccharomyces cerevisiae.* Current research is aimed at engineering microorganisms to use both 6- and 5-carbon sugars for more complete carbon conversion (Dien et al., 2003). There has been some success with engineering bacteria to convert 5-carbon sugars (Bothast et al., 1994). However, the few organisms now capable of converting complex-sugars through bioengineering are now being studied more in depth to see if they can efficiently convert sugars in the lignocellulosic-biomass to ethanol process (Bothast et al., 1999; Zaldivar et al., 2000).

Process parameters necessary for the maximum conversion of biomass to ethanol through hydrolysis and fermentation make it costly for large-scale industrial applications. Chemical and physical pretreatments including extreme temperature, pressure, and pH environments create additional costs with the need for filtration and waste management (Kamm et al., 2006). In addition, pretreatment can generate toxic byproducts, phenolic and

furfural compounds, reducing enzyme activities and growth and productivity of downstream cultures (Mussatto and Roberto, 2004; Pamqvist and Hahn-Hägerdal, 2000). Purification and separation of these byproducts also increase the overall production cost of ethanol from lignocellulosic biomass. The efficiency of this method of conversion to ethanol needs to be improved in order for lignocellulosic biomass to be considered as a practical source for sustainable fuel.

1.6 Gasification and Fermentation

1.6.1 Gasification

The continued development of sustainable energy resources has led researchers to look at past energy production approaches. Gasifiers were developed for coal combustion to produce H_2, CO_2, N_2, and CO gases when German fuel supplies were cut off and diminished during WWII (TONDU, 2006). Recent research has shown that down-draft gasifiers in series are capable of burning biomass to produce a mixture of H_2, CO_2, N_2, and CO gases with residual CH_4, char and ash (Datar et al., 2004, Demirbaş, 2002; and Maschio et al., 1994). This process converts all the available carbon from the biomass to single-carbon gases that can be easily fermented by autotrophic bacteria (Huhnke et al., 2002). The end products of gasification can be shifted toward a desired product by varying the gasification parameters (air input to biomass ratio, temperature, stages, and catalysts) and by downstream thermo-chemical processing (Walawender et al., 1985; Wang et al., 2005; Yan et al., 2006). By modifying process parameters of gasification (upstream and downstream) high-value gases such as H_2 or NH_3 can be produced in high concentrations (de Jong et al., 2003; Demirbaş, 2004b). Gasification can possibly provide a valuable step in converting biomass to ethanol by supplying a carbon-rich gaseous substrate available for downstream fermentation.

The Fischer-Tropsch cycle utilizes chemical catalysts to convert synthesis gas components to valuable products including fuels (methanol, C3 and C4 hydrocarbons), lubricants and waxes (Syntroleum Corporation, 2006). Chemical catalysts are required to convert synthesis gas to specified end products such as high-value hydrocarbons (Simrad et al., 1995). However, the conversion of synthesis gas to ethanol through the Fischer-Tropsch synthesis is not an efficient process. Therefore biological conversion of synthesis gas to ethanol has become a promising area of research.

1.6.2 Fermentation of Synthesis Gas with Homoacetogenic Autotrophs

The use of microbial catalysts for synthesis gas conversion is a more recently studied area of research. The overall process idea is to convert synthesis gas produced from biomass gasification to ethanol through fermentation with an autotrophic bacterium as the microbial catalyst (Figure 1.2). Homoacetogenic autotrophs are anaerobic bacteria that metabolize CO and CO_2 plus H_2 to acetyl-CoA which is then further reduced to acetate, cellular carbon, or ethanol (Diekert and

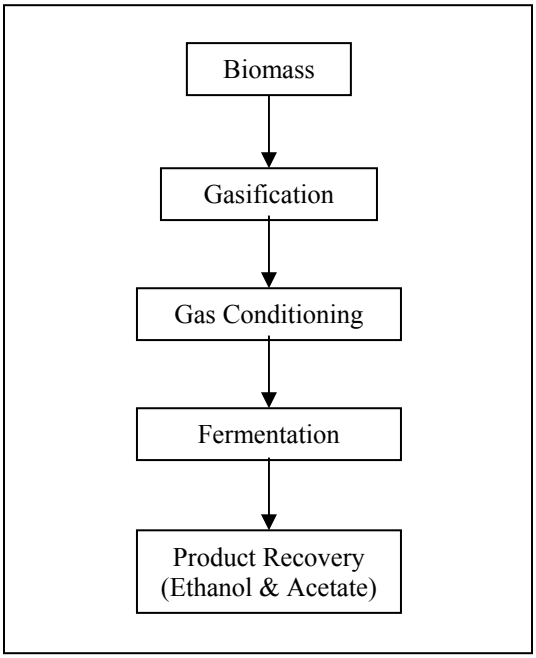

Figure 1.2 Combined gasification and fermentation process for chemical production from plant biomass

Wohlfarth, 1994; Ljungdahl, 1986). One advantage to this process as an alternative approach to hydrolysis and fermentation is the requirement of only one biological processing step: fermentation of synthesis gas.

Bioethanol production through autotrophic/acetogenic fermentation of the gaseous substrates CO and CO_2 plus H_2 follows the Wood-Ljungdahl and acetyl-CoA pathways. The Wood-ljungdahl pathway reduces single carbon substrates to acetyl-CoA with carbon monoxide dehydrogenase and acetyl-CoA synthase (Hegg, 2004). The acetyl-CoA pathway then uses the two branches (methyl and carbonyl) of acetyl-CoA to form cell carbon or reduction products (Wood et al., 1986). The end products vary depending on the organism, substrate, and environmental conditions. Some examples of autotrophs with biotechnological significance and their common end products are listed in Table 1.1. The general stoichiometry of ethanol (CH_3CH_2OH) and acetate (CH_3COOH) production from CO and CO_2 plus H_2 has been described by the following equations (Vega et al., 1989):

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

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

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

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

Table 1.1 List of autotrophic bacteria: substrate use and products formed.

Microorganism	Substrate	Product	Reference
Acetobacterium woodii	Fructose, glucose, CO, methanol	Acetate	Balch et al., 1977; Diekert and Ritter, 1983; Buschhorn et al., 1989
Clostridium autoethanogenum	Xylose, fructose, CO, $CO_2 + H_2$	Ethanol, acetate, CO_2, and H_2	Abrini et al., 1994
Clostridium ljungdahlii	Fructose, CO, $CO_2 + H_2$	Ethanol, acetate, CO_2, and H_2	Barik et al., 1988; Vega et al., 1989
Clostridium thermoaceticum	Glucose, CO, $CO_2 + H_2$	Acetate, and CO_2	Diekert and Thauer, 1978; Kerby and Zeikus 1983
Clostridium thermoautotroophicum	Glucose, CO, $CO_2 + H_2$	Acetate + H_2	Weigel et al., 1981
Eubacterium limosum	CO	Acetate and butyrate	Genthner and Bryant, 1982; Chang et al., 1999
Moorella sp. HUC22-1	Fructose, $CO_2 + H_2$	Acetate and ethanol	Sakai et al., 2004
P7	CO, $CO_2 + H_2$	Ethanol, acetate, butanol, and butyrate	Rajagopalan et al., 2002; Datar et al., 2004

Although acetate is a more common end product, several research groups have found autotrophic homoacetogens capable of producing significant amounts of ethanol (Abrini et al., 1994; Barik et al., 1988; Buschhorn, 1989; Rajagopalan et al., 2002; Tanner et al., 1993). The possible use of these homoacetogenic bacteria for biological conversion of synthesis gas components to ethanol could be promising for several reasons: 1) there is a greater potential for complete conversion of the available carbon in biomass 2) the process is independent of the biomass source and 3) there is a reduced number of biological processing steps compared to the hydrolysis and fermentation of lignocellulosic biomass.

This research focuses on the use of *Clostridium ljungdahlii* (ATCC 55383) and *Clostridium autoethanogenum* (DSM 10061) as the biocatalysts for conversion of synthesis gas to ethanol. Past research developments on each of these organisms that have led to this work's specific objectives are discussed below.

1.6.2.1 Clostridium ljungdahlii

The homoacetogenic bacterium *Clostridium ljungdahlii*, a spore-forming,motile, rod-shaped bacterium was originally isolated from chicken yard waste (Barik et al., 1988). *C. ljungdahlii* as well as other autotrophic homoacetogens have been shown to grow on various substrates including but not limited to gaseous substrates (H_2-CO_2 and CO), sugar substrates (arabinose, cellibiose, fructose, and xylose) as well as ethanol and pyruvate (Buschhorn et al., 1989; Diekert and Wohlfarth, 1994; and Tanner et al., 1993).

Barik et al. (1988) demonstrated *C. ljungdahlii's* ability to produce ethanol and acetate from CO. Supporting growth medium has been reported to contain Pfennig's minerals (5.0 mL), Pfennig's trace minerals (0.1 mL), B-vitamins (0.1 mL), yeast extract (0.1 g), resazurin (0.5 mL) per 100 mL of distilled or deionized water at pH levels between 5.0 and 7.0 and an optimum temperature of 37ºC (Barik et al., 1988; Gaddy et al., 1992). As the main nitrogen source, yeast extract plays an important role in the metabolic pathways converting substrates to biomass or liquid products. Early studies illustrated a relationship between the yeast extract concentration in the growth medium to the ethanol to acetate production ratio (Barik et al., 1988; Vega et al., 1989). The conversion of yeast extract to cellular nitrogen for the purpose of building amino acids for cell growth leads to a minimum amount of yeast extract necessary in the medium for healthy cell growth (Barik et al., 1988). Following studies with *C. ljungdahlii* indicated that low ethanol to acetate production ratios are seen during the cell growth (Vega et al., 1989).

Initial experiments with *C. ljungdahlii* in liquid, gas batch cultures on CO and H_2-CO_2 showed a low ethanol to acetate production ratio of 0.05 with less than 1 g/L final ethanol concentration (Gaddy et al., 1992). However in liquid batch cultures with continuous

gas substrate (65 % CO, 24 % H_2, and 11 % CO_2; flow rate data not shown), *C. ljungdahlii* was able to produce up to 7 g/L ethanol with a product ratio favoring ethanol production over acetate production 7:1 (Gaddy et al., 1992). Ethanol production mainly occurred in the stationary phase of metabolism in yeast-limited, low pH (between 4.0 and 4.5) medium, while acetate was shown to be the main product during growth (Gaddy et al., 1992; Klasson et al., 1992). However, subsequent studies have not been able to reproduce such high levels of ethanol production or favorable product ratios.

After initial studies, research groups centered their research efforts towards increasing the ethanol to acetate production ratio. Klasson et al, (1992) also found that the concentration of yeast extract in the growth medium had a significant affect on increasing the ethanol to acetate production ratio (mmol ethanol : mmol acetate) from 0.05 under 'normal' laboratory conditions (0.1% yeast extract) to 0.14 under yeast extract limited conditions (0.005% yeast extract). Further studies focused on using chemicals instead of nutrient availability to shift the metabolism from acid to solvent production. The effect of several different electron carriers including sodium thioglycolate, ascorbic acid, methyl viologen, and benzyl viologen on the ethanol to acetate production ratio was studied by Klasson et al. (1992). The electron carriers were used in an attempt to direct the electron flow of the acetyl-CoA pathway towards ethanol production which requires more reducing power than acetate production from acetyl-CoA. With a 30 ppm concentration of benzyl viologen and the addition of cellibiose in the growth medium, it was found that ethanol to acetate ratios (mmol ethanol : mmol acetate) could be enhanced to favor ethanol production 1.10:1 on synthesis gas constituents as the main carbon source.

The most promising study demonstrating the ability of *C. ljungdahlii* to produce significant quantities of ethanol used a continuous stirred tank reactor (CSTR) with cell recycle to produce up to 48 g/L ethanol with a corresponding acetate production of only 3 g/L on bottled gas (23-35 % H_2, 40-65 % CO, 1-20 % CO_2, 0-7 % CH_4) (Klasson et al., 1993). Phillips et al. (1993) utilized cell recycle to achieve cell densities of up to 4 g/L, at least 20 times more dense than their previous research with electron carriers. This robust autotrophic growth demonstrated that *C. ljungdahlii* could be used as a viable biocatalyst to produce ethanol from bottled CO, and $CO_2 + H_2$ with conditions that favor high mass transfer (high gas flow rate and mixing) in addition to nitrogen-limited growth conditions at a low pH with high cell densities (Phillips et al., 1993; Klasson et al., 1993). However, studies at the same time by Tanner et al., (1993) demonstrated that acetate was the sole end product for growth of *C. ljungdahlii* on synthesis gas constituents in batch reactions. These inconsistencies in the early literature present the need for a standard measure for the organism's ability to use CO, and $CO_2 + H_2$ to produce ethanol as a viable process.

More recent studies have examined *C. ljungdahlii* in a batch culture setting with CO_2 plus H_2 as the sole carbon substrates (Younesi et al., 2005). This group focused on trying to increase *C. ljungdahlii*'s ethanol to acetate production and were able to achieve an approximate ratio (calculated from presented data) of 0.70 mM ethanol mM^{-1} acetate in batch cultures by altering the internal pressures of CO_2 and H_2 (Najafpour and Younesi, 2006; Younesi et al., 2005). However, they reported a more favorable production ratio of 5:1 with similar studies in data that was not offered.

The only known group to date to convert actual synthesis gas to ethanol with a microbial catalyst was Datar et al., (2004). Experiments with the autotrophic bacterium P7

demonstrated that cell growth was inhibited by exposure to actual synthesis gas. This research was important in identifying that research needs to be focused on achieving healthy cells able to withstand impure substrates in addition to enhancing the ethanol production capabilities of autotrophic microorganisms. This work also identified ethanol as a non-growth associated product. These findings suggest a more robust cell system needs to be created to make syngas conversion using bacteria a more feasible process.

In order for an autotrophic organism such as the well-studied *C. ljungdahlii*, to be used as a biocatalyst for fermentation of synthesis gas to ethanol, more in depth experiments on how environmental factors and processing conditions including metabolic state (growing or non-growing), initial pH, presence of electron donors, and gas substrate flow rate affect culture stability, viability, and end product formation need to be completed.

1.6.2.2 Clostridium autoethanogenum

The autotrophic homoacetogen *Clostridium autoethanogenum* isolated from rabbit feces was found to metabolize the following carbon sources: CO, CO_2 plus H_2, pyruvate, xylose, arabinose, fructose, rhamnose, and L-glutamate; and produce ethanol, acetate, and CO_2 end products (Abrini et al., 1994). These initial studies by Abini et al. (1994) found that the ethanol to acetate ratio increased from 0.27 to 0.97 mmol ethanol per mmol acetate with increasing CO partial pressures from 33 to 60 %, respectively. The culture was shown to grow best at 37 ºC and pH levels between 4.5 and 6.5. In a genetic analysis comparing various clostridium species, Stackebrandt et al. (1999) found that *C. ljungdahlii* and *C. autoethanogenum* were 'genetically indistinguishable'. Contrary to this finding, cultivation studies found that *C. ljungdahlii* will not grow without the addition of yeast extract or Casamino acids while *C. autoethanogenum* does not require yeast extract or Casamino acids

(Abrini et al., 1994; Tanner et al., 1993). However, further studies comparing the growing metabolism and physiology of the two cultures would help to verify the uniqueness of *C. autoethanogenum* from *C. ljungdahlii*. Additionally, the growth metabolism of *C. autoethanogenum* on both sugar and on synthesis gas constituents need to be studied for this relatively unexplored bacterium.

1.7 Objectives

The recently developing method of gasification and fermentation of biomass is a potentially viable alternative for bioethanol production. A major advantage of this process is that it involves a single biological processing step of fermentation for the direct conversion of synthesis gas to ethanol. There are inconsistencies among past research on whether ethanol is a primary or secondary metabolite of autotrophic homoacetogens grown on synthesis gas. Based on past research, there are some unique opportunities for research on the fermentation of synthesis gas. In particular, how the metabolic stage (growing versus resting) affects product selectivity, and overall culture stability. The effects of various fermentation factors including, medium pH and synthesis gas flow rate on product selectivity (ethanol production versus acetate production) and culture growth need to be studied for synthesis gas fermentation.

The overall goal of this research project was to look at synthesis gas fermentation technology through investigation of the *C. ljungdahlii* and *C. autoethanogenum* cultures for improved microbial stability, increased ethanol to acetate production ratios and greater understanding of processing conditions on growth and product formation. The specific objectives of this effort were to: 1) Develop methods for inducing resting cell cultures and study the effect of electron carriers and pH on the performance of resting cells; and 2) Study

the effects of synthesis gas flow rate and medium pH on substrate use and metabolic end product formation by growing cultures.

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Chapter 2: Ethanol and Acetate Production by *Clostridium ljungdahlii* and *Clostridium autoethanogenum* using Resting Cells

2.1 Introduction

The demand for ethanol has dramatically risen in the past decade due to the recent alternative fuel incentives and the demand for ethanol as a fuel oxygenate (RFA 2005a). In the U.S., ethanol is produced from the hydrolysis and fermentation of corn. Ethanol production has increased over the last decade from 1.1 to 4.0 billion gallons per year (RFA 2005b). However, the U.S. corn market cannot sustain the rising demand for ethanol production (Baker and Zahniser, 2006). Therefore, extensive research has been focused on alternative methods for ethanol production from readily available biomass.

Biomass derived from any plant material provides carbon-dense stock that can be converted to ethanol. Current research in the field of biomass conversion to ethanol includes fermentation of biomass after the breakdown of complex plant structures to simple sugars. Lignocellulosic biomass consists of lignin, hemicellulose and cellulose. For direct hydrolysis of cellulose to glucose and subsequent fermentation of glucose to ethanol a number of steps are necessary. These include 1) pretreatment which involves extreme pH levels (acidic or alkaline), temperature and/or pressure to remove lignin and improve enzyme accessibility to cellulose; 2) hydrolysis with enzymes to breakdown cellulose to glucose; and 3) subsequent fermentation of sugars with yeast to yield ethanol. Challenges exist with this sugar based approach in creating more cost effective, complete and environmentally friendly pretreatment and hydrolysis processes apart from more efficient use of C_6 and C_5 sugars available for ethanol production (Eriksson et al., 2002; Mielenz, 2001).

Another alternative method being developed for conversion of biomass to ethanol involves the stepwise process of gasification and fermentation. Gasification processes involve the pyrolysis and reduction of carbon material (coal or biomass) into synthesis gas (syngas) products. Useful synthesis gas components include H_2, CO, and CO_2 and there are residual amounts of CH_4, O_2, tars and ash (Phillips et al., 1994). The composition of synthesis gas ranges depending on substrate composition, moisture content, and the presence of catalysts (Datar et al., 2004).

Synthesis gas can be used to generate chemicals through chemical catalysts (Kini and Lahiri, 1975). However, ethanol production from synthesis gas by chemical catalysts has not proven to be an efficient process (Saha and Sivasanker, 1992; Stiles et al., 1991). The conversion of synthesis gas to liquid fuels using microbial catalysts is an alternative approach being studied as a viable alternative method for ethanol production from renewable biomass.

Discoveries of autotrophic bacteria that use single-carbon gases, such as CO and CO_2, have sparked interest in the biological conversion of synthesis gas to solvents and acids. A majority of studies have focused on *Clostridum lungdahlii* and have shown varying results on ethanol production capabilities from synthesis gas components (Gaddy, 1992; Klasson et al., 1992, Najafpour and Younesi, 2006; Phillips et al., 1994; and Younesi et al., 2005). Isolation experiments with *C. ljungdahlii* demonstrated that ethanol and acetate were the main end products found from growth on CO, with product concentrations of 1.14 and 4.62 g/L for ethanol and acetate, respectively (Barik et al., 1988). Studies by Gaddy et al. (1992) showed ethanol to acetate product ratios of 1:20 during batch fermentations with bottled synthesis gas provided in the headspace (73 % CO, 10 % CO_2, 15 % H_2 and 2 % CH_4).

Subsequent experiments with *C. ljungdahlii* on liquid-batch, continuous-gas fermentations (73 % CO, 10 % CO_2, 15 % H_2 and 2 % CH_4) were performed by this group at a lower pH and reduced yeast extract concentration (0.005 % w/v) to improve solvent production. These studies resulted in a favorable ethanol to acetate production ratio of 7:1 (g/L basis).

In order to achieve higher ethanol to acetate production ratios process parameters have been manipulated to isolate the acetogenic and solventogenic metabolic stages. Klasson et al., (1992) suggested that ethanol was a non-growth associated metabolite by demonstrating that the production of acetate was associated with ATP production while ethanol production corresponded to a decrease in cellular ATP. More recent studies completed by Datar et al. (2004) with another autotrophic bacterium, P7, have also shown a correlation between product selectivity and growth stages. This group grew P7 on artificial synthesis gas components in a 4 L CSTR to achieve a dense culture. Upon switching to an actual synthesis gas stream, the cells entered a non-growing state leading to cell wash out, yet improved ethanol production. When cells were reconditioned with artificial syngas stable growth occurred. These results suggest that ethanol may be a non-growth associated product and exposure to actual synthesis gas inhibits growth while maintaining cell viability. A more robust cell system needs to be developed before fermentation of actual synthesis gas can be evaluated as a viable process.

Challenges in the overall process of gasification and fermentation of biomass to ethanol indicate the need for a more stable microbial catalyst that can use actual synthesis gas to produce significant quantities of ethanol. The use of resting cell cultures can offer increased stability and a shift in metabolism toward solventogenesis and away from biomass (cell carbon) production and acetogenesis (Förberg et al., 1983; Monot and Engasser 1983;

Thomsson et al., 2003). Resting cells are cultures that are not growing but are still metabolically active. These non-growing cells have been used specifically for product selectivity and metabolic regulation and are generated through transferring dense cell cultures from growth media into various nutrient-limited media (e.g., carbon-, phosphate-, and nitrogen-limited media) (Förberg et al., 1983; Fordyce et al., 1984). Resting cell cultures have been also been used to enhance culture robustness in extreme environments (Berberich et al., 2000). The use of resting cell cultures could prove to enhance both culture stability and ethanol production levels for key autotrophic bacteria.

The medium pH can strongly affect both growth rate and product formation. As the external pH begins to drop an organism may begin to produce alcohols as the main fermentative product to prevent a further drop in pH (Padan et al., 1981). However if the initial pH level is too acidic, the organism may not be able to maintain a neutral internal pH (Vasconcelos et al., 1994; Terracciano and Kashket, 1986). Klasson et al. (1992) used an initial pH of 5.0 to grow a dense culture then shifted to pH levels between 4.0-4.5 to enhance ethanol production by *C. ljungdahlii*. It would be beneficial to study the effects of lowering the pH during a controlled metabolic shift from a growing to non-growing culture on ethanol production enhancement.

Additionally, alcohol production levels by *C. ljungdahlii* and *Clostridium thermoaceticum* have been shown to increase with the addition of electron mediators in both growing (Klasson et al., 1992) and non-growing cultures (White et al., 1987). Electron carriers can transfer electrons from reducers to oxidizers in order to replenish NADH levels in the medium which are needed for ethanol production from acetyl-CoA. In early studies by Rao and Mutharasan (1987) there was a direct correlation with the addition of viologen dyes

to a rapid switch from acid to alcohol production in continuous cultures of *Clostridium acetobutylicum*. Klasson et al. (1992) tested various electron mediators (benzyl and methyl viologen) in batch cultures of *C. ljungdahlii*. They found a favorable ethanol to acetate production ratio (mM EtOH/ mM ACH) of 1.10 for benzyl viologen addition to the growth medium compared to the control with a product ratio of only 0.24; further suggesting that a switch to solventogenesis from acidogenesis is enhanced with the addition of electron carriers.

The goal of this work was to induce resting cell cultures of *Clostridium ljungdahlii* and *Clostridium autoethanogenum*. *C. ljungdahlii* is a well-studied autotroph while little is known about the fermentation capabilities of *C. autotethanogenum* for syngas conversion. Resting cell performance was evaluated on culture stability over time, ethanol and acetate production, and culture viability. The effects of benzyl viologen and medium pH on resting cell performance of *C. ljungdahlii* cultures were also evaluated.

2.2 Materials and Methods

2.2.1 Organisms and Inoculum Preparation

Clostridium ljungdahlii (ATCC 55383) was grown on a modified Reinforced Clostridial Medium without agar and with additional salts, vitamins, and trace elements from ATCC 1754 PETC medium (RCM.NA.SVE). This basal medium contained (per liter, pH 6.8): 10.0 g proteose peptone (no. 5), 10.0 g beef extract, 3.0 g yeast extract, 0.5 g cysteine-HCl, and 0.5 ml 0.1% resazurin, 50 ml PETC salt solution, 10 ml modified PETC trace elements, and 10 ml modified Wolfe's vitamin solution (see Appendix A for exact recipes of salt, vitamin, and trace element solutions in the RCM.NA.SVE medium preparation protocol). The medium was prepared and dispensed anaerobically under a nitrogen

atmosphere into Balch tubes or serum bottles using a modified Hungate method (Bryant, 1972) and autoclaved for 20 minutes (121°C, 15 psig). Initial cell preparations of *C. ljungdahlii* were transferred aseptically from frozen culture stocks (-80 ºC) into Balch tubes containing 10 ml of RCM medium (Difco 218081), prepared anaerobically as described above and supplemented with an additional 0.1 ml of 3% w/v cysteine-HCl. The cultures (5% inoculum) were incubated for 48 hours (37ºC). A second transfer (5%) to Balch tubes containing 10 ml of basal medium, 5 g/L fructose and 0.1 ml 3% w/v cysteine-HCL was completed and incubated for 48 hours (37ºC). After the second transfer, cells were subsequently transferred and grown to the appropriate density and quantities for experimental purposes on a 24 hour cycle.

C. autoethanogenum (DSMZ 10061) was grown on DSMZ medium 640. This basal medium contained (per liter, pH 6.0): 2.0 g trypticase peptone, 1.0 g yeast extract, 0.75 g cysteine-HCl, 0.5 ml 0.1% resazurin, 1.0 ml trace elements and 100 ml salt solution (see Appendix A for trace element and salt solution recipes in the 640 medium preparation protocol). The medium was prepared and dispensed anaerobically under a nitrogen atmosphere into Balch tubes or serum bottles using a modified Hungate method (Bryant, 1972) and autoclaved for 20 minutes (121°C, 15 psig). Initial cell preparations of *C. autoethanogenum* were transferred aseptically (5% inoculum) from frozen culture stocks (-80 ºC) into Balch tubes containing 10 ml of 640 basal medium, and 5 g/L xylose and incubated for 72 hours (37ºC). A second transfer (5%) to Balch tubes (10 ml, 5 g/L xylose) was completed and incubated for 36 hours (37ºC). After the second transfer, cells were subsequently transferred and grown to the appropriate density and quantities for experimental purposes. Anoxic conditions were maintained for all cell transfers.

2.2.2 Cell Dry Weight and Optical Density Relationships

Growing cultures of *C. ljungdahlii* were completed on RCM.NA.SVE with 5 g/l of fructose and 0.01% of 3%w/v cysteine-HCL. *C. autoethanogenum* growing cultures were completed on 640 basal medium with 5 g/L of xylose. All cultures were inoculated with a 5% growing culture and incubated at 37 ºC in a water bath. To determine a relationship between optical density and dry cell weight both bacteria were grown in serum bottles (80 ml, triplicates). The cultures were sampled (11 ml) approximately every 4 hours, for optical density and dry weight measurements. Optical density was read with a spectrophotometer (Shimadzu Model UV-1700, Kyoto, Japan) at 600 nm and culture extracts (10 ml) were centrifuged (10 min, 13,000 × *g*, 4 ºC). The supernatant was removed, and the pellet was washed with deionized, distilled water and centrifuged (10 min, 13,000 × *g*, 4 ºC). The washing step was completed twice. The cell pellet was resuspended in deionized, distilled water (10 ml) and dispensed into a pre-weighed tin and dried in a 105 ºC oven for 24 hours. The dried sample and tin were then weighed, and the cell weight was calculated. The optical densities were plotted against their corresponding dry cell weights yielding a linear relationship between measured optical density at 600 nm and culture densities (mg dry cells/L).

The performance of growing *C. ljungdahlii* and *C. autoethnaogenum* cultures was evaluated in the appropriate growth medium (80 ml serum bottles). Samples (4 ml) were removed from triplicate cultures of each bacterium every 3 to 12 hours for a period of 48 to 72 hours, depending on the rate of change in optical density. Optical density (600 nm) was measured and aliquots of sample were stored at -80 ºC for ethanol and acetate concentration measurements.

2.2.3 Experimental Design and Statistical Analyses

All work for this manuscript includes fermentation studies on sugar substrates instead of synthesis gas. Theses studies were used as a starting point, as it is known that these organisms grow well on sugar substrates at higher growth rates and with more predictable growth curves than on synthesis gas substrates.

2.2.3.1 Clostridium ljungdahlii

To investigate the effects of nitrogen limitation on inducing the resting state, culture stability and end product formation, three non-growth medium formulations were used. The medium levels included: 1) RCM.NA.SVE without vitamins, elements and any of its major nitrogen sources: proteose peptone, yeast extract, and beef extract (NG.RCM.NA.S); 2) RCM.NA.SVE without the major nitrogen sources (NG.RCM.NA.SVE); and 3) NG.RCM.NA.S with the addition of an NH_4Cl solution that doubled the final concentration in the broth (NG.RCM.NA.S 2X NH_4Cl). Experiments were conducted to examine the effects of initial pH on resting cell stability and ethanol and acetate production over time and three initial pH factor levels (6.8, 5.5, and 4.5) were tested using NG.RCM.NA.SVE. Similarly, the effects of electron carriers on ethanol formation in resting cultures were determined. Non-growing cultures on NG.RCM.NA.SVE were supplemented with three levels of benzyl viologen (0, 50, and 100 ppm).

2.2.3.2 Clostridium autoethanogenum

The effect of nitrogen limitation on inducing the resting state and ethanol and acetate production were also examined for *C. autoethanogenum*. Six medium formulations omitting various nitrogen sources from the 640 basal medium at different levels were studied in order to achieve a suitable non-growing medium. The non-growth media included: 1) exclusion of

all major nitrogen sources from the 640 medium: NH_4Cl (found in the 640 salt solution), trypticase peptone, and yeast extract (640.NG.1); 2) exclusion of both the trypticase peptone and yeast extract (640.NG.2); 3) removal of only yeast extract (640.NG.3); 4) removal of only trypticase peptone (640.NG.4); 5) omission of trypticase peptone and addition of yeast extract (0.1 g/L) and 0.9 g/L NH_4Cl (640.NG.5); and 6) omission of trypticase peptone and addition of yeast extract (0.1 g/L) and 0.45 g/L NH_4Cl (640.NG.6)

Each non-growth medium/treatment culture was conducted in triplicate and the response variables measured over time were the primary end products, ethanol and acetate, cell density and culture viability. The change in ethanol and acetate concentrations were evaluated using an analysis of variance (ANOVA) test with time included as a factor (general linear model) and paired t-test comparisons in SAS® (SAS Institute, Cary, NC). Assessment of statistical significance for treatment comparisons of non-growth media type, pH, and benzyl viologen concentration were made at an α level of 0.05.

2.2.4 Resting Cell Experiments

2.2.4.1 Non-growing Culture Media Preparation

The three non-growth media examined for *C. ljungdahlii* resting cell induction, NG.RCM.NA.S, NG.RCM.NA.SVE, and NG.RCM.NA.S 2X NH_4Cl, and the six non-growth media studied for *C. autoethanogenum* resting cultures, 640.NG.1, 640.NG.2, 640.NG.3, 640.NG.4, 640.NG.5 and 640.NG.6, were prepared and dispensed anaerobically under a nitrogen atmosphere into 80 ml serum bottles and autoclaved for 20 minutes (121°C, 15 psig). The list of components included in each medium for *C. ljungdahlii* and *C. autoethanogenum* are shown in Table 2.1 and Table 2.2, respectively. The composition of the

various salt, vitamin and trace element solutions can be found in Appendix A within the media preparation protocols.

Table 2.1 Media compositions used for *C.ljungdahlii* studies

Component	RCM.NA	RCM.NA. SVE	NG.RCM.NA.S	NG.RCM.NA.S 2X NH4Cl	NG.RCM.NA. SVE
Proteose Peptone No. 3	10.0 g	10.0 g	---	---	---
Beef Extract	10.0 g	10.0 g	---	---	---
Yeast Extract	3.0 g	3.0 g	----	----	----
PETC salts	---	50 ml	50 ml	50 ml	50 ml
NH4Cl stock solution	---	---	---	50 ml	
PETC Modified Trace Elements	---	10 ml	---	---	10 ml
Modified Wolfe's Vitamin Solution	---	10 ml	---	---	10 ml
Cysteine-HCl	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g
Resazurin Stock	0.50 ml	0.50 ml	0.50 ml	0.50 ml	0.50 ml
Deionized H_2O	1000 ml	930 ml	950 ml	900 ml	930 ml

Table 2.2 DSM 640 media variations for growth and non-growth studies

Component	DSMZ 640	640.NG.1	640.NG.2	640.NG.3	640.NG.4	640.NG.5	640.NG.6
640 Salts	100 mL	---	100 ml	100 ml	100 ml	100 ml	---
640 Salts (-) [1]	---	100 ml	---	---	---	---	100 ml
NH_4Cl stock solution (20 g/L)	---	---	---	---	---	---	22.5 ml
Trypticase Peptone	2 g	---	---	2 g	---	---	---
Yeast Extract	1 g	---	---	---	1 g	0.1 g	0.1 g
Trace Element Solution	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml	1 ml
Cysteine-CHl.H_2O	0.75 g	0.75 g	0.75 g	0.75 g	0.75 g	0.75 g	0.75 g
Resazurin Stock	0.50 mL	0.50 mL	0.50 mL	0.50 mL	0.50 mL	0.50 mL	0.50 mL
Distilled water	900 mL	900 ml	900 ml	900 ml	900 ml	900 ml	876 ml

[1]640 Salts (-) is identical to 640 Salts except made without NH_4Cl

2.2.4.2 Resting Cell Preparation

Growing cultures (80 ml) were grown to late log phase (600-700 mg dry cells/L) and aseptically transferred under a nitrogen atmosphere into sterile 85 ml centrifuge tubes and centrifuged (20 min, 13,000 × *g*, 4ºC). Supernatants were removed and cell pellets were resuspended in the appropriate non-growth medium (30 ml). Culture suspensions were transferred to serum bottles containing the appropriate non-growth medium (50 ml) to achieve a uniform culture density between all non-growing fermentation vessels and a predetermined final volume of 80 ml. Calculations based on optical density (600 nm) to culture density (mg dry cells/L) ratios were used to determine the proportion of cell suspension needed to be transferred to non-growth medium (see Appendix B for optical density versus dry cell weight calibration curves). Sterile, non-growth medium was used to make up any differences in transfer volumes due to varying densities of the growing cultures (see Appendix C for MS Excel files with these calculations). All cell transfers and resuspensions were completed under anoxic conditions.

C. ljungdahlii non-growing cultures were provided with 5 g/L of fructose and supplemented with 0.1% of 3% w/v cysteine-HCL. Similarly, *C. autoethanogenum* cultures were provided with 5 g/L of xylose. All cultures were incubated in a water bath (37 °C) and sampled every 24 hours, starting at time zero. *C. ljungdahlii* non-growth experiments were conducted for 144 hours and *C. autoethanogenum* non-growth experiments were conducted for 96 hours. Optical density (600 nm) measurements were made and culture extracts (3 ml) were stored at -80 ºC for ethanol and acetate analysis. Every 48 hours, including time zero, 0.5 ml of culture was removed and inoculated into the appropriate basal growth medium for each organism (10 ml Balch tube, 5 g/L sugar) to test for culture viability. Viability tests

were performed for each replicate of all treatment levels throughout the experimental time period for a total of 12 viability tubes for each non-growing experiment with *C. ljungdahlii* (3 replications per treatment × 4 test times @ t = 0, 48, 96, and 144 hours) and 9 tubes for *C. autoethanogenum* (3 replications per treatment × 3 test times @ t = 0, 48, and 144 hours). Culture viability was analyzed qualitatively and given a percent viability based on the number of viability tubes that grew above 0.25 OD_{600} after inoculation with treated non-growing cells.

2.2.5 End Product Analyses

Ethanol and acetate concentrations were determined in acidified samples by gas chromatography (Shimadzu GC 17A, Kyoto, Japan). The acidified samples were prepared by combining samples (500 μl) with 25% meta-phosphoric acid (125 μl). The acidified mixture was centrifuged (10 min, 16,000 × *g*, 25 °C) and the clarified supernatant was transferred to crimp top vials. The chromatography column was packed with Supelco SP 1000 (1% $H_3 PO_4$, 100/120 mesh). The carrier gas was nitrogen (36 ml/min) and the inlet and detector temperatures were 185 °C and 190 °C, respectively. The column temperature was programmed to run isothermally at 125 ºC for 1 minute, ramp at a rate of 20 ºC/min and held at 160 ºC for 3.5 minutes.

2.3 Results

2.3.1 Growth of *C. ljungdahlii* and *C. autoethanogenum*

Typical growth of *C. ljungdahlii* from a 5 % inoculum of late-exponential phase cells (600 – 800 mg dry cells/L) supplemented with 5 g/L fructose in RCM.NA.SVE (pH 6.8) was able to achieve its stock culture inoculum density within 24 hours and held a stable culture density of 1 g/L after approximately 36 hours of growth (Figure 2.1). The relationship between optical density and dry cell weight was found to be 472 mg dry cells/L per OD_{600} unit for *C. ljungdahlii*.

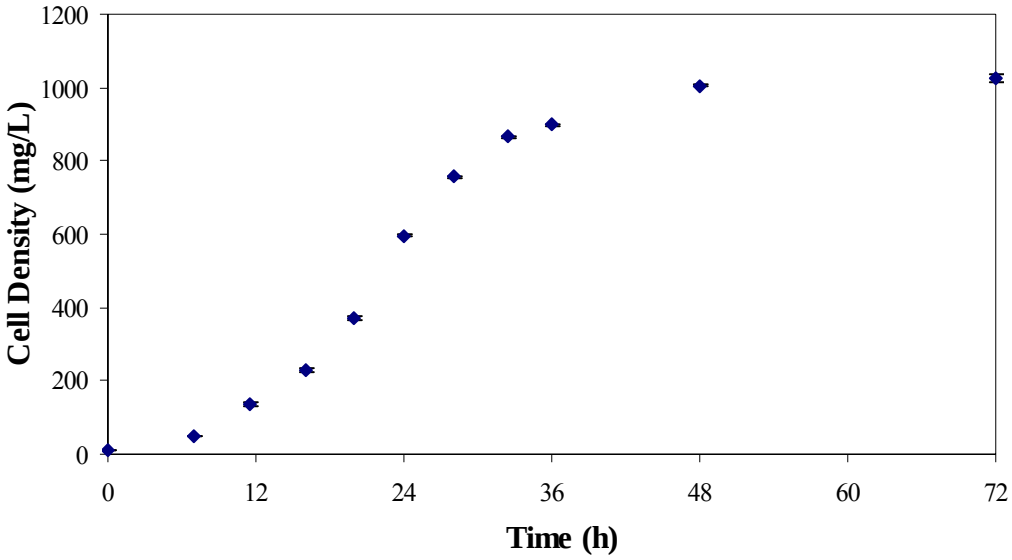

Figure 2.1 Culture density of *C. ljungdahlii* over time during growth on RCM.NA.SVE

Growth of *C. ljungdahlii* was accompanied by ethanol and acetate production during the exponential growth period (Figure 2.2). Average production levels of 13.11 ± 0.05 and 88.99 ± 2.95 mM were observed for ethanol and acetate, respectively after 48 hours. These values represent an approximate ethanol to acetate production ratio of 1:7 during growth on RCM.NA.SVE (pH 6.8).

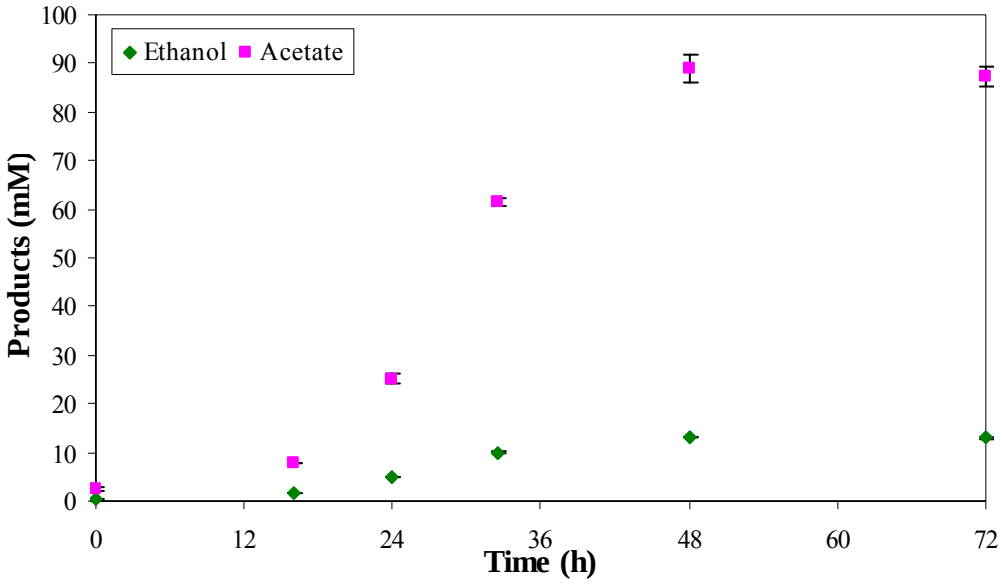

Figure 2.2. Ethanol and acetate production over time during growth of *Clostridium ljungdahliii* on RCM.NA.SVE

Typical growth of *C. autoethanogenum* from a 5 % inoculum of late-exponential cells (200 mg dry cells/L) supplemented with 5 g/L xylose in DSM 640 medium (pH 6.0) was able to achieve its stock inoculum density within 24 to 36 hours and held a stable culture density of 250 mg dry cells/L after 48 hours of growth (Figure 2.3). The relationship between dry cell weight and optical density was found to be 317 mg dry cells/L per OD_{600} unit.

Growth of *C. autoethanogenum* was accompanied by ethanol and acetate production during the exponential growth period (Figure 2.4). An approximate ethanol to acetate production ratio of 1:8 was observed during growth of *C. autoethanogenum* on xylose with maximum ethanol and acetate concentrations of 5.23 ± 0.87 and 40.14 ± 5.79 mM, respectively.

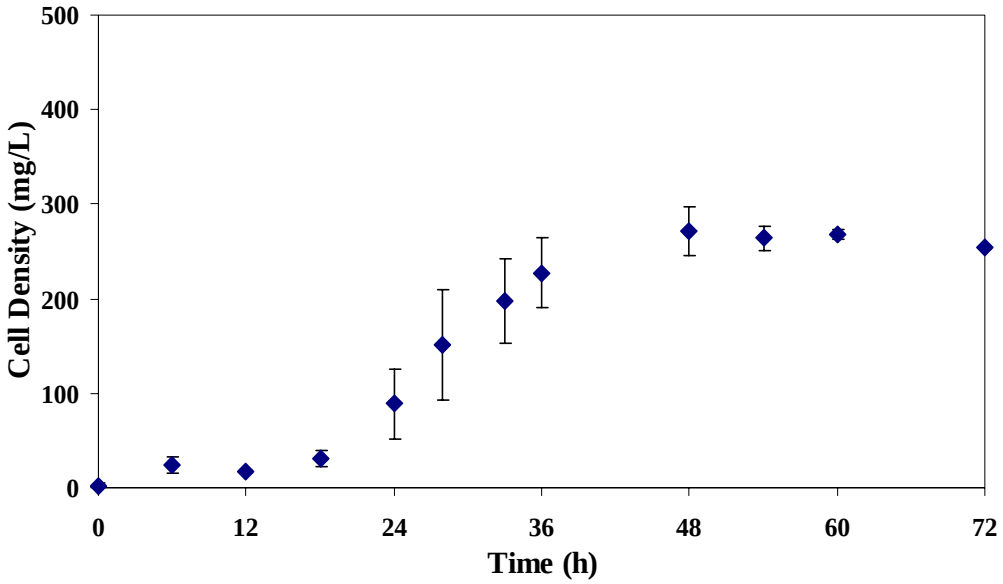

Figure 2.3 Culture density of *C. autoethanogenum* over time during growth DSMZ 640

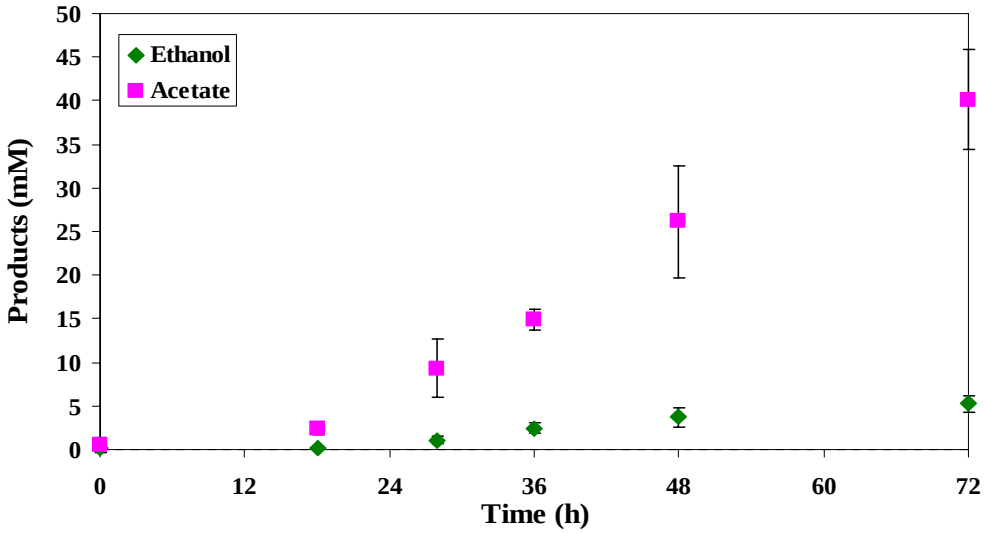

Figure 2.4 Ethanol and acetate production of *C. autoethanogenum* on DSMZ 640

2.3.2 *Clostridium ljungdahlii* Resting Cell Performance

2.3.2.1. Non-growth Media

Initial studies comparing the ability of non-growth media to induce a resting state with viable cells demonstrated that the salts, vitamins, and trace elements from ATCC 1754 PETC medium were essential for cell maintenance. There was a substantial difference in culture stability between NG.RCM.NA.S, NG.RCM.NA.S with 2 X NH_4Cl, and NG.RCM.NA.SVE based on the observed trends seen in Figure 2.5a. Cultures transferred to NG.RCM.NA.S and NG.RCM.NA.S 2X NH_4Cl media experienced a drop (approximately 15 to 20 %) in cell density during the first 48 hours. The addition of the vitamins and trace elements had a positive effect on enabling cultures to maintain high cell densities in nitrogen deficient media. The cell viability was also affected by the addition of PETC vitamins and trace elements to the non-growth medium. Only 50 % of cultures from NG.RCM.NA.S with 2X NH_4Cl (6 of 12 viability test cultures grew ≥ 0.25 OD within 3 days) and 50 % of cultures from NG.RCM.NA.S remained viable (6 of 12 viability test cultures grew ≥ 0.25 OD within 3 days), while 83.3 % of cultures from NG.RCM.NA.SVE remained viable (10 of 12 viability test cultures grew ≥ 0.25 OD within 3 days). Ethanol production levels were statistically affected by media type but not time indicating that there was no difference in the change in ethanol concentration for all media. Ethanol production levels were significantly higher in the NG.RCM.NA.SVE cultures compared to both the NG.RCM.NA.S (p-value = 0.0327) and NG.RCM.NA.S 2XNH_4Cl (p-value = 0.0029) (see Appendix D for statistical analysis). Although there were significant levels of ethanol produced in NG.RCM.NA.SVE cultures, the 0.097 mM ethanol produced in 144 hours was less than ethanol production in growing *C. ljungdahlii* cultures. Acetate production levels were less than 1 mM and not

significantly affected by time (p-value = 0.9297), media (p-value = 0.2759), or the interaction of time and media (p-value = 0.8247).

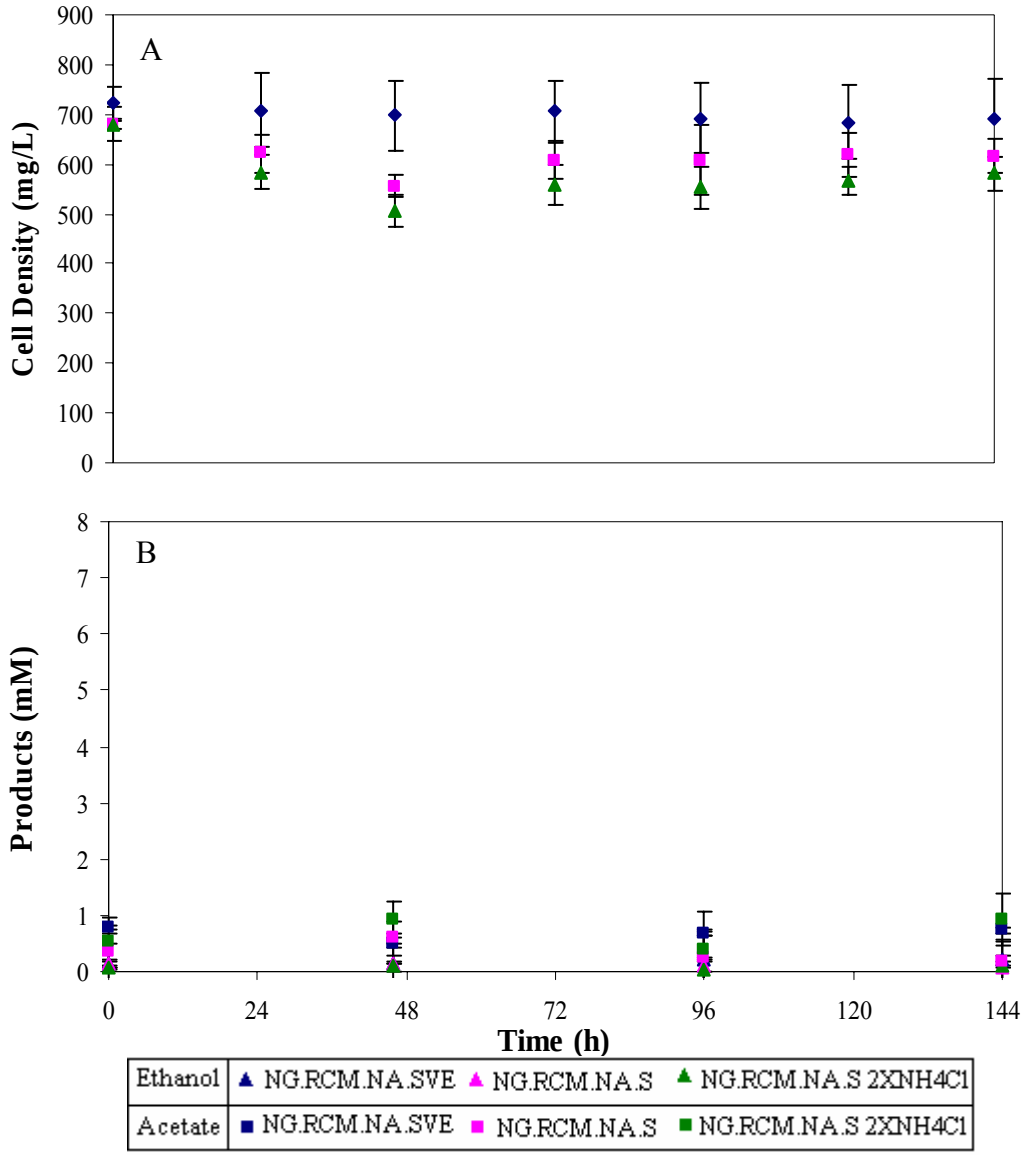

Figure 2.5 Initial non-growing studies of *C. ljungdahlii* on NG.RCM.NA.SVE, NG.RCM.NA.S and NG.RCM.NA.S 2XNH_4Cl over time. A) Culture density; B) ethanol and acetate production.

2.3.2.2 Effects of Initial pH on Resting Cell Performance

Lowering the initial pH of the non-growth medium did not affect culture stability as seen in Figure 2.6 (see Appendix D for statistical analysis). However, the lower pH values had an adverse affect on culture viability. Only 11.1 % of cultures from pH 4.5 non-growth medium remained viable (1 of 9 viability test cultures grew ≥ 0.25 OD within 3 days). Non-growth medium at pH 5.5 proved to be more viable with 44.4 % viability (4 of 9 viability test cultures grew ≥ 0.25 OD in up to 5 days). The control set (pH 6.8) remained 100 % viable over the course of the 144 hour experiment (9 of 9 viability test cultures grew to ≥ 0.25 OD within 5 days).

The main and interaction effects of pH and time were significant for ethanol production but not acetate (Table 2.3). The end products formed in the non-growing *C. ljungdahlii* pH study are shown in Figure 2.6. There was a considerably large concentration of ethanol present in each triplicate of the non-growing medium at pH 6.8. The reasoning for this phenomenon might be due to possible ethanol carryover from the growing starter cultures. The product formation for the non-growing cultures was evaluated statistically as the change in ethanol concentration over time. Therefore, this discrepancy in initial ethanol concentrations should not have affected the statistical outcome. Ethanol production levels were significantly affected by time, medium pH, and by their interaction. Cultures at pH 4.5 produced 0.52 mM ethanol over 144 hours. The control cultures at pH 6.8 were able to produce 1 mM ethanol within the first 2 days which was statistically more than what was produced at pH 4.5. There was no significant increase in ethanol production over time at the pH 5.5 level.

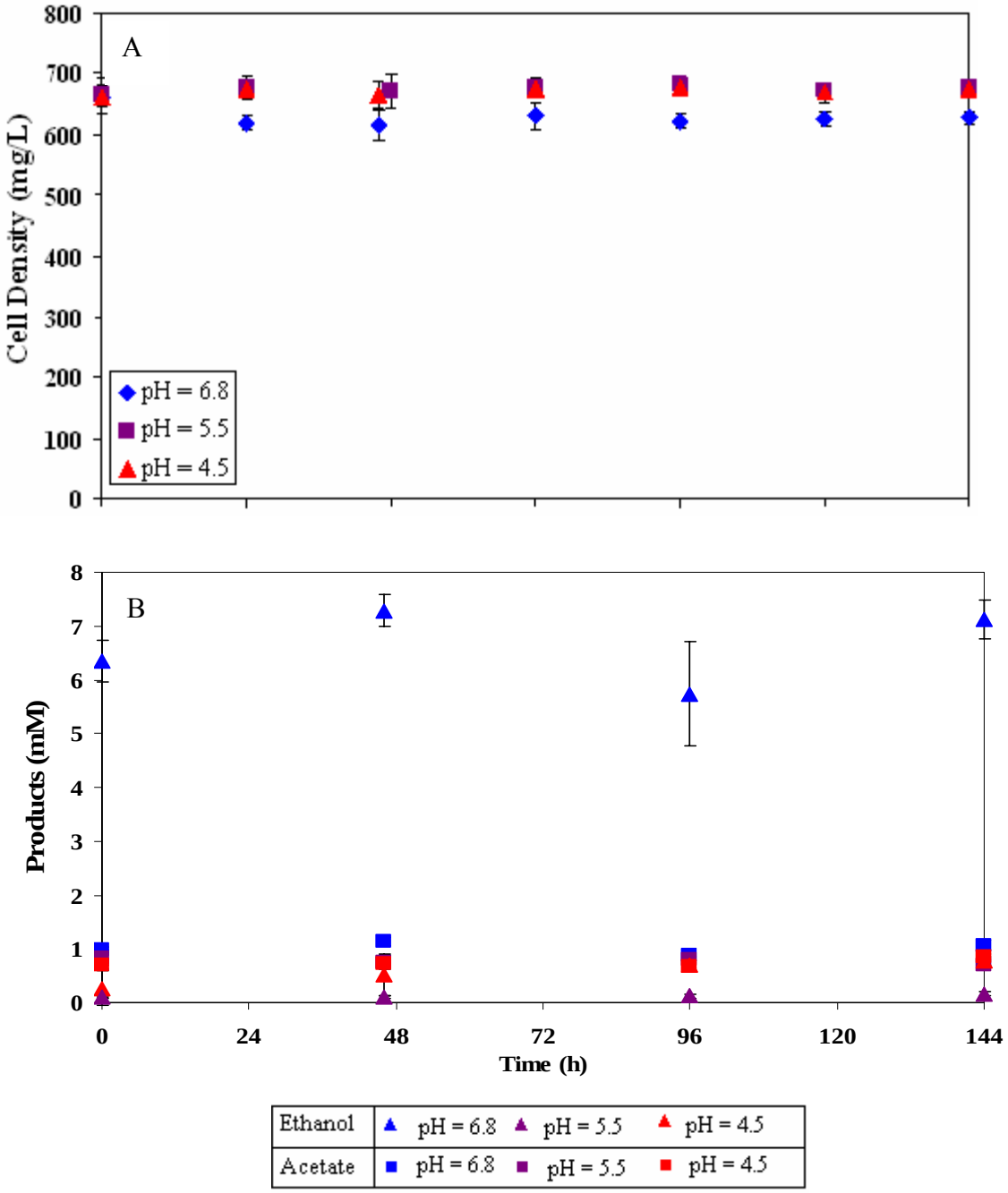

Figure 2.6 *C. ljungdahlii* metabolism over time on NG.RCM.NA.SVE at different pH levels. A) culture density; B) ethanol and acetate production.

Table 2.3 ANOVA table for pH and time effects on ethanol and acetate production of *C. ljungdahlii* in NG.RCM.NA.SVE

Product	Source	DF	Mean Square	F Value	Pr > F
Ethanol	pH	2	0.52228115	9.84	0.0008
	Time	3	0.37975162	7.16	0.0015
	pH*Time	6	0.23818278	4.49	0.0038
Acetate	pH	2	0.03575222	1.27	0.3004
	Time	3	0.03975776	1.41	0.2654
	pH*Time	6	0.03511165	1.24	0.3206

2.3.2.3 Effects of Benzyl Viologen on Resting Cell Performance

Previous studies have shown that the addition of electron carriers, specifically benzyl viologen, to growing *C. ljungdahlii* cultures improved ethanol production (Klasson et al., 1992). The results from our experiments on the effects of benzyl viologen in 50 and 100 ppm quantities on non-growing cultures are shown below in Figure 2.7. The culture stability of *C. ljungdahlii* was not significantly affected by the benzyl viologen. Viability tests for this experiment were only performed every 72 hours beginning with time zero. Therefore resulting viability values reflect the outcomes of 9 viability inoculations over time per treatment level. The culture viability was severely decreased with the addition of benzyl viologen under non-growing conditions. Only 33 % (3 of 9 viability test cultures grew ≥ 0.25 OD within 3 days) of the non-growing cultures exposed to 100 ppm benzyl viologen remained viable. There was some improvement for cultures with only 50 ppm benzyl viologen addition which were 55.5% viable (1 of 9 viability test cultures grew ≥ 0.25 OD in up to 7 days), yet overall both were less viable than the control cultures with 0 ppm benzyl viologen (77.7 % viability) over the course of the experiment. Time, benzyl viologen concentration, and the interaction of time and benzyl viologen concentration were not

significant factors for ethanol and acetate production (Table 2.4). There were no significant changes in product formation within each treatment over time (p-values > 0.05).

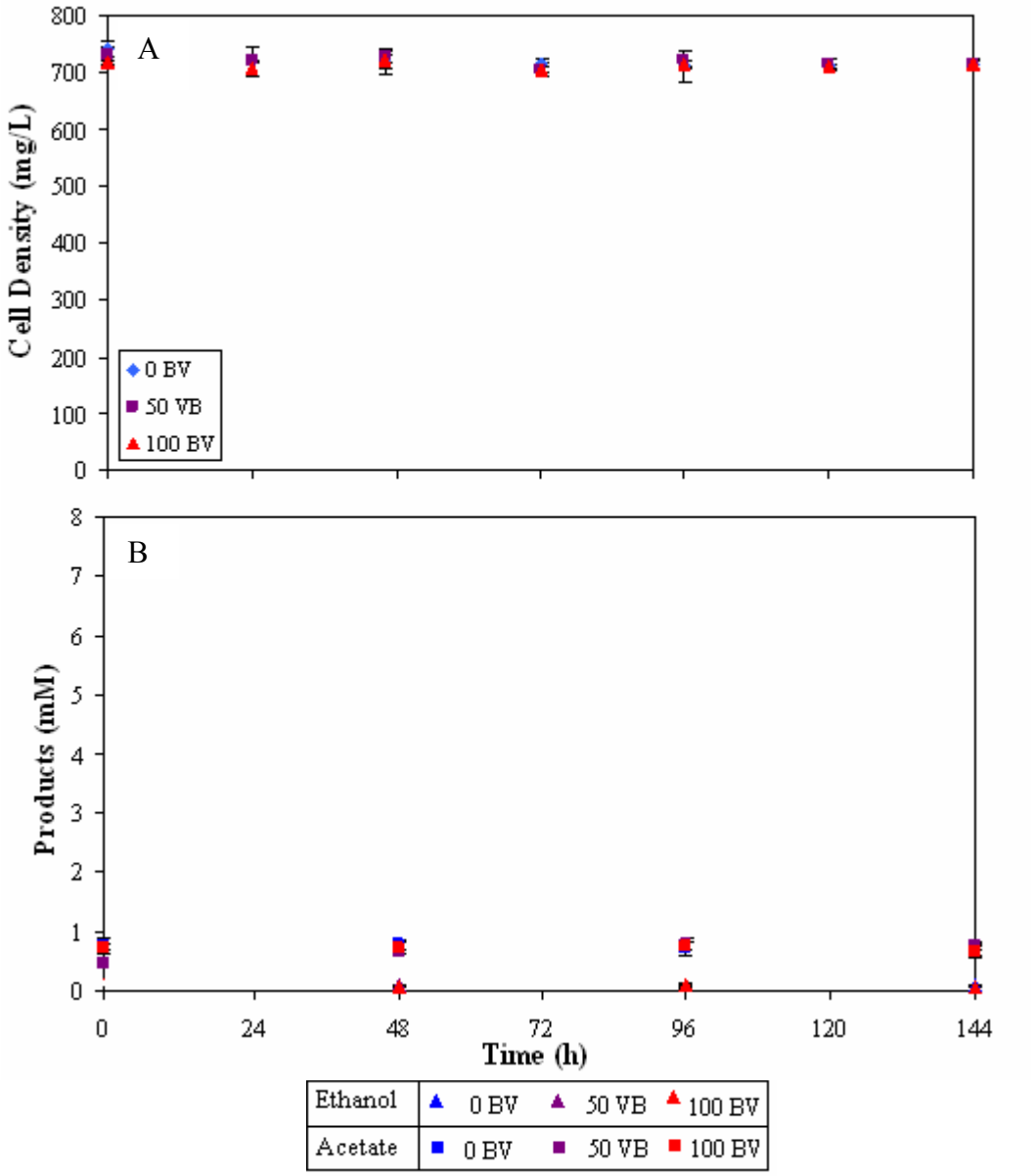

Figure 2.7 *C. ljungdahlii* metabolism over time on NG.RCM.NA.SVE with varying concentrations of benzyl viologen. A) culture density; B) ethanol and acetate production.

Table 2.4 ANOVA table for benzyl viologen and time effects on ethanol and acetate production by *C. ljungdahlii* in NG.RCM.NA.SVE

Product	Source	DF	Mean Square	F Value	Pr > F
Ethanol	BV	2	0.00030833	1.06	0.3631
	Time	3	0.00040278	1.38	0.2726
	BV*Time	6	0.0010833	0.37	0.8899
Acetate	BV	2	0.12360833	2.09	0.1455
	Time	3	0.04626296	0.78	0.5154
	Time*BV	6	0.02776019	0.47	0.8240

2.3.3 *Clostridium autoethanogenum* Resting Cell Performance

Initial studies completed with four of the six 640 non-growth media (640.NG 1, 2, 3 and 4) demonstrated the sensitivity of *C. autoethanogenum* to total nitrogen concentration and the type of nitrogen source (Figure 2.8). Each of the media types proved significantly unstable compared to what was expected from a functional non-growing culture by either promoting cell growth, or causing a severe decrease in culture density. The culture densities of both DSM.NG.1 and DSM.NG.2 decreased 83 and 60 % respectively within 36 hours after being transferred from growth media. Cultures transferred to 640.NG.3 and 640. NG.4 increased in density by 112 and 136 % respectively within 48 hours after the transfer from DSMZ 640. For cultures that decreased in cell density, *C. autoethanogenum* proved to be much more resilient to nitrogen limited media than *C. ljungdahlii*. Viability tests resulted in 100 % viability (9 of 9 viability cultures grew above 0.5 OD within 72 hours for all nitrogen-limited media tested).

The ethanol and acetate production trends from *C. autoethanogenum* cultures in nitrogen-deficient media NG.640.1 through NG.640.4 are shown in Figure 2.8b. In general, the level of product formation appears to be correlated with the quantity of nitrogen in the non-growth media.

Although similar levels of culture density are seen over time for 640.NG.3 and 640.NG.4, there are significant differences in the product formation observed by *C. autoethanogenum* cultures in these two media. Table 2.5 presents the significant differences between maximum ethanol and acetate production values between all NG.640 media. The highest level of ethanol produced in the initial non-growth experiment with *C. autoethanogenum* was observed in NG.640.3 media which did not include yeast extract but did contain trypticase peptone (9.43 mM compared to 5.11mM in a growing culture). More importantly, this level of ethanol production corresponds to an ethanol to acetate product ratio of 1 to 4.5 (mM ethanol per mM acetate) which is greater than what was observed in the growing culture (1:7.8). A favorable change in the ethanol to acetate product ratios was also observed in NG.640.1 and NG.640.2 (from 1:7.8 in growth DSMZ 640 to 1:3.2 and 1:2.4 in NG.640.1 and NG.640.2, respectively).

Table 2.5 Significance levels for ethanol and acetate production over time in all 640 media types (Non growth NG.640.1 – NG.640.6 and growth 640).

Medium	Ethanol (mM)	Acetate (mM)	Ethanol : Acetate
NG.640.1	0.35 ^{A,E}	1.11 ^{A}	1: 3.2
NG.640.2	2.34 ^{B,E}	5.64 ^{A}	1: 2.4
NG.640.3	9.43 ^{C}	41.99 ^{B}	1: 4.5
NG.640.4	6.06 ^{D}	30.54 ^{C}	1: 5.0
NG.640.5	0.92 ^{E}	6.45 ^{A}	1: 7.0
NG.640.6	0.96 ^{E}	7.89 ^{A}	1: 8.2
Growth 640	5.11 ^{D}	39.66 ^{B}	1: 7.8

$^{A-E}$ Values with similar superscripts were not statistically different within each end product response variable.

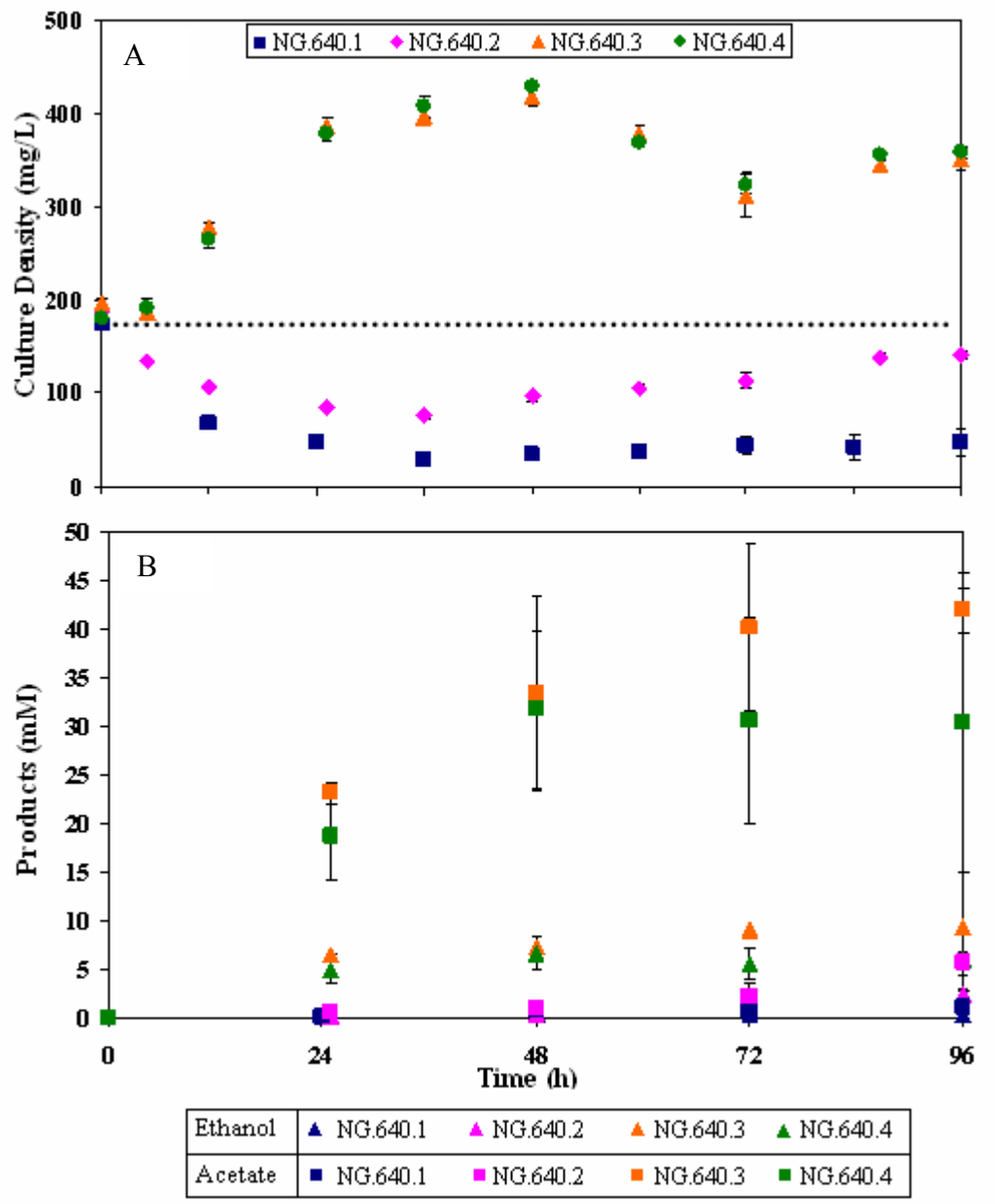

Figure 2.8 *C. autoethanogenum* metabolism over time on non-growth media DSMZ 640 with varying concentrations of yeast extract and trypticase peptone. A) culture density; B) ethanol and acetate production.

Subsequent non-growth media studies on *C. autoethanogenum* using media NG.640.5 and NG.640.6 led to higher levels of culture stability with minimal (0.1 g/L) yeast extract concentrations (Figure 2.9a). Although this figure represents only duplicate cultures for each medium, it is clear that a yeast extract concentration between 0 and 1 g/L without trypticase peptone as found in NG.640.1 and NG.640.2, was more suitable for resting culture induction. Both of these non-growth media formulations (NG.640.5 and NG.640.6) kept the cells viable throughout the study.

Although these media yielded more stable non-growing cultures, the ethanol to acetate production ratios were not improved by the 0.1 g/L yeast extract in NG.640.5 and NG.640.6 compared to resting cell performance of *C. autoethanogenum* in NG.640.2 (Table 2.5). The control growth medium contained 0.9 g/L NH_4Cl as did NG.640.5. There was no significant difference in culture growth or product formation between NG.640.5 and NG.640.6 with half of the standard NH_4Cl (0.45 g/L). Therefore, the NH_4Cl found in the growth medium does not have a significant effect on the solvent-production capabilities of *C. autoethanogenum.*

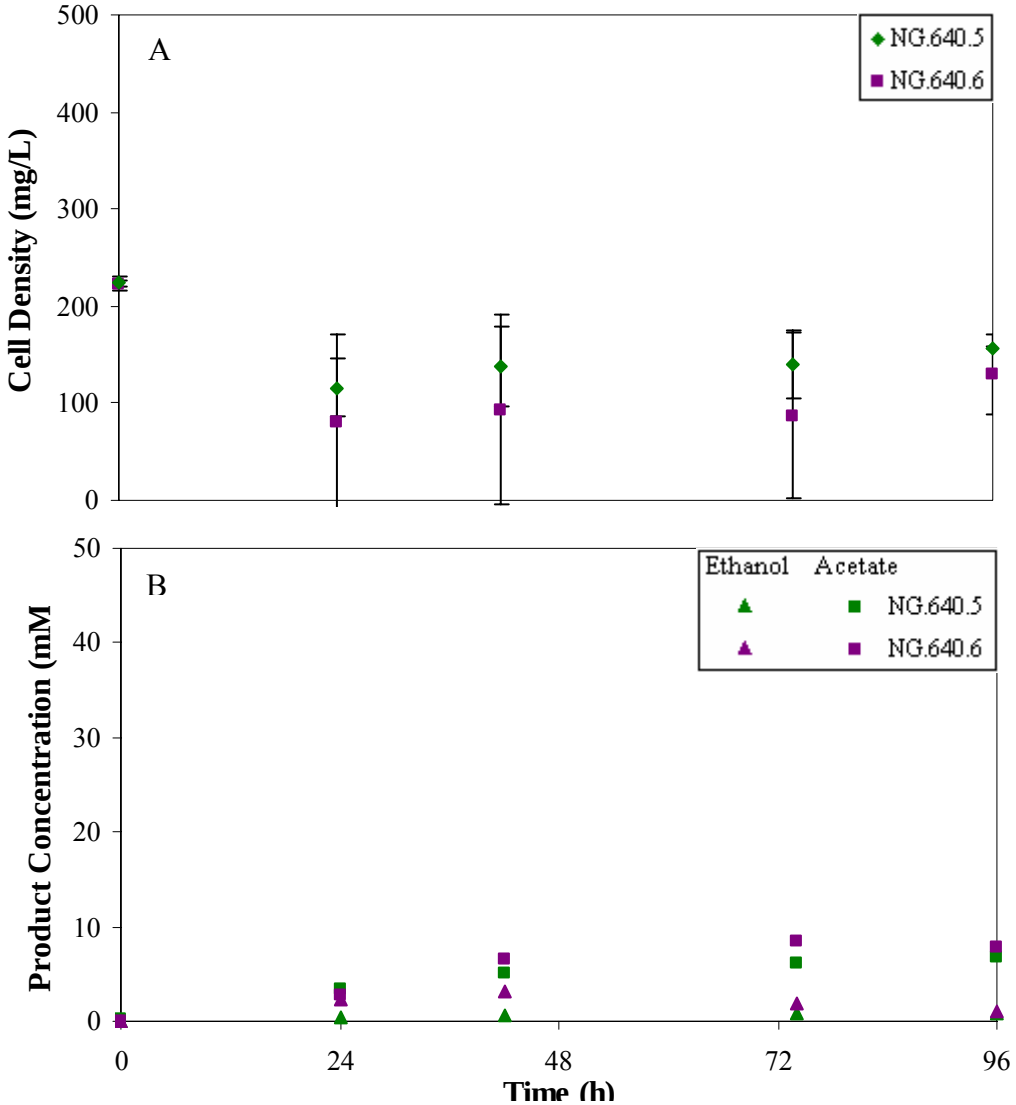

Figure 2.9 *Clostridium autoethanogenum* over time in NG.640.5 and NG.640.6 with varying concentrations of NH_4Cl and yeast extract. A: culture density and B: product formation.

2.4 Discussion

Past work with *C. ljungdahlii* suggests that ethanol is a secondary metabolite and enhanced ethanol production levels occur at lower growth rates in nitrogen-limited media (Klasson et al., 1992). For these studies, observed increases in ethanol concentration were associated with a diminished level of acetate fermentation suggesting a shift in metabolism from acid to alcohol production. However, our studies aimed at enhancing the ethanol

production levels of *C. ljungdahlii* with non-growing cultures in nitrogen-deficient media were not successful. Our results indicate that ethanol is not a secondary metabolite for *C. ljungdahlii* on sugar substrates based on the observances from growing cultures where ethanol was mainly produced during the logarithmic growth stage.

Yeast extract concentration in the basal medium for *C. ljungdahlii* has been shown to have a significant affect on cell growth as well as solvent and acid metabolism by other research groups (Barik et al., 1988; Klasson et al., 1992). The original isolation studies of *C. ljungdahlii* demonstrated that removal of yeast extract from the media enhanced the ethanol produced by 300 % (Barik et al., 1988). However, it was found that a minimum of 0.005 % of yeast extract was needed to maintain useful amino acid levels (Barik et al., 1988).

Under conditions completely deficient in yeast extract and trypticase peptone (NG.640.1 and NG.640.2) *C. autoethanogenum* was not able to maintain a stable culture density. As soon as the culture was introduced into these nitrogen-limited conditions the culture density dropped severely from 200 mg/L to as the cells were not able to maintain their structural integrity without nitrogen. With the control quantities of either trypticase peptone (2 g) or yeast extract (1 g) as found in NG.640.3 and NG.640.4, respectively, *C. autoethanogenum* was found to double the initial cell density and was not able to maintain a true resting culture. The cultures with 0.1 g/L yeast extract were able to maintain a more stable culture without severe cell lysis. The minimal yeast extract in the non-growth media enabled the culture to maintain cell density while not affecting the metabolic behavior of the organism.

Under certain growing conditions, yeast extract limitation is said to have a positive influence on ethanol production in autotrophic and homoacetogenic bacteria. (Barik et al.,

1988; Klasson et al., 1992; Monot and Engasser, 1983; Vega et al., 1989). However, these results are based on growing cultures. This idea is supported by the performance of growing *C. autoethanogenum* in medium without yeast extract (NG.640.3 and NG.640.4).

Removing the nitrogen source while maintaining a consistent level of carbon alters the C/N ratio available to the microorganism in the medium. Limitations in metabolism can be caused by excess C/N ratios (Larsson et al., 1993; Lebloas et al., 1993). This ratio of nutrient availability could explain the issues seen with both *C. ljungdahlii* and *C. autoethanogenum* in nitrogen-limited media. *C. ljungdahlii* had reduced overall metabolic activity levels where culture densities remained consistent, but neither ethanol or acetate was produced, while *C. autoethanogenum* was unable to maintain steady culture densities over time and end product formation was minimal. Nitrogen seems necessary to generate key proteins to metabolism for these microbial catalysts. However, minimal amounts of nitrogen in the medium promotes growth. Therefore, inducing a resting state metabolism through nitrogen-limitation does not seem feasible for these organisms. Alternative methods for resting-stage induction may need to be explored such as decouplers or growth arrestors.

In addition to nitrogen limitation, the medium pH for *C. ljungdahlii* cultures were adjusted to more acidic levels after dense growth in an attempt to initiate solvent production. The onset of solventogenesis has been associated with a drop in pH for homoacetogenic fermentation (Barik et al., 1988, Vega et al., 1989; Klasson et al., 1992; Datar et al., 2004; Terracciano and Kashket 1986; Vasconcelos et al., 1994). These studies by various research groups report either an intentional drop in pH in order to promote alcohol production or a natural pH drop associated with the induction of solventogenesis. Gottwald and Gottschalk (1985) stated that a controlled drop in pH during the acid production phase must be

controlled in order to initiate solventogenesis. These studies suggest that although a drop in internal pH is necessary for solvent production, the medium pH level must be maintained at a more neutral pH after solvent production is induced in order for cultures to maintain their ethanol production capabilities (Gottwald and Gottschalk, 1985). It is possible that the mechanisms used to turn on ethanol production enzymes may be triggered with low pH levels (4 – 4.5). However, the overall fermentation capabilities may be negatively affected by the acidic environment. In our studies, the sudden change to a more acidic environment may have been detrimental to the ability of *C. ljungdahlii* to reduce fructose to any fermentative products.

In fermentation cultures the pH will become more acidic after the growth phase. This is due to the acid production associated with culture growth (Förberg et al., 1983; Vasconcelos et al., 1994; Gottwald and Gottschalk, 1985). The pH levels dropped for both *C. ljungdahlii* and *C. autoethanogenum* in nitrogen limited media (NG.RCM.SVE and 640.NG.1) from 6.8 and 6.0 to approximately 5.3 and 4.5, respectively throughout the non-growth studies (data not shown). These drops in pH do not correlate with the low levels of acetate production observed. The change in pH in nitrogen-limited media may be coming from an acidic product appearing in the gas chromatography spectrums sample analyses. The retention time of the product was between acetate and proprionate. There was an initial thought that it may be formate yet discussions regarding the chemistry of the column and analysis conditions suggested that formate was not detectable. Previous work with these cultures has not shown formate to be an end product. Identification of this product may need to be completed with mass spectrometry.

The ethanol production levels of *C. ljungdahlii* cultures with large C/N ratios in the non-growth state were not affected by the addition of benzyl viologen. The theory behind past research with electron carriers to enhance alcohol production was based on the greater need of reducing power to convert acetyl-CoA to ethanol rather than acetate.

If an electron mediator could provide excess reducing power within the cell, then regulation of reduced end products may shift towards ethanol (Klasson et al., 1992; Rao and Mutharasan, 1987; Rao and Mutharasan, 1988). A similar shift toward ethanol production with *C. ljungdahlii* and *C. autoethanogenum* was not observed most likely due to an overall lack of metabolic activity in the cells. Although the cultures were stable and somewhat viable if their metabolic activity levels were too low the cells may not have been able to generate the correct cofactors (e.g. acetaldehyde dehydrogenase, acetate kinase and NADH) to uptake carbon substrates of convert pathyway intermediates. These limitations probably affected the ability of electron carriers to react effectively for ethanol production. However, the lowered culture viability does give some insight that the use of electron mediators to enhance ethanol production during synthesis gas fermentation might have detrimental effects to the already compromised environmental conditions.

2.5 Conclusions

C. ljungdahlii was found to produce considerable quantities of ethanol and acetate with product formation favoring acetate as an end product over ethanol and an ethanol to acetate production ratio of 1:7 in a growing culture. Achieving resting cell cultures of *C. ljungdahlii* with no significant change in culture density over time was feasible. After testing various non-growth media it was clear that *C. ljungdahlii* is capable of maintaining culture density in a nitrogen-limited environment if given the appropriate amount of salts, vitamins

and trace elements. However, there was little to no ethanol and acetate production observed in resting cell cultures. Lowering the pH in non-growing cells did not improve culture viability or ethanol production for non-growing *C. ljungdahlii* cultures. The addition of electron carriers to the non-growth medium did not lead to improvements in either ethanol or acetate production and significantly decreased the culture viability in non-growing cells.

The relatively unstudied *C. autoethanogenum* was found to produce comparable quantities of ethanol and acetate to *C. ljungdahlii* with an ethanol to acetate product ratio of 1:8 in a growing culture. Achieving resting cell cultures of *C. autoethanogenum* was not as straightforward for this culture compared to *C. ljungdahlii*. *C. autoethanogenum* proved to be extremely sensitive to nitrogen concentration in non-growth media. Similar to results with other autotrophs such as *C. acetobutylicum*, nitrogen limitation led to increased ethanol production capabilities in growing cultures of *C. autoethanogenum* (Gottwald and Gottschalk, 1985**)**. Alternative methods for resting cell induction for both *C. autoethanogenum* and *C. ljungdahlii* need to be explored in order to study functional resting culture.

2.6 Future Work

Testing effects of the ratio of carbon to nitrogen in the media, both growth and non-growth, would help to determine what role this balance plays in culture maintenance and overall metabolic function. Medium pH levels in *C. ljungdahlii*, *C. autoethanogenum*, and other solvent-producing clostridia have been shown to affect performance, although similar results were not seen in this work. Closer examination of pH regulation for these organisms may help elucidate metabolic pathways for ethanol production.

Considering the potential of resting cultures and their impact on syngas fermentation for ethanol, it would be worthwhile to look at alternative techniques for generating functional non-growing cells. Uncoupling of the anabolic and catabolic metabolism through adjustment of the C/N ratio in resting cultures would provide more information on the actual ability of resting cells to produce ethanol in non-overflow media conditions. In addition, the investigation of other methods used to control microbial metabolism, such as the use of growth arrestors could be tested on *C. autoethanogenum* and *C. ljungdahlii* in order to achieve steady non-growth metabolism. Testing the effects of adding growth arrestors to growing cultures of these organisms during different stages, especially when intracellular ethanol production enzyme levels are highest, would help to determine what conditions are most suitable for resting solventogenesis. Once the methodology for achieving a functional resting cell culture was established in sugar fermentations, it would be beneficial to characterize the non-growth metabolism using synthesis gas substrates.

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Chapter 3: Influence of Process Parameters on Synthesis Gas Fermentation by *Clostridium ljungdahlii* and *Clostridium autoethanogenum*

3.1 Introduction

The use of ethanol as an energy source has the potential to decrease the U.S. dependency on foreign oil and is more environmentally friendly. Current U.S. ethanol supplies are produced from corn starch through a mature hydrolysis and fermentation process (RFA, 2005a). However, the U.S. corn market cannot support the projected 87.5 % increase in ethanol demand over the next 6 years. Due to the rising demand for ethanol as both a fuel alternative and fuel additive, there has been a renewed interest in the development of new ethanol production methods (RFA, 2005b).

Technology advancements for biomass conversion to ethanol have been focused on the pretreatment, enzymatic hydrolysis and fermentation of renewable lignocellulosic biomass. This process has limitations due to the complex polymers that make up lignocellulosic plant material. Pretreatment methods currently employed involve extreme process conditions such as acidic/alkali slurries, high temperatures and/or pressures to degrade the lignin fractions in biomass and expose cellulosic portions to applicable enzymes for hydrolysis to glucose. Pretreatment can generate toxic byproducts including phenolic and furfural compounds that have been found to reduce enzyme activities and the growth and productivity of downstream fermentation cultures (Mussatto and Roberto, 2004, Pamqvist and Han-Hägerdal, 2000). The purification and separation of these byproducts increase the overall production cost of ethanol from lignocellulosic biomass. In addition, the cellulolytic enzymes used to produce fermentable sugars are expensive and can be considered limited in activity performance. Therefore, the cost effectiveness of this sugar-based approach to

lignocellulosic conversion needs to be improved for consideration as a practical ethanol production method.

An alternative ethanol production approach is the conversion of biomass using the hybrid technologies of gasification and fermentation. Gasification is a controlled pyrolysis and reduction process in which biomass can be converted into synthesis gas, composed primarily of N_2, CO, CO_2, and H_2, with some residual tars, C_2 compounds NO_x and O_2 (Datar et al., 2004; Demibraş, 2004). Some autotrophs, including a recent isolate P7, *Clostridium ljungdahlii*, and *Clostridium autoethanogenum* have been shown to ferment single carbon gases such as CO and CO_2 plus H_2 into ethanol and acetate through the Acetyl-CoA pathway (Abrini et al., 1993; Barik et al., 1988; Datar et al., 2004).

However, challenges exist in improving the efficiency of the gasification and fermentation process. A major set-back to this process for solvent production is the bacteria produce acetate as their main product over ethanol. Work from various research groups have been focused on increasing the ethanol to acetate production ratios as well as the overall ethanol yield from available substrates.

Gaddy et al. (1992) demonstrated the ability of *C. ljungdahlii* to produce significant quantities of ethanol in a continuous stirred tank reactor (CSTR). Concentrations of up to 2 g/L ethanol were achieved through an 18-day experiment in a single CSTR on bottled pure synthesis gas components (55.35 % CO, 10.61 % CO_2, 19.11 % H_2, and 15.78 % Ar, flow rates not specified). This group also used two CSTRs in series to improve the ethanol to acetate production ratio. They attempted to isolate the growth stage of the *C. ljungdahlii* in the first CSTR followed by a drop in both pH and dilution rate in the second CSTR in order to promote ethanol production. The pH in the first reactor was held at the growing pH (5.0).

Then a shift in pH to 4.0 – 4.5 was used to reduce growth and enhance ethanol production. This combination of CSTR conditions led to increased ethanol to acetate ratios of up to nearly 1.5:1 from 1:1 in a single CSTR. One study with the autotrophic bacterium P7 using synthesis gas showed that ethanol production was correlated to medium pH. A liquid-batch, continuous gas fermentation (flow rate 180 ml/min, 4 L reactor, pure gas mixture of 57 % N_2, 15 % CO, 17 % CO_2, 5 % H_2, and 4 % CH_4) was able to produce 5 mM ethanol and 75 mM acetate after 6.5 days of growth. It was noted that there was a natural decrease of 0.5 pH units in the medium from the initial growing pH of 5.0 before ethanol production occurred (Datar et al., 2004). Overall, the effects of medium pH on culture growth rates and end product formation during autotrophic growth on synthesis gas components needs to be more clearly defined.

Klasson et al. (1993) showed the results of studies on *C. ljungdahlii* substrate use in liquid and gas batch fermentations on pure CO bottled gas. Different levels of CO partial pressures ranging from 1.01 to 1.62 Nm^{-2} in the headspace did not affect the exponential growth rate of the organism; it did however have an effect on the final culture density (Klasson et al., 1993). This indicated that CO utilization rates in batch cultures were not mass-transfer limited. More recent research with *C. ljungdahlii* also reported the consumption of CO over time in a batch culture (Younesi et al., 2005). This work presented the effects of substrate concentration on total culture growth, CO and H_2 utilization, CO_2 production, ethanol and acetate yields and ethanol to acetate production ratios. This work agrees with the results of Klasson et al. (1993) in that the growth rate was not affected by substrate concentration, yet this group did not report a difference in final culture density. They also reported a 30% increase in substrate concentration (from 1.4 atm to 1.8 atm of gas

pressure in the headspace) gave rise to an approximately 300 % increase in ethanol production. The acetate production however was not affected as strongly by increases in substrate availability, improving corresponding ethanol to acetate ratios.

The effects of substrate availability on batch culture ethanol and acetate production is well documented. However, it is not clear how gas flow rates in continuous-gas reactors influence fermentation metabolism. Experiments with fermentations on continuous gas feeds may be supplying gas substrates at rates significantly different than the maximum uptake rate of the organism due to the fact that the CO uptake rate is mass-transfer limited (Klasson et al., 1991). In order to maximize overall process efficiency of biomass gasification and fermentation, the relationship between gas flow rate and ethanol production levels needs to be understood.

Work with the autotroph *C. autoethanogenum* has been limited to the isolation research by Abrini et al. (1994). *C. autoethanogenum* was found to metabolize CO and CO_2 plus H_2 gaseous substrates as well as other carbon substrates including xylose, pyruvate, rhamnose, fructose, and glutamate to synthesize ethanol, acetate, H_2 and CO_2 (Abrini et al., 1994). It was found that this organism also yielded higher ethanol to acetate ratios with increases in CO concentration when in batch mode with gaseous substrates in the headspace (Abrini et al., 1994). *C. autoethanogenum* is a promising organism for use in bioconversion of synthesis gas to ethanol. However, little is known about the culture's overall metabolism on synthesis gas substrates.

The objectives of this research effort were to 1) study the effects of pH and synthesis gas flow rate (in compositions typical for a downdraft gasifier) on substrate utilization and metabolic end product formation by *C. ljungdahlii* in liquid-batch, continuous-gas

fermentation; and 2) examine the overall culture metabolism of *C. autoethanogenum* grown on bottled synthesis gas supplied at different flow rates. Ethanol and acetate production were measured and changes in headspace gas composition (CO, CO_2 and H_2) were quantified.

3.2 Materials and Methods

3.2.1 Organisms and Inoculum Preparation

C. ljungdahlii (ATCC 55383), an anaerobe isolated from chicken yard waste by Tanner et al. (1993), was obtained from the American Type Culture Collection. A modified Differential Reinforced Clostridial Medium (Sigma Aldrich, St. Louis, MO) containing (per liter, pH 6.8): 10 g proteose peptone, 10 g beef extract, 3 g yeast extract, 0.5 g cysteine-HCl, and 0.5 ml of a 0.1 % w/v resazurin stock solution was supplemented with modified ATCC 1754 salt, vitamin, and trace element solutions (see Appendix A for medium preparation protocol and modified ATCC 1754 supplement solutions) was used as the basal medium for maintaining cultures. The medium was prepared and dispensed using anaerobic techniques and autoclaved for 20 minutes (121 ºC, 15 psig) (Bryant et al., 1972). Initial preparations of *C. ljungdahlii* were transferred aseptically and anaerobicallly from frozen stocks (-80 ºC) into Balch tubes containing anaerobically prepared 9.4 ml of RCM medium (Difco 218081) and 0.1 ml 3 % w/v cysteine-HCl (5% inoculum). The cultures were incubated in a 37 ºC water bath for 48 hours. Subsequent transfers (5% inoculum) into 8.9 ml of basal growth medium, 0.1 ml 3 % w/v cysteine-HCl, and 0.5 ml 10 % w/v fructose were completed every 24 hours when dense growth was achieved.

C. autoethanogenum (DSM 10061), an anaerobe isolated from rabbit feces by Abrini et al. (1991) was obtained from the DSMZ culture collection. Growth medium DSMZ 640 contained (per liter, pH 6.0): 2 g trypticase peptone, 1 g yeast extract, 0.75 g cysteine-HCl,

0.5 ml of a 0.1 % w/v resazurin stock solution, 100 ml 640 salt solution, and 1 ml DSM 640 SL-10 trace element solution. Recipes for 640 salt and SL-10 trace element solutions are in Appendix A. The medium was prepared and dispensed using anaerobic techniques and autoclaved for 20 minutes (121 ºC, 15 psig) (Bryant et al., 1972). Initial cell preparations of *C. autoethanogenum* were transferred from frozen stocks (-80 ºC) into Balch tubes containing 9.0 ml growth medium and 0.5 ml 10 % w/v xylose with a 0.5 ml inoculum (5 %). The cultures were incubated in a 37 ºC water bath for 72 hours. A second transfer to Balch tubes was completed and incubated for 36 hours (37 ºC). Subsequent transfers to Balch tubes or serum bottles were incubated for 24 hours to reach optimum inoculum density.

Inoculum cultures for synthesis gas fermentation experiments were grown in the appropriate growth basal medium (250 ml) on pure synthesis gas components (50% N_2, 20% CO, 20% CO_2, 10% H_2) to account for any metabolism shifts from growth on a sugar substrate to gaseous substrates. Cultures were inoculated (5%) and were incubated in an air convection incubator (37 ºC) for 24-36 hours.

3.2.2 Experimental Design and Statistical Analyses

The effect of flow rate (5, 7.5 and 10 ml/min) and pH (5.5 and 6.8) on the growth of *C. ljungdahlii* on bottled synthesis gas constituents, similar to those produced from a downdraft gasifier, was investigated over time in liquid-batch, continuous gas bioreactors. The effect of syngas flow rate (5, 7.5 and 10 ml/min) on growth and metabolism of *C. autoethanogenum* was also investigated over time in liquid-batch, continuous gas bioreactors. All treatment combinations were completed in triplicate. Response variables for both sets of experiments were culture density, CO_2, CO and H_2 composition in the headspace, and primary fermentation end products.

The main and interaction effects of flow rate and pH on ethanol, acetate, CO_2, CO and H_2 changes over time in *C. ljungdahlii* cultures were evaluated using the General Linear Model (GLM) in SAS® version 9.1 (SAS Inc., Cary, NC). Similarly, the main effect of flow rate on response variable changes over time for *C. autoethanogenum* was completed using GLM in SAS® version 9.1 (SAS Inc., Cary, NC). Pairwise t-test comparisons (α, 0.05) were completed to determine if the treatment effects were significantly different and time was included as a factor. All SAS programs and output are located in Appendix F.

3.2.3 Bioreactor Design and Synthesis Gas Fermentations

Fermentation reactors with liquid media (batch) and a continuous feed of the gaseous substrate were chosen for this study to enhance substrate utilization while minimizing the risk of cell wash out.

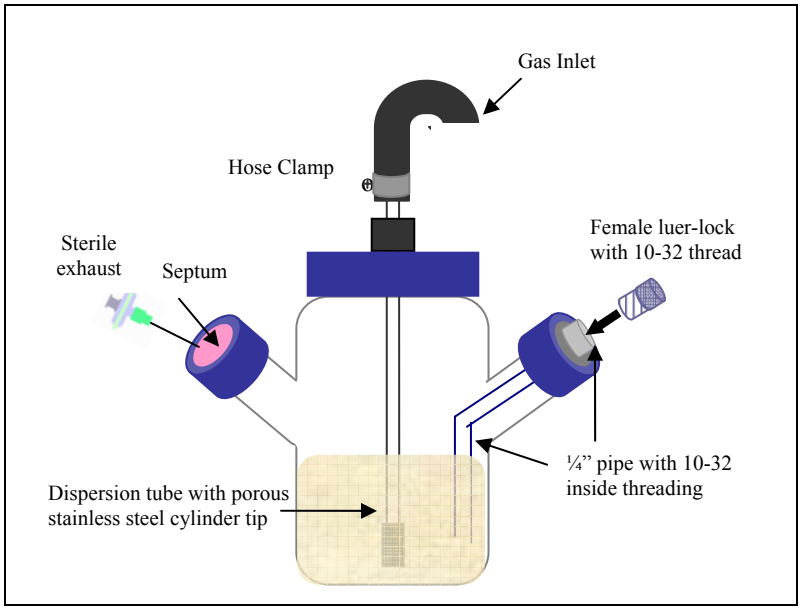

Figure 3.1 Fermentation bioreactor set-up.

A diagram of the reactor set-up used for experimentation is shown in Figure 3.1. The reactors were modified 250 ml spinner flasks (Bellco Glass, San Diego, Ca.). The 'spinner' was removed and replaced with a 7 mm stainless steel tube. The stainless steel tube was connected to a 7 mm diameter stainless steel porous gas dispersion cylinder (part no. 6500-1/4-1/8-1, Mott Corp, Farmington, CT), exited the top of the flask

through a compression fitting and was connected to butyl-rubber tubing for inlet gas flow. The side-arms of the flasks were modified so that liquid and gas sampling could be completed without compromising the reactor's anaerobicity or sterility. One side-arm was adapted with a female lure lock (part no. 6313, Popper and Sons, New Hyde Park, NY,) welded to a flanged stainless steel tube so that liquid samples could be removed during the experiment with a syringe. The other side arm was fitted with a rubber septum for inoculation, gas sampling, and an exhaust through a sterile syringe filter. Preliminary experiments were performed with both organisms on sugar substrates in the modified spinner-flask set-up to test the function of the reactors in maintaining anaerobic and sterile conditions.

The pure bottled gas substrate contained 50% N_2, 20% CO, 20% CO_2, and 10 % H_2 (Machine Welding and Supply Company, Raleigh, NC). Gas was directed from the tank through three flow meters (set at the chosen test flow rate multiplied by the number of experimental flasks at that flow rate). Butyl-rubber tubing directed flow from the exit of each flow meter through a sterilized in-line gas filter (ED025037, Randolph Austin, Austin, TX). From the in-line filters, gas flow was split by sterilized tubing and tubing connectors with equal flow distances between the filter and each flask. The entire experimental set-up (flow meters, filters, tubing and connectors, and reactors) was contained in a 37 ºC incubator.

3.2.3.1 Clostridium ljungdahlii

C. ljungdahlii growth experiments were completed with treatment combinations of three flow rates and two pH levels. The basal medium was prepared as previously described for RCM.NA.SVE (Appendix A). After pH adjustments were made, media were degassed (boiled for 15 minutes while being sparged with N_2 gas). Media was then randomly

dispensed into the individual reactors (235 ml). The gas tubes were sealed with a hose-cock clamp and covered with aluminum foil. Syringe filters attached to needles were inserted into the rubber septum and covered with aluminum foil to prevent 'filter wetting' in the autoclave. Male luer-lock caps were attached to the open female luer of the liquid sampling port. All reactors were autoclaved for 20 minutes (121 ºC, 15 psig). Syringe filters were removed once the autoclave cycle was complete to maintain a closed system.

Reactors were left in room temperature conditions overnight to ensure anoxic conditions. Prior to inoculation, reactors were sparged with the bottled syngas to remove any residual oxygen and to saturate the media with gaseous substrate. Cultures were inoculated (5%, 12.5 ml inoculum per reactor) from a single fermentation grown on bottled syngas and supplemented with 2.5 ml 0.1 % cysteine-HCl.

Liquid samples were taken every four hours over a 44 hour growth period for analyses of culture density and end product formation (quantified every 8 hours and at the final sampling time) for each treatment combination. The minimum time between gas sampling was limited to the gas chromatography analysis time (~34 minutes for each sample) and the availability of gas-tight syringes. Therefore, gas sample analyses were performed only on two of the three flow rate sample sets (5 and 7.5 ml/min) at both pH levels for each replicate. Gas sampling times varied between treatments, but remained consistent within treatments, and were analyzed over time within treatments (~every 6 hours throughout the 44 hour experiment).

3.2.3.1 Clostridium autoethanogenum

C. autoethanogenum growth experiments on bottled synthesis gas were investigated at three flow rates (5, 7.5, and 10 ml/min) in basal medium prepared as previously described for *C. ljungdahlii* in modified reactors. Liquid samples were taken every 4 hours over a 60 hour growth period for analyses of culture density and end product formation (quantified every 8 hours) for each treatment replicate. Gas samples were taken every 8 hours beginning with time zero.

Culture density was measured spectrophotometrically at a wavelength of 600 nm. *C. ljungdahlii* and *C. autoethanogenum* cultures had a cell dry weight to OD ratio of 472 mg dry cells/OD and 317 mg dry cells/OD, respectively (Appendix B). Culture purity was checked at the end of each experimental period. Reactors containing foreign cells under microscopic observation were considered contaminated and not included in the results.

3.2.4 Gas and End Product Analyses

Headspace gases were analyzed by gas chromatography using a thermal conductivity detector (TCD, Shimadzu GC-17A). Samples were collected in gas-tight syringes (Hamilton Company, Reno, NV, part no. 81330) and injected into a gas actuating valve (Valco Instruments Co. Inc., Houston, TX) for precise injections (250 ul) into the gas chromatograph. H_2, CO and CO_2 concentrations were quantified on a Carbosieve S-II, 100/120 mesh stainless steel column (10' x 1/8": 3.05 m x 0.3175 cm). The oven temperature was held at 35 °C for 7 minutes and then ramped to 225°C at a rate of 32°C/min and held isothermally for 6 minutes. The carrier gas (He) flow rate was 30 ml/min, the current was set at 50 mA and the injector and detector (TCD) temperatures were 200°C and 250°C, respectively. Ethanol and acetate concentrations were also determined by gas

chromatography using a flame ionization detector (FID, Shimadzu GC-17A). Liquid samples (500 µL) were acidified with the addition of 125 µL 25 % meta-phosphoric acid for clarification. The samples were centrifuged for 10 minutes (25ºC, 14,000 × *g*). The supernatant was then analyzed with a Supelco SP 1000 (1 % H_3PO_4, 100/120 mesh) column. Nitrogen was the carrier gas (36 ml/min) and the inlet and detector temperatures were 185 ºC and 190 ºC respectively. The column temperature was programmed to run isothermally for 1 minute at 125 ºC, rise to 160ºC at 20 ºC/min, then held isothermally at 160 ºC for 3.5 minutes.

3.3 Results and Discussion

3.3.1 *Clostridium ljungdahlii*

C. ljungdahlii was able to grow and produce metabolic end products on bottled synthesis gas. A high level of variance was seen in culture growth at each time within treatments, resulting in few significant differences in product formation between flow rate and pH levels (Figure 3.2). The cells reached a final density of 500 mg dry cells/L with an exponential growth rate of 22 mg dry cells/L/hr (pH 6.8, 7.5 ml/min gas flow rate). This density and growth rate were lower in comparison to preliminary tests completed with *C. ljungdahlii* cultures grown on bottled synthesis gas (similar conditions) inoculated with a culture grown on fructose as opposed to syngas (Appendix E; 850 mg dry cells/L; 27 mg dry cells/L/hr). This difference in culture performance may be due to a greater accumulation of intracellular cofactors and enzymes used for cell growth in cultures adapted to sugar substrates. Comparing growth of *C. ljungdahlii* on synthesis gas and fructose, performance with sugar based carbons resulted in higher final culture densities (1000 mg dry cell/L), exponential growth rates (42 mg dry cell/L/hr) and ethanol concentrations (13 mM, Chapter

2). These differences are most likely related to mechanisms used for carbon fixation, where uptake rates and substrate transport may not be as efficient for gases.

As seen in Figure 3.2a and 3.2b, the rate of ethanol production appears to slow as culture growth decreases within the treatments. Acetate production however, consistently increased through the end of the experimental time frame (Figure 3.2c). The basis for this phenomenon is not well understood. It has been reported that ethanol is a non-growth associated metabolite and that acetate is growth associated (Datar et al., 2004; Klasson et al., 1992). The reduction of acetyl-CoA to acetate forms ATP which explains the acetate production during growth. Unfortunately, this experiment was not conducted long enough into the stationary phase of growth to detect changes in the rate of acetate formation nor a paralleled increase in ethanol concentration. However, these results do suggest that ethanol production on syngas is a growth associated product as opposed to a secondary metabolite. Similar results were found with *C. ljungdahlii* cultures grown on sugar (Chapter 2).

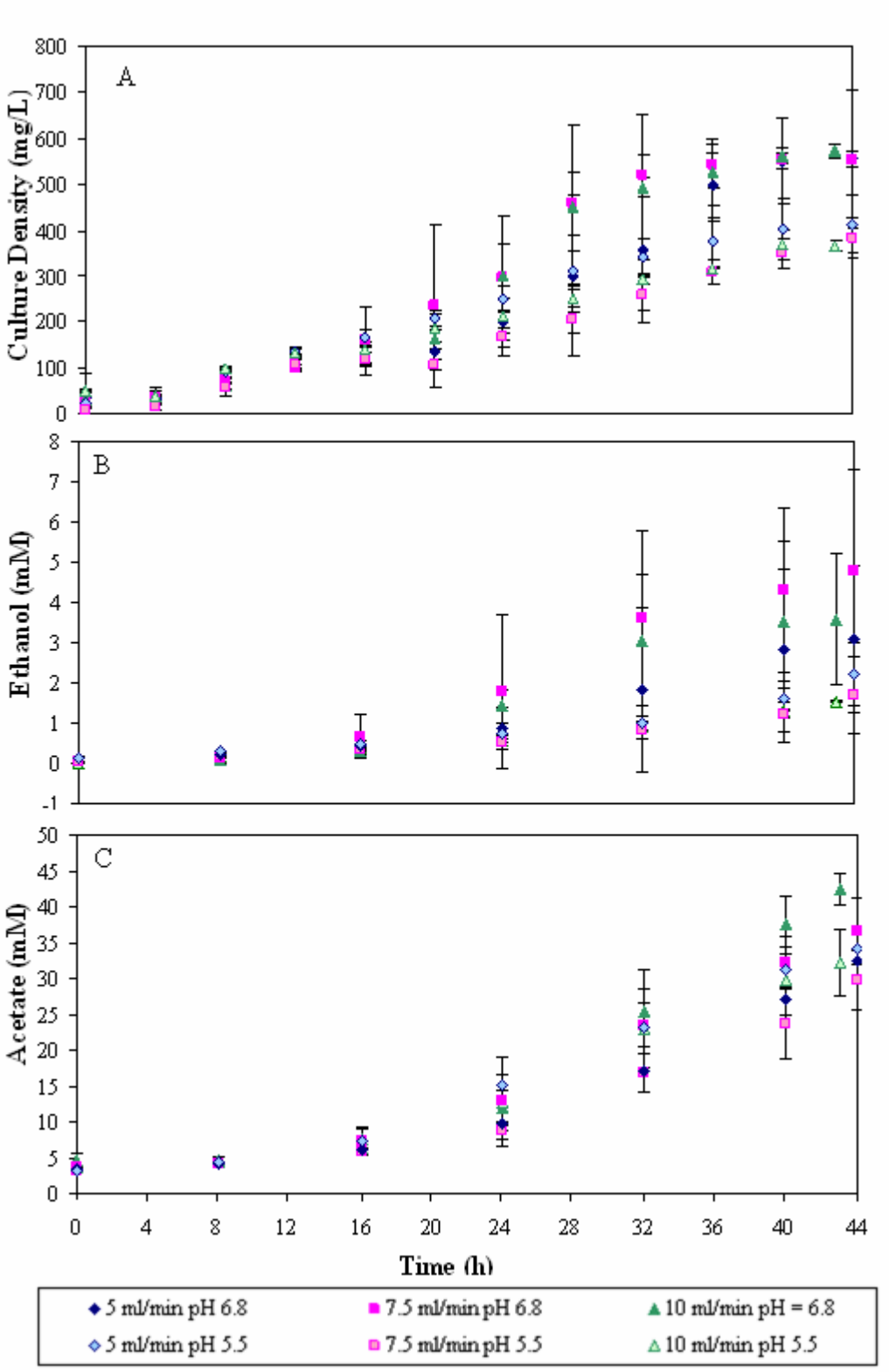

Figure 3.2 *Clostridium ljungdahlii* metabolism over time during growth on bottled syngas. A) culture growth; B) ethanol production; and C) acetate production

The ANOVA tables for ethanol and acetate are shown in Table 3.1. Ethanol production by *C. ljungdahlii* on syngas was significantly affected by the main effects of media pH, time and the interaction of pH and time (p-value < 0.05). The average ethanol concentrations at the end of the fermentation across all flow rates were 3.81 and 1.81 mM for media pH 6.8 and 5.5, respectively. Lowering the initial medium pH had a negative effect on ethanol production capabilities where cultures at the lower pH produced significantly less ethanol on average than cultures in pH 6.8 media (p-value < 0.05). The lower ethanol production levels observed in the pH 5.5 treatments could have been associated with slower growth. Transferring dense cultures into lower pH media without the expectation of significant growth has been shown to improve solvent production (Datar et al., 2004; Klasson et al., 1992; Vega et al., 1989). Klasson et al. (1993) used medium at pH 5.0 to grow a dense culture, then lowered the medium pH to initiate ethanol production. However, cells transferred for growth into media with low pH levels, as reported here, did not demonstrate improved solvent producing capabilities. These cells were better suited for pH levels closer to neutral for growth and solvent production despite reports that *C. ljungdahlii* cultures gave optimal performance on PETC medium at pH 5.5 (Phillips et al., 1993; Younesi et al., 2006).

Acetate production by *C. ljungdahlii* on syngas fermentation was significantly affected by the main effects of time, media pH, and flow rate and the interactions of pH and flow, pH and time, and pH, flow and time (p-values < 0.05). At a synthesis gas flow rate of 10 ml/min, there was statistically more acetate produced on average (17.6 mM) than at flow rates 5 (15.6 mM) and 7.5 ml/min (15.1 mM) (p-values < 0.05). Significantly higher acetate values were seen at pH 6.8 than 5.5 for all flow rates. The highest levels of acetate production were seen at the neutral pH (6.8) at 10 ml/min synthesis gas flow rate.

The ethanol to acetate production ratios (mM ethanol / mM acetate) were highest during the growth phase of fermentation. The cultures grown at pH 6.8 had higher ethanol to acetate ratios than their pH 5.5 counterparts. During the period with the highest rate of both ethanol and acetate production (32 hours), the ethanol to acetate ratios were 0.127 ± 0.24 and 0.044 ± 0.005 (averaged across all flow rates) for cultures at pH 6.8 and 5.5, respectively. This is related to the minimal growth and end product formation in *C. ljungdahlii* cultures at pH 5.5.

Lower flow rates were generally associated with lower growth rates yet improved ethanol to acetate ratios for *C. ljungdahlii* fermentation cultures. Growth rates were 2 to 3 times higher (within each flow rate) at pH 6.8 than at pH 5.5. Ethanol to acetate ratios ranged from 1:15.5 to 1:21.0 at pH 5.5 and from 1:8.2 to 1:10.8 at pH 6.8 with the best distribution occurring at the lower flow rates. Similar results have been observed by others (Datar et al., 2004; Klasson et al., 1992) where reduced growth has been associated with improved product ratios.

Table. 3.1 *C. ljungdahlii* ANOVA tables for final ethanol and acetate values

Product	Source	DF	Mean Square	F Value	Pr > F
Ethanol	Time	7	20.3563810	18.62	<.0001
	Flow	2	1.0298077	0.94	0.3947
	Time*Flow	11	0.2374563	0.22	0.9971
	pH	1	26.4617286	24.21	<.0001
	Time*pH	7	4.1379369	3.79	0.0025
	Flow*pH	2	3.0844673	2.82	0.0663
	Time*Flow*pH	11	0.3553784	0.33	0.9823
Acetate	Time	7	2525.97537	238.88	<.0001
	Flow	2	52.41916	4.96	0.0097
	Time*Flow	11	7.41645	0.70	0.7448
	pH	1	37.45672	3.54	0.0640
	Time*pH	7	15.98095	1.51	0.1872
	Flow*pH	2	129.14645	12.21	<.0001
	Time*Flow*pH	11	15.59747	1.48	0.1546

Results on the composition of the gaseous headspace over time for the 5 ml/min and 7.5 ml/min flow rate treatments are visually represented in Figure 3.3. Although there was a considerable amount of variance at each sample time, the overall trends are still noteworthy. The percent CO_2 in the headspace increased over time for all treatments. In general, the highest CO_2 levels for all treatments were observed between 22 and 38 hours which correspond to the exponential growth period (Figure 3.3a). As a whole, the H_2 and CO % composition values decreased over time (Figure 3.3b and c). Both H_2 and CO levels fell below the starting composition (represented by the dashed line across each graph) at each level of flow and media pH. The level of CO_2 in the headspace was statistically higher during exponential growth than the initial composition (p-value < 0.05). This is an indication that the microorganism was using CO as the primary carbon substrate as seen by the increase in CO_2 (Eq. 1). Uptake of CO_2 requires an associated uptake in H_2. The decreases in CO_2 (seen after 38 hours) and H_2 levels signified that CO_2 was used by *C. ljungdahlii* through the end of the study even as culture growth slowed, implying that CO_2 was being used as a secondary carbon source. The following equations relate the use of CO and CO_2 plus H_2 for ethanol and acetate production during autotrophic growth as reported by Vega et al. (1989).

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

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

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

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

In order for microbial cells to ‘consume’ or ‘produce’ gas, the molecules (CO, CO_2, and H_2) have to diffuse into the culture medium from the bubbles. The rate of diffusion depends on physical properties such as the temperature, density, and gas concentration of the

medium as well as the bubble size and relative density of gas bubbles in the medium (Garci-Ochoa and Gomez, 2005). Unless the medium is saturated with dissolved gas at the lowest flow rate, using a higher flow rate would provide higher concentrations of dissolved gas in the culture medium. Once the gases are dissolved into the medium, the individual gas molecules flow into the cells based on the concentration gradient through the cell boundary layer. Inside the cell, the organism uses CO as an electron acceptor to gain reducing power and produces CO_2, acetate, and ethanol as byproducts (equations 1 and 3). The CO_2 can be further reduced (in the presence of H_2) with ethanol and acetate as the major end products (equations 2 and 4) (Wood, 1986; Vega et al., 1989).

The ANOVA results for each gas component measured in the 5 ml/min and 7.5 ml/min treatments at pH levels 5.5 and 6.8 are provided in Tables 3.2 through 3.4, and show the main factors and factorial interactions that had significant effects. There were no significant main or interaction effects for time, flow rate, or pH for the H_2 concentration in the headspace. CO headspace concentrations were affected by flow, where a higher percent of CO was consumed at the lower flow rate of 5 ml/min compared to CO consumption at 7.5 ml/min. CO_2 levels were significantly affected by time, flow rate, media pH and the interaction of time and flow. Although gas consumption was affected by flow rate, overall performance of the cultures were not affected by flow rate, indicating that trends observed in gas consumption and production may be extended to all flow rate levels examined.

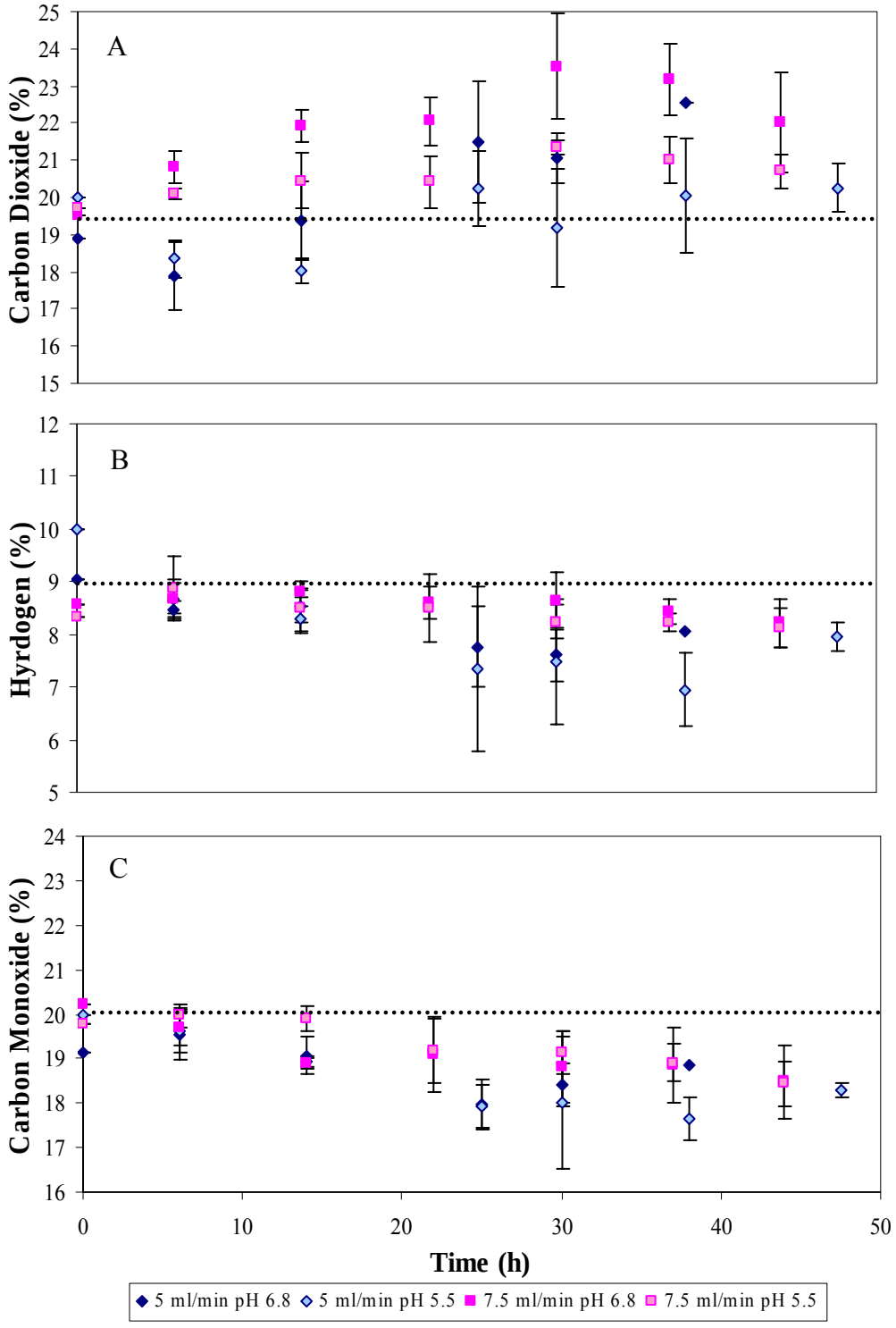

Figure 3.3 *Clostridium ljungdahlii* headspace gas composition over time during growth on bottled gas. A) % CO_2; b) H_2 %; c) % CO. Dashed lines show approximate average % composition at t = 0.

Table 3.2 ANOVA table for *C. ljungdahlii* H_2

Source	DF	Mean Square	F value	Pr > F
Time	6	1.35985161	0.63	0.7021
Flow	1	8.42116918	3.93	0.0532
Time * Flow	1	2.73583626	1.28	0.2642
pH	6	1.32453566	0.62	0.7149
Time*pH	6	0.74225500	0.35	0.9086
Flow*pH	5	0.61759537	0.29	0.9173
Time*Flow*pH	1	1.32549537	0.62	0.4355

Table 3.3 ANOVA table for *C. ljungdahlii* CO

Source	DF	Mean Square	F value	Pr > F
Time	6	3.49060417	1.80	0.1196
Flow	1	18.14433246	9.34	0.0037
Time * Flow	1	0.67179052	0.35	0.5592
pH	6	1.52018767	0.78	0.5876
Time*pH	6	0.54662118	0.28	0.9430
Flow*pH	1	3.93967342	2.03	0.1608
Time*Flow*pH	5	1.71306843	0.88	0.5004

Table 3.4 ANOVA table for *C. ljungdahlii* CO_2

Source	DF	Mean Square	F value	Pr > F
Time	10	13.47670774	10.41	< 0.0001
Flow	1	53.32878543	41.21	< 0.0001
Time * Flow	1	29.95625177	23.15	< 0.0001
pH	3	3.37073979	2.60	0.0288
Time*pH	7	2.87893484	2.22	0.0566
Flow*pH	1	0.00180053	0.00	0.9707
Time*Flow*pH	3	0.56313800	0.44	0.8218

Within flow rate, higher levels of CO_2 consumption were observed at pH 5.5. (Figure 3.3a). This may be related to the solubility characteristics of CO_2 at lower pH levels. Although this may affect the carbon use values, it does not affect the overall trends in the CO_2 profiles or ethanol production. From a processing standpoint, CO_2 compounds in synthesis gas fermentation act as a natural buffer and have the potential to offer benefits such as, minimizing acetate inhibition levels, pH drops, and the energy requirements for internal pH regulation.

When CO_2 was being produced, maximum levels of CO_2 concentrations at 5 ml/min were 19.2 and 21.0 % for pH 5.5 and 6.8 respectively, which were significantly lower than

the 21.4 and 23.5 % CO_2 levels observed at 7.5 ml/min for pH 5.5 and 6.8 (p-values < 0.05). The increasing levels of CO_2 observed correspond to periods of growth and significant product formation, and help demonstrate the enhanced growth rates of cells at pH 6.8 and higher flow levels.

3.3.2 *Clostridium autoethanogenum*

The changes in culture density and end product concentrations over time for *C. autoethanogenum* are shown in Figure 3.4. Culture densities around 150 mg dry cells/L were achieved on bottled synthesis gas. However, the cultures did not reach a distinct stationary phase within the 72 hour experiment. Based on preliminary experiments (Appendix E), *C. autoethanogenum* cultures reached a stationary phase within 72 hours with similar culture densities to these syngas studies. Therefore, the cultures presented here may be just shy of reaching stationary growth. Due to this, it is unknown whether ethanol and acetate production would have continued at the same rates during the stationary phase as observed in the growth phase. Similar to the growth of *C. ljungdahlii* on syngas, a longer than expected lag phase was observed in comparison to preliminary synthesis gas fermentation studies for *C. autoethanogenum*. In the preliminary experiments, reactors were inoculated with dense cultures grown on xylose. Cultures had completed exponential growth within 18 hours of inoculation (Appendix E). The reduced growth rate (5.6 compared to 4.2 mg dry cells/L/hr) for fermentations inoculated with cultures grown previously on synthesis gas as opposed to sugar suggest that *C. autoethanogenum* may prefer sugar substrates for optimal growth. An advantage to inoculating with cultures grown on sugar may be the build-up of intracellular enzymes, cofactors and maintenance energy from sugar fermentation. Differences in growth performance compared to sugars substrates may also be related to diffusion limitations at the

gas-liquid interface and/or efficiency of the cellular uptake mechanisms for gaseous substrates. However, additional experimental replications may be needed to establish the repeatability of this performance for both *C. autoethanogenum* and *C. ljungdahlii* cultures.

C. autoethanogenum was capable of fermenting synthesis gas constituents to ethanol and acetate (Figure 3.4b and c). Significant production of ethanol and acetate occur after the observed 36 hour lag phase. Both ethanol and acetate were produced during the growth of *C. autoethanogenum*. Interestingly, the production of these end products continued while culture growth slowed after 60 hours (during stationary phase for cultures at 5 and 10 ml/min flow rates). Final ethanol concentrations of 1.23, 0.83, and 1.45 mM were achieved for flow rates 5, 7.5, and 10 ml/min, respectively. Final acetate concentrations for the 5, 7.5 and 10 ml/min flow rate treatments were 18.23, 8.64, and 23.3 mM, respectively. These results were not intuitive in that the middle flow rate had the lowest level of product formation. The cultures at 7.5 ml/min flow rates were significantly less dense than the cultures at both 5 and 10 ml/min. This indicated that the overall health of the organisms at the middle flow rate was compromised which would prevent the cells from performing at their maximum potential. This organism appears to be especially sensitive to its environment when inoculated with a low density culture.

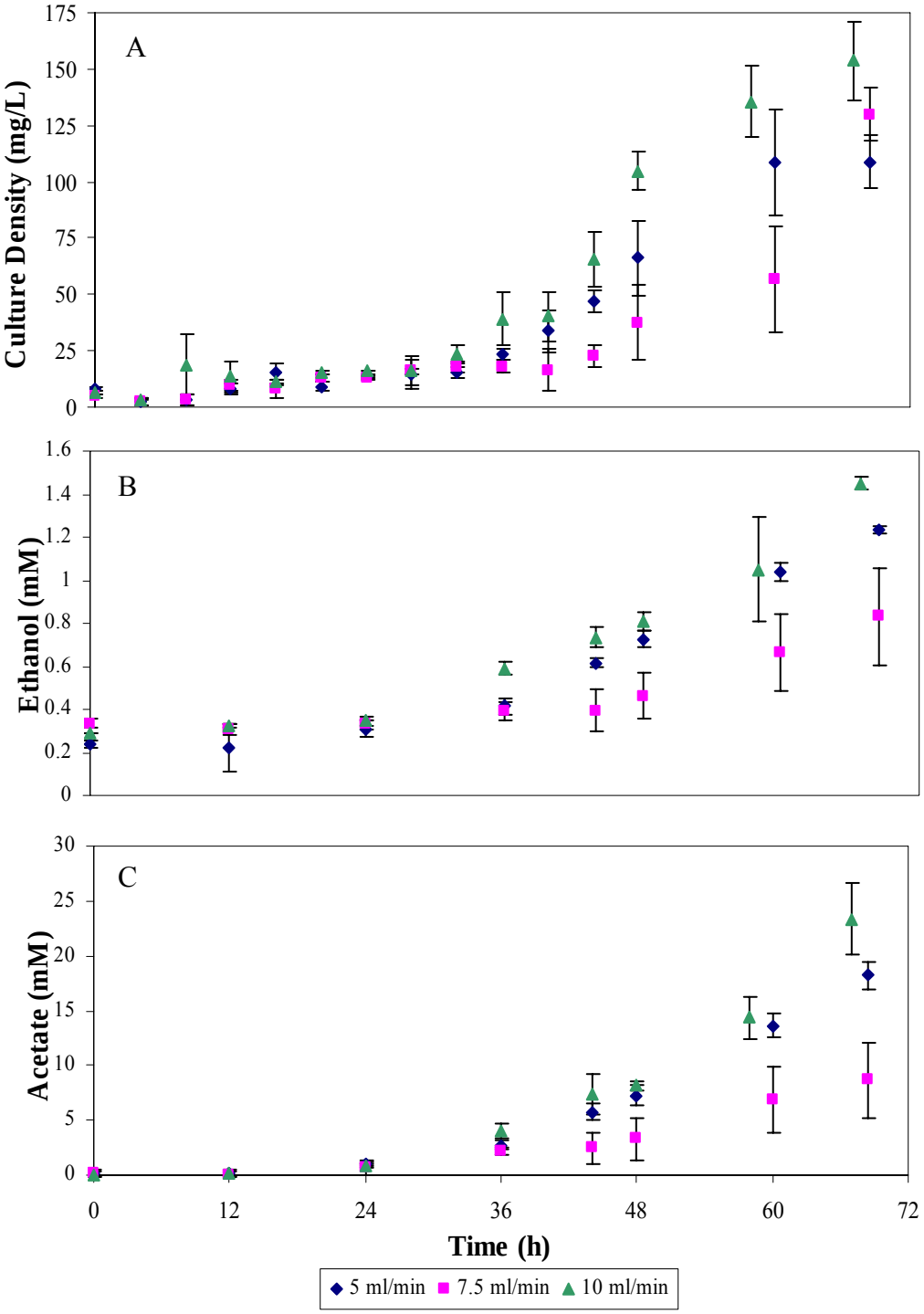

Figure 3.4 *C. autoethanogenum* metabolism over time on bottled syngas. A) ethanol production; B) acetate production; C) culture growth.

A possible explanation for the lower growth rate at 7.5 ml/min might have been from oxygen contamination. Yet during growth, there was no observed change in the medium color due to the presence of oxygen (from the resazurin indicator) nor were there higher levels of nitrogen in the gas composition signifying air contamination in the reactor. Inoculation times were off-set in order to enable consistent sampling times throughout the experimental period. Due to the different inoculum times, oxygen contamination could have occurred in the inoculum culture between the inoculation of the 5 and 10 ml/min cultures and the 7.5 ml/min cultures. Exposure to oxygen would cause a decrease in the overall quality of the microorganism and the observed longer lag phase.

Table 3.5 summarizes the ANOVA results for ethanol and acetate production by *C. autoethanogenum* on synthesis gas. Both ethanol and acetate were significantly affected by the flow rate and time, with a significant interaction effect (p-value <0.05). Final ethanol product concentrations for flow rates 5 and 10 ml/min were statistically different from one another at 1.23 and 1.45 mM ethanol, respectively (p-value < 0.05) which could be attributed to the increased flow rate if the data for 7.5 ml/min fermentations are excluded. Values for ethanol and acetate for all three replications at 7.5 ml/min were statistically lower than the corresponding values from 5 and 10 ml/min fermentations and are most likely related to the health of the inoculum culture for those reactors.

There was no difference in the ethanol to acetate production ratios (mM ethanol / mM acetate) between 5 and 10 ml/min flow rate treatments. The ethanol to acetate production ratios (approximately 1:15) for *C. autoethanogenum* cultures favor acetate more than *C. ljungdahlii* (approximately 1:4 for control cultures) fermentations on synthesis gas. As acetate was produced at higher rates than ethanol, the ethanol to acetate production ratios

dropped equally across all three flow rates. This indicated that the flow rate did not affect the metabolic direction of the reduction of acetyl-CoA to either acetate or ethanol.

Table. 3.5 *C. autoethanogenum* ANOVA tables for final ethanol and acetate concentration values

Product	Source	DF	Mean Square	F Value	Pr > F
Ethanol	Flow	2	0.27946068	36.29	<.0001
	Time	7	0.77125513	100.16	<.0001
	Flow*time	14	0.04485353	5.82	<.0001
Acetate	Flow	2	101.167058	49.05	<.0001
	Time	7	261.182000	126.64	<.0001
	Flow*time	14	17.903048	8.68	<.0001

The ANOVA results for each synthesis gas species measured during the growth of *C. autoethanogenum* are presented in Table 3.6. The concentration of CO was significantly affected by the main effects of time and flow and by the interaction of time and flow rate (p-value < 0.05). The general trend of lower CO % in the headspace over time indicate that the organism was using CO as a growth substrate at significant levels. There was significantly less CO (p-values < 0.05) observed at 7.5 ml/min compared to 5 and 10 ml/min, respectively between 32 and 40 hours. CO_2 concentration levels were affected by the main effect of flow and not time. The more dense cultures at 10 ml/min produced more CO_2 than cultures at 5 ml/min. Although there are differences in CO and CO_2 uses between flow rates, the general trends of gas uses (consumption and production) are similar across all flow rates.

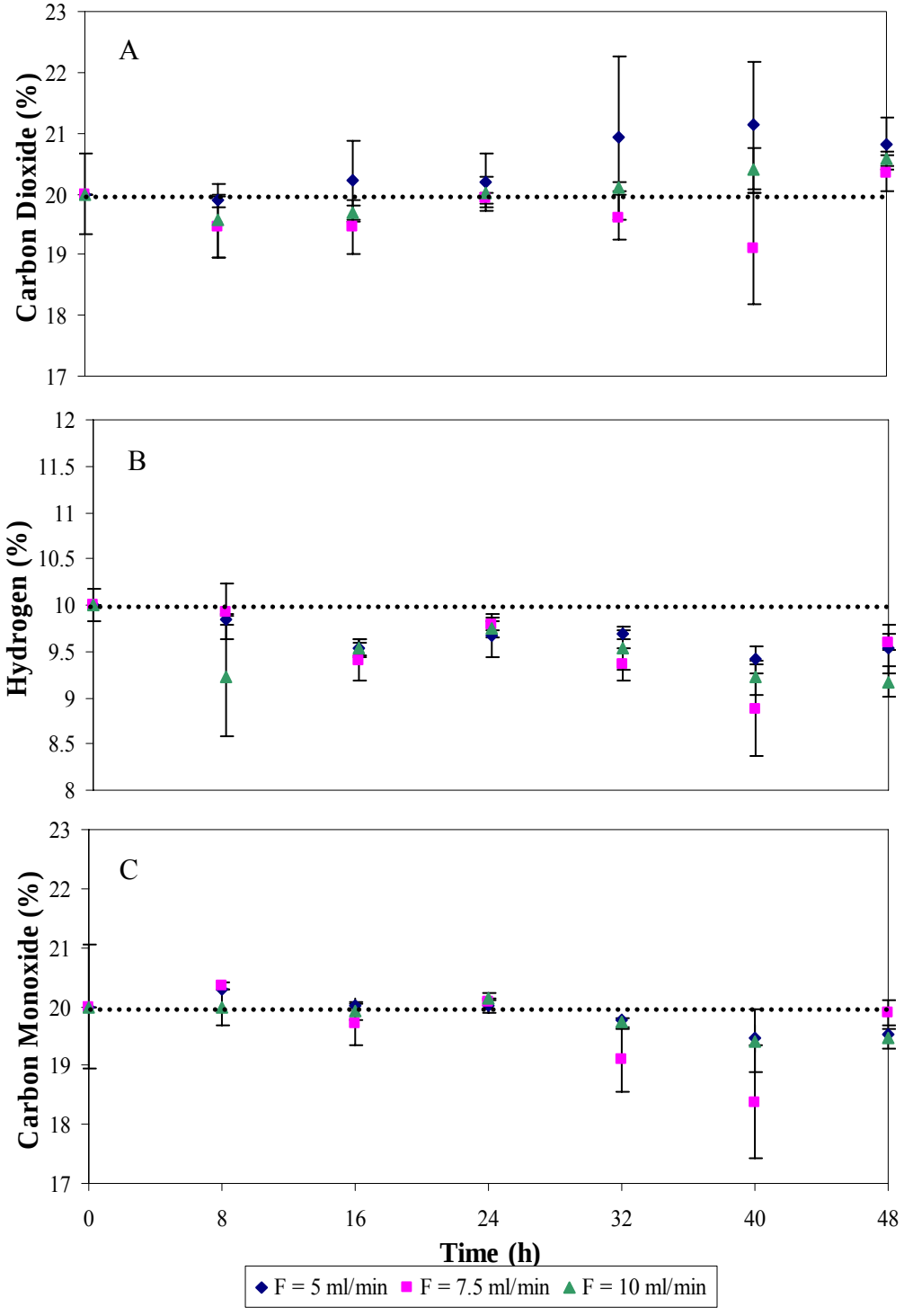

Figure 3.5 *C. autoethanogenum* headspace gas composition over time during growth on bottled synthesis gas. A) % CO_2; B) % H_2; C) % CO. Dashed lines show approximate average % composition at t = 0.

The concentration of H_2 was significantly affected by time, but not flow rate. As seen in Figure 3.5b, the H_2 concentration in the headspace generally decreased over time for all flow rates. From 8 to 40 hours the average H_2 compositions for all flow rates changed from 9.70 to 9.20 % (p-value < 0.0001).

Consumption of CO by autotrophic organisms is often associated with production of CO_2. Although there were not significant increases in CO_2 over time, the fact that the H_2 levels decreased over time signifies that CO_2 was being consumed by *C. autoethanogenum* as it was being produced. This indicated that both CO and CO_2 were used as the primary carbon sources for growth and product formation by *C. autoethanogenum*.

Table 3.6 *C. autoethanogenum* combined ANOVA tables for gas compositions showing main and interaction effects of time and flow

Gas	Source	DF	Mean Square	F Value	Pr > F
H_2	Time	5	0.29831111	4.86	0.0017
	Flow	2	0.10545000	1.72	0.1936
	Time*Flow	10	0.12467444	2.03	0.0585
CO	Time	5	1.36218852	12.17	<.0001
	Flow	2	0.44805185	4.00	0.0269
	Time*Flow	10	0.33192963	2.97	0.0080
CO_2	Time	5	0.66134519	1.96	0.1076
	Flow	2	3.91419074	11.63	0.0001
	Time*Flow	10	0.32364852	0.96	0.4921

3.4 Conclusions

The autotrophic bacteria *C. ljungdahlii* and *C. autoethanogenum* are capable of producing measurable amounts ethanol and significant quantities of acetate through synthesis gas fermentation. Culture growth on synthesis gas substrates leads to a longer lag phase than seen on sugar substrates or growth on gas with a sugar adapted culture. *C. ljungdahlii* yielded comparable ethanol to acetate production ratios (mM ethanol : mM acetate) for fermentations on syngas (averaged across all flow rates) at pH 6.8 and fermentations on fructose, 1:8 and

1:7, respectively. This was not the case for *C. autoethanogenum* where the ethanol to acetate product ratio decreased from 1:8 on sugar to 1:13 on syngas (averaged across all flow rates).

Initial medium pH had significant affects on metabolism of growing cultures of *C. ljungdahlii*. More acidic pH levels lead to slower growth rates, and lower ethanol yields. There was no significant effect of flow rate on ethanol and acetate production for *C. ljungdahlii*. Therefore, higher flow rates do not directly correspond to higher product yields. The flow rate results have implications on the evaluation of the overall process of gasification and fermentation for ethanol production. If low flow rates can be used to achieve similar levels of ethanol, then the conversion of biomass to ethanol could be maximized at low gas flow rates.

However, flow rate did play an important role for *C. autoethanogenum* growth rates and corresponding product formation levels, despite the trends observed for the 7.5 ml/min culture which were not intuitive.

3.5 Future Work

Future work on substrate use and correlations to product formation and biomass yield needs to be further studied for both *C. ljungdahlii* and *C. autoethanogenum* using various substrate type combinations. Testing the effects of mixed sugar and gas substrates on performance and effectiveness of pre-adapted inoculum sources would be beneficial. Growth on sugar could possibly affect the overall culture stability by decreasing the fermentation lag phase and increasing growth rate. Testing various ratios of useful syngas components in the gas feed on growth and product formation would help determine the ideal gas compositions needed from an upstream gasifier. Such studies would help determine the feasibility of synthesis gas fermentation as a commercial ethanol production method. Additionally, the

mass-transfer limitations and cellular substrate transport mechanisms affecting the microorganisms' abilities to access useful synthesis gas components need to be further studied in order to better understand the approaches necessary to enhance carbon fixation and ultimately ethanol and acetate yields.

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Chapter 4: Gasification and Fermentation Process Assessment

There are several key issues with the technology of gasification and fermentation that would be valuable to future work including upstream gasification conditioning, intracellular enzyme and cofactor use and characterization, and culture growth-stage studies to control metabolic pathways and to increase stability.

The most unstable variable in the technology of gasification and fermentation for ethanol production is the biocatalyst. The microorganisms used to convert synthesis gas are sensitive to their environment and minor changes in the feed-gas stream can have significant affects on culture stability. It would be valuable to study the optimal gas composition and contamination tolerance levels for culture performance and stability. This would allow gasification research to focus processing requirements for subsequent fermentation.

Another area of research used to enhance culture stability is cell immobilization which has been used to increase tolerance levels of toxic chemicals and other harsh environmental conditions (Ayabe et al., 1986; Bajpai et al., 1989; Freeman et al., 1998). However, cell immobilization of homoacetogenic autotrophs has not been fully studied. It would be valuable to characterize the culture performance of these clostridia organisms in cell immobilization systems on actual synthesis gas streams to quantify improvements in culture stability.

One important question for the purpose of enhancing fermentation performance and stability is how the production and use of intracellular enzymes correlates with culture growth. Measuring enzyme levels during synthesis gas fermentation over time would help to understand the necessary conditions for preparing a dense culture with high levels of ethanol production enzymes. Additionally, genetic sequence determination and annotation of these

microorganisms is needed to better understand their metabolic capabilities on a molecular level and to allow for possible future genetic modifications.

Metabolic regulation of autotrophic clostridia to enhance ethanol production through use of resting cells may still hold promise. Studies in this work demonstrated that using nitrogen-limitation to induce a non-growth stage for the purposes of solventogenesis initiation might not be the most feasible option. Possible alternative methods to induce resting cells include the use of growth arrestors and metabolic decouplers. Antibiotics could be added to fermentation cultures at desired growth stages to prevent further growth. The use of decouplers enables the isolation of anabolic and catabolic pathways to shift metabolism towards product formation and away from cellular biomass production. Studying the effects that resting-cell induction methods have on culture performance will help determine if resting-cell fermentation of synthesis gas is a viable approach.

The microorganism acts as a chemical reactor converting synthesis gas components to ethanol. At this point, studies aimed at increasing the process stability of ethanol production from synthesis gas fermentation need to focus on the biological catalysts. Analyzing fermentation parameters on a cellular level will help answer the most important questions for further development of the gasification and fermentation technology.

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Appendix A: Media Preparation Protocols

Appendix A1: *Clostridium ljungdahlii* Media Protocols

Appendix A1.1: RCM.NA.SVE Growth Medium

RCM.NA.SVE General Recipe

Component	Quantity per 1L
Proteose Peptone No. 3	10.0 g
Beef Extract	10.0 g
Yeast Extract	3.00 g
PETC salts	50 ml
PETC Modified Trace Elements	10 ml
Modified Wolfe's Vitamin Solution	10 ml
Cysteine-HCl	0.5 g
Resazurin Stock	0.50 ml
Distilled H_2O	930 ml

1. Prepare general recipe and adjust medium pH to 6.8 ± 0.2.
2. Autoclave for 10 minutes to degas medium. (Liquid cycle slow exhaust)
3. Bubble with N_2 until medium cools to room temperature.
4. Anaerobically transfer medium to Balch tubes and/or to serum bottles. Place under gas stream for ~5 minutes if medium appears oxidized.
5. Autoclave for 15 minutes at 121ºC (liquid cycle).

Prior to Inoculation:
If transferring to a 10 ml Balch tube sample add:
0.5 ml of 10% (w/v) fructose stock solution
0.1 ml of 3% (w/v) Cysteine Hydrochloride stock solution
0.5 ml inoculation of cell culture
(8.9 media + 0.5 ml fructose + 0.1 ml Cysteine Hydrochloride + 0.5 cell culture ~ 10.0 ml/ Balch tube)

If transferring to an 80 ml serum bottle sample add:
4 ml of 10% (w/v) fructose stock solution
0.8 ml 3% (w/v) Cysteine Hydrochloride stock solution
4 ml of cell culture
(71 ml media + 4 ml fructose + 1.3 ml Cysteine Hydrochloride + 4 ml cell culture ~ 80 ml/ serum bottle)

Appendix A1.2: Non-growth Media

NG.RCM.NA.S General Recipe

Component	Quantity per 1L	Per 150 L
PETC salts	50 ml	7.5 ml
Cysteine-HCl	0.5 g	0.075 g
Resazurin Stock	0.50 ml	75 µl
Distilled H_2O	950 ml	142.5 ml

NG.RCM.NA.S 2X NH_4Cl General Recipe

Component	Quantity per 1L	Per 150 L
PETC salts	50 ml	7.5 ml
Cysteine-HCl	0.5 g	0.075 g
Resazurin Stock	0.50 ml	75 µl
NH_4Cl Stock	50 ml	7.5 ml
Distilled H_2O	900 ml	135 ml

NG.RCM.NA.SVE General Recipe

Component	Quantity per 1L
PETC salts	50 ml
PETC Modified Trace Elements	10 ml
Modified Wolfe's Vitamin Solution	10 ml
Cysteine-HCl	0.5 g
Resazurin Stock	0.50 ml
Distilled H_2O	950 ml

1. Prepare general recipe and adjust medium pH to 6.8
2. Autoclave for 10 minutes to degas medium. (Liquid cycle slow exhaust)
3. Bubble with N_2 until medium cools to room temperature.
4. Anaerobically dispense appropriate amount of media into serum bottles according to 'Growth to Non-growth' protocol calculations (Appendix C). Place under gas stream for ~5 minutes if medium appears oxidized.
5. Autoclave for 15 minutes at 121ºC (liquid cycle).

NG.RCM.NA.SVE pH 5.5 and 4.5

Prepare with NG.RCM.NA.SVE recipe above. Adjust medium desired pH (5.5 or 4.5) prior to degassing step

NG.RCM.NA.SVE with 50 or 100 ppm Benzyl Violgoen

Prepare with NG.RCM.NA.SVE recipe above. Prior to incubation for non-growth studies add 0.5 ml or 1.0 ml 8 % w/v benzyl viologen for 50 and 100 ppm final benzyl viologen concentrations

Appendix A1.3: Stock Solutions

Resazurin Stock (0.1% w/v)
Add 0.1 g of resazurin to 100 ml distilled water.

Fructose Stock Solution (100 g/L or 10% w/v)
Weigh 4 g of fructose in a serum bottle and place under a N_2 atmosphere. Boil distilled water under a N_2 atmosphere in a round-bottom flask and anaerobically transfer 40 ml to the xylose substrate. Autoclave stopped and sealed bottles for 20 minutes.

Cysteine Hydrochloride Stock (3% w/v)
Weigh 3 g of Cysteine hydrochloride in a serum bottle and place under a N_2 atmosphere. Boil distilled water under a N_2 atmosphere in a round-bottom flask and anaerobically transfer 100 ml to the xylose substrate. Autoclave stopped and sealed bottles for 20 minutes.

Soluble Starch Stock Solution ***(1 g/L or 0.1% w/v)***
Weigh 0.04 g of soluble starch in a serum bottle and place under a N_2 atmosphere. Boil distilled water under a N_2 atmosphere in a round-bottom flask and anaerobically transfer 40 ml to the soluble starch substrate. Autoclave stopped and sealed bottles for 20 minutes.

Benzyl Viologen Stock Solution ***(0.8 g/L)***
Weigh 3.20 g of benzyl viologen in serum bottle and place under a N_2 atmosphere. Boil distilled water under a N_2 atmosphere (make sure water comes to a rolling boil for at least 40 seconds) in a round-bottom flask and anaerobically transfer 40 ml to the serum bottle containing the benzyl viologen. Autoclave stopped and sealed bottle for 20 minutes at 121ºC.

NH_4CL Stock Solution **(20 g/L)**
Weigh 4.0 g NH_4CL in a 250 ml container. Add 200 ml distilled water, fasten the top onto the container, and shake until NH_4Cl is completely dissolved.

PETC Salts (44.4 g/L or 4.44% w/v)

Component	Quantity
NH_4Cl	20 g
KCl	2 g
$MgSO_4.7H_2O$	4 g
NaCl	16 g
KH_2PO_4	2 g
$CaCl_2$	0.4 g
Distilled H_2O	1 L

Wolfe's Vitamin Solution (49 g/L or 4.9% w/v)

Component	Quantity
Biotin	2 mg
Folic acid	2 mg
Pyridoxine	10 mg
Thiamine HCl	5 mg
Riboflavin	5 mg
Nicotinic acid	5 mg
Calcium D(+) pantothenate	5 mg
Vitamin B12	5 mg
p-Aminobenzoic acid	5 mg
Lipoic acid (thioctic)	5 mg
Distilled water	1000ml

Store in 4ºC refrigerator

Trace Element Solution (4.25 g/L or 0.425% w/v)

Component	Quantity
Nitrilotriacetic acid*	2 g
$MnCl_2.4H_2O$	1.3 g
$FeSO_4.7H_2O$	0.4 g
$CoCl_2.6H_2O$	0.2 g
$ZnSO_4.7H_2O$	0.2 g
$CuCl_2.2H_2O$	0.02 g
$NiCl_2.6H_2O$	0.02 g
$Na_2MoO_4.2H_2O$	0.02 g
Na_2SeO_3	0.02 g
$Na_2WO_4.2H_2O$	0.025 g
Distilled H_2O	1000ml

**Add nitrilotriacetic acid to the water first, adjust to pH 6.0 with KOH, and then add remaining ingredients. Cover container in tin foil and store in 4ºC refrigerator.*

Appendix A2: *Clostridium autoethanogenum* Media Protocols

DSMZ 640 Growth Medium

DSMZ 640 General Recipe

Component	Quantity (per L)
640 Salts	100 mL
Trypticase Peptone	2 g
Yeast Extract	1 g
Trace Element Solution	1.00 mL
Cysteine-CHl.H_2O	0.75 g
Resazurin Stock	0.50 mL
Distilled water	900 mL

1. Prepare general recipe and adjust pH to 6.0.
2. Autoclave for 10 minutes to degas medium. (Liquid cycle slow exhaust)
3. Bubble with N_2 until medium cools to room temperature.
4. Anaerobically transfer 9 ml of medium to Balch tubes and 72 ml to serum bottles. Place under gas stream for ~5 minutes if medium appears oxidized.
5. Autoclave for 15 minutes at 121ºC (liquid cycle).

Prior to Inoculation:
If transferring to a 10 ml Balch tube sample add:
0.5 ml of 10% (w/v) xylose stock solution
0.5 ml inoculation of cell culture
(9 media + 0.5 ml xylose + 0.5 cell culture = 10.0 ml/ Balch tube)

If transferring to an 80 ml serum bottle sample add:
4 ml of 10% (w/v) xylose stock solution
4 ml of cell culture
(72 ml media + 4 ml fructose + 1.3 ml Cysteine Hydrochloride + 0.4 ml cell culture ~ 80 ml/ serum bottle)

Non-growth media for C. autoethanogenum

640.NG.1 General Recipe

Component	Quantity (per L)
640 Salts **(-)**	100 mL
Trypticase Peptone	0 g
Yeast Extract	0 g
Trace Element Solution	1.00 mL
Cysteine-CHl.H_2O	0.75 g
Resazurin Stock	0.50 mL
Distilled water	900 mL

640.NG.2 General Recipe

Component	Quantity (per L)
640 Salts	100 mL
Trypticase Peptone	0 g
Yeast Extract	0 g
Trace Element Solution	1.00 mL
Cysteine-CHl.H_2O	0.75 g
Resazurin Stock	0.50 mL
Distilled water	900 mL

640.NG.3 General Recipe

Component	Quantity (per L)
640 Salts	100 mL
Trypticase Peptone	2 g
Yeast Extract	0 g
Trace Element Solution	1.00 mL
Cysteine-CHl.H_2O	0.75 g
Resazurin Stock	0.50 mL
Distilled water	900 mL

640.NG.4 General Recipe

Component	Quantity (per L)
640 Salts	100 mL
Trypticase Peptone	0 g
Yeast Extract	1 g
Trace Element Solution	1.00 mL
Cysteine-CHl.H_2O	0.75 g
Resazurin Stock	0.50 mL
Distilled water	900 mL

640.NG.5 General Recipe

Component	Quantity (per L)
640 Salts	100 mL
Trypticase Peptone	0 g
Yeast Extract	0.1 g
Trace Element Solution	1.00 mL
Cysteine-CHl.H_2O	0.75 g
Resazurin Stock	0.50 mL
Distilled water	900 mL

640.NG.6 General Recipe

Component	Quantity (per L)
40 Salts (-)	100 mL
Trypticase Peptone	0 g
Yeast Extract	0.1 g
Trace Element Solution	1.00 mL
Cysteine-CHl.H_2O	0.75 g
Resazurin Stock	0.50 mL
Distilled water	900 mL

1. Prepare general recipe adjust pH to 6.0.
2. Autoclave for 10 minutes to degas medium. (Liquid cycle slow exhaust)
3. Bubble with N_2 until medium cools to room temperature.
4. Anaerobically dispense appropriate amount of media into serum bottles according to 'Growth to Non-growth' protocol calculations (Appendix C). Place under gas stream for ~5 minutes if medium appears oxidized.
5. Autoclave for 15 minutes at 121ºC (liquid cycle).

Stock Solutions

Resazurin Stock (0.1% w/v)
Add 0.1 g of resazurin to 100 ml distilled water.

Xylose Stock Solution (100 g/L or 10% w/v)
Weigh 4 g of xylose in a serum bottle and place under a N_2 atmosphere. Boil distilled water under a N_2 atmosphere in a round-bottom flask and anaerobically transfer 40 ml to the xylose substrate. Autoclave stopped and sealed bottles for 20 minutes.

Medium 640 Salt Solution

Component	Quantity
NH_4Cl	9 g
NaCl	9 g
$MgCl_2.6H_2O$	4 g
KH_2PO_4	7.5 g
K_2HPO_4	15 g
$FeCl_3.6H_2O$	0.025 g
Distilled water	1 L

*If using $FeCl_3$, add 0.015 g

Medium 640 Trace element solution SL-10

Component	Quantity
HCl (25%; 7.7M)	10.00 ml
$FeCl_2.4H_2O$	1.50 g
$ZnCl_2$	70.00 mg
$MnCl_2.4H_2O$	100.00 mg
H_2BO_3	6.00 mg
$CoCl_2.6H_2O$	190.00 mg
$CuCl_2.2H_2O$	2.00 mg
$NiCl_2.6H_2O$	24.00 mg
$Na_2MoO_4.2H_2O$	36.00 mg
Distilled water	990.00 ml

*First dissolve $FeCl_2$ in the HCl, then dilute into water and add/dissolve other salts. (For 7.7 M HCl solution: Dilute 152 ml 37% (w/v) into 48 ml H_2O

NH_4CL Stock Solution **(20 g/L)**
Weigh 4.0 g NH_4CL in a 250 ml container. Add 200 ml distilled water, fasten the top onto the container, and shake until NH_4Cl is completely dissolved.

Appendix B: Optical Weight vs. Dry Cell Weight Studies

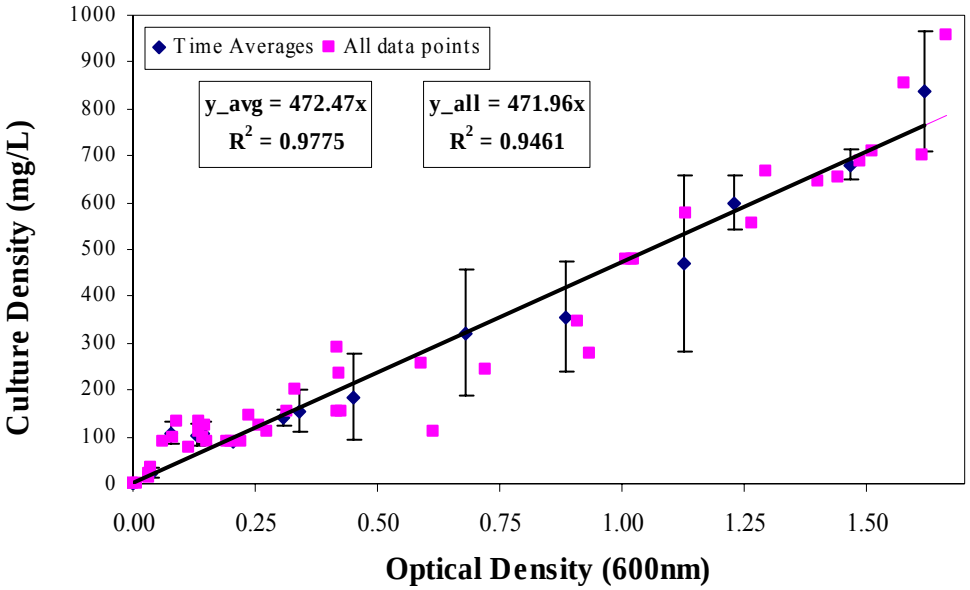

Figure B.1 Optical Density versus Dry Cell Weight Study for *C. ljungdahlii*

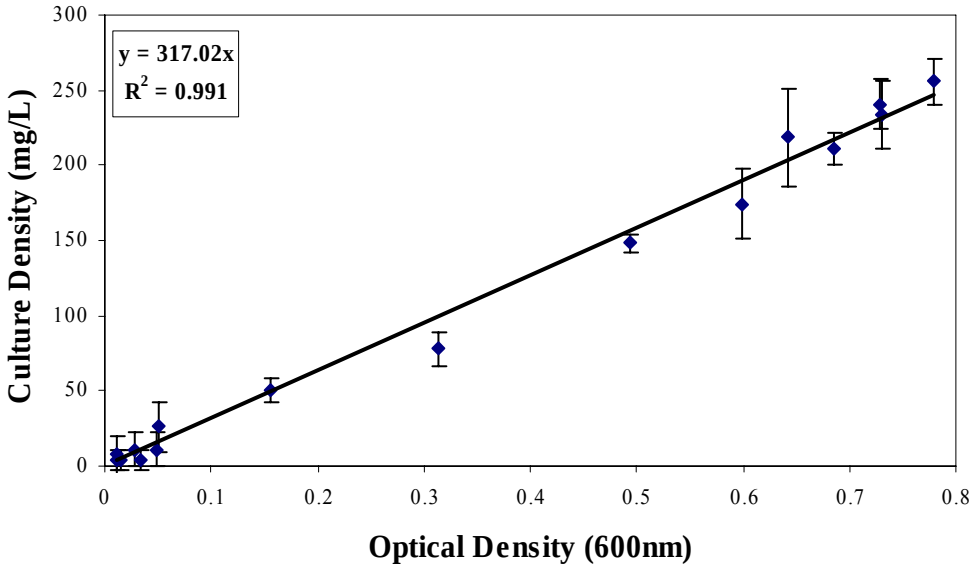

Figure B.2 Optical Density versus Dry Cell Weight for *C. autoethanogenum*

Appendix C: Growing to Non-growing Transfer Calculations & Spreadsheet

Table C1 Example Spreadsheet for growing to non-growing transfer calculations

	R1				R2		
Description	**symbol**	**units**	**value**		**symbol**	**units**	**value**
Properties of Growing Cultures	1st growing stock culture				2nd growing stock culture		
Volume of growing culture to be put into c. tube	V1	ml	70		V2	ml	70
OD of growing culture	OD1		0.816		OD2		0.822
		(mg/L)^-				(mg/L)^-	
OD to DCW conversion factor	m	1	317		m	1	317
Growing culture final density	γ1	mg/L	258.672		γ2	mg/L	260.574
Centrifuge Growing Culture - Remove Supernatant & Replace with Non-growing media							
Cell Weight in Centrifuge tubes	CWC	mg	18.10704		CWC	mg	18.24018
Volume of non-growth media added to c. tube	Va	ml	30		Va	ml	30
Concentration of culture in c.tube	γc	mg/L	603.568		γc	mg/L	608.006
Properties of Non-growing Cultures							
Non-growing inoculation OD	ODng		0.65		ODng		0.65
Non-growing inoculation density	γng	mg/L	206.05		γng	mg/L	206.05
Volume predispensed into non-growing serum bottle	Vng,0	ml	49		Vng,0	ml	49
Volume of non-growth media to transfer	Vt	ml	27.31		Vt	ml	27.11
Final volume of non-growth media culture	Vng,f	ml	80		Vng,f	ml	80
Media to add to bottle to get vinal volume = 80 ml			3.69				3.89
number of non-growing inoculations possible	n	-	1.10		n	-	1.11
	NG 640 + 0.1g/L YE + 0.9g/L NH4C1						

Constant values ~ determined before experimentation

Input values ~ used to calculate remaining values

JLC 3/2/2006

Clostridium autoethanogenum

Media = DSM 640 with no Yeast Extract & No Peptone + 2X [NH4C1]

Appendix D: SAS®Analyses of for Non-growth Studies

Appendix D.1 Non-growth Studies on *C. ljungdahlii*

Appendix D.1.1 Initial non-growth studies with C. ljungdahlii

```
Title1 'Initial Non-growth studies with Clostridium ljungdahlii';
Title2 'medium 1 = RCM.NA.SVE
 medium 2 = RCM.NA.S
 medium 3 = RCM.NA.S 2X NH4Cl';
Title3 'Ethanol and Acetate Production over time';
Data practice;
Input Medium ethanol acetate time;
Cards;
1     0.18  1.19  0
1     0.28  0.98  46
1     0.21  0.48  96
1     0.25  0.62  144
1     0.10  0.60  0
1     0.18  0.53  46
1     0.18  0.76  96
1     0.18  0.75  144
1     0.11  0.54  0
1     0.00  0.00  46
1     0.26  0.82  96
1     0.25  0.82  144
2     0.19  0.45  0
2     0.16  0.66  46
2     0.21  0.70  96
2     0.11  0.57  144
2     0.16  0.63  0
2     0.16  0.63  46
2     0.07  0.00  96
2     0.07  0.00  144
2     0.06  0.00  0
2     0.14  0.49  46
2     0.00  0.00  96
2     0.07  0.00  144
3     0.13  0.72  0
3     0.12  0.85  46
3     0.00  0.00  96
3     0.07  0.57  144
3     0.00  0.04  0
3     0.15  1.27  46
3     0.00  0.00  96
3     0.06  0.78  144
3     0.08  0.85  0
3     0.10  0.64  46
3     0.16  1.17  96
3     0.17  1.45  144
;
OPTIONS NODATE PAGENO = 1;
Proc GLM Data = practice;
```

```
Class Medium time;

model ethanol acetate /*put in desired gas to analyze*/ =  Medium|time;
lsmeans Medium|time /pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
```

Initial Non-growth studies with Clostridium ljungdahlii
medium 1 = RCM.NA.SVE medium 2 = RCM.NA.S medium 3 = RCM.NA.S 2X NH4Cl
Ethanol and Acetate Production over time

The GLM Procedure

Class Level Information

Class	Levels	Values
Medium	3	1 2 3
time	4	0 46 96 144

Number of Observations Read	36
Number of Observations Used	36

Initial Non-growth studies with Clostridium ljungdahlii
medium 1 = RCM.NA.SVE medium 2 = RCM.NA.S medium 3 = RCM.NA.S 2X NH4Cl
Ethanol and Acetate Production over time

The GLM Procedure

Dependent Variable: ethanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	0.09570000	0.00870000	1.76	0.1187
Error	24	0.11840000	0.00493333		
Corrected Total	35	0.21410000			

R-Square	Coeff Var	Root MSE	ethanol Mean
0.446987	54.73067	0.070238	0.128333

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Medium	2	0.05660000	0.02830000	5.74	0.0092
time	3	0.00545556	0.00181852	0.37	0.7763
Medium*time	6	0.03364444	0.00560741	1.14	0.3717

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Medium	2	0.05660000	0.02830000	5.74	0.0092
time	3	0.00545556	0.00181852	0.37	0.7763
Medium*time	6	0.03364444	0.00560741	1.14	0.3717

Dependent Variable: acetate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	2.03028889	0.18457172	1.26	0.3042
Error	24	3.51546667	0.14647778		
Corrected Total	35	5.54575556			

R-Square	Coeff Var	Root MSE	acetate Mean
0.366098	67.01396	0.382724	0.571111

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Medium	2	0.92967222	0.46483611	3.17	0.0599
time	3	0.27588889	0.09196296	0.63	0.6041
Medium*time	6	0.82472778	0.13745463	0.94	0.4862

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Medium	2	0.92967222	0.46483611	3.17	0.0599
time	3	0.27588889	0.09196296	0.63	0.6041
Medium*time	6	0.82472778	0.13745463	0.94	0.4862

Initial Non-growth studies with Clostridium ljungdahlii
medium 1 = RCM.NA.SVE medium 2 = RCM.NA.S medium 3 = RCM.NA.S 2X NH4Cl
Ethanol and Acetate Production over time

The GLM Procedure
Least Squares Means

Medium	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0.18166667	0.02027588	<.0001	1
2	0.11666667	0.02027588	<.0001	2
3	0.08666667	0.02027588	0.0003	3

Least Squares Means for effect Medium
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3
1		0.0327	0.0029
2	0.0327		0.3059
3	0.0029	0.3059	

Medium	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0.67416667	0.11048295	<.0001	1
2	0.34416667	0.11048295	0.0047	2
3	0.69500000	0.11048295	<.0001	3

Least Squares Means for effect Medium
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3
1		0.0453	0.8950
2	0.0453		0.0342
3	0.8950	0.0342	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.11222222	0.02341256	<.0001	1
46	0.14333333	0.02341256	<.0001	2
96	0.12111111	0.02341256	<.0001	3
144	0.13666667	0.02341256	<.0001	4

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4
1		0.3568	0.7906	0.4675
2	0.3568		0.5085	0.8421
3	0.7906	0.5085		0.6427
4	0.4675	0.8421	0.6427	

Initial Non-growth studies with Clostridium ljungdahlii
medium 1 = RCM.NA.SVE medium 2 = RCM.NA.S medium 3 = RCM.NA.S 2X NH4Cl
Ethanol and Acetate Production over time

The GLM Procedure
Least Squares Means

time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.55777778	0.12757472	0.0002	1
46	0.67222222	0.12757472	<.0001	2
96	0.43666667	0.12757472	0.0022	3
144	0.61777778	0.12757472	<.0001	4

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4
1		0.5319	0.5085	0.7424
2	0.5319		0.2041	0.7654
3	0.5085	0.2041		0.3255
4	0.7424	0.7654	0.3255	

Medium	time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	0.13000000	0.04055175	0.0038	1
1	46	0.15333333	0.04055175	0.0009	2
1	96	0.21666667	0.04055175	<.0001	3
1	144	0.22666667	0.04055175	<.0001	4
2	0	0.13666667	0.04055175	0.0025	5
2	46	0.15333333	0.04055175	0.0009	6
2	96	0.09333333	0.04055175	0.0303	7
2	144	0.08333333	0.04055175	0.0509	8
3	0	0.07000000	0.04055175	0.0972	9
3	46	0.12333333	0.04055175	0.0056	10
3	96	0.05333333	0.04055175	0.2009	11
3	144	0.10000000	0.04055175	0.0212	12

Least Squares Means for effect Medium*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.6877	0.1438	0.1048	0.9084	0.6877	0.5286	0.4238	0.3059	0.9084	0.1938	0.6057
2	0.6877		0.2804	0.2132	0.7738	1.0000	0.3059	0.2341	0.1591	0.6057	0.0940	0.3616
3	0.1438	0.2804		0.8630	0.1758	0.2804	0.0418	0.0289	0.0173	0.1167	0.0089	0.0531
4	0.1048	0.2132	0.8630		0.1297	0.2132	0.0289	0.0197	0.0116	0.0841	0.0059	0.0370
5	0.9084	0.7738	0.1758	0.1297		0.7738	0.4572	0.3616	0.2565	0.8181	0.1591	0.5286
6	0.6877	1.0000	0.2804	0.2132	0.7738		0.3059	0.2341	0.1591	0.6057	0.0940	0.3616
7	0.5286	0.3059	0.0418	0.0289	0.4572	0.3059		0.8630	0.6877	0.6057	0.4922	0.9084
8	0.4238	0.2341	0.0289	0.0197	0.3616	0.2341	0.8630		0.8181	0.4922	0.6057	0.7738
9	0.3059	0.1591	0.0173	0.0116	0.2565	0.1591	0.6877	0.8181		0.3616	0.7738	0.6057
10	0.9084	0.6057	0.1167	0.0841	0.8181	0.6057	0.6057	0.4922	0.3616		0.2341	0.6877
11	0.1938	0.0940	0.0089	0.0059	0.1591	0.0940	0.4922	0.6057	0.7738	0.2341		0.4238
12	0.6057	0.3616	0.0531	0.0370	0.5286	0.3616	0.9084	0.7738	0.6057	0.6877	0.4238	

Initial Non-growth studies with Clostridium ljungdahlii
medium 1 = RCM.NA.SVE medium 2 = RCM.NA.S medium 3 = RCM.NA.S 2X NH4Cl
Ethanol and Acetate Production over time

The GLM Procedure
Least Squares Means

Medium	time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	0.77666667	0.22096589	0.0018	1
1	46	0.50333333	0.22096589	0.0319	2
1	96	0.68666667	0.22096589	0.0048	3
1	144	0.73000000	0.22096589	0.0030	4
2	0	0.36000000	0.22096589	0.1163	5
2	46	0.59333333	0.22096589	0.0129	6
2	96	0.23333333	0.22096589	0.3015	7
2	144	0.19000000	0.22096589	0.3984	8
3	0	0.53666667	0.22096589	0.0230	9
3	46	0.92000000	0.22096589	0.0003	10
3	96	0.39000000	0.22096589	0.0903	11
3	144	0.93333333	0.22096589	0.0003	12

Least Squares Means for effect Medium*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.3904	0.7758	0.8825	0.1949	0.5629	0.0949	0.0727	0.4500	0.6506	0.2279	0.6207
2	0.3904		0.5629	0.4753	0.6506	0.7758	0.3961	0.3260	0.9159	0.1949	0.7200	0.1815
3	0.7758	0.5629		0.8909	0.3063	0.7678	0.1598	0.1251	0.6356	0.4625	0.3519	0.4376
4	0.8825	0.4753	0.8909		0.2480	0.6658	0.1251	0.0968	0.5420	0.5489	0.2874	0.5214
5	0.1949	0.6506	0.3063	0.2480		0.4625	0.6888	0.5915	0.5771	0.0857	0.9243	0.0790
6	0.5629	0.7758	0.7678	0.6658	0.4625		0.2607	0.2091	0.8576	0.3063	0.5214	0.2874
7	0.0949	0.3961	0.1598	0.1251	0.6888	0.2607		0.8909	0.3414	0.0379	0.6207	0.0346
8	0.0727	0.3260	0.1251	0.0968	0.5915	0.2091	0.8909		0.2783	0.0282	0.5282	0.0257
9	0.4500	0.9159	0.6356	0.5420	0.5771	0.8576	0.3414	0.2783		0.2318	0.6431	0.2165
10	0.6506	0.1949	0.4625	0.5489	0.0857	0.3063	0.0379	0.0282	0.2318		0.1028	0.9663
11	0.2279	0.7200	0.3519	0.2874	0.9243	0.5214	0.6207	0.5282	0.6431	0.1028		0.0949
12	0.6207	0.1815	0.4376	0.5214	0.0790	0.2874	0.0346	0.0257	0.2165	0.9663	0.0949	

Appendix D.1.2 C. ljungdahlii NG.RCM.NA.SVE pH Study

```
Title1 'pH effects on NG C. ljungdahlii  Study';
Title2 'Ethanol and Acetate Production over time';
Data practice;
Input pH time ethanol acetate;
Cards;
4.5   0     0.06  0
4.5   46    0.07  0
4.5   96    0.77  0.77
4.5   144   0.65  0.65
4.5   0     0.64  0.64
4.5   46    0.73  0.73
4.5   96    0.72  0.72
4.5   144   0.85  0.84
4.5   0     0.09  0.77
4.5   46    0.74  0.74
4.5   96    0.63  0.63
4.5   144   0.84  0.84
5.5   0     0.09  0.73
5.5   46    0.13  0.98
5.5   96    0.15  1.02
5.5   144   0.22  0.65
5.5   0     0.09  0.73
5.5   46    0.08  0.65
5.5   96    0.09  0.84
5.5   144   0.13  0.70
5.5   0     0.11  0.94
5.5   46    0.11  0.84
5.5   96    0.11  0.75
5.5   144   0.15  0.68
6.8   0     6.50  0.95
6.8   46    7.08  1.08
6.8   96    6.85  1.01
6.8   144   7.20  1.08
6.8   0     6.63  1.03
6.8   96    5.22  0.84
6.8   144   7.45  1.16
6.8   0     5.91  0.93
6.8   46    7.50  1.18
6.8   96    5.13  0.82
6.8   144   6.74  0.97
;
OPTIONS NODATE PAGENO = 1;
Proc GLM Data = practice;
Class pH time ;
model ethanol acetate /*put in desired gas to analyze*/ =  pH|time;
lsmeans pH|time /pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
end;
```

pH effects on NG C. ljungdahlii Study
Ethanol and Acetate Production over time

The GLM Procedure

Class Level Information

Class	Levels	Values
pH	3	4.5 5.5 6.8
time	4	0 46 96 144

Number of Observations Read	35
Number of Observations Used	35

pH effects on NG C. ljungdahlii Study
Ethanol and Acetate Production over time

The GLM Procedure

Dependent Variable: ethanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	297.7949543	27.0722686	202.95	<.0001
Error	23	3.0680000	0.1333913		
Corrected Total	34	300.8629543			

R-Square	Coeff Var	Root MSE	ethanol Mean
0.989803	15.88736	0.365228	2.298857

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pH	2	293.0794232	146.5397116	1098.57	<.0001
time	3	1.6572486	0.5524162	4.14	0.0174
pH*time	6	3.0582825	0.5097137	3.82	0.0087

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pH	2	292.5370696	146.2685348	1096.54	<.0001
time	3	1.8263852	0.6087951	4.56	0.0119
pH*time	6	3.0582825	0.5097137	3.82	0.0087

Dependent Variable: acetate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	1.25188762	0.11380797	2.88	0.0155
Error	23	0.90806667	0.03948116		
Corrected Total	34	2.15995429			

R-Square	Coeff Var	Root MSE	acetate Mean
0.579590	24.93529	0.198699	0.796857

Source	DF	Type I SS	Mean Square	F Value	Pr > F
pH	2	0.88996489	0.44498245	11.27	0.0004
time	3	0.04501495	0.01500498	0.38	0.7683
pH*time	6	0.31690778	0.05281796	1.34	0.2810

Source	DF	Type III SS	Mean Square	F Value	Pr > F
pH	2	0.92302179	0.46151090	11.69	0.0003
time	3	0.04569630	0.01523210	0.39	0.7642
pH*time	6	0.31690778	0.05281796	1.34	0.2810

pH effects on NG C. ljungdahlii Study
Ethanol and Acetate Production over time

The GLM Procedure
Least Squares Means

pH	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
4.5	0.56583333	0.10543217	<.0001	1
5.5	0.12166667	0.10543217	0.2604	2
6.8	6.62500000	0.11182770	<.0001	3

Least Squares Means for effect pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3
1		0.0067	<.0001
2	0.0067		<.0001
3	<.0001	<.0001	

pH	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
4.5	0.61083333	0.05735936	<.0001	1
5.5	0.79250000	0.05735936	<.0001	2
6.8	1.01500000	0.06083879	<.0001	3

Least Squares Means for effect pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3
1		0.0351	<.0001
2	0.0351		0.0140
3	<.0001	0.0140	

time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	2.23555556	0.12174258	<.0001	1
46	2.63666667	0.13149702	<.0001	2
96	2.18555556	0.12174258	<.0001	3
144	2.69222222	0.12174258	<.0001	4

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4
1		0.0352	0.7741	0.0142
2	0.0352		0.0192	0.7593
3	0.7741	0.0192		0.0073
4	0.0142	0.7593	0.0073	

pH effects on NG C. ljungdahlii Study
Ethanol and Acetate Production over time

The GLM Procedure
Least Squares Means

time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.74666667	0.06623289	<.0001	1
46	0.81444444	0.07153970	<.0001	2
96	0.82222222	0.06623289	<.0001	3
144	0.84111111	0.06623289	<.0001	4

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4
1		0.4939	0.4281	0.3238
2	0.4939		0.9371	0.7869
3	0.4281	0.9371		0.8420
4	0.3238	0.7869	0.8420	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

pH	time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
4.5	0	0.26333333	0.21086434	0.2243	1
4.5	46	0.51333333	0.21086434	0.0231	2
4.5	96	0.70666667	0.21086434	0.0028	3
4.5	144	0.78000000	0.21086434	0.0012	4
5.5	0	0.09666667	0.21086434	0.6509	5
5.5	46	0.10666667	0.21086434	0.6178	6
5.5	96	0.11666667	0.21086434	0.5854	7
5.5	144	0.16666667	0.21086434	0.4374	8

pH effects on NG C. ljungdahlii Study
Ethanol and Acetate Production over time
The GLM Procedure
Least Squares Means

pH	time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
6.8	0	6.34666667	0.21086434	<.0001	9
6.8	46	7.29000000	0.25825501	<.0001	10
6.8	96	5.73333333	0.21086434	<.0001	11
6.8	144	7.13000000	0.21086434	<.0001	12

Least Squares Means for effect pH*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.4105	0.1507	0.0966	0.5816	0.6044	0.6275	0.7487	<.0001	<.0001	<.0001	<.0001
2	0.4105		0.5232	0.3805	0.1757	0.1859	0.1965	0.2570	<.0001	<.0001	<.0001	<.0001
3	0.1507	0.5232		0.8079	0.0524	0.0561	0.0600	0.0833	<.0001	<.0001	<.0001	<.0001
4	0.0966	0.3805	0.8079		0.0314	0.0337	0.0362	0.0512	<.0001	<.0001	<.0001	<.0001
5	0.5816	0.1757	0.0524	0.0314		0.9735	0.9471	0.8165	<.0001	<.0001	<.0001	<.0001
6	0.6044	0.1859	0.0561	0.0337	0.9735		0.9735	0.8423	<.0001	<.0001	<.0001	<.0001
7	0.6275	0.1965	0.0600	0.0362	0.9471	0.9735		0.8683	<.0001	<.0001	<.0001	<.0001
8	0.7487	0.2570	0.0833	0.0512	0.8165	0.8423	0.8683		<.0001	<.0001	<.0001	<.0001
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0095	0.0512	0.0151
10	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0095		0.0001	0.6358
11	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0512	0.0001		0.0001
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0151	0.6358	0.0001	

pH	time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
4.5	0	0.47000000	0.11471873	0.0004	1
4.5	46	0.49000000	0.11471873	0.0003	2
4.5	96	0.70666667	0.11471873	<.0001	3
4.5	144	0.77666667	0.11471873	<.0001	4
5.5	0	0.80000000	0.11471873	<.0001	5
5.5	46	0.82333333	0.11471873	<.0001	6
5.5	96	0.87000000	0.11471873	<.0001	7
5.5	144	0.67666667	0.11471873	<.0001	8
6.8	0	0.97000000	0.11471873	<.0001	9
6.8	46	1.13000000	0.14050117	<.0001	10
6.8	96	0.89000000	0.11471873	<.0001	11
6.8	144	1.07000000	0.11471873	<.0001	12

Least Squares Means for effect pH*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.9030	0.1582	0.0714	0.0536	0.0399	0.0216	0.2154	0.0053	0.0014	0.0164	0.0012
2	0.9030		0.1948	0.0905	0.0686	0.0514	0.0282	0.2617	0.0070	0.0018	0.0216	0.0016
3	0.1582	0.1948		0.6701	0.5707	0.4793	0.3245	0.8549	0.1182	0.0287	0.2701	0.0351
4	0.0714	0.0905	0.6701		0.8869	0.7762	0.5707	0.5437	0.2455	0.0637	0.4918	0.0837
5	0.0536	0.0686	0.5707	0.8869		0.8869	0.6701	0.4549	0.3056	0.0819	0.5844	0.1096
6	0.0399	0.0514	0.4793	0.7762	0.8869		0.7762	0.3754	0.3754	0.1044	0.6849	0.1420
7	0.0216	0.0282	0.3245	0.5707	0.6701	0.7762		0.2455	0.5437	0.1652	0.9030	0.2301
8	0.2154	0.2617	0.8549	0.5437	0.4549	0.3754	0.2455		0.0837	0.0200	0.2015	0.0236
9	0.0053	0.0070	0.1182	0.2455	0.3056	0.3754	0.5437	0.0837		0.3869	0.6266	0.5437
10	0.0014	0.0018	0.0287	0.0637	0.0819	0.1044	0.1652	0.0200	0.3869		0.1988	0.7438
11	0.0164	0.0216	0.2701	0.4918	0.5844	0.6849	0.9030	0.2015	0.6266	0.1988		0.2787
12	0.0012	0.0016	0.0351	0.0837	0.1096	0.1420	0.2301	0.0236	0.5437	0.7438	0.2787	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix D1.3 C. ljungdahlii NG.RCM.NA.SVE BV Study

```
Title1 'C. ljungdahlii Benzyl Viologen Study';
Title3 'Maximun Ethanol and Acetate Production';
Data practice;
Input BV time ethanol acetate;
Cards;
0     0     0.07  0.67
0     46    0.15  0.72
0     96    0.07  0.64
0     144   0.07  0.59
0     0     0.11  0.85
0     46    0.11  0.84
0     96    0.11  0.84
0     144   0.11  0.82
0     0     0.06
0     46    0.04
0     96    0.09  0.65
0     144   0.09  0.68
50    0     0.06  0.00
50    46    0.09  0.68
50    96    0.09  0.90
50    144   0.07  0.72
50    0     0.07  0.68
50    46    0.09  0.65
50    96    0.09  0.77
50    144   0.10  0.80
50    0     0.07  0.73
50    46    0.07  0.62
50    96    0.08  0.69
50    144   0.07  0.72
100   0     0.09  0.73
100   46    0.08  0.75
100   96    0.08  0.72
100   144   0.07  0.58
100   0     0.11  0.79
100   46    0.08  0.82
100   96    0.10  0.72
100   144   0.09  0.77
100   0     0.06  0.69
100   46    0.07  0.60
100   96    0.08  0.82
100   144   0.07  0.65
;
OPTIONS NODATE PAGENO = 1;
Proc GLM Data = practice;
Class time BV;

model ethanol acetate /*put in desired gas to analyze*/ =  time|BV;
lsmeans time|BV /pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
end;
```

C. ljungdahlii Benzyl Viologen Study

Maximun Ethanol and Acetate Production

The GLM Procedure

Class Level Information

Class	Levels	Values
time	4	0 46 96 144
BV	3	0 50 100

Number of Observations Read	35
Number of Observations Used	35

C. ljungdahlii Benzyl Viologen Study

Maximun Ethanol and Acetate Production

The GLM Procedure

Dependent Variable: ethanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	0.00580762	0.00052797	1.82	0.1087
Error	23	0.00666667	0.00028986		
Corrected Total	34	0.01247429			

R-Square	Coeff Var	Root MSE	ethanol Mean
0.465567	20.06329	0.017025	0.084857

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	3	0.00105762	0.00035254	1.22	0.3262
BV	2	0.00170009	0.00085005	2.93	0.0734
time*BV	6	0.00304991	0.00050832	1.75	0.1536

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	3	0.00164033	0.00054678	1.89	0.1601
BV	2	0.00219263	0.00109631	3.78	0.0380
time*BV	6	0.00304991	0.00050832	1.75	0.1536

Dependent Variable: acetate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	11	0.32778095	0.02979827	0.78	0.6555
Error	23	0.87733333	0.03814493		
Corrected Total	34	1.20511429			

R-Square	Coeff Var	Root MSE	acetate Mean
0.271992	28.60148	0.195307	0.682857

Source	DF	Type I SS	Mean Square	F Value	Pr > F
time	3	0.16262540	0.05420847	1.42	0.2622
BV	2	0.02425037	0.01212519	0.32	0.7308
time*BV	6	0.14090519	0.02348420	0.62	0.7156

Source	DF	Type III SS	Mean Square	F Value	Pr > F
time	3	0.16606008	0.05535336	1.45	0.2539
BV	2	0.02174872	0.01087436	0.29	0.7546
time*BV	6	0.14090519	0.02348420	0.62	0.7156

C. ljungdahlii Benzyl Viologen Study

Maximun Ethanol and Acetate Production

The GLM Procedure
Least Squares Means

time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.07777778	0.00567504	<.0001	1
46	0.09666667	0.00612975	<.0001	2
96	0.08777778	0.00567504	<.0001	3
144	0.08222222	0.00567504	<.0001	4

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4
1		0.0335	0.2253	0.5851
2	0.0335		0.2983	0.0972
3	0.2253	0.2983		0.4957
4	0.5851	0.0972	0.4957	

time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.57111111	0.06510242	<.0001	1
46	0.71777778	0.07031865	<.0001	2
96	0.75000000	0.06510242	<.0001	3
144	0.70333333	0.06510242	<.0001	4

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4
1		0.1395	0.0644	0.1644
2	0.1395		0.7397	0.8815
3	0.0644	0.7397		0.6171
4	0.1644	0.8815	0.6171	

BV	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.09750000	0.00521286	<.0001	1
50	0.07916667	0.00491473	<.0001	2
100	0.08166667	0.00491473	<.0001	3

Least Squares Means for effect BV
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3
1		0.0175	0.0373
2	0.0175		0.7224
3	0.0373	0.7224	

C. ljungdahlii Benzyl Viologen Study

Maximun Ethanol and Acetate Production

The GLM Procedure
Least Squares Means

BV	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.67333333	0.05980039	<.0001	1
50	0.66333333	0.05638035	<.0001	2
100	0.72000000	0.05638035	<.0001	3

Least Squares Means for effect BV
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3
1		0.9042	0.5757
2	0.9042		0.4844
3	0.5757	0.4844	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

time	BV	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0	0.08000000	0.00982946	<.0001	1
0	50	0.06666667	0.00982946	<.0001	2
0	100	0.08666667	0.00982946	<.0001	3
46	0	0.13000000	0.01203859	<.0001	4
46	50	0.08333333	0.00982946	<.0001	5
46	100	0.07666667	0.00982946	<.0001	6
96	0	0.09000000	0.00982946	<.0001	7
96	50	0.08666667	0.00982946	<.0001	8
96	100	0.08666667	0.00982946	<.0001	9
144	0	0.09000000	0.00982946	<.0001	10
144	50	0.08000000	0.00982946	<.0001	11
144	100	0.07666667	0.00982946	<.0001	12

Least Squares Means for effect time*BV
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.3475	0.6361	0.0038	0.8126	0.8126	0.4792	0.6361	0.6361	0.4792	1.0000	0.8126
2	0.3475		0.1637	0.0005	0.2428	0.4792	0.1068	0.1637	0.1637	0.1068	0.3475	0.4792
3	0.6361	0.1637		0.0104	0.8126	0.4792	0.8126	1.0000	1.0000	0.8126	0.6361	0.4792
4	0.0038	0.0005	0.0104		0.0064	0.0023	0.0170	0.0104	0.0104	0.0170	0.0038	0.0023
5	0.8126	0.2428	0.8126	0.0064		0.6361	0.6361	0.8126	0.8126	0.6361	0.8126	0.6361
6	0.8126	0.4792	0.4792	0.0023	0.6361		0.3475	0.4792	0.4792	0.3475	0.8126	1.0000
7	0.4792	0.1068	0.8126	0.0170	0.6361	0.3475		0.8126	0.8126	1.0000	0.4792	0.3475
8	0.6361	0.1637	1.0000	0.0104	0.8126	0.4792	0.8126		1.0000	0.8126	0.6361	0.4792
9	0.6361	0.1637	1.0000	0.0104	0.8126	0.4792	0.8126	1.0000		0.8126	0.6361	0.4792
10	0.4792	0.1068	0.8126	0.0170	0.6361	0.3475	1.0000	0.8126	0.8126		0.4792	0.3475
11	1.0000	0.3475	0.6361	0.0038	0.8126	0.8126	0.4792	0.6361	0.6361	0.4792		0.8126
12	0.8126	0.4792	0.4792	0.0023	0.6361	1.0000	0.3475	0.4792	0.4792	0.3475	0.8126	

C. ljungdahlii Benzyl Viologen Study

Maximun Ethanol and Acetate Production

The GLM Procedure
Least Squares Means

time	BV	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0	0.50666667	0.11276070	0.0002	1
0	50	0.47000000	0.11276070	0.0004	2
0	100	0.73666667	0.11276070	<.0001	3
46	0	0.78000000	0.13810309	<.0001	4
46	50	0.65000000	0.11276070	<.0001	5
46	100	0.72333333	0.11276070	<.0001	6
96	0	0.71000000	0.11276070	<.0001	7
96	50	0.78666667	0.11276070	<.0001	8
96	100	0.75333333	0.11276070	<.0001	9
144	0	0.69666667	0.11276070	<.0001	10
144	50	0.74666667	0.11276070	<.0001	11
144	100	0.66666667	0.11276070	<.0001	12

Least Squares Means for effect time*BV
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.8202	0.1627	0.1389	0.3781	0.1874	0.2150	0.0924	0.1356	0.2456	0.1459	0.3261
2	0.8202		0.1080	0.0955	0.2706	0.1258	0.1459	0.0591	0.0888	0.1686	0.0961	0.2299
3	0.1627	0.1080		0.8101	0.5920	0.9341	0.8687	0.7567	0.9177	0.8042	0.9505	0.6648
4	0.1389	0.0955	0.8101		0.4733	0.7535	0.6982	0.9705	0.8824	0.6446	0.8533	0.5313
5	0.3781	0.2706	0.5920	0.4733		0.6499	0.7102	0.4003	0.5234	0.7724	0.5503	0.9177
6	0.1874	0.1258	0.9341	0.7535	0.6499		0.9341	0.6949	0.8524	0.8687	0.8849	0.7256
7	0.2150	0.1459	0.8687	0.6982	0.7102	0.9341		0.6352	0.7882	0.9341	0.8202	0.7882
8	0.0924	0.0591	0.7567	0.9705	0.4003	0.6949	0.6352		0.8363	0.5780	0.8042	0.4594
9	0.1356	0.0888	0.9177	0.8824	0.5234	0.8524	0.7882	0.8363		0.7256	0.9670	0.5920
10	0.2456	0.1686	0.8042	0.6446	0.7724	0.8687	0.9341	0.5780	0.7256		0.7567	0.8524
11	0.1459	0.0961	0.9505	0.8533	0.5503	0.8849	0.8202	0.8042	0.9670	0.7567		0.6207
12	0.3261	0.2299	0.6648	0.5313	0.9177	0.7256	0.7882	0.4594	0.5920	0.8524	0.6207	

Appendix D.2 *C. autoethanogenum* NG640.1 – NG.640.6

```
Title1 'C. autoethanogenum Non-growth Media';
Title2 'Ethanol and Acetate Production over time';
Data practice;
Input NGMedia time ethanol acetate;
Cards;

1     0     0     0
1     24    0.15  0
1     48    0.21  0.58
1     72    0.17  0
1     96    0.2   0.48
1     0     0     0
1     24    0.12  0
1     48    0.37  0.96
1     72    0.6   1.44
1     96    0.43  1.17
1     0     0     0
1     24    0.19  0.55
1     48    0.37  0.62
1     72    0.6   0.82
1     96    0.43  1.68
3     0     0     0
3     24    6.37  22.59
3     48    4.52  24.64
3     72    7.18  30.28
3     96    8.69  39.46
3     0     0     0
3     24    6.23  22.3
3     48    7.06  31.55
3     72    10.01 45.27
3     96    9.44  42.54
3     0     0.03  0
3     24    7.04  24.43
3     48    10.36 44.1
3     72    10.09 45.07
3     96    10.19 43.97
4     0     0     0
4     24    3.57  13.81
4     48    5.05  24.37
4     72    3.84  18.57
4     96    2.63  12.77
4     0     0     0
4     24    5.87  22.58
4     48    8.55  40.47
4     72    6.92  38.66
4     96    8.41  40.17
4     0     0     0
4     24    5.21  19.87
4     48    6.43  30.42
4     72    5.94  34.55
4     96    7.14  38.68
2     0     0     0
2     24    0.22  0.64
```

```
2     48    0.55  1.03
2     72    0.33  0.78
2     96    2.24  4.66
2     0     0     0
2     24    0.3   0.79
2     48    0.29  0.62
2     72    1.63  3.83
2     96    2.87  6.9
2     0     0.02  0
2     24    0.19  0.62
2     48    0.55  1.38
2     72    0.7   1.85
2     96    1.95  5.35
5     0     0.04  0.44
5     24    0.42  4.05
5     42    0.77  6.37
5     74    1.07  8.34
5     96    0.56  4.03
5     0     0     0
5     24    0.44  4.25
5     42    0.74  5.86
5     74    0.96  6.76
5     96    1.31  9.3
6     0     0     0
6     24    0.16  1.39
6     42    0.24  1.95
6     74    0.38  2.74
6     96    0.47  3
6     0     0     0
6     24    0.39  4.04
6     42    1.19  11.25
6     74    1.57  13.93
6     96    1.45  12.75

;
OPTIONS NODATE PAGENO = 1;
Proc GLM Data = practice;
Class NGMedia time;

model ethanol acetate/*put in desired gas to analyze*/ =  NGMedia|time;
lsmeans NGMedia|time /pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
end;
```

C. autoethanogenum Non-growth Media
Ethanol and Acetate Production over time

The GLM Procedure

Class Level Information

Class	Levels	Values
NGMedia	6	1 2 3 4 5 6
time	7	0 24 42 48 72 74 96

Number of Observations Read	80
Number of Observations Used	80

C. autoethanogenum Non-growth Media
Ethanol and Acetate Production over time

The GLM Procedure

Dependent Variable: ethanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	770.8282488	26.5802844	22.11	<.0001
Error	50	60.0957000	1.2019140		
Corrected Total	79	830.9239488			

R-Square	Coeff Var	Root MSE	ethanol Mean
0.927676	45.06730	1.096318	2.432625

Source	DF	Type I SS	Mean Square	F Value	Pr > F
NGMedia	5	496.8779688	99.3755938	82.68	<.0001
time	6	136.2685144	22.7114191	18.90	<.0001
NGMedia*time	18	137.6817656	7.6489870	6.36	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
NGMedia	5	446.5481324	89.3096265	74.31	<.0001
time	6	115.5564748	19.2594125	16.02	<.0001
NGMedia*time	18	137.6817656	7.6489870	6.36	<.0001

The GLM Procedure

Dependent Variable: acetate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	29	15713.32640	541.83884	19.21	<.0001
Error	50	1410.48572	28.20971		
Corrected Total	79	17123.81212			

R-Square	Coeff Var	Root MSE	acetate Mean
0.917630	47.83215	5.311282	11.10400

Source	DF	Type I SS	Mean Square	F Value	Pr > F
NGMedia	5	9725.550230	1945.110046	68.95	<.0001
time	6	3095.247042	515.874507	18.29	<.0001
NGMedia*time	18	2892.529131	160.696063	5.70	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
NGMedia	5	9126.923573	1825.384715	64.71	<.0001
time	6	2752.582627	458.763771	16.26	<.0001
NGMedia*time	18	2892.529131	160.696063	5.70	<.0001

C. autoethanogenum Non-growth Media
Ethanol and Acetate Production over time

The GLM Procedure
Least Squares Means

NGMedia	time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	0.00000000	0.63295971	1.0000	1
1	24	0.15333333	0.63295971	0.8096	2
1	48	0.31666667	0.63295971	0.6191	3
1	72	0.45666667	0.63295971	0.4740	4
1	96	0.35333333	0.63295971	0.5792	5
2	0	0.00666667	0.63295971	0.9916	6
2	24	0.23666667	0.63295971	0.7101	7
2	48	0.46333333	0.63295971	0.4676	8
2	72	0.88666667	0.63295971	0.1674	9
2	96	2.35333333	0.63295971	0.0005	10
3	0	0.01000000	0.63295971	0.9875	11
3	24	6.54666667	0.63295971	<.0001	12
3	48	7.31333333	0.63295971	<.0001	13
3	72	9.09333333	0.63295971	<.0001	14
3	96	9.44000000	0.63295971	<.0001	15
4	0	0.00000000	0.63295971	1.0000	16
4	24	4.88333333	0.63295971	<.0001	17
4	48	6.67666667	0.63295971	<.0001	18
4	72	5.56666667	0.63295971	<.0001	19
4	96	6.06000000	0.63295971	<.0001	20
5	0	0.02000000	0.77521416	0.9795	21
5	24	0.43000000	0.77521416	0.5816	22
5	42	0.75500000	0.77521416	0.3348	23
5	74	1.01500000	0.77521416	0.1964	24
5	96	0.93500000	0.77521416	0.2334	25
6	0	-0.00000000	0.77521416	1.0000	26
6	24	0.27500000	0.77521416	0.7243	27
6	42	0.71500000	0.77521416	0.3608	28
6	74	0.97500000	0.77521416	0.2143	29
6	96	0.96000000	0.77521416	0.2214	30

C. autoethanogenum Non-growth Media
Ethanol and Acetate Production over time
The GLM Procedure
Least Squares Means
east Squares Means for effect NGMedia*time
Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10
1		0.8647	0.7250	0.6122	0.6947	0.9941	0.7926	0.6070	0.3267	0.0113
2	0.8647		0.8560	0.7361	0.8241	0.8705	0.9262	0.7306	0.4165	0.0175
3	0.7250	0.8560		0.8763	0.9675	0.7306	0.9291	0.8705	0.5272	0.0272
4	0.6122	0.7361	0.8763		0.9086	0.6174	0.8069	0.9941	0.6331	0.0391
5	0.6947	0.8241	0.9675	0.9086		0.7002	0.8968	0.9027	0.5540	0.0300
6	0.9941	0.8705	0.7306	0.6174	0.7002		0.7983	0.6122	0.3303	0.0116
7	0.7926	0.9262	0.9291	0.8069	0.8968	0.7983		0.8011	0.4711	0.0220
8	0.6070	0.7306	0.8705	0.9941	0.9027	0.6122	0.8011		0.6383	0.0398
9	0.3267	0.4165	0.5272	0.6331	0.5540	0.3303	0.4711	0.6383		0.1076
10	0.0113	0.0175	0.0272	0.0391	0.0300	0.0116	0.0220	0.0398	0.1076	
11	0.9911	0.8734	0.7333	0.6200	0.7029	0.9970	0.8011	0.6148	0.3321	0.0117
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	1.0000	0.8647	0.7250	0.6122	0.6947	0.9941	0.7926	0.6070	0.3267	0.0113
17	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0068
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0008
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001
21	0.9841	0.8945	0.7681	0.6645	0.7405	0.9894	0.8295	0.6597	0.3906	0.0238
22	0.6693	0.7833	0.9103	0.9788	0.9392	0.6741	0.8476	0.9736	0.6501	0.0603
23	0.4541	0.5504	0.6633	0.7669	0.6899	0.4581	0.6068	0.7719	0.8959	0.1166
24	0.3154	0.3934	0.4885	0.5794	0.5116	0.3185	0.4404	0.5839	0.8985	0.1872
25	0.3547	0.4385	0.5395	0.6348	0.5637	0.3581	0.4885	0.6395	0.9617	0.1626
26	1.0000	0.8788	0.7530	0.6501	0.7255	0.9947	0.8140	0.6454	0.3799	0.0227
27	0.7846	0.9037	0.9670	0.8567	0.9379	0.7897	0.9696	0.8515	0.5438	0.0430
28	0.4783	0.5772	0.6923	0.7974	0.7193	0.4824	0.6348	0.8025	0.8645	0.1079
29	0.3346	0.4155	0.5137	0.6068	0.5373	0.3379	0.4641	0.6114	0.9300	0.1746
30	0.3421	0.4240	0.5233	0.6172	0.5471	0.3454	0.4732	0.6219	0.9419	0.1700

i/j	11	12	13	14	15	16	17	18	19	20
1	0.9911	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001
2	0.8734	<.0001	<.0001	<.0001	<.0001	0.8647	<.0001	<.0001	<.0001	<.0001
3	0.7333	<.0001	<.0001	<.0001	<.0001	0.7250	<.0001	<.0001	<.0001	<.0001
4	0.6200	<.0001	<.0001	<.0001	<.0001	0.6122	<.0001	<.0001	<.0001	<.0001
5	0.7029	<.0001	<.0001	<.0001	<.0001	0.6947	<.0001	<.0001	<.0001	<.0001
6	0.9970	<.0001	<.0001	<.0001	<.0001	0.9941	<.0001	<.0001	<.0001	<.0001
7	0.8011	<.0001	<.0001	<.0001	<.0001	0.7926	<.0001	<.0001	<.0001	<.0001
8	0.6148	<.0001	<.0001	<.0001	<.0001	0.6070	<.0001	<.0001	<.0001	<.0001
9	0.3321	<.0001	<.0001	<.0001	<.0001	0.3267	<.0001	<.0001	<.0001	<.0001
10	0.0117	<.0001	<.0001	<.0001	<.0001	0.0113	0.0068	<.0001	0.0008	0.0001
11		<.0001	<.0001	<.0001	<.0001	0.9911	<.0001	<.0001	<.0001	<.0001
12	<.0001		0.3958	0.0064	0.0022	<.0001	0.0690	0.8851	0.2788	0.5891
13	<.0001	0.3958		0.0522	0.0214	<.0001	0.0091	0.4802	0.0566	0.1676
14	<.0001	0.0064	0.0522		0.7002	<.0001	<.0001	0.0094	0.0003	0.0014
15	<.0001	0.0022	0.0214	0.7002		<.0001	<.0001	0.0033	<.0001	0.0004
16	0.9911	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
17	<.0001	0.0690	0.0091	<.0001	<.0001	<.0001		0.0506	0.4488	0.1947
18	<.0001	0.8851	0.4802	0.0094	0.0033	<.0001	0.0506		0.2208	0.4941
19	<.0001	0.2788	0.0566	0.0003	<.0001	<.0001	0.4488	0.2208		0.5840
20	<.0001	0.5891	0.1676	0.0014	0.0004	<.0001	0.1947	0.4941	0.5840	
21	0.9921	<.0001	<.0001	<.0001	<.0001	0.9841	<.0001	<.0001	<.0001	<.0001
22	0.6765	<.0001	<.0001	<.0001	<.0001	0.6693	<.0001	<.0001	<.0001	<.0001
23	0.4601	<.0001	<.0001	<.0001	<.0001	0.4541	0.0001	<.0001	<.0001	<.0001
24	0.3201	<.0001	<.0001	<.0001	<.0001	0.3154	0.0003	<.0001	<.0001	<.0001
25	0.3598	<.0001	<.0001	<.0001	<.0001	0.3547	0.0002	<.0001	<.0001	<.0001
26	0.9921	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001
27	0.7923	<.0001	<.0001	<.0001	<.0001	0.7846	<.0001	<.0001	<.0001	<.0001
28	0.4844	<.0001	<.0001	<.0001	<.0001	0.4783	0.0001	<.0001	<.0001	<.0001
29	0.3396	<.0001	<.0001	<.0001	<.0001	0.3346	0.0003	<.0001	<.0001	<.0001
30	0.3471	<.0001	<.0001	<.0001	<.0001	0.3421	0.0003	<.0001	<.0001	<.0001

Dependent Variable: ethanol

C. autoethanogenum Non-growth Media
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Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	21	22	23	24	25	26	27	28	29	30
1	0.9841	0.6693	0.4541	0.3154	0.3547	1.0000	0.7846	0.4783	0.3346	0.3421
2	0.8945	0.7833	0.5504	0.3934	0.4385	0.8788	0.9037	0.5772	0.4155	0.4240
3	0.7681	0.9103	0.6633	0.4885	0.5395	0.7530	0.9670	0.6923	0.5137	0.5233
4	0.6645	0.9788	0.7669	0.5794	0.6348	0.6501	0.8567	0.7974	0.6068	0.6172
5	0.7405	0.9392	0.6899	0.5116	0.5637	0.7255	0.9379	0.7193	0.5373	0.5471
6	0.9894	0.6741	0.4581	0.3185	0.3581	0.9947	0.7897	0.4824	0.3379	0.3454
7	0.8295	0.8476	0.6068	0.4404	0.4885	0.8140	0.9696	0.6348	0.4641	0.4732
8	0.6597	0.9736	0.7719	0.5839	0.6395	0.6454	0.8515	0.8025	0.6114	0.6219
9	0.3906	0.6501	0.8959	0.8985	0.9617	0.3799	0.5438	0.8645	0.9300	0.9419
10	0.0238	0.0603	0.1166	0.1872	0.1626	0.0227	0.0430	0.1079	0.1746	0.1700
11	0.9921	0.6765	0.4601	0.3201	0.3598	0.9921	0.7923	0.4844	0.3396	0.3471
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	0.9841	0.6693	0.4541	0.3154	0.3547	1.0000	0.7846	0.4783	0.3346	0.3421
17	<.0001	<.0001	0.0001	0.0003	0.0002	<.0001	<.0001	0.0001	0.0003	0.0003
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21		0.7100	0.5057	0.3685	0.4079	0.9855	0.8170	0.5290	0.3879	0.3953
22	0.7100		0.7681	0.5960	0.6471	0.6966	0.8881	0.7960	0.6213	0.6309
23	0.5057	0.7681		0.8135	0.8702	0.4942	0.6634	0.9710	0.8418	0.8524
24	0.3685	0.5960	0.8135		0.9421	0.3590	0.5028	0.7855	0.9710	0.9602
25	0.4079	0.6471	0.8702	0.9421		0.3978	0.5499	0.8418	0.9710	0.9819
26	0.9855	0.6966	0.4942	0.3590	0.3978		0.8030	0.5173	0.3781	0.3854
27	0.8170	0.8881	0.6634	0.5028	0.5499	0.8030		0.6899	0.5261	0.5349
28	0.5290	0.7960	0.9710	0.7855	0.8418	0.5173	0.6899		0.8135	0.8241
29	0.3879	0.6213	0.8418	0.9710	0.9710	0.3781	0.5261	0.8135		0.9891
30	0.3953	0.6309	0.8524	0.9602	0.9819	0.3854	0.5349	0.8241	0.9891	

C. autoethanogenum Non-growth Media
Ethanol and Acetate Production over time

The GLM Procedure
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Least Squares Means for effect NGMedia*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

NGMedia	time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	0	-0.0000000	3.0664700	1.0000	1
1	24	0.1833333	3.0664700	0.9526	2
1	48	0.7200000	3.0664700	0.8153	3
1	72	0.7533333	3.0664700	0.8069	4
1	96	1.1100000	3.0664700	0.7189	5
2	0	0.0000000	3.0664700	1.0000	6
2	24	0.6833333	3.0664700	0.8246	7
2	48	1.0100000	3.0664700	0.7433	8
2	72	2.1533333	3.0664700	0.4858	9
2	96	5.6366667	3.0664700	0.0720	10
3	0	0.0000000	3.0664700	1.0000	11
3	24	23.1066667	3.0664700	<.0001	12
3	48	33.4300000	3.0664700	<.0001	13
3	72	40.2066667	3.0664700	<.0001	14
3	96	41.9900000	3.0664700	<.0001	15
4	0	0.0000000	3.0664700	1.0000	16
4	24	18.7533333	3.0664700	<.0001	17
4	48	31.7533333	3.0664700	<.0001	18
4	72	30.5933333	3.0664700	<.0001	19
4	96	30.5400000	3.0664700	<.0001	20
5	0	0.2200000	3.7556434	0.9535	21
5	24	4.1500000	3.7556434	0.2744	22
5	42	6.1150000	3.7556434	0.1098	23
5	74	7.5500000	3.7556434	0.0498	24
5	96	6.6650000	3.7556434	0.0820	25
6	0	-0.0000000	3.7556434	1.0000	26
6	24	2.7150000	3.7556434	0.4731	27
6	42	6.6000000	3.7556434	0.0850	28
6	74	8.3350000	3.7556434	0.0310	29
6	96	7.8750000	3.7556434	0.0411	30

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Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8	9	10
1		0.9664	0.8688	0.8628	0.7990	1.0000	0.8754	0.8168	0.6217	0.1996
2	0.9664		0.9020	0.8960	0.8317	0.9664	0.9087	0.8496	0.6516	0.2144
3	0.8688	0.9020		0.9939	0.9287	0.8688	0.9933	0.9470	0.7424	0.2623
4	0.8628	0.8960	0.9939		0.9348	0.8628	0.9872	0.9530	0.7482	0.2655
5	0.7990	0.8317	0.9287	0.9348		0.7990	0.9220	0.9817	0.8109	0.3016
6	1.0000	0.9664	0.8688	0.8628	0.7990		0.8754	0.8168	0.6217	0.1996
7	0.8754	0.9087	0.9933	0.9872	0.9220	0.8754		0.9403	0.7361	0.2588
8	0.8168	0.8496	0.9470	0.9530	0.9817	0.8168	0.9403		0.7931	0.2912
9	0.6217	0.6516	0.7424	0.7482	0.8109	0.6217	0.7361	0.7931		0.4256
10	0.1996	0.2144	0.2623	0.2655	0.3016	0.1996	0.2588	0.2912	0.4256	
11	1.0000	0.9664	0.8688	0.8628	0.7990	1.0000	0.8754	0.8168	0.6217	0.1996
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0002
13	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	1.0000	0.9664	0.8688	0.8628	0.7990	1.0000	0.8754	0.8168	0.6217	0.1996
17	<.0001	<.0001	0.0001	0.0001	0.0002	<.0001	0.0001	0.0002	0.0004	0.0039
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21	0.9640	0.9940	0.9183	0.9129	0.8551	0.9640	0.9243	0.8712	0.6918	0.2693
22	0.3961	0.4172	0.4826	0.4868	0.5335	0.3961	0.4779	0.5202	0.6822	0.7604
23	0.2131	0.2269	0.2712	0.2741	0.3069	0.2131	0.2679	0.2974	0.4178	0.9218
24	0.1257	0.1350	0.1651	0.1672	0.1901	0.1257	0.1629	0.1835	0.2710	0.6948
25	0.1754	0.1873	0.2259	0.2285	0.2574	0.1754	0.2231	0.2490	0.3566	0.8329
26	1.0000	0.9700	0.8825	0.8772	0.8199	1.0000	0.8885	0.8358	0.6589	0.2505
27	0.5780	0.6039	0.6825	0.6875	0.7420	0.5780	0.6770	0.7266	0.9082	0.5495
28	0.1795	0.1917	0.2309	0.2335	0.2629	0.1795	0.2281	0.2544	0.3635	0.8433
29	0.0918	0.0989	0.1226	0.1242	0.1425	0.0918	0.1208	0.1371	0.2082	0.5803
30	0.1106	0.1190	0.1463	0.1481	0.1691	0.1106	0.1443	0.1630	0.2435	0.6463

i/j	11	12	13	14	15	16	17	18	19	20
1	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001
2	0.9664	<.0001	<.0001	<.0001	<.0001	0.9664	<.0001	<.0001	<.0001	<.0001
3	0.8688	<.0001	<.0001	<.0001	<.0001	0.8688	0.0001	<.0001	<.0001	<.0001
4	0.8628	<.0001	<.0001	<.0001	<.0001	0.8628	0.0001	<.0001	<.0001	<.0001
5	0.7990	<.0001	<.0001	<.0001	<.0001	0.7990	0.0002	<.0001	<.0001	<.0001
6	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001
7	0.8754	<.0001	<.0001	<.0001	<.0001	0.8754	0.0001	<.0001	<.0001	<.0001
8	0.8168	<.0001	<.0001	<.0001	<.0001	0.8168	0.0002	<.0001	<.0001	<.0001
9	0.6217	<.0001	<.0001	<.0001	<.0001	0.6217	0.0004	<.0001	<.0001	<.0001
10	0.1996	0.0002	<.0001	<.0001	<.0001	0.1996	0.0039	<.0001	<.0001	<.0001
11		<.0001	<.0001	<.0001	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001
12	<.0001		0.0211	0.0003	<.0001	<.0001	0.3203	0.0516	0.0905	0.0927
13	<.0001	0.0211		0.1244	0.0539	<.0001	0.0014	0.7007	0.5160	0.5082
14	<.0001	0.0003	0.1244		0.6827	<.0001	<.0001	0.0569	0.0312	0.0303
15	<.0001	<.0001	0.0539	0.6827		<.0001	<.0001	0.0222	0.0114	0.0110
16	1.0000	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001
17	<.0001	0.3203	0.0014	<.0001	<.0001	<.0001		0.0042	0.0087	0.0090
18	<.0001	0.0516	0.7007	0.0569	0.0222	<.0001	0.0042		0.7902	0.7808
19	<.0001	0.0905	0.5160	0.0312	0.0114	<.0001	0.0087	0.7902		0.9902
20	<.0001	0.0927	0.5082	0.0303	0.0110	<.0001	0.0090	0.7808	0.9902	
21	0.9640	<.0001	<.0001	<.0001	<.0001	0.9640	0.0004	<.0001	<.0001	<.0001
22	0.3961	0.0003	<.0001	<.0001	<.0001	0.3961	0.0041	<.0001	<.0001	<.0001
23	0.2131	0.0010	<.0001	<.0001	<.0001	0.2131	0.0120	<.0001	<.0001	<.0001
24	0.1257	0.0023	<.0001	<.0001	<.0001	0.1257	0.0250	<.0001	<.0001	<.0001
25	0.1754	0.0014	<.0001	<.0001	<.0001	0.1754	0.0160	<.0001	<.0001	<.0001
26	1.0000	<.0001	<.0001	<.0001	<.0001	1.0000	0.0003	<.0001	<.0001	<.0001
27	0.5780	0.0001	<.0001	<.0001	<.0001	0.5780	0.0017	<.0001	<.0001	<.0001
28	0.1795	0.0013	<.0001	<.0001	<.0001	0.1795	0.0155	<.0001	<.0001	<.0001
29	0.0918	0.0037	<.0001	<.0001	<.0001	0.0918	0.0365	<.0001	<.0001	<.0001
30	0.1106	0.0028	<.0001	<.0001	<.0001	0.1106	0.0293	<.0001	<.0001	<.0001

The GLM Procedure
Least Squares Means

Least Squares Means for effect NGMedia*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	21	22	23	24	25	26	27	28	29	30
1	0.9640	0.3961	0.2131	0.1257	0.1754	1.0000	0.5780	0.1795	0.0918	0.1106
2	0.9940	0.4172	0.2269	0.1350	0.1873	0.9700	0.6039	0.1917	0.0989	0.1190
3	0.9183	0.4826	0.2712	0.1651	0.2259	0.8825	0.6825	0.2309	0.1226	0.1463
4	0.9129	0.4868	0.2741	0.1672	0.2285	0.8772	0.6875	0.2335	0.1242	0.1481
5	0.8551	0.5335	0.3069	0.1901	0.2574	0.8199	0.7420	0.2629	0.1425	0.1691
6	0.9640	0.3961	0.2131	0.1257	0.1754	1.0000	0.5780	0.1795	0.0918	0.1106
7	0.9243	0.4779	0.2679	0.1629	0.2231	0.8885	0.6770	0.2281	0.1208	0.1443
8	0.8712	0.5202	0.2974	0.1835	0.2490	0.8358	0.7266	0.2544	0.1371	0.1630
9	0.6918	0.6822	0.4178	0.2710	0.3566	0.6589	0.9082	0.3635	0.2082	0.2435
10	0.2693	0.7604	0.9218	0.6948	0.8329	0.2505	0.5495	0.8433	0.5803	0.6463
11	0.9640	0.3961	0.2131	0.1257	0.1754	1.0000	0.5780	0.1795	0.0918	0.1106
12	<.0001	0.0003	0.0010	0.0023	0.0014	<.0001	0.0001	0.0013	0.0037	0.0028
13	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	0.9640	0.3961	0.2131	0.1257	0.1754	1.0000	0.5780	0.1795	0.0918	0.1106
17	0.0004	0.0041	0.0120	0.0250	0.0160	0.0003	0.0017	0.0155	0.0365	0.0293
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21		0.4628	0.2724	0.1737	0.2307	0.9671	0.6406	0.2353	0.1328	0.1557
22	0.4628		0.7130	0.5250	0.6379	0.4383	0.7881	0.6466	0.4344	0.4863
23	0.2724	0.7130		0.7881	0.9179	0.2551	0.5250	0.9276	0.6778	0.7417
24	0.1737	0.5250	0.7881		0.8683	0.1614	0.3670	0.8588	0.8831	0.9515
25	0.2307	0.6379	0.9179	0.8683		0.2154	0.4605	0.9903	0.7545	0.8207
26	0.9671	0.4383	0.2551	0.1614	0.2154		0.6115	0.2198	0.1229	0.1444
27	0.6406	0.7881	0.5250	0.3670	0.4605	0.6115		0.4679	0.2951	0.3360
28	0.2353	0.6466	0.9276	0.8588	0.9903	0.2198	0.4679		0.7453	0.8113
29	0.1328	0.4344	0.6778	0.8831	0.7545	0.1229	0.2951	0.7453		0.9313
30	0.1557	0.4863	0.7417	0.9515	0.8207	0.1444	0.3360	0.8113	0.9313	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix E: Preliminary Growth Studies on Synthesis Gas

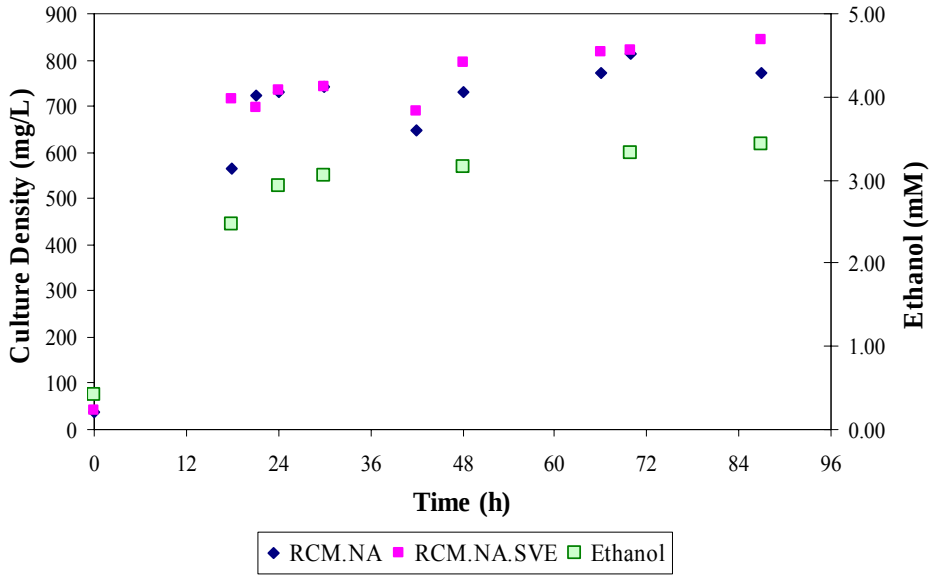

Figure E.1 *C. ljungdahlii* growth and ethanol concentrations over time on syngas in RCM.NA and RCM.NA.SVE (from RCM.NA.SVE culture)

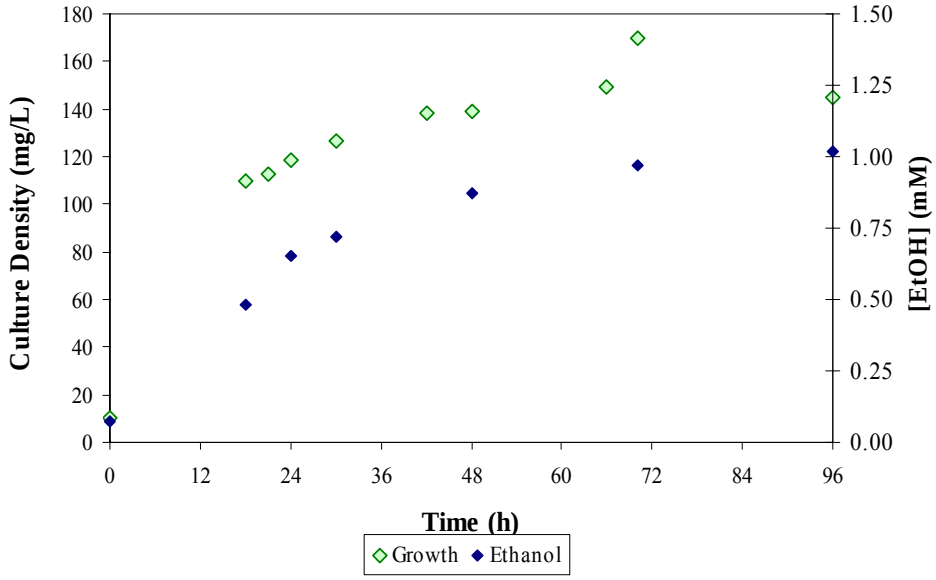

Figure E.2 *C. autoethanogenum* growth and ethanol production over time on syngas in DSMZ 640 medium

Appendix F: SAS®Analyses of Synthesis Gas Fermentation

Appendix F.1: SAS Analysis of *C. ljungdahlii*: Ethanol and Acetate Over Time

```
Title1 'C. autoethanogenum on Syngas 06/27/2006';
Title2 'LSMeans for Main and Interaction Affects';
Title3 'Gas Composition over Time';


Data practice;
Input H2 N2 CO CO2 Flow Time ;
Cards;
9.8   48.52 20.27 19.96 5     1
9.71  48.8  20.32 20.76 5     1
9.88  48.54 20.3  19.81 5     1
9.89  48.47 20.42 19.93 7.5   1
9.65  49.91 20.31 18.94 7.5   1
10.25 48.94 20.33 19.49 7.5   1
9.69  48.63 20.19 19.99 10    1
9.68  48.67 20.27 19.93 10    1
8.77  50.23 19.78 19.13 10    1
9.63  48.46 20.16 20.14 5     2
9.53  48.5  19.98 20.91 5     2
9.44  49.05 19.91 19.6  5     2
9.67  48.87 20.01 19.89 7.5   2
9.31  49.15 19.83 19.5  7.5   2
9.24  49.85 19.31 19    7.5   2
9.66  48.43 20.21 19.91 10    2
9.57  48.77 20    19.77 10    2
9.48  49.21 19.82 19.58 10    2
9.68  48.05 19.98 20.15 5     3
9.91  48.24 20.11 20.69 5     3
9.44  48.66 19.92 19.75 5     3
9.83  48.7  19.91 19.9  7.5   3
9.73  48.42 20.22 19.87 7.5   3
9.78  48.39 20.06 20.03 7.5   3
9.83  47.84 20.12 20.2  10    3
9.28  48.79 19.85 19.96 10    3
9.68  48.35 20.13 19.85 10    3
9.65  47.99 19.72 20.24 5     4
9.66  48.27 19.68 22.46 5     4
9.74  48.16 19.89 20.06 5     4
9.17  49.73 18.5  19.48 7.5   4
9.42  48.96 19.52 19.32 7.5   4
9.5   49.07 19.25 20.02 7.5   4
9.37  48.96 19.68 20.17 10    4
9.79  48.21 20.2  20.09 10    4
9.7   47.99 19.79 20.06 10    4
9.54  48.09 19.4  22.32 5     5
9.26  48.41 19.27 20.61 5     5
9.44  48.46 19.71 20.44 5     5
9.11  49.78 18.82 18.88 7.5   5
9.19  49.16 19.04 20.12 7.5   5
8.3   51.56 17.29 18.31 7.5   5
```

```
9.34  48.31 19.79 20.65 10    5
9.55  48.12 19.89 20.01 10    5
9.09  49.15 19.04 20.12 10    5
9.42  48.58 19.49 20.78 5     6
9.83  48.7  19.71 21.26 5     6
9.34  48.84 19.41 20.42 5     6
9.69  48.33 20.14 20.13 7.5   6
9.58  48.69 19.82 20.67 7.5   6
9.52  48.69 19.71 20.19 7.5   6
9.05  49.33 19.34 20.65 10    6
9.29  48.8  19.57 20.48 10    6
9.47  48.79 19.9  19.9  10    6
;
OPTIONS NODATE PAGENO = 1;
Proc GLM Data = practice;
Class Time Flow;

model N2 H2 CO CO2/*put in desired gas to analyze*/ = Time|Flow ;
lsmeans Time Flow Time*Flow /pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
end;
```

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Ethanol and Acetate Production

The GLM Procedure

Class Level Information

Class	Levels	Values
Time	7	1 2 3 4 5 6 7
Flow	3	5 7.5 10
pH	2	5.5 6.8

Number of Observations Read	112
Number of Observations Used	112

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Dependent Variable: ethanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	41	188.7702024	4.6041513	4.21	<.0001
Error	70	76.5088833	1.0929840		
Corrected Total	111	265.2790857			

R-Square	Coeff Var	Root MSE	ethanol Mean
0.711591	82.78520	1.045459	1.262857

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	6	122.1382857	20.3563810	18.62	<.0001
Flow	2	2.0596155	1.0298077	0.94	0.3947
Time*Flow	12	2.8494762	0.2374563	0.22	0.9971
pH	1	26.4617286	26.4617286	24.21	<.0001
Time*pH	6	24.8276214	4.1379369	3.79	0.0025
Flow*pH	2	6.1689345	3.0844673	2.82	0.0663
Time*Flow*pH	12	4.2645405	0.3553784	0.33	0.9823

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	6	117.0812116	19.5135353	17.85	<.0001
Flow	2	2.0596155	1.0298077	0.94	0.3947
Time*Flow	12	2.8494762	0.2374563	0.22	0.9971
pH	1	25.7756328	25.7756328	23.58	<.0001
Time*pH	6	24.3787327	4.0631221	3.72	0.0029
Flow*pH	2	6.1689345	3.0844673	2.82	0.0663
Time*Flow*pH	12	4.2645405	0.3553784	0.33	0.9823

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Dependent Variable: acetate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	41	15928.49286	388.49983	36.74	<.0001
Error	70	740.19743	10.57425		
Corrected Total	111	16668.69029			

R-Square	Coeff Var	Root MSE	acetate Mean
0.955594	20.42077	3.251807	15.92402

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	6	15155.85220	2525.97537	238.88	<.0001
Flow	2	104.83832	52.41916	4.96	0.0097
Time*Flow	12	88.99746	7.41645	0.70	0.7448
pH	1	37.45672	37.45672	3.54	0.0640
Time*pH	6	95.88568	15.98095	1.51	0.1872
Flow*pH	2	258.29289	129.14645	12.21	<.0001
Time*Flow*pH	12	187.16958	15.59747	1.48	0.1546

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	6	14997.78543	2499.63091	236.39	<.0001
Flow	2	104.83832	52.41916	4.96	0.0097
Time*Flow	12	88.99746	7.41645	0.70	0.7448
pH	1	47.49846	47.49846	4.49	0.0376
Time*pH	6	119.99015	19.99836	1.89	0.0944
Flow*pH	2	258.29289	129.14645	12.21	<.0001
Time*Flow*pH	12	187.16958	15.59747	1.48	0.1546

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The GLM Procedure
Least Squares Means

Flow	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	15.6109524	0.5017647	<.0001	1
7.5	15.1445238	0.5017647	<.0001	2
10	17.5628571	0.6145338	<.0001	3

Least Squares Means for effect Flow
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3
1		0.5131	0.0164
2	0.5131		0.0032
3	0.0164	0.0032	

Time	pH	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5.5	0.06444444	0.37640818	0.8646	1
1	6.8	0.06555556	0.37640818	0.8622	2
2	5.5	0.21222222	0.37640818	0.5747	3
2	6.8	0.14333333	0.37640818	0.7045	4
3	5.5	0.40055556	0.37640818	0.2909	5
3	6.8	0.46000000	0.37640818	0.2258	6
4	5.5	0.60277778	0.37640818	0.1138	7
4	6.8	1.35166667	0.37640818	0.0006	8
5	5.5	0.90944444	0.37640818	0.0183	9
5	6.8	2.83000000	0.37640818	<.0001	10
6	5.5	1.36944444	0.37640818	0.0005	11
6	6.8	3.54833333	0.37640818	<.0001	12
7	5.5	1.81333333	0.37640818	<.0001	13
7	6.8	3.81277778	0.37640818	<.0001	14

Least Squares Means for effect Time*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7
1		0.9983	0.7821	0.8826	0.5298	0.4599	0.3154
2	0.9983		0.7837	0.8843	0.5312	0.4612	0.3164
3	0.7821	0.7837		0.8974	0.7246	0.6430	0.4656
4	0.8826	0.8843	0.8974		0.6305	0.5538	0.3910
5	0.5298	0.5312	0.7246	0.6305		0.9114	0.7052
6	0.4599	0.4612	0.6430	0.5538	0.9114		0.7893
7	0.3154	0.3164	0.4656	0.3910	0.7052	0.7893	
8	0.0182	0.0183	0.0358	0.0263	0.0783	0.0984	0.1639
9	0.1169	0.1174	0.1946	0.1546	0.3424	0.4014	0.5664
10	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	0.0167	0.0168	0.0331	0.0242	0.0730	0.0920	0.1543
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	0.0016	0.0016	0.0037	0.0025	0.0098	0.0132	0.0260
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

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The GLM Procedure
Least Squares Means

Least Squares Means for effect Time*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	8	9	10	11	12	13	14
1	0.0182	0.1169	<.0001	0.0167	<.0001	0.0016	<.0001
2	0.0183	0.1174	<.0001	0.0168	<.0001	0.0016	<.0001
3	0.0358	0.1946	<.0001	0.0331	<.0001	0.0037	<.0001
4	0.0263	0.1546	<.0001	0.0242	<.0001	0.0025	<.0001
5	0.0783	0.3424	<.0001	0.0730	<.0001	0.0098	<.0001
6	0.0984	0.4014	<.0001	0.0920	<.0001	0.0132	<.0001
7	0.1639	0.5664	<.0001	0.1543	<.0001	0.0260	<.0001
8		0.4089	0.0070	0.9735	0.0001	0.3888	<.0001
9	0.4089		0.0006	0.3905	<.0001	0.0939	<.0001
10	0.0070	0.0006		0.0077	0.1815	0.0602	0.0691
11	0.9735	0.3905	0.0077		0.0001	0.4072	<.0001
12	0.0001	<.0001	0.1815	0.0001		0.0017	0.6209
13	0.3888	0.0939	0.0602	0.4072	0.0017		0.0004
14	<.0001	<.0001	0.0691	<.0001	0.6209	0.0004	

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The GLM Procedure
Least Squares Means

Least Squares Means for effect Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Flow	pH	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	5.5	16.9652381	0.7096025	<.0001	1
5	6.8	14.2566667	0.7096025	<.0001	2
7.5	5.5	13.1428571	0.7096025	<.0001	3
7.5	6.8	17.1461905	0.7096025	<.0001	4
10	5.5	16.2207143	0.8690820	<.0001	5
10	6.8	18.9050000	0.8690820	<.0001	6

Least Squares Means for effect Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4	5	6
1		0.0087	0.0003	0.8574	0.5091	0.0882
2	0.0087		0.2708	0.0053	0.0844	<.0001
3	0.0003	0.2708		0.0002	0.0077	<.0001
4	0.8574	0.0053	0.0002		0.4123	0.1215
5	0.5091	0.0844	0.0077	0.4123		0.0323
6	0.0882	<.0001	<.0001	0.1215	0.0323	

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Least Squares Means

Time	Flow	pH	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5	5.5	0.14333333	0.60359590	0.8130	1
1	5	6.8	0.14666667	0.60359590	0.8087	2
1	7.5	5.5	0.05000000	0.60359590	0.9342	3
1	7.5	6.8	0.05000000	0.60359590	0.9342	4
1	10	5.5	0.00000000	0.73925099	1.0000	5
1	10	6.8	0.00000000	0.73925099	1.0000	6
2	5	5.5	0.30000000	0.60359590	0.6207	7
2	5	6.8	0.23333333	0.60359590	0.7002	8
2	7.5	5.5	0.16666667	0.60359590	0.7833	9
2	7.5	6.8	0.12666667	0.60359590	0.8344	10
2	10	5.5	0.17000000	0.73925099	0.8188	11
2	10	6.8	0.07000000	0.73925099	0.9248	12
3	5	5.5	0.47000000	0.60359590	0.4388	13
3	5	6.8	0.41666667	0.60359590	0.4923	14
3	7.5	5.5	0.34666667	0.60359590	0.5676	15
3	7.5	6.8	0.65333333	0.60359590	0.2828	16
3	10	5.5	0.38500000	0.73925099	0.6042	17
3	10	6.8	0.31000000	0.73925099	0.6762	18
4	5	5.5	0.73666667	0.60359590	0.2264	19
4	5	6.8	0.86333333	0.60359590	0.1571	20
4	7.5	5.5	0.51666667	0.60359590	0.3949	21
4	7.5	6.8	1.77666667	0.60359590	0.0044	22
4	10	5.5	0.55500000	0.73925099	0.4553	23
4	10	6.8	1.41500000	0.73925099	0.0597	24
5	5	5.5	1.00333333	0.60359590	0.1009	25
5	5	6.8	1.82000000	0.60359590	0.0036	26
5	7.5	5.5	0.82000000	0.60359590	0.1787	27
5	7.5	6.8	3.61000000	0.60359590	<.0001	28
5	10	5.5	0.90500000	0.73925099	0.2250	29
5	10	6.8	3.06000000	0.73925099	<.0001	30
6	5	5.5	1.60333333	0.60359590	0.0098	31
6	5	6.8	2.81666667	0.60359590	<.0001	32
6	7.5	5.5	1.20000000	0.60359590	0.0507	33
6	7.5	6.8	4.30333333	0.60359590	<.0001	34
6	10	5.5	1.30500000	0.73925099	0.0819	35
6	10	6.8	3.52500000	0.73925099	<.0001	36
7	5	5.5	2.21333333	0.60359590	0.0005	37
7	5	6.8	3.08666667	0.60359590	<.0001	38
7	7.5	5.5	1.69666667	0.60359590	0.0064	39
7	7.5	6.8	4.76666667	0.60359590	<.0001	40
7	10	5.5	1.53000000	0.73925099	0.0422	41
7	10	6.8	3.58500000	0.73925099	<.0001	42

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10	11
1		0.9969	0.9132	0.9132	0.8810	0.8810	0.8549	0.9163	0.9783	0.9845	0.9778
2	0.9969		0.9102	0.9102	0.8783	0.8783	0.8580	0.9194	0.9814	0.9814	0.9806
3	0.9132	0.9102		1.0000	0.9584	0.9584	0.7705	0.8306	0.8917	0.9287	0.9003
4	0.9132	0.9102	1.0000		0.9584	0.9584	0.7705	0.8306	0.8917	0.9287	0.9003
5	0.8810	0.8783	0.9584	0.9584		1.0000	0.7542	0.8076	0.8619	0.8948	0.8713
6	0.8810	0.8783	0.9584	0.9584	1.0000		0.7542	0.8076	0.8619	0.8948	0.8713
7	0.8549	0.8580	0.7705	0.7705	0.7542	0.7542		0.9380	0.8763	0.8397	0.8920
8	0.9163	0.9194	0.8306	0.8306	0.8076	0.8076	0.9380		0.9380	0.9009	0.9473
9	0.9783	0.9814	0.8917	0.8917	0.8619	0.8619	0.8763	0.9380		0.9628	0.9972
10	0.9845	0.9814	0.9287	0.9287	0.8948	0.8948	0.8397	0.9009	0.9628		0.9639
11	0.9778	0.9806	0.9003	0.9003	0.8713	0.8713	0.8920	0.9473	0.9972	0.9639	
12	0.9390	0.9362	0.9833	0.9833	0.9468	0.9468	0.8103	0.8646	0.9196	0.9528	0.9241
13	0.7031	0.7060	0.6242	0.6242	0.6239	0.6239	0.8427	0.7824	0.7234	0.6888	0.7542
14	0.7498	0.7527	0.6688	0.6688	0.6638	0.6638	0.8917	0.8306	0.7705	0.7351	0.7968
15	0.8124	0.8154	0.7292	0.7292	0.7175	0.7175	0.9566	0.8948	0.8336	0.7974	0.8537

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The GLM Procedure
Least Squares Means

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

16	0.5521	0.5547	0.4820	0.4820	0.4959	0.4959	0.6802	0.6242	0.5704	0.5392	0.6141
17	0.8008	0.8035	0.7266	0.7266	0.7138	0.7138	0.9293	0.8742	0.8197	0.7874	0.8377
18	0.8619	0.8646	0.7861	0.7861	0.7677	0.7677	0.9917	0.9362	0.8810	0.8482	0.8939
19	0.4893	0.4917	0.4239	0.4239	0.4428	0.4428	0.6106	0.5573	0.5065	0.4772	0.5546
20	0.4018	0.4040	0.3440	0.3440	0.3688	0.3688	0.5115	0.4630	0.4172	0.3911	0.4700
21	0.6632	0.6660	0.5863	0.5863	0.5900	0.5900	0.8004	0.7409	0.6830	0.6492	0.7175
22	0.0598	0.0603	0.0469	0.0469	0.0669	0.0669	0.0881	0.0749	0.0634	0.0573	0.0967
23	0.6675	0.6701	0.5984	0.5984	0.5972	0.5972	0.7901	0.7371	0.6853	0.6550	0.7138
24	0.1870	0.1882	0.1571	0.1571	0.1803	0.1803	0.2466	0.2198	0.1951	0.1814	0.2377
25	0.3172	0.3190	0.2679	0.2679	0.2967	0.2967	0.4128	0.3701	0.3304	0.3080	0.3856
26	0.0535	0.0539	0.0418	0.0418	0.0606	0.0606	0.0793	0.0673	0.0568	0.0512	0.0882
27	0.4306	0.4329	0.3701	0.3701	0.3932	0.3932	0.5444	0.4942	0.4466	0.4194	0.4981
28	0.0001	0.0001	<.0001	<.0001	0.0003	0.0003	0.0002	0.0002	0.0001	0.0001	0.0006

Dependent Variable: ethanol

i/j	12	13	14	15	16	17	18	19	20	21	22
1	0.9390	0.7031	0.7498	0.8124	0.5521	0.8008	0.8619	0.4893	0.4018	0.6632	0.0598
2	0.9362	0.7060	0.7527	0.8154	0.5547	0.8035	0.8646	0.4917	0.4040	0.6660	0.0603
3	0.9833	0.6242	0.6688	0.7292	0.4820	0.7266	0.7861	0.4239	0.3440	0.5863	0.0469
4	0.9833	0.6242	0.6688	0.7292	0.4820	0.7266	0.7861	0.4239	0.3440	0.5863	0.0469
5	0.9468	0.6239	0.6638	0.7175	0.4959	0.7138	0.7677	0.4428	0.3688	0.5900	0.0669
6	0.9468	0.6239	0.6638	0.7175	0.4959	0.7138	0.7677	0.4428	0.3688	0.5900	0.0669
7	0.8103	0.8427	0.8917	0.9566	0.6802	0.9293	0.9917	0.6106	0.5115	0.8004	0.0881
8	0.8646	0.7824	0.8306	0.8948	0.6242	0.8742	0.9362	0.5573	0.4630	0.7409	0.0749
9	0.9196	0.7234	0.7705	0.8336	0.5704	0.8197	0.8810	0.5065	0.4172	0.6830	0.0634
10	0.9528	0.6888	0.7351	0.7974	0.5392	0.7874	0.8482	0.4772	0.3911	0.6492	0.0573
11	0.9241	0.7542	0.7968	0.8537	0.6141	0.8377	0.8939	0.5546	0.4700	0.7175	0.0967
12		0.6764	0.7175	0.7728	0.5430	0.7641	0.8191	0.4872	0.4087	0.6412	0.0781
13	0.6764		0.9504	0.8855	0.8306	0.9293	0.8673	0.7557	0.6464	0.9566	0.1303
14	0.7175	0.9504		0.9349	0.7824	0.9736	0.9113	0.7089	0.6024	0.9071	0.1156
15	0.7728	0.8855	0.9349		0.7205	0.9681	0.9695	0.6492	0.5470	0.8427	0.0984
16	0.5430	0.8306	0.7824	0.7205		0.7794	0.7201	0.9225	0.8064	0.8733	0.1925
17	0.7641	0.9293	0.9736	0.9681	0.7794		0.9430	0.7136	0.6178	0.8907	0.1493
18	0.8191	0.8673	0.9113	0.9695	0.7201	0.9430		0.6562	0.5639	0.8292	0.1289
19	0.4872	0.7557	0.7089	0.6492	0.9225	0.7136	0.6562		0.8825	0.7974	0.2272
20	0.4087	0.6464	0.6024	0.5470	0.8064	0.6178	0.5639	0.8825		0.6859	0.2883
21	0.6412	0.9566	0.9071	0.8427	0.8733	0.8907	0.8292	0.7974	0.6859		0.1444
22	0.0781	0.1303	0.1156	0.0984	0.1925	0.1493	0.1289	0.2272	0.2883	0.1444	
23	0.6442	0.9293	0.8852	0.8278	0.9182	0.8713	0.8154	0.8496	0.7476	0.9681	0.2047
24	0.2025	0.3255	0.2991	0.2668	0.4275	0.3279	0.2942	0.4796	0.5651	0.3498	0.7059
25	0.3315	0.5341	0.4942	0.4443	0.6830	0.5192	0.4700	0.7557	0.8702	0.5704	0.3681
26	0.0710	0.1183	0.1047	0.0888	0.1761	0.1372	0.1181	0.2086	0.2662	0.1313	0.9597
27	0.4346	0.6830	0.6380	0.5810	0.8458	0.6499	0.5948	0.9225	0.9597	0.7234	0.2662
28	0.0004	0.0005	0.0004	0.0003	0.0009	0.0012	0.0009	0.0012	0.0020	0.0005	0.0352

Dependent Variable: ethanol

i/j	23	24	25	26	27	28	29	30	31	32	33
1	0.6675	0.1870	0.3172	0.0535	0.4306	0.0001	0.4275	0.0032	0.0916	0.0025	0.2199
2	0.6701	0.1882	0.3190	0.0539	0.4329	0.0001	0.4295	0.0032	0.0924	0.0026	0.2213
3	0.5984	0.1571	0.2679	0.0418	0.3701	<.0001	0.3734	0.0024	0.0731	0.0018	0.1823
4	0.5984	0.1571	0.2679	0.0418	0.3701	<.0001	0.3734	0.0024	0.0731	0.0018	0.1823
5	0.5972	0.1803	0.2967	0.0606	0.3932	0.0003	0.3896	0.0046	0.0974	0.0043	0.2128
6	0.5972	0.1803	0.2967	0.0606	0.3932	0.0003	0.3896	0.0046	0.0974	0.0043	0.2128
7	0.7901	0.2466	0.4128	0.0793	0.5444	0.0002	0.5282	0.0051	0.1313	0.0043	0.2954
8	0.7371	0.2198	0.3701	0.0673	0.4942	0.0002	0.4839	0.0042	0.1130	0.0035	0.2613
9	0.6853	0.1951	0.3304	0.0568	0.4466	0.0001	0.4418	0.0034	0.0968	0.0028	0.2301
10	0.6550	0.1814	0.3080	0.0512	0.4194	0.0001	0.4175	0.0030	0.0881	0.0024	0.2128
11	0.7138	0.2377	0.3856	0.0882	0.4981	0.0006	0.4844	0.0073	0.1376	0.0071	0.2842
12	0.6442	0.2025	0.3315	0.0710	0.4346	0.0004	0.4272	0.0056	0.1126	0.0053	0.2404

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Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

13	0.9293	0.3255	0.5341	0.1183	0.6830	0.0005	0.6499	0.0084	0.1886	0.0076	0.3954
14	0.8852	0.2991	0.4942	0.1047	0.6380	0.0004	0.6105	0.0072	0.1689	0.0064	0.3619
15	0.8278	0.2668	0.4443	0.0888	0.5810	0.0003	0.5604	0.0059	0.1455	0.0051	0.3209
16	0.9182	0.4275	0.6830	0.1761	0.8458	0.0009	0.7928	0.0140	0.2696	0.0135	0.5240
17	0.8713	0.3279	0.5192	0.1372	0.6499	0.0012	0.6205	0.0127	0.2060	0.0130	0.3960
18	0.8154	0.2942	0.4700	0.1181	0.5948	0.0009	0.5711	0.0105	0.1797	0.0106	0.3543
19	0.8496	0.4796	0.7557	0.2086	0.9225	0.0012	0.8605	0.0175	0.3135	0.0174	0.5890
20	0.7476	0.5651	0.8702	0.2662	0.9597	0.0020	0.9653	0.0243	0.3890	0.0251	0.6945
21	0.9681	0.3498	0.5704	0.1313	0.7234	0.0005	0.6853	0.0096	0.2072	0.0088	0.4261
22	0.2047	0.7059	0.3681	0.9597	0.2662	0.0352	0.3642	0.1831	0.8397	0.2272	0.5015
23		0.4135	0.6400	0.1893	0.7821	0.0021	0.7388	0.0192	0.2758	0.0206	0.5014
24	0.4135		0.6675	0.6726	0.5350	0.0244	0.6272	0.1201	0.8441	0.1464	0.8224
25	0.6400	0.6675		0.3420	0.8306	0.0032	0.9182	0.0346	0.4845	0.0372	0.8185
26	0.1893	0.6726	0.3420		0.2454	0.0396	0.3410	0.1981	0.8004	0.2469	0.4701
27	0.7821	0.5350	0.8306	0.2454		0.0017	0.9293	0.0218	0.3619	0.0222	0.6576
28	0.0021	0.0244	0.0032	0.0396	0.0017		0.0060	0.5663	0.0216	0.3559	0.0062

Dependent Variable: ethanol

i/j	34	35	36	37	38	39	40	41	42
1	<.0001	0.2276	0.0007	0.0179	0.0010	0.0731	<.0001	0.1507	0.0006
2	<.0001	0.2289	0.0007	0.0181	0.0010	0.0737	<.0001	0.1517	0.0006
3	<.0001	0.1928	0.0005	0.0135	0.0007	0.0578	<.0001	0.1255	0.0004
4	<.0001	0.1928	0.0005	0.0135	0.0007	0.0578	<.0001	0.1255	0.0004
5	<.0001	0.2161	0.0012	0.0233	0.0019	0.0798	<.0001	0.1478	0.0010
6	<.0001	0.2161	0.0012	0.0233	0.0019	0.0798	<.0001	0.1478	0.0010
7	<.0001	0.2959	0.0012	0.0282	0.0017	0.1063	<.0001	0.2017	0.0010
8	<.0001	0.2653	0.0010	0.0233	0.0013	0.0909	<.0001	0.1786	0.0008
9	<.0001	0.2370	0.0008	0.0192	0.0010	0.0774	<.0001	0.1576	0.0006
10	<.0001	0.2211	0.0007	0.0170	0.0009	0.0701	<.0001	0.1459	0.0005
11	<.0001	0.2814	0.0020	0.0358	0.0032	0.1142	<.0001	0.1976	0.0017
12	<.0001	0.2415	0.0015	0.0279	0.0023	0.0927	<.0001	0.1670	0.0013
13	<.0001	0.3846	0.0021	0.0449	0.0031	0.1552	<.0001	0.2705	0.0017
14	<.0001	0.3552	0.0017	0.0389	0.0026	0.1382	<.0001	0.2473	0.0014
15	<.0001	0.3188	0.0014	0.0321	0.0020	0.1183	<.0001	0.2191	0.0011
16	<.0001	0.4970	0.0036	0.0719	0.0057	0.2257	<.0001	0.3615	0.0030
17	0.0001	0.3819	0.0037	0.0595	0.0061	0.1737	<.0001	0.2772	0.0031
18	<.0001	0.3445	0.0030	0.0500	0.0049	0.1507	<.0001	0.2472	0.0025
19	<.0001	0.5534	0.0047	0.0881	0.0075	0.2646	<.0001	0.4087	0.0039
20	0.0001	0.6450	0.0068	0.1183	0.0112	0.3323	<.0001	0.4872	0.0057
21	<.0001	0.4116	0.0024	0.0508	0.0036	0.1713	<.0001	0.2920	0.0020
22	0.0042	0.6227	0.0712	0.6106	0.1294	0.9256	0.0008	0.7968	0.0623
23	0.0002	0.4755	0.0059	0.0867	0.0099	0.2356	<.0001	0.3542	0.0050
24	0.0035	0.9165	0.0474	0.4057	0.0842	0.7688	0.0008	0.9127	0.0416
25	0.0002	0.7529	0.0102	0.1608	0.0172	0.4194	<.0001	0.5828	0.0086
26	0.0049	0.5912	0.0783	0.6464	0.1423	0.8855	0.0009	0.7621	0.0686
27	0.0001	0.6129	0.0060	0.1071	0.0098	0.3080	<.0001	0.4594	0.0050
28	0.4194	0.0183	0.9293	0.1063	0.5418	0.0282	0.1798	0.0327	0.9792

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Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10	11
29	0.4275	0.4295	0.3734	0.3734	0.3896	0.3896	0.5282	0.4839	0.4418	0.4175	0.4844
30	0.0032	0.0032	0.0024	0.0024	0.0046	0.0046	0.0051	0.0042	0.0034	0.0030	0.0073
31	0.0916	0.0924	0.0731	0.0731	0.0974	0.0974	0.1313	0.1130	0.0968	0.0881	0.1376
32	0.0025	0.0026	0.0018	0.0018	0.0043	0.0043	0.0043	0.0035	0.0028	0.0024	0.0071
33	0.2199	0.2213	0.1823	0.1823	0.2128	0.2128	0.2954	0.2613	0.2301	0.2128	0.2842
34	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
35	0.2276	0.2289	0.1928	0.1928	0.2161	0.2161	0.2959	0.2653	0.2370	0.2211	0.2814
36	0.0007	0.0007	0.0005	0.0005	0.0012	0.0012	0.0012	0.0010	0.0008	0.0007	0.0020
37	0.0179	0.0181	0.0135	0.0135	0.0233	0.0233	0.0282	0.0233	0.0192	0.0170	0.0358
38	0.0010	0.0010	0.0007	0.0007	0.0019	0.0019	0.0017	0.0013	0.0010	0.0009	0.0032
39	0.0731	0.0737	0.0578	0.0578	0.0798	0.0798	0.1063	0.0909	0.0774	0.0701	0.1142
40	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
41	0.1507	0.1517	0.1255	0.1255	0.1478	0.1478	0.2017	0.1786	0.1576	0.1459	0.1976
42	0.0006	0.0006	0.0004	0.0004	0.0010	0.0010	0.0010	0.0008	0.0006	0.0005	0.0017

Dependent Variable: ethanol

i/j	12	13	14	15	16	17	18	19	20	21	22
29	0.4272	0.6499	0.6105	0.5604	0.7928	0.6205	0.5711	0.8605	0.9653	0.6853	0.3642
30	0.0056	0.0084	0.0072	0.0059	0.0140	0.0127	0.0105	0.0175	0.0243	0.0096	0.1831
31	0.1126	0.1886	0.1689	0.1455	0.2696	0.2060	0.1797	0.3135	0.3890	0.2072	0.8397
32	0.0053	0.0076	0.0064	0.0051	0.0135	0.0130	0.0106	0.0174	0.0251	0.0088	0.2272
33	0.2404	0.3954	0.3619	0.3209	0.5240	0.3960	0.3543	0.5890	0.6945	0.4261	0.5015
34	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001	<.0001	<.0001	0.0001	<.0001	0.0042
35	0.2415	0.3846	0.3552	0.3188	0.4970	0.3819	0.3445	0.5534	0.6450	0.4116	0.6227
36	0.0015	0.0021	0.0017	0.0014	0.0036	0.0037	0.0030	0.0047	0.0068	0.0024	0.0712
37	0.0279	0.0449	0.0389	0.0321	0.0719	0.0595	0.0500	0.0881	0.1183	0.0508	0.6106
38	0.0023	0.0031	0.0026	0.0020	0.0057	0.0061	0.0049	0.0075	0.0112	0.0036	0.1294
39	0.0927	0.1552	0.1382	0.1183	0.2257	0.1737	0.1507	0.2646	0.3323	0.1713	0.9256
40	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0008
41	0.1670	0.2705	0.2473	0.2191	0.3615	0.2772	0.2472	0.4087	0.4872	0.2920	0.7968
42	0.0013	0.0017	0.0014	0.0011	0.0030	0.0031	0.0025	0.0039	0.0057	0.0020	0.0623

Dependent Variable: ethanol

i/j	23	24	25	26	27	28	29	30	31	32	33
29	0.7388	0.6272	0.9182	0.3410	0.9293	0.0060		0.0430	0.4668	0.0490	0.7582
30	0.0192	0.1201	0.0346	0.1981	0.0218	0.5663	0.0430		0.1314	0.7995	0.0553
31	0.2758	0.8441	0.4845	0.8004	0.3619	0.0216	0.4668	0.1314		0.1596	0.6380
32	0.0206	0.1464	0.0372	0.2469	0.0222	0.3559	0.0490	0.7995	0.1596		0.0624
33	0.5014	0.8224	0.8185	0.4701	0.6576	0.0062	0.7582	0.0553	0.6380	0.0624	
34	0.0002	0.0035	0.0002	0.0049	0.0001	0.4194	0.0007	0.1969	0.0023	0.0860	0.0005
35	0.4755	0.9165	0.7529	0.5912	0.6129	0.0183	0.7032	0.0977	0.7555	0.1177	0.9127
36	0.0059	0.0474	0.0102	0.0783	0.0060	0.9293	0.0145	0.6579	0.0479	0.4604	0.0174
37	0.0867	0.4057	0.1608	0.6464	0.1071	0.1063	0.1748	0.3780	0.4772	0.4820	0.2392
38	0.0099	0.0842	0.0172	0.1423	0.0098	0.5418	0.0253	0.9778	0.0867	0.7527	0.0304
39	0.2356	0.7688	0.4194	0.8855	0.3080	0.0282	0.4096	0.1576	0.9132	0.1938	0.5625
40	<.0001	0.0008	<.0001	0.0009	<.0001	0.1798	0.0001	0.0781	0.0004	0.0254	<.0001
41	0.3542	0.9127	0.5828	0.7621	0.4594	0.0327	0.5519	0.1478	0.9390	0.1819	0.7305
42	0.0050	0.0416	0.0086	0.0686	0.0050	0.9792	0.0125	0.6171	0.0415	0.4235	0.0148

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Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	34	35	36	37	38	39	40	41	42
29	0.0007	0.7032	0.0145	0.1748	0.0253	0.4096	0.0001	0.5519	0.0125
30	0.1969	0.0977	0.6579	0.3780	0.9778	0.1576	0.0781	0.1478	0.6171
31	0.0023	0.7555	0.0479	0.4772	0.0867	0.9132	0.0004	0.9390	0.0415
32	0.0860	0.1177	0.4604	0.4820	0.7527	0.1938	0.0254	0.1819	0.4235
33	0.0005	0.9127	0.0174	0.2392	0.0304	0.5625	<.0001	0.7305	0.0148
34		0.0025	0.4175	0.0169	0.1585	0.0032	0.5890	0.0049	0.4542
35	0.0025		0.0373	0.3445	0.0661	0.6828	0.0005	0.8302	0.0326
36	0.4175	0.0373		0.1737	0.6474	0.0595	0.1975	0.0605	0.9544
37	0.0169	0.3445	0.1737		0.3098	0.5470	0.0038	0.4764	0.1551
38	0.1585	0.0661	0.6474	0.3098		0.1079	0.0530	0.1074	0.6032
39	0.0032	0.6828	0.0595	0.5470	0.1079		0.0006	0.8619	0.0518
40	0.5890	0.0005	0.1975	0.0038	0.0530	0.0006		0.0011	0.2198
41	0.0049	0.8302	0.0605	0.4764	0.1074	0.8619	0.0011		0.0533
42	0.4542	0.0326	0.9544	0.1551	0.6032	0.0518	0.2198	0.0533	

The GLM Procedure

Least Squares Means

Time	Flow	pH	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5	5.5	3.1366667	1.8774317	0.0992	1
1	5	6.8	3.2966667	1.8774317	0.0835	2
1	7.5	5.5	3.1833333	1.8774317	0.0944	3
1	7.5	6.8	3.5566667	1.8774317	0.0623	4
1	10	5.5	3.6450000	2.2993748	0.1174	5
1	10	6.8	4.5700000	2.2993748	0.0508	6
2	5	5.5	4.4466667	1.8774317	0.0206	7
2	5	6.8	4.1233333	1.8774317	0.0314	8
2	7.5	5.5	4.0800000	1.8774317	0.0332	9
2	7.5	6.8	4.2166667	1.8774317	0.0279	10
2	10	5.5	4.6950000	2.2993748	0.0449	11
2	10	6.8	4.2900000	2.2993748	0.0663	12
3	5	5.5	7.3533333	1.8774317	0.0002	13
3	5	6.8	6.0866667	1.8774317	0.0018	14
3	7.5	5.5	5.7933333	1.8774317	0.0029	15
3	7.5	6.8	7.2700000	1.8774317	0.0002	16
3	10	5.5	7.5250000	2.2993748	0.0017	17
3	10	6.8	6.2350000	2.2993748	0.0084	18
4	5	5.5	15.1300000	1.8774317	<.0001	19
4	5	6.8	9.8000000	1.8774317	<.0001	20
4	7.5	5.5	8.7533333	1.8774317	<.0001	21
4	7.5	6.8	12.8266667	1.8774317	<.0001	22
4	10	5.5	12.7650000	2.2993748	<.0001	23
4	10	6.8	11.9550000	2.2993748	<.0001	24
5	5	5.5	23.2833333	1.8774317	<.0001	25
5	5	6.8	17.1066667	1.8774317	<.0001	26
5	7.5	5.5	16.7533333	1.8774317	<.0001	27
5	7.5	6.8	23.4466667	1.8774317	<.0001	28
5	10	5.5	22.9700000	2.2993748	<.0001	29
5	10	6.8	25.4050000	2.2993748	<.0001	30
6	5	5.5	31.1400000	1.8774317	<.0001	31
6	5	6.8	27.0600000	1.8774317	<.0001	32
6	7.5	5.5	23.6966667	1.8774317	<.0001	33
6	7.5	6.8	32.1300000	1.8774317	<.0001	34
6	10	5.5	29.7500000	2.2993748	<.0001	35
6	10	6.8	37.4450000	2.2993748	<.0001	36
7	5	5.5	34.2666667	1.8774317	<.0001	37
7	5	6.8	32.3233333	1.8774317	<.0001	38
7	7.5	5.5	29.7400000	1.8774317	<.0001	39
7	7.5	6.8	36.5766667	1.8774317	<.0001	40
7	10	5.5	32.1950000	2.2993748	<.0001	41
7	10	6.8	42.4350000	2.2993748	<.0001	42

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Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8	9	10	11
1		0.9521	0.9860	0.8748	0.8645	0.6307	0.6233	0.7113	0.7234	0.6854	0.6013
2	0.9521		0.9661	0.9223	0.9069	0.6693	0.6663	0.7565	0.7688	0.7300	0.6391
3	0.9860	0.9661		0.8886	0.8769	0.6419	0.6357	0.7244	0.7366	0.6983	0.6122
4	0.8748	0.9223	0.8886		0.9763	0.7339	0.7385	0.8316	0.8443	0.8044	0.7025
5	0.8645	0.9069	0.8769	0.9763		0.7769	0.7879	0.8724	0.8839	0.8478	0.7477
6	0.6307	0.6693	0.6419	0.7339	0.7769		0.9670	0.8808	0.8694	0.9056	0.9694
7	0.6233	0.6663	0.6357	0.7385	0.7879	0.9670		0.9034	0.8906	0.9312	0.9336
8	0.7113	0.7565	0.7244	0.8316	0.8724	0.8808	0.9034		0.9870	0.9721	0.8478
9	0.7234	0.7688	0.7366	0.8443	0.8839	0.8694	0.8906	0.9870		0.9591	0.8365
10	0.6854	0.7300	0.6983	0.8044	0.8478	0.9056	0.9312	0.9721	0.9591		0.8724
11	0.6013	0.6391	0.6122	0.7025	0.7477	0.9694	0.9336	0.8478	0.8365	0.8724	
12	0.6988	0.7389	0.7104	0.8056	0.8433	0.9316	0.9581	0.9554	0.9438	0.9804	0.9012
13	0.1168	0.1310	0.1208	0.1572	0.2157	0.3517	0.2774	0.2279	0.2218	0.2415	0.3736
14	0.2703	0.2970	0.2779	0.3439	0.4136	0.6110	0.5388	0.4621	0.4523	0.4836	0.6407
15	0.3205	0.3503	0.3290	0.4024	0.4717	0.6815	0.6136	0.5314	0.5208	0.5545	0.7125
16	0.1240	0.1390	0.1283	0.1664	0.2261	0.3662	0.2913	0.2400	0.2336	0.2541	0.3887
17	0.1438	0.1588	0.1481	0.1856	0.2368	0.3666	0.3033	0.2557	0.2498	0.2689	0.3871
18	0.3002	0.3257	0.3075	0.3700	0.4284	0.6102	0.5488	0.4792	0.4703	0.4988	0.6373
19	<.0001	<.0001	<.0001	<.0001	0.0002	0.0007	0.0001	<.0001	<.0001	0.0001	0.0008
20	0.0144	0.0168	0.0151	0.0215	0.0418	0.0825	0.0476	0.0360	0.0347	0.0391	0.0899
21	0.0380	0.0436	0.0395	0.0543	0.0897	0.1632	0.1093	0.0856	0.0828	0.0919	0.1760
22	0.0005	0.0006	0.0005	0.0008	0.0028	0.0069	0.0024	0.0016	0.0016	0.0018	0.0078
23	0.0018	0.0021	0.0019	0.0028	0.0065	0.0140	0.0066	0.0048	0.0046	0.0053	0.0155
24	0.0041	0.0047	0.0043	0.0061	0.0128	0.0262	0.0137	0.0103	0.0099	0.0112	0.0288
25	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
26	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
27	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	0.0001
28	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Dependent Variable: acetate

i/j	12	13	14	15	16	17	18	19	20	21	22
1	0.6988	0.1168	0.2703	0.3205	0.1240	0.1438	0.3002	<.0001	0.0144	0.0380	0.0005
2	0.7389	0.1310	0.2970	0.3503	0.1390	0.1588	0.3257	<.0001	0.0168	0.0436	0.0006
3	0.7104	0.1208	0.2779	0.3290	0.1283	0.1481	0.3075	<.0001	0.0151	0.0395	0.0005
4	0.8056	0.1572	0.3439	0.4024	0.1664	0.1856	0.3700	<.0001	0.0215	0.0543	0.0008
5	0.8433	0.2157	0.4136	0.4717	0.2261	0.2368	0.4284	0.0002	0.0418	0.0897	0.0028
6	0.9316	0.3517	0.6110	0.6815	0.3662	0.3666	0.6102	0.0007	0.0825	0.1632	0.0069
7	0.9581	0.2774	0.5388	0.6136	0.2913	0.3033	0.5488	0.0001	0.0476	0.1093	0.0024
8	0.9554	0.2279	0.4621	0.5314	0.2400	0.2557	0.4792	<.0001	0.0360	0.0856	0.0016
9	0.9438	0.2218	0.4523	0.5208	0.2336	0.2498	0.4703	<.0001	0.0347	0.0828	0.0016
10	0.9804	0.2415	0.4836	0.5545	0.2541	0.2689	0.4988	0.0001	0.0391	0.0919	0.0018
11	0.9012	0.3736	0.6407	0.7125	0.3887	0.3871	0.6373	0.0008	0.0899	0.1760	0.0078
12		0.3056	0.5470	0.6141	0.3189	0.3232	0.5517	0.0005	0.0676	0.1372	0.0053
13	0.3056		0.6348	0.5587	0.9751	0.9540	0.7075	0.0046	0.3600	0.5997	0.0430
14	0.5470	0.6348		0.9123	0.6572	0.6295	0.9603	0.0011	0.1664	0.3187	0.0134
15	0.6141	0.5587	0.9123		0.5799	0.5615	0.8822	0.0008	0.1358	0.2687	0.0100
16	0.3189	0.9751	0.6572	0.5799		0.9318	0.7284	0.0042	0.3439	0.5782	0.0400
17	0.3232	0.9540	0.6295	0.5615	0.9318		0.6928	0.0126	0.4460	0.6803	0.0784
18	0.5517	0.7075	0.9603	0.8822	0.7284	0.6928		0.0038	0.2338	0.3991	0.0296
19	0.0005	0.0046	0.0011	0.0008	0.0042	0.0126	0.0038		0.0486	0.0190	0.3886
20	0.0676	0.3600	0.1664	0.1358	0.3439	0.4460	0.2338	0.0486		0.6946	0.2582
21	0.1372	0.5997	0.3187	0.2687	0.5782	0.6803	0.3991	0.0190	0.6946		0.1295
22	0.0053	0.0430	0.0134	0.0100	0.0400	0.0784	0.0296	0.3886	0.2582	0.1295	
23	0.0112	0.0726	0.0276	0.0217	0.0684	0.1116	0.0485	0.4283	0.3213	0.1809	0.9835
24	0.0212	0.1256	0.0520	0.0416	0.1190	0.1775	0.0829	0.2885	0.4703	0.2845	0.7699
25	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0030	<.0001	<.0001	0.0002
26	<.0001	0.0005	<.0001	<.0001	0.0004	0.0019	0.0005	0.4591	0.0075	0.0024	0.1115
27	<.0001	0.0007	0.0001	<.0001	0.0006	0.0027	0.0007	0.5429	0.0108	0.0036	0.1436
28	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0025	<.0001	<.0001	0.0002

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Dependent Variable: acetate

i/j	23	24	25	26	27	28	29	30	31	32	33
1	0.0018	0.0041	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	0.0021	0.0047	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	0.0019	0.0043	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	0.0028	0.0061	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	0.0065	0.0128	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
6	0.0140	0.0262	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
7	0.0066	0.0137	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
8	0.0048	0.0103	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
9	0.0046	0.0099	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
10	0.0053	0.0112	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	0.0155	0.0288	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
12	0.0112	0.0212	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	0.0726	0.1256	<.0001	0.0005	0.0007	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	0.0276	0.0520	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	0.0217	0.0416	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	0.0684	0.1190	<.0001	0.0004	0.0006	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	0.1116	0.1775	<.0001	0.0019	0.0027	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18	0.0485	0.0829	<.0001	0.0005	0.0007	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	0.4283	0.2885	0.0030	0.4591	0.5429	0.0025	0.0102	0.0009	<.0001	<.0001	0.0019
20	0.3213	0.4703	<.0001	0.0075	0.0108	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21	0.1809	0.2845	<.0001	0.0024	0.0036	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
22	0.9835	0.7699	0.0002	0.1115	0.1436	0.0002	0.0011	<.0001	<.0001	<.0001	0.0001
23		0.8040	0.0007	0.1481	0.1834	0.0006	0.0025	0.0002	<.0001	<.0001	0.0005
24	0.8040		0.0003	0.0871	0.1105	0.0002	0.0012	<.0001	<.0001	<.0001	0.0002
25	0.0007	0.0003		0.0229	0.0164	0.9511	0.9162	0.4772	0.0042	0.1593	0.8767
26	0.1481	0.0871	0.0229		0.8945	0.0196	0.0522	0.0067	<.0001	0.0004	0.0155
27	0.1834	0.1105	0.0164	0.8945		0.0140	0.0399	0.0048	<.0001	0.0002	0.0109
28	0.0006	0.0002	0.9511	0.0196	0.0140		0.8729	0.5116	0.0050	0.1779	0.9253

Dependent Variable: acetate

i/j	34	35	36	37	38	39	40	41	42
1	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
10	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
21	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
22	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
23	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
25	0.0014	0.0327	<.0001	<.0001	0.0011	0.0176	<.0001	0.0037	<.0001
26	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
27	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
28	0.0017	0.0373	<.0001	0.0001	0.0013	0.0205	<.0001	0.0044	<.0001

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Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8	9	10	11
29	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
30	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
31	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
34	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
37	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
38	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
39	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
40	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
41	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
42	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	12	13	14	15	16	17	18	19	20	21	22
29	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0102	<.0001	<.0001	0.0011
30	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0009	<.0001	<.0001	<.0001
31	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
32	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
33	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0019	<.0001	<.0001	0.0001
34	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
35	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
36	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
37	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
38	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
39	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
40	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
41	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
42	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

Dependent Variable: acetate

i/j	23	24	25	26	27	28	29	30	31	32	33
29	0.0025	0.0012	0.9162	0.0522	0.0399	0.8729		0.4565	0.0075	0.1727	0.8073
30	0.0002	<.0001	0.4772	0.0067	0.0048	0.5116	0.4565		0.0574	0.5789	0.5668
31	<.0001	<.0001	0.0042	<.0001	<.0001	0.0050	0.0075	0.0574		0.1289	0.0065
32	<.0001	<.0001	0.1593	0.0004	0.0002	0.1779	0.1727	0.5789	0.1289		0.2094
33	0.0005	0.0002	0.8767	0.0155	0.0109	0.9253	0.8073	0.5668	0.0065	0.2094	
34	<.0001	<.0001	0.0014	<.0001	<.0001	0.0017	0.0029	0.0266	0.7104	0.0603	0.0022
35	<.0001	<.0001	0.0327	<.0001	<.0001	0.0373	0.0407	0.1858	0.6411	0.3679	0.0452
36	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0004	0.0372	0.0008	<.0001
37	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001	0.0003	0.0039	0.2429	0.0084	0.0002
38	<.0001	<.0001	0.0011	<.0001	<.0001	0.0013	0.0024	0.0227	0.6572	0.0514	0.0018
39	<.0001	<.0001	0.0176	<.0001	<.0001	0.0205	0.0256	0.1487	0.5997	0.3163	0.0259
40	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003	0.0443	0.0006	<.0001
41	<.0001	<.0001	0.0037	<.0001	<.0001	0.0044	0.0060	0.0404	0.7234	0.0881	0.0055
42	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003	<.0001	<.0001

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LSMeans for Main and Interaction Affects
Ethanol and Acetate Production

The GLM Procedure
Least Squares Means

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	34	35	36	37	38	39	40	41	42
29	0.0029	0.0407	<.0001	0.0003	0.0024	0.0256	<.0001	0.0060	<.0001
30	0.0266	0.1858	0.0004	0.0039	0.0227	0.1487	0.0003	0.0404	<.0001
31	0.7104	0.6411	0.0372	0.2429	0.6572	0.5997	0.0443	0.7234	0.0003
32	0.0603	0.3679	0.0008	0.0084	0.0514	0.3163	0.0006	0.0881	<.0001
33	0.0022	0.0452	<.0001	0.0002	0.0018	0.0259	<.0001	0.0055	<.0001
34		0.4254	0.0777	0.4237	0.9422	0.3711	0.0984	0.9826	0.0009
35	0.4254		0.0207	0.1326	0.3890	0.9973	0.0245	0.4546	0.0002
36	0.0777	0.0207		0.2880	0.0889	0.0115	0.7708	0.1109	0.1294
37	0.4237	0.1326	0.2880		0.4667	0.0926	0.3873	0.4876	0.0075
38	0.9422	0.3890	0.0889	0.4667		0.3339	0.1137	0.9656	0.0011
39	0.3711	0.9973	0.0115	0.0926	0.3339		0.0121	0.4110	<.0001
40	0.0984	0.0245	0.7708	0.3873	0.1137	0.0121		0.1444	0.0524
41	0.9826	0.4546	0.1109	0.4876	0.9656	0.4110	0.1444		0.0024
42	0.0009	0.0002	0.1294	0.0075	0.0011	<.0001	0.0524	0.0024	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix F.2: **SAS Analysis of *C. ljungdahlii*: Gas Composition over Time**

```
Title1 'C. autoethanogenum on Syngas 06/27/2006';
Title2 'LSMeans for Main and Interaction Affects';
Title3 'Gas Composition over Time';


Data practice;
Input H2 N2 CO CO2 Flow Time ;
Cards;
9.8   48.52 20.27 19.96 5     1
9.71  48.8  20.32 20.76 5     1
9.88  48.54 20.3  19.81 5     1
9.89  48.47 20.42 19.93 7.5   1
9.65  49.91 20.31 18.94 7.5   1
10.25 48.94 20.33 19.49 7.5   1
9.69  48.63 20.19 19.99 10    1
9.68  48.67 20.27 19.93 10    1
8.77  50.23 19.78 19.13 10    1
9.63  48.46 20.16 20.14 5     2
9.53  48.5  19.98 20.91 5     2
9.44  49.05 19.91 19.6  5     2
9.67  48.87 20.01 19.89 7.5   2
9.31  49.15 19.83 19.5  7.5   2
9.24  49.85 19.31 19    7.5   2
9.66  48.43 20.21 19.91 10    2
9.57  48.77 20    19.77 10    2
9.48  49.21 19.82 19.58 10    2
9.68  48.05 19.98 20.15 5     3
9.91  48.24 20.11 20.69 5     3
9.44  48.66 19.92 19.75 5     3
9.83  48.7  19.91 19.9  7.5   3
9.73  48.42 20.22 19.87 7.5   3
9.78  48.39 20.06 20.03 7.5   3
9.83  47.84 20.12 20.2  10    3
9.28  48.79 19.85 19.96 10    3
9.68  48.35 20.13 19.85 10    3
9.65  47.99 19.72 20.24 5     4
9.66  48.27 19.68 22.46 5     4
9.74  48.16 19.89 20.06 5     4
9.17  49.73 18.5  19.48 7.5   4
9.42  48.96 19.52 19.32 7.5   4
9.5   49.07 19.25 20.02 7.5   4
9.37  48.96 19.68 20.17 10    4
9.79  48.21 20.2  20.09 10    4
9.7   47.99 19.79 20.06 10    4
9.54  48.09 19.4  22.32 5     5
9.26  48.41 19.27 20.61 5     5
9.44  48.46 19.71 20.44 5     5
9.11  49.78 18.82 18.88 7.5   5
9.19  49.16 19.04 20.12 7.5   5
8.3   51.56 17.29 18.31 7.5   5
9.34  48.31 19.79 20.65 10    5
9.55  48.12 19.89 20.01 10    5
9.09  49.15 19.04 20.12 10    5
```

```
9.42  48.58 19.49 20.78 5     6
9.83  48.7  19.71 21.26 5     6
9.34  48.84 19.41 20.42 5     6
9.69  48.33 20.14 20.13 7.5   6
9.58  48.69 19.82 20.67 7.5   6
9.52  48.69 19.71 20.19 7.5   6
9.05  49.33 19.34 20.65 10    6
9.29  48.8  19.57 20.48 10    6
9.47  48.79 19.9  19.9  10    6
;
OPTIONS NODATE PAGENO = 1;
Proc GLM Data = practice;
Class Time Flow;

model N2 H2 CO CO2/*put in desired gas to analyze*/ = Time|Flow ;
lsmeans Time Flow Time*Flow /pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
end;
```

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The GLM Procedure

Class Level Information

Class	Levels	Values
Time	7	1 2 3 4 5 6 7
Flow	2	5 7.5
pH	2	5.5 6.8

Number of Observations Read	75
Number of Observations Used	75

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Gas Composition over Time

The GLM Procedure

Dependent Variable: N2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	314.8273620	12.1087447	2.97	0.0005
Error	48	195.9996167	4.0833253		
Corrected Total	74	510.8269787			

R-Square	Coeff Var	Root MSE	N2 Mean
0.616309	4.151558	2.020724	48.67387

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	6	93.8540498	15.6423416	3.83	0.0034
Flow	1	138.4285855	138.4285855	33.90	<.0001
pH	1	26.3235990	26.3235990	6.45	0.0144
Time*Flow	6	29.8149887	4.9691648	1.22	0.3142
Time*pH	6	13.8115085	2.3019181	0.56	0.7569
Flow*pH	1	3.2476800	3.2476800	0.80	0.3769
Time*Flow*pH	5	9.3469505	1.8693901	0.46	0.8055

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	6	86.91488605	14.48581434	3.55	0.0055
Flow	1	99.52647902	99.52647902	24.37	<.0001
pH	1	37.96567613	37.96567613	9.30	0.0037
Time*Flow	6	29.14666016	4.85777669	1.19	0.3278
Time*pH	6	15.79144680	2.63190780	0.64	0.6941
Flow*pH	1	4.19643601	4.19643601	1.03	0.3158
Time*Flow*pH	5	9.34695053	1.86939011	0.46	0.8055

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The GLM Procedure

Dependent Variable: H2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	36.1303313	1.3896281	0.65	0.8821
Error	48	102.9009833	2.1437705		
Corrected Total	74	139.0313147			

R-Square	Coeff Var	Root MSE	H2 Mean
0.259872	17.88239	1.464162	8.187733

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	6	8.15910967	1.35985161	0.63	0.7021
Flow	1	8.42116918	8.42116918	3.93	0.0532
pH	1	2.73583626	2.73583626	1.28	0.2642
Time*Flow	6	7.94721397	1.32453566	0.62	0.7149
Time*pH	6	4.45353003	0.74225500	0.35	0.9086
Flow*pH	1	1.32549537	1.32549537	0.62	0.4355
Time*Flow*pH	5	3.08797686	0.61759537	0.29	0.9173

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	6	6.81227151	1.13537859	0.53	0.7830
Flow	1	6.03334291	6.03334291	2.81	0.0999
pH	1	3.81346609	3.81346609	1.78	0.1886
Time*Flow	6	6.51488687	1.08581448	0.51	0.8004
Time*pH	6	4.41238623	0.73539771	0.34	0.9105
Flow*pH	1	1.54307411	1.54307411	0.72	0.4004
Time*Flow*pH	5	3.08797686	0.61759537	0.29	0.9173

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Gas Composition over Time

The GLM Procedure

Dependent Variable: CO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	64.6656167	2.4871391	1.28	0.2249
Error	48	93.2165833	1.9420122		
Corrected Total	74	157.8822000			

R-Square	Coeff Var	Root MSE	CO Mean
0.409581	7.400749	1.393561	18.83000

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	6	20.94362500	3.49060417	1.80	0.1196
Flow	1	18.14433246	18.14433246	9.34	0.0037
pH	1	0.67179052	0.67179052	0.35	0.5592
Time*Flow	6	9.12112604	1.52018767	0.78	0.5876
Time*pH	6	3.27972709	0.54662118	0.28	0.9430
Flow*pH	1	3.93967342	3.93967342	2.03	0.1608
Time*Flow*pH	5	8.56534213	1.71306843	0.88	0.5004

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	6	18.41095558	3.06849260	1.58	0.1735
Flow	1	12.04728537	12.04728537	6.20	0.0163
pH	1	2.08665053	2.08665053	1.07	0.3051
Time*Flow	6	7.19146695	1.19857783	0.62	0.7155
Time*pH	6	3.21512776	0.53585463	0.28	0.9456
Flow*pH	1	4.79290744	4.79290744	2.47	0.1228
Time*Flow*pH	5	8.56534213	1.71306843	0.88	0.5004

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Gas Composition over Time

The GLM Procedure

Dependent Variable: CO2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	26	204.4608220	7.8638778	6.08	<.0001
Error	48	62.1190500	1.2941469		
Corrected Total	74	266.5798720			

R-Square	Coeff Var	Root MSE	CO2 Mean
0.766978	5.575078	1.137606	20.40520

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	6	80.86024644	13.47670774	10.41	<.0001
Flow	1	53.32878543	53.32878543	41.21	<.0001
pH	1	29.95625177	29.95625177	23.15	<.0001
Time*Flow	6	20.22443875	3.37073979	2.60	0.0288
Time*pH	6	17.27360904	2.87893484	2.22	0.0566
Flow*pH	1	0.00180053	0.00180053	0.00	0.9704
Time*Flow*pH	5	2.81569002	0.56313800	0.44	0.8218

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	6	71.27257877	11.87876313	9.18	<.0001
Flow	1	37.60500361	37.60500361	29.06	<.0001
pH	1	34.70491864	34.70491864	26.82	<.0001
Time*Flow	6	20.21593496	3.36932249	2.60	0.0289
Time*pH	6	16.81572740	2.80262123	2.17	0.0628
Flow*pH	1	0.00696696	0.00696696	0.01	0.9418
Time*Flow*pH	5	2.81569002	0.56313800	0.44	0.8218

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LSMeans for Main and Interaction Affects
Gas Composition over Time

The GLM Procedure
Least Squares Means

Time	Flow	pH	H2 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5	5.5	8.42000000	0.84533435	<.0001	1
1	5	6.8	9.05333333	0.84533435	<.0001	2
1	7.5	5.5	8.31666667	0.84533435	<.0001	3
1	7.5	6.8	8.57666667	0.84533435	<.0001	4
2	5	5.5	8.68333333	0.84533435	<.0001	5
2	5	6.8	8.47666667	0.84533435	<.0001	6
2	7.5	5.5	8.88000000	0.84533435	<.0001	7
2	7.5	6.8	8.68666667	0.84533435	<.0001	8
3	5	5.5	5.74000000	0.84533435	<.0001	9
3	5	6.8	8.54000000	1.03531891	<.0001	10
3	7.5	5.5	8.50333333	0.84533435	<.0001	11
3	7.5	6.8	8.79000000	1.03531891	<.0001	12
4	5	5.5	7.34666667	0.84533435	<.0001	13
4	5	6.8	7.75666667	0.84533435	<.0001	14
4	7.5	5.5	8.50666667	0.84533435	<.0001	15
4	7.5	6.8	8.60666667	0.84533435	<.0001	16
5	5	5.5	7.47500000	1.03531891	<.0001	17
5	5	6.8	7.60000000	1.03531891	<.0001	18
5	7.5	5.5	8.24000000	0.84533435	<.0001	19
5	7.5	6.8	8.64666667	0.84533435	<.0001	20
6	5	5.5	6.95333333	0.84533435	<.0001	21
6	5	6.8	8.05000000	1.46416204	<.0001	22
6	7.5	5.5	8.23000000	0.84533435	<.0001	23
6	7.5	6.8	8.43333333	0.84533435	<.0001	24
7	5	5.5	7.97000000	0.84533435	<.0001	25
7	7.5	5.5	8.13000000	0.84533435	<.0001	26
7	7.5	6.8	8.25000000	0.84533435	<.0001	27

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: H2

i/j	1	2	3	4	5	6	7	8	9
1		0.5987	0.9315	0.8963	0.8266	0.9624	0.7021	0.8244	0.0296
2	0.5987		0.5407	0.6919	0.7583	0.6317	0.8853	0.7604	0.0079
3	0.9315	0.5407		0.8288	0.7604	0.8941	0.6396	0.7583	0.0362
4	0.8963	0.6919	0.8288		0.9293	0.9337	0.8008	0.9271	0.0217
5	0.8266	0.7583	0.7604	0.9293		0.8635	0.8700	0.9978	0.0175
6	0.9624	0.6317	0.8941	0.9337	0.8635		0.7373	0.8613	0.0265
7	0.7021	0.8853	0.6396	0.8008	0.8700	0.7373		0.8722	0.0115
8	0.8244	0.7604	0.7583	0.9271	0.9978	0.8613	0.8722		0.0173
9	0.0296	0.0079	0.0362	0.0217	0.0175	0.0265	0.0115	0.0173	
10	0.9288	0.7026	0.8680	0.9782	0.9150	0.9624	0.8003	0.9131	0.0415
11	0.9447	0.6475	0.8766	0.9513	0.8809	0.9823	0.7541	0.8788	0.0251
12	0.7831	0.8446	0.7248	0.8739	0.9367	0.8157	0.9466	0.9387	0.0270
13	0.3738	0.1599	0.4211	0.3087	0.2691	0.3493	0.2058	0.2679	0.1853
14	0.5816	0.2835	0.6416	0.4961	0.4421	0.5498	0.3521	0.4404	0.0981
15	0.9425	0.6495	0.8744	0.9536	0.8831	0.9801	0.7562	0.8809	0.0250
16	0.8766	0.7103	0.8094	0.9801	0.9491	0.9139	0.8201	0.9469	0.0204
17	0.4830	0.2435	0.5319	0.4139	0.3705	0.4573	0.2984	0.3692	0.2005
18	0.5424	0.2823	0.5943	0.4685	0.4216	0.5150	0.3430	0.4202	0.1705
19	0.8809	0.4996	0.9491	0.7794	0.7124	0.8439	0.5949	0.7103	0.0418
20	0.8504	0.7352	0.7837	0.9536	0.9757	0.8875	0.8461	0.9734	0.0188
21	0.2259	0.0854	0.2598	0.1808	0.1544	0.2087	0.1136	0.1536	0.3152
22	0.8277	0.5557	0.8753	0.7568	0.7096	0.8018	0.6257	0.7081	0.1782
23	0.8744	0.4943	0.9425	0.7731	0.7062	0.8374	0.5892	0.7042	0.0426
24	0.9911	0.6064	0.9227	0.9051	0.8352	0.9712	0.7103	0.8331	0.0289
25	0.7083	0.3694	0.7731	0.6142	0.5535	0.6736	0.4503	0.5517	0.0683
26	0.8094	0.4437	0.8766	0.7103	0.6456	0.7731	0.5334	0.6436	0.0513
27	0.8875	0.5048	0.9558	0.7858	0.7186	0.8504	0.6006	0.7165	0.0410

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The GLM Procedure
Least Squares Means

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: H2

i/j	10	11	12	13	14	15	16	17	18
1	0.9288	0.9447	0.7831	0.3738	0.5816	0.9425	0.8766	0.4830	0.5424
2	0.7026	0.6475	0.8446	0.1599	0.2835	0.6495	0.7103	0.2435	0.2823
3	0.8680	0.8766	0.7248	0.4211	0.6416	0.8744	0.8094	0.5319	0.5943
4	0.9782	0.9513	0.8739	0.3087	0.4961	0.9536	0.9801	0.4139	0.4685
5	0.9150	0.8809	0.9367	0.2691	0.4421	0.8831	0.9491	0.3705	0.4216
6	0.9624	0.9823	0.8157	0.3493	0.5498	0.9801	0.9139	0.4573	0.5150
7	0.8003	0.7541	0.9466	0.2058	0.3521	0.7562	0.8201	0.2984	0.3430
8	0.9131	0.8788	0.9387	0.2679	0.4404	0.8809	0.9469	0.3692	0.4202
9	0.0415	0.0251	0.0270	0.1853	0.0981	0.0250	0.0204	0.2005	0.1705
10		0.9782	0.8651	0.3764	0.5606	0.9802	0.9604	0.4705	0.5239
11	0.9782		0.8311	0.3381	0.5352	0.9978	0.9315	0.4454	0.5024
12	0.8651	0.8311		0.2856	0.4432	0.8330	0.8915	0.3736	0.4204
13	0.3764	0.3381	0.2856		0.7331	0.3368	0.2972	0.9239	0.8505
14	0.5606	0.5352	0.4432	0.7331		0.5334	0.4805	0.8340	0.9072
15	0.9802	0.9978	0.8330	0.3368	0.5334		0.9337	0.4440	0.5008
16	0.9604	0.9315	0.8915	0.2972	0.4805	0.9337		0.4014	0.4550
17	0.4705	0.4454	0.3736	0.9239	0.8340	0.4440	0.4014		0.9323
18	0.5239	0.5024	0.4204	0.8505	0.9072	0.5008	0.4550	0.9323	
19	0.8234	0.8266	0.6825	0.4586	0.6878	0.8244	0.7604	0.5698	0.6342
20	0.9367	0.9051	0.9150	0.2823	0.4602	0.9073	0.9734	0.3851	0.4374
21	0.2410	0.2010	0.1758	0.7436	0.5048	0.2000	0.1731	0.6980	0.6307
22	0.7858	0.7897	0.6817	0.6793	0.8630	0.7882	0.7434	0.7499	0.8029
23	0.8176	0.8201	0.6771	0.4636	0.6939	0.8180	0.7541	0.5748	0.6395
24	0.9367	0.9536	0.7907	0.3679	0.5740	0.9513	0.8853	0.4768	0.5359
25	0.6717	0.6575	0.5424	0.6045	0.8591	0.6555	0.5968	0.7128	0.7831
26	0.7604	0.7562	0.6237	0.5154	0.7562	0.7541	0.6919	0.6263	0.6935
27	0.8292	0.8331	0.6880	0.4536	0.6817	0.8309	0.7667	0.5647	0.6290

Dependent Variable: H2

i/j	19	20	21	22	23	24	25	26	27
1	0.8809	0.8504	0.2259	0.8277	0.8744	0.9911	0.7083	0.8094	0.8875
2	0.4996	0.7352	0.0854	0.5557	0.4943	0.6064	0.3694	0.4437	0.5048
3	0.9491	0.7837	0.2598	0.8753	0.9425	0.9227	0.7731	0.8766	0.9558
4	0.7794	0.9536	0.1808	0.7568	0.7731	0.9051	0.6142	0.7103	0.7858
5	0.7124	0.9757	0.1544	0.7096	0.7062	0.8352	0.5535	0.6456	0.7186
6	0.8439	0.8875	0.2087	0.8018	0.8374	0.9712	0.6736	0.7731	0.8504
7	0.5949	0.8461	0.1136	0.6257	0.5892	0.7103	0.4503	0.5334	0.6006
8	0.7103	0.9734	0.1536	0.7081	0.7042	0.8331	0.5517	0.6436	0.7165
9	0.0418	0.0188	0.3152	0.1782	0.0426	0.0289	0.0683	0.0513	0.0410
10	0.8234	0.9367	0.2410	0.7858	0.8176	0.9367	0.6717	0.7604	0.8292
11	0.8266	0.9051	0.2010	0.7897	0.8201	0.9536	0.6575	0.7562	0.8331
12	0.6825	0.9150	0.1758	0.6817	0.6771	0.7907	0.5424	0.6237	0.6880
13	0.4586	0.2823	0.7436	0.6793	0.4636	0.3679	0.6045	0.5154	0.4536
14	0.6878	0.4602	0.5048	0.8630	0.6939	0.5740	0.8591	0.7562	0.6817
15	0.8244	0.9073	0.2000	0.7882	0.8180	0.9513	0.6555	0.7541	0.8309
16	0.7604	0.9734	0.1731	0.7434	0.7541	0.8853	0.5968	0.6919	0.7667
17	0.5698	0.3851	0.6980	0.7499	0.5748	0.4768	0.7128	0.6263	0.5647
18	0.6342	0.4374	0.6307	0.8029	0.6395	0.5359	0.7831	0.6935	0.6290
19		0.7352	0.2872	0.9110	0.9934	0.8722	0.8223	0.9271	0.9934
20	0.7352		0.1631	0.7257	0.7290	0.8591	0.5740	0.6675	0.7415
21	0.2872	0.1631		0.5197	0.2909	0.2217	0.3993	0.3299	0.2835
22	0.9110	0.7257	0.5197		0.9157	0.8216	0.9625	0.9625	0.9063
23	0.9934	0.7290	0.2909	0.9157		0.8657	0.8288	0.9337	0.9867
24	0.8722	0.8591	0.2217	0.8216	0.8657		0.7000	0.8008	0.8788
25	0.8223	0.5740	0.3993	0.9625	0.8288	0.7000		0.8941	0.8158
26	0.9271	0.6675	0.3299	0.9625	0.9337	0.8008	0.8941		0.9205
27	0.9934	0.7415	0.2835	0.9063	0.9867	0.8788	0.8158	0.9205	

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Time	Flow	pH	CO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5	5.5	18.6233333	0.8045728	<.0001	1
1	5	6.8	19.1466667	0.8045728	<.0001	2
1	7.5	5.5	19.7933333	0.8045728	<.0001	3
1	7.5	6.8	20.2266667	0.8045728	<.0001	4
2	5	5.5	19.6000000	0.8045728	<.0001	5
2	5	6.8	19.5333333	0.8045728	<.0001	6
2	7.5	5.5	19.9600000	0.8045728	<.0001	7
2	7.5	6.8	19.7166667	0.8045728	<.0001	8
3	5	5.5	15.7066667	0.8045728	<.0001	9
3	5	6.8	19.0600000	0.9853964	<.0001	10
3	7.5	5.5	19.9033333	0.8045728	<.0001	11
3	7.5	6.8	18.9000000	0.9853964	<.0001	12
4	5	5.5	17.9300000	0.8045728	<.0001	13
4	5	6.8	17.9633333	0.8045728	<.0001	14
4	7.5	5.5	19.1766667	0.8045728	<.0001	15
4	7.5	6.8	19.1000000	0.8045728	<.0001	16
5	5	5.5	18.0250000	0.9853964	<.0001	17
5	5	6.8	18.4200000	0.9853964	<.0001	18
5	7.5	5.5	19.1366667	0.8045728	<.0001	19
5	7.5	6.8	18.8233333	0.8045728	<.0001	20
6	5	5.5	17.6466667	0.8045728	<.0001	21
6	5	6.8	18.8400000	1.3935610	<.0001	22
6	7.5	5.5	18.9066667	0.8045728	<.0001	23
6	7.5	6.8	18.8533333	0.8045728	<.0001	24
7	5	5.5	18.2866667	0.8045728	<.0001	25
7	7.5	5.5	18.4400000	0.8045728	<.0001	26
7	7.5	6.8	18.3933333	0.8045728	<.0001	27

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: CO

i/j	1	2	3	4	5	6	7	8	9
1		0.6476	0.3090	0.1652	0.3950	0.4278	0.2459	0.3414	0.0136
2	0.6476		0.5725	0.3473	0.6921	0.7355	0.4782	0.6187	0.0040
3	0.3090	0.5725		0.7050	0.8658	0.8202	0.8842	0.9466	0.0008
4	0.1652	0.3473	0.7050		0.5844	0.5452	0.8157	0.6560	0.0002
5	0.3950	0.6921	0.8658	0.5844		0.9535	0.7531	0.9188	0.0013
6	0.4278	0.7355	0.8202	0.5452	0.9535		0.7093	0.8727	0.0015
7	0.2459	0.4782	0.8842	0.8157	0.7531	0.7093		0.8316	0.0005
8	0.3414	0.6187	0.9466	0.6560	0.9188	0.8727	0.8316		0.0009
9	0.0136	0.0040	0.0008	0.0002	0.0013	0.0015	0.0005	0.0009	
10	0.7329	0.9460	0.5670	0.3637	0.6731	0.7115	0.4827	0.6081	0.0113
11	0.2662	0.5092	0.9234	0.7775	0.7909	0.7465	0.9605	0.8704	0.0006
12	0.8288	0.8471	0.4859	0.3022	0.5847	0.6209	0.4088	0.5240	0.0155
13	0.5452	0.2903	0.1080	0.0492	0.1487	0.1652	0.0807	0.1229	0.0565
14	0.5646	0.3036	0.1143	0.0524	0.1568	0.1740	0.0857	0.1299	0.0531
15	0.6290	0.9791	0.5904	0.3607	0.7115	0.7553	0.4945	0.6372	0.0037
16	0.6771	0.9675	0.5452	0.3271	0.6623	0.7050	0.4535	0.5904	0.0045
17	0.6402	0.3823	0.1709	0.0899	0.2217	0.2416	0.1348	0.1899	0.0746
18	0.8737	0.5705	0.2857	0.1620	0.3583	0.3858	0.2320	0.3132	0.0381
19	0.6539	0.9930	0.5666	0.3429	0.6857	0.7289	0.4728	0.6126	0.0041
20	0.8612	0.7775	0.3982	0.2235	0.4982	0.5356	0.3228	0.4362	0.0086
21	0.3950	0.1937	0.0653	0.0279	0.0925	0.1038	0.0476	0.0751	0.0947
22	0.8935	0.8497	0.5563	0.3931	0.6389	0.6685	0.4898	0.5884	0.0574
23	0.8044	0.8338	0.4397	0.2517	0.5452	0.5844	0.3592	0.4800	0.0071
24	0.8407	0.7977	0.4128	0.2334	0.5148	0.5529	0.3356	0.4517	0.0080
25	0.7686	0.4535	0.1917	0.0947	0.2541	0.2787	0.1479	0.2149	0.0279
26	0.8727	0.5375	0.2401	0.1229	0.3131	0.3414	0.1879	0.2674	0.0202
27	0.8407	0.5111	0.2245	0.1137	0.2942	0.3214	0.1749	0.2506	0.0223

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Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: CO

i/j	10	11	12	13	14	15	16	17	18
1	0.7329	0.2662	0.8288	0.5452	0.5646	0.6290	0.6771	0.6402	0.8737
2	0.9460	0.5092	0.8471	0.2903	0.3036	0.9791	0.9675	0.3823	0.5705
3	0.5670	0.9234	0.4859	0.1080	0.1143	0.5904	0.5452	0.1709	0.2857
4	0.3637	0.7775	0.3022	0.0492	0.0524	0.3607	0.3271	0.0899	0.1620
5	0.6731	0.7909	0.5847	0.1487	0.1568	0.7115	0.6623	0.2217	0.3583
6	0.7115	0.7465	0.6209	0.1652	0.1740	0.7553	0.7050	0.2416	0.3858
7	0.4827	0.9605	0.4088	0.0807	0.0857	0.4945	0.4535	0.1348	0.2320
8	0.6081	0.8704	0.5240	0.1229	0.1299	0.6372	0.5904	0.1899	0.3132
9	0.0113	0.0006	0.0155	0.0565	0.0531	0.0037	0.0045	0.0746	0.0381
10		0.5106	0.9091	0.3788	0.3929	0.9273	0.9750	0.4613	0.6481
11	0.5106		0.4342	0.0893	0.0947	0.5261	0.4836	0.1463	0.2494
12	0.9091	0.4342		0.4495	0.4651	0.8288	0.8757	0.5331	0.7320
13	0.3788	0.0893	0.4495		0.9768	0.2787	0.3090	0.9408	0.7018
14	0.3929	0.0947	0.4651	0.9768		0.2916	0.3228	0.9615	0.7212
15	0.9273	0.5261	0.8288	0.2787	0.2916		0.9466	0.3698	0.5548
16	0.9750	0.4836	0.8757	0.3090	0.3228	0.9466		0.4023	0.5954
17	0.4613	0.1463	0.5331	0.9408	0.9615	0.3698	0.4023		0.7781
18	0.6481	0.2494	0.7320	0.7018	0.7212	0.5548	0.5954	0.7781	
19	0.9522	0.5037	0.8532	0.2942	0.3076	0.9721	0.9744	0.3865	0.5758
20	0.8532	0.3473	0.9522	0.4362	0.4535	0.7575	0.8089	0.5333	0.7526
21	0.2721	0.0531	0.3295	0.8044	0.7820	0.1851	0.2076	0.7674	0.5461
22	0.8980	0.5119	0.9721	0.5744	0.5884	0.8352	0.8723	0.6352	0.8067
23	0.9046	0.3854	0.9958	0.3950	0.4112	0.8134	0.8658	0.4916	0.7037
24	0.8716	0.3607	0.9709	0.4211	0.4379	0.7775	0.8293	0.5181	0.7349
25	0.5461	0.1618	0.6319	0.7553	0.7775	0.4379	0.4782	0.8379	0.9170
26	0.6282	0.2046	0.7192	0.6560	0.6771	0.5204	0.5646	0.7457	0.9875
27	0.6027	0.1908	0.6922	0.6857	0.7072	0.4945	0.5375	0.7734	0.9834

Dependent Variable: CO

i/j	19	20	21	22	23	24	25	26	27
1	0.6539	0.8612	0.3950	0.8935	0.8044	0.8407	0.7686	0.8727	0.8407
2	0.9930	0.7775	0.1937	0.8497	0.8338	0.7977	0.4535	0.5375	0.5111
3	0.5666	0.3982	0.0653	0.5563	0.4397	0.4128	0.1917	0.2401	0.2245
4	0.3429	0.2235	0.0279	0.3931	0.2517	0.2334	0.0947	0.1229	0.1137
5	0.6857	0.4982	0.0925	0.6389	0.5452	0.5148	0.2541	0.3131	0.2942
6	0.7289	0.5356	0.1038	0.6685	0.5844	0.5529	0.2787	0.3414	0.3214
7	0.4728	0.3228	0.0476	0.4898	0.3592	0.3356	0.1479	0.1879	0.1749
8	0.6126	0.4362	0.0751	0.5884	0.4800	0.4517	0.2149	0.2674	0.2506
9	0.0041	0.0086	0.0947	0.0574	0.0071	0.0080	0.0279	0.0202	0.0223
10	0.9522	0.8532	0.2721	0.8980	0.9046	0.8716	0.5461	0.6282	0.6027
11	0.5037	0.3473	0.0531	0.5119	0.3854	0.3607	0.1618	0.2046	0.1908
12	0.8532	0.9522	0.3295	0.9721	0.9958	0.9709	0.6319	0.7192	0.6922
13	0.2942	0.4362	0.8044	0.5744	0.3950	0.4211	0.7553	0.6560	0.6857
14	0.3076	0.4535	0.7820	0.5884	0.4112	0.4379	0.7775	0.6771	0.7072
15	0.9721	0.7575	0.1851	0.8352	0.8134	0.7775	0.4379	0.5204	0.4945
16	0.9744	0.8089	0.2076	0.8723	0.8658	0.8293	0.4782	0.5646	0.5375
17	0.3865	0.5333	0.7674	0.6352	0.4916	0.5181	0.8379	0.7457	0.7734
18	0.5758	0.7526	0.5461	0.8067	0.7037	0.7349	0.9170	0.9875	0.9834
19		0.7842	0.1966	0.8545	0.8407	0.8044	0.4587	0.5432	0.5167
20	0.7842		0.3063	0.9918	0.9419	0.9791	0.6393	0.7377	0.7072
21	0.1966	0.3063		0.4619	0.2737	0.2942	0.5764	0.4890	0.5148
22	0.8545	0.9918	0.4619		0.9671	0.9934	0.7324	0.8047	0.7825
23	0.8407	0.9419	0.2737	0.9671		0.9628	0.5883	0.6835	0.6539
24	0.8044	0.9791	0.2942	0.9934	0.9628		0.6207	0.7180	0.6878
25	0.4587	0.6393	0.5764	0.7324	0.5883	0.6207		0.8934	0.9257
26	0.5432	0.7377	0.4890	0.8047	0.6835	0.7180	0.8934		0.9675
27	0.5167	0.7072	0.5148	0.7825	0.6539	0.6878	0.9257	0.9675	

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Time	Flow	pH	CO2 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5	5.5	18.8733333	0.6567970	<.0001	1
1	5	6.8	18.8933333	0.6567970	<.0001	2
1	7.5	5.5	19.7100000	0.6567970	<.0001	3
1	7.5	6.8	19.5133333	0.6567970	<.0001	4
2	5	5.5	18.3566667	0.6567970	<.0001	5
2	5	6.8	17.8933333	0.6567970	<.0001	6
2	7.5	5.5	20.1100000	0.6567970	<.0001	7
2	7.5	6.8	20.8266667	0.6567970	<.0001	8
3	5	5.5	16.3233333	0.6567970	<.0001	9
3	5	6.8	19.3700000	0.8044088	<.0001	10
3	7.5	5.5	20.4466667	0.6567970	<.0001	11
3	7.5	6.8	21.9100000	0.8044088	<.0001	12
4	5	5.5	20.2466667	0.6567970	<.0001	13
4	5	6.8	21.4966667	0.6567970	<.0001	14
4	7.5	5.5	20.4100000	0.6567970	<.0001	15
4	7.5	6.8	22.0500000	0.6567970	<.0001	16
5	5	5.5	19.1900000	0.8044088	<.0001	17
5	5	6.8	21.0350000	0.8044088	<.0001	18
5	7.5	5.5	21.3633333	0.6567970	<.0001	19
5	7.5	6.8	23.5266667	0.6567970	<.0001	20
6	5	5.5	20.0466667	0.6567970	<.0001	21
6	5	6.8	22.5400000	1.1376058	<.0001	22
6	7.5	5.5	21.0033333	0.6567970	<.0001	23
6	7.5	6.8	23.1533333	0.6567970	<.0001	24
7	5	5.5	20.2633333	0.6567970	<.0001	25
7	7.5	5.5	20.7066667	0.6567970	<.0001	26
7	7.5	6.8	23.0666667	0.6567970	<.0001	27

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: CO2

i/j	1	2	3	4	5	6	7	8	9
1		0.9829	0.3722	0.4941	0.5806	0.2967	0.1893	0.0407	0.0085
2	0.9829		0.3837	0.5077	0.5661	0.2870	0.1965	0.0428	0.0080
3	0.3722	0.3837		0.8332	0.1516	0.0563	0.6687	0.2352	0.0007
4	0.4941	0.5077	0.8332		0.2191	0.0875	0.5237	0.1638	0.0012
5	0.5806	0.5661	0.1516	0.2191		0.6202	0.0651	0.0106	0.0335
6	0.2967	0.2870	0.0563	0.0875	0.6202		0.0210	0.0027	0.0975
7	0.1893	0.1965	0.6687	0.5237	0.0651	0.0210		0.4442	0.0002
8	0.0407	0.0428	0.2352	0.1638	0.0106	0.0027	0.4442		<.0001
9	0.0085	0.0080	0.0007	0.0012	0.0335	0.0975	0.0002	<.0001	
10	0.6346	0.6483	0.7448	0.8908	0.3341	0.1615	0.4796	0.1671	0.0051
11	0.0968	0.1010	0.4316	0.3200	0.0291	0.0084	0.7186	0.6843	<.0001
12	0.0053	0.0055	0.0393	0.0254	0.0013	0.0003	0.0895	0.3021	<.0001
13	0.1458	0.1516	0.5661	0.4337	0.0474	0.0146	0.8836	0.5353	0.0001
14	0.0069	0.0073	0.0604	0.0379	0.0014	0.0003	0.1420	0.4742	<.0001
15	0.1046	0.1090	0.4548	0.3392	0.0319	0.0093	0.7481	0.6558	<.0001
16	0.0013	0.0014	0.0151	0.0088	0.0002	<.0001	0.0421	0.1941	<.0001
17	0.7617	0.7764	0.6189	0.7569	0.4262	0.2179	0.3801	0.1216	0.0082
18	0.0427	0.0446	0.2081	0.1494	0.0130	0.0040	0.3775	0.8418	<.0001
19	0.0100	0.0106	0.0814	0.0521	0.0022	0.0005	0.1836	0.5661	<.0001
20	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	0.0006	0.0055	<.0001
21	0.2126	0.2204	0.7186	0.5685	0.0751	0.0247	0.9459	0.4052	0.0002
22	0.0075	0.0078	0.0363	0.0256	0.0025	0.0009	0.0705	0.1983	<.0001
23	0.0263	0.0276	0.1702	0.1152	0.0064	0.0016	0.3410	0.8500	<.0001
24	<.0001	<.0001	0.0005	0.0003	<.0001	<.0001	0.0020	0.0157	<.0001
25	0.1411	0.1468	0.5542	0.4234	0.0456	0.0140	0.8696	0.5471	0.0001
26	0.0542	0.0568	0.2886	0.2050	0.0147	0.0039	0.5237	0.8977	<.0001
27	<.0001	<.0001	0.0007	0.0004	<.0001	<.0001	0.0026	0.0198	<.0001

C. ljungdahlii: syngass 06/21/2006
LSMeans for Main and Interaction Affects
Gas Composition over Time

The GLM Procedure
Least Squares Means

Least Squares Means for effect Time*Flow*pH
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: CO2

i/j	10	11	12	13	14	15	16	17	18
1	0.6346	0.0968	0.0053	0.1458	0.0069	0.1046	0.0013	0.7617	0.0427
2	0.6483	0.1010	0.0055	0.1516	0.0073	0.1090	0.0014	0.7764	0.0446
3	0.7448	0.4316	0.0393	0.5661	0.0604	0.4548	0.0151	0.6189	0.2081
4	0.8908	0.3200	0.0254	0.4337	0.0379	0.3392	0.0088	0.7569	0.1494
5	0.3341	0.0291	0.0013	0.0474	0.0014	0.0319	0.0002	0.4262	0.0130
6	0.1615	0.0084	0.0003	0.0146	0.0003	0.0093	<.0001	0.2179	0.0040
7	0.4796	0.7186	0.0895	0.8836	0.1420	0.7481	0.0421	0.3801	0.3775
8	0.1671	0.6843	0.3021	0.5353	0.4742	0.6558	0.1941	0.1216	0.8418
9	0.0051	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	0.0082	<.0001
10		0.3050	0.0303	0.4028	0.0461	0.3216	0.0130	0.8749	0.1498
11	0.3050		0.1653	0.8304	0.2639	0.9687	0.0908	0.2322	0.5737
12	0.0303	0.1653		0.1158	0.6924	0.1551	0.8933	0.0208	0.4456
13	0.4028	0.8304	0.1158		0.1847	0.8612	0.0581	0.3140	0.4515
14	0.0461	0.2639	0.6924	0.1847		0.2478	0.5542	0.0311	0.6586
15	0.3216	0.9687	0.1551	0.8612	0.2478		0.0838	0.2459	0.5501
16	0.0130	0.0908	0.8933	0.0581	0.5542	0.0838		0.0083	0.3333
17	0.8749	0.2322	0.0208	0.3140	0.0311	0.2459	0.0083		0.1114
18	0.1498	0.5737	0.4456	0.4515	0.6586	0.5501	0.3333	0.1114	
19	0.0609	0.3286	0.6010	0.2352	0.8865	0.3099	0.4633	0.0417	0.7532
20	0.0002	0.0017	0.1261	0.0009	0.0338	0.0016	0.1185	0.0001	0.0204
21	0.5178	0.6687	0.0791	0.8304	0.1251	0.6974	0.0361	0.4135	0.3460
22	0.0274	0.1176	0.6532	0.0872	0.4310	0.1115	0.7108	0.0201	0.2855
23	0.1223	0.5518	0.3870	0.4193	0.5978	0.5260	0.2654	0.0872	0.9758
24	0.0007	0.0054	0.2371	0.0030	0.0808	0.0049	0.2407	0.0004	0.0469
25	0.3939	0.8444	0.1194	0.9858	0.1905	0.8752	0.0604	0.3065	0.4611
26	0.2042	0.7807	0.2523	0.6227	0.3993	0.7508	0.1546	0.1507	0.7532
27	0.0008	0.0069	0.2709	0.0039	0.0975	0.0063	0.2792	0.0005	0.0563

Dependent Variable: CO2

i/j	19	20	21	22	23	24	25	26	27
1	0.0100	<.0001	0.2126	0.0075	0.0263	<.0001	0.1411	0.0542	<.0001
2	0.0106	<.0001	0.2204	0.0078	0.0276	<.0001	0.1468	0.0568	<.0001
3	0.0814	0.0002	0.7186	0.0363	0.1702	0.0005	0.5542	0.2886	0.0007
4	0.0521	<.0001	0.5685	0.0256	0.1152	0.0003	0.4234	0.2050	0.0004
5	0.0022	<.0001	0.0751	0.0025	0.0064	<.0001	0.0456	0.0147	<.0001
6	0.0005	<.0001	0.0247	0.0009	0.0016	<.0001	0.0140	0.0039	<.0001
7	0.1836	0.0006	0.9459	0.0705	0.3410	0.0020	0.8696	0.5237	0.0026
8	0.5661	0.0055	0.4052	0.1983	0.8500	0.0157	0.5471	0.8977	0.0198
9	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	0.0001	<.0001	<.0001
10	0.0609	0.0002	0.5178	0.0274	0.1223	0.0007	0.3939	0.2042	0.0008
11	0.3286	0.0017	0.6687	0.1176	0.5518	0.0054	0.8444	0.7807	0.0069
12	0.6010	0.1261	0.0791	0.6532	0.3870	0.2371	0.1194	0.2523	0.2709
13	0.2352	0.0009	0.8304	0.0872	0.4193	0.0030	0.9858	0.6227	0.0039
14	0.8865	0.0338	0.1251	0.4310	0.5978	0.0808	0.1905	0.3993	0.0975
15	0.3099	0.0016	0.6974	0.1115	0.5260	0.0049	0.8752	0.7508	0.0063
16	0.4633	0.1185	0.0361	0.7108	0.2654	0.2407	0.0604	0.1546	0.2792
17	0.0417	0.0001	0.4135	0.0201	0.0872	0.0004	0.3065	0.1507	0.0005
18	0.7532	0.0204	0.3460	0.2855	0.9758	0.0469	0.4611	0.7532	0.0563
19		0.0241	0.1628	0.3749	0.7000	0.0599	0.2421	0.4830	0.0729
20	0.0241		0.0005	0.4562	0.0091	0.6895	0.0010	0.0039	0.6227
21	0.1628	0.0005		0.0637	0.3082	0.0016	0.8165	0.4808	0.0021
22	0.3749	0.4562	0.0637		0.2478	0.6427	0.0895	0.1692	0.6902
23	0.7000	0.0091	0.3082	0.2478		0.0250	0.4296	0.7508	0.0311
24	0.0599	0.6895	0.0016	0.6427	0.0250		0.0031	0.0113	0.9260
25	0.2421	0.0010	0.8165	0.0895	0.4296	0.0031		0.6353	0.0041
26	0.4830	0.0039	0.4808	0.1692	0.7508	0.0113	0.6353		0.0143
27	0.0729	0.6227	0.0021	0.6902	0.0311	0.9260	0.0041	0.0143	

Appendix F.3: SAS Analysis of *C. autoethanogenum*: Ethanol and Acetate over Time

```
Title1 'C. autoethanogenum on Syngas 06/27/2006 ';
Title2 'LSMeans for Main and Interaction Affects';
Title3 'Ethanol and Acetate Production';
Data practice;
Input ethanol acetate time flow;
Cards;
0.24      0.47      0.0       5
0.22      0.00      0.0       5
0.25      0.00      0.0       5
0.27      0.00      12.0      5
0.30      0.00      12.0      5
0.10      0.38      12.0      5
0.35      1.00      24.0      5
0.30      0.85      24.0      5
0.28      0.95      24.0      5
0.45      3.09      36.0      5
0.38      2.27      36.0      5
0.41      2.84      36.0      5
0.61      6.49      44.0      5
0.60      5.01      44.0      5
0.64      5.81      44.0      5
0.77      8.09      48.0      5
0.70      6.31      48.0      5
0.71      7.43      48.0      5
1.00      14.14     60.0      5
1.08      12.41     60.0      5
1.03      14.38     60.0      5
1.23      18.58     68        5
1.25      16.79     68        5
1.22      19.31     68        5
0.35      0.46      0.0       7.5
0.34      0.00      0.0       7.5
0.31      0.00      0.0       7.5
0.30      0.00      12.0      7.5
0.33      0.00      12.0      7.5
0.28      0.00      12.0      7.5
0.31      0.00      24.0      7.5
0.35      1.16      24.0      7.5
0.33      0.93      24.0      7.5
0.35      1.87      36.0      7.5
0.43      2.59      36.0      7.5
0.40      2.15      36.0      7.5
0.29      0.96      44.0      7.5
0.48      3.73      44.0      7.5
0.41      2.70      44.0      7.5
0.35      1.33      48.0      7.5
0.56      5.30      48.0      7.5
0.48      3.18      48.0      7.5
0.49      3.63      60.0      7.5
0.84      9.69      60.0      7.5
0.66      7.15      60.0      7.5
0.63      5.41      68        7.5
1.07      12.23     68        7.5
0.79      8.28      68        7.5
0.29      0.00      0.0       10
0.29      0.00      0.0       10
0.32      0.42      12.0      10
0.33      0.00      12.0      10
0.33      0.75      24.0      10
0.36      1.09      24.0      10
0.57      4.42      36.0      10
0.61      3.48      36.0      10
0.77      8.71      44.0      10
0.70      6.10      44.0      10
0.78      7.86      48.0      10
0.84      8.41      48.0      10
0.88      13.01     60.0      10
1.22      15.71     60.0      10
1.47      25.65     68.0      10
1.43      21.01     68.0      10

;
Options NODATE PAGENO = 1;
Proc GLM Data = practice;
Class flow time;

model ethanol acetate/*put in desired gas to analyze*/ = Flow|time ;

lsmeans Flow|time/pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
```

LSMeans for Main and Interaction Affects
Ethanol and Acetate Production

The GLM Procedure

Class Level Information

Class	Levels	Values
flow	3	5 7.5 10
time	8	0 12 24 36 44 48 60 68

Number of Observations Read 64
Number of Observations Used 64

The GLM Procedure

Dependent Variable: ethanol

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	6.58565677	0.28633290	37.18	<.0001
Error	40	0.30801667	0.00770042		
Corrected Total	63	6.89367344			

R-Square	Coeff Var	Root MSE	ethanol Mean
0.955319	15.29864	0.087752	0.573594

Source	DF	Type I SS	Mean Square	F Value	Pr > F
flow	2	0.55892135	0.27946068	36.29	<.0001
time	7	5.39878594	0.77125513	100.16	<.0001
flow*time	14	0.62794948	0.04485353	5.82	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
flow	2	0.55892135	0.27946068	36.29	<.0001
time	7	5.55407113	0.79343873	103.04	<.0001
flow*time	14	0.62794948	0.04485353	5.82	<.0001

Dependent Variable: acetate

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	23	2281.250786	99.184817	48.09	<.0001
Error	40	82.497900	2.062447		
Corrected Total	63	2363.748686			

R-Square	Coeff Var	Root MSE	acetate Mean
0.965099	27.35715	1.436122	5.249531

Source	DF	Type I SS	Mean Square	F Value	Pr > F
flow	2	202.334115	101.167058	49.05	<.0001
time	7	1828.273998	261.182000	126.64	<.0001
flow*time	14	250.642672	17.903048	8.68	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
flow	2	202.334115	101.167058	49.05	<.0001
time	7	1930.909624	275.844232	133.75	<.0001
flow*time	14	250.642672	17.903048	8.68	<.0001

The GLM Procedure
Least Squares Means

flow	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	0.59958333	0.01791231	<.0001	1
7.5	0.46375000	0.01791231	<.0001	2
10	0.69937500	0.02193800	<.0001	3

Least Squares Means for effect flow
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3
1		<.0001	0.0011
2	<.0001		<.0001
3	0.0011	<.0001	

flow	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	6.10833333	0.29314725	<.0001	1
7.5	3.03125000	0.29314725	<.0001	2
10	7.28875000	0.35903060	<.0001	3

Least Squares Means for effect flow
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3
1		<.0001	0.0148
2	<.0001		<.0001
3	0.0148	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.28666667	0.03159434	<.0001	1
12	0.28388889	0.03159434	<.0001	2
24	0.32833333	0.03159434	<.0001	3
36	0.46555556	0.03159434	<.0001	4
44	0.58166667	0.03159434	<.0001	5
48	0.66666667	0.03159434	<.0001	6
60	0.91666667	0.03159434	<.0001	7
68	1.17111111	0.03159434	<.0001	8

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8
1		0.9507	0.3567	0.0003	<.0001	<.0001	<.0001	<.0001
2	0.9507		0.3259	0.0002	<.0001	<.0001	<.0001	<.0001
3	0.3567	0.3259		0.0038	<.0001	<.0001	<.0001	<.0001
4	0.0003	0.0002	0.0038		0.0130	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.0130		0.0643	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	0.0643		<.0001	<.0001

7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	

LSMeans for Main and Interaction Affects
Ethanol and Acetate Production

time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
0	0.1033333	0.5170632	0.8426	1
12	0.1122222	0.5170632	0.8293	2
24	0.8500000	0.5170632	0.1080	3
36	2.9622222	0.5170632	<.0001	4
44	5.2127778	0.5170632	<.0001	5
48	6.2272222	0.5170632	<.0001	6
60	11.6088889	0.5170632	<.0001	7
68	16.7322222	0.5170632	<.0001	8

Least Squares Means for effect time
Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8
1		0.9904	0.3133	0.0003	<.0001	<.0001	<.0001	<.0001
2	0.9904		0.3191	0.0004	<.0001	<.0001	<.0001	<.0001
3	0.3133	0.3191		0.0062	<.0001	<.0001	<.0001	<.0001
4	0.0003	0.0004	0.0062		0.0038	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	0.0038		0.1730	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	0.1730		<.0001	<.0001
7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	

flow	time	ethanol LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	0	0.23666667	0.05066365	<.0001	1
5	12	0.22333333	0.05066365	<.0001	2
5	24	0.31000000	0.05066365	<.0001	3
5	36	0.41333333	0.05066365	<.0001	4
5	44	0.61666667	0.05066365	<.0001	5
5	48	0.72666667	0.05066365	<.0001	6
5	60	1.03666667	0.05066365	<.0001	7
5	68	1.23333333	0.05066365	<.0001	8
7.5	0	0.33333333	0.05066365	<.0001	9
7.5	12	0.30333333	0.05066365	<.0001	10
7.5	24	0.33000000	0.05066365	<.0001	11
7.5	36	0.39333333	0.05066365	<.0001	12
7.5	44	0.39333333	0.05066365	<.0001	13
7.5	48	0.46333333	0.05066365	<.0001	14
7.5	60	0.66333333	0.05066365	<.0001	15
7.5	68	0.83000000	0.05066365	<.0001	16
10	0	0.29000000	0.06205005	<.0001	17
10	12	0.32500000	0.06205005	<.0001	18
10	24	0.34500000	0.06205005	<.0001	19
10	36	0.59000000	0.06205005	<.0001	20
10	44	0.73500000	0.06205005	<.0001	21
10	48	0.81000000	0.06205005	<.0001	22
10	60	1.05000000	0.06205005	<.0001	23
10	68	1.45000000	0.06205005	<.0001	24

Least Squares Means for effect flow*time
Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.8533	0.3122	0.0181	<.0001	<.0001	<.0001	<.0001	0.1849	0.3577	0.2001	0.0347
2	0.8533		0.2335	0.0114	<.0001	<.0001	<.0001	<.0001	0.1326	0.2708	0.1444	0.0226
3	0.3122	0.2335		0.1570	0.0001	<.0001	<.0001	<.0001	0.7464	0.9263	0.7816	0.2517
4	0.0181	0.0114	0.1570		0.0071	<.0001	<.0001	<.0001	0.2708	0.1326	0.2517	0.7816
5	<.0001	<.0001	0.0001	0.0071		0.1326	<.0001	<.0001	0.0003	<.0001	0.0003	0.0034
6	<.0001	<.0001	<.0001	<.0001	0.1326		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0090	<.0001	<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0090		<.0001	<.0001	<.0001	<.0001
9	0.1849	0.1326	0.7464	0.2708	0.0003	<.0001	<.0001	<.0001		0.6777	0.9631	0.4073
10	0.3577	0.2708	0.9263	0.1326	<.0001	<.0001	<.0001	<.0001	0.6777		0.7117	0.2164
11	0.2001	0.1444	0.7816	0.2517	0.0003	<.0001	<.0001	<.0001	0.9631	0.7117		0.3820

LSMeans for Main and Interaction Affects
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The GLM Procedure
Least Squares Means

Least Squares Means for effect flow*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	1	2	3	4	5	6	7	8	9	10	11	12
12	0.0347	0.0226	0.2517	0.7816	0.0034	<.0001	<.0001	<.0001	0.4073	0.2164	0.3820	
13	0.0347	0.0226	0.2517	0.7816	0.0034	<.0001	<.0001	<.0001	0.4073	0.2164	0.3820	1.0000
14	0.0030	0.0018	0.0385	0.4893	0.0385	0.0007	<.0001	<.0001	0.0771	0.0312	0.0701	0.3345
15	<.0001	<.0001	<.0001	0.0012	0.5186	0.3820	<.0001	<.0001	<.0001	<.0001	<.0001	0.0005
16	<.0001	<.0001	<.0001	<.0001	0.0049	0.1570	0.0063	<.0001	<.0001	<.0001	<.0001	<.0001
17	0.5094	0.4102	0.8041	0.1315	0.0002	<.0001	<.0001	<.0001	0.5915	0.8686	0.6203	0.2045
18	0.2767	0.2117	0.8524	0.2767	0.0008	<.0001	<.0001	<.0001	0.9177	0.7882	0.9505	0.3987
19	0.1839	0.1367	0.6645	0.3987	0.0016	<.0001	<.0001	<.0001	0.8849	0.6058	0.8524	0.5497
20	<.0001	<.0001	0.0012	0.0332	0.7410	0.0957	<.0001	<.0001	0.0027	0.0009	0.0024	0.0185
21	<.0001	<.0001	<.0001	0.0003	0.1475	0.9177	0.0005	<.0001	<.0001	<.0001	<.0001	0.0001
22	<.0001	<.0001	<.0001	<.0001	0.0205	0.3045	0.0073	<.0001	<.0001	<.0001	<.0001	<.0001
23	<.0001	<.0001	<.0001	<.0001	<.0001	0.0002	0.8686	0.0275	<.0001	<.0001	<.0001	<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0100	<.0001	<.0001	<.0001	<.0001

Least Squares Means for effect flow*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	13	14	15	16	17	18	19	20	21	22	23	24
1	0.0347	0.0030	<.0001	<.0001	0.5094	0.2767	0.1839	<.0001	<.0001	<.0001	<.0001	<.0001
2	0.0226	0.0018	<.0001	<.0001	0.4102	0.2117	0.1367	<.0001	<.0001	<.0001	<.0001	<.0001
3	0.2517	0.0385	<.0001	<.0001	0.8041	0.8524	0.6645	0.0012	<.0001	<.0001	<.0001	<.0001
4	0.7816	0.4893	0.0012	<.0001	0.1315	0.2767	0.3987	0.0332	0.0003	<.0001	<.0001	<.0001
5	0.0034	0.0385	0.5186	0.0049	0.0002	0.0008	0.0016	0.7410	0.1475	0.0205	<.0001	<.0001
6	<.0001	0.0007	0.3820	0.1570	<.0001	<.0001	<.0001	0.0957	0.9177	0.3045	0.0002	<.0001
7	<.0001	<.0001	<.0001	0.0063	<.0001	<.0001	<.0001	<.0001	0.0005	0.0073	0.8686	<.0001

Least Squares Means for effect flow*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: ethanol

i/j	13	14	15	16	17	18	19	20	21	22	23	24
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0275	0.0100
9	0.4073	0.0771	<.0001	<.0001	0.5915	0.9177	0.8849	0.0027	<.0001	<.0001	<.0001	<.0001
10	0.2164	0.0312	<.0001	<.0001	0.8686	0.7882	0.6058	0.0009	<.0001	<.0001	<.0001	<.0001
11	0.3820	0.0701	<.0001	<.0001	0.6203	0.9505	0.8524	0.0024	<.0001	<.0001	<.0001	<.0001
12	1.0000	0.3345	0.0005	<.0001	0.2045	0.3987	0.5497	0.0185	0.0001	<.0001	<.0001	<.0001
13		0.3345	0.0005	<.0001	0.2045	0.3987	0.5497	0.0185	0.0001	<.0001	<.0001	<.0001
14	0.3345		0.0080	<.0001	0.0365	0.0919	0.1475	0.1217	0.0016	<.0001	<.0001	<.0001
15	0.0005	0.0080		0.0252	<.0001	0.0001	0.0003	0.3654	0.3763	0.0746	<.0001	<.0001
16	<.0001	<.0001	0.0252		<.0001	<.0001	<.0001	0.0047	0.2426	0.8041	0.0090	<.0001
17	0.2045	0.0365	<.0001	<.0001		0.6921	0.5344	0.0015	<.0001	<.0001	<.0001	<.0001
18	0.3987	0.0919	0.0001	<.0001	0.6921		0.8209	0.0044	<.0001	<.0001	<.0001	<.0001
19	0.5497	0.1475	0.0003	<.0001	0.5344	0.8209		0.0080	<.0001	<.0001	<.0001	<.0001
20	0.0185	0.1217	0.3654	0.0047	0.0015	0.0044	0.0080		0.1063	0.0163	<.0001	<.0001
21	0.0001	0.0016	0.3763	0.2426	<.0001	<.0001	<.0001	0.1063		0.3978	0.0009	<.0001
22	<.0001	<.0001	0.0746	0.8041	<.0001	<.0001	<.0001	0.0163	0.3978		0.0093	<.0001
23	<.0001	<.0001	<.0001	0.0090	<.0001	<.0001	<.0001	<.0001	0.0009	0.0093		<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	

LSMeans for Main and Interaction Affects
Ethanol and Acetate Production

The GLM Procedure
Least Squares Means

flow	time	acetate LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	0	0.1566667	0.8291456	0.8511	1
5	12	0.1266667	0.8291456	0.8793	2
5	24	0.9333333	0.8291456	0.2670	3
5	36	2.7333333	0.8291456	0.0021	4
5	44	5.7700000	0.8291456	<.0001	5
5	48	7.2766667	0.8291456	<.0001	6
5	60	13.6433333	0.8291456	<.0001	7
5	68	18.2266667	0.8291456	<.0001	8
7.5	0	0.1533333	0.8291456	0.8542	9
7.5	12	-0.0000000	0.8291456	1.0000	10
7.5	24	0.6966667	0.8291456	0.4058	11
7.5	36	2.2033333	0.8291456	0.0113	12
7.5	44	2.4633333	0.8291456	0.0050	13
7.5	48	3.2700000	0.8291456	0.0003	14
7.5	60	6.8233333	0.8291456	<.0001	15
7.5	68	8.6400000	0.8291456	<.0001	16
10	0	-0.0000000	1.0154919	1.0000	17
10	12	0.2100000	1.0154919	0.8372	18
10	24	0.9200000	1.0154919	0.3704	19
10	36	3.9500000	1.0154919	0.0004	20
10	44	7.4050000	1.0154919	<.0001	21
10	48	8.1350000	1.0154919	<.0001	22
10	60	14.3600000	1.0154919	<.0001	23
10	68	23.3300000	1.0154919	<.0001	24

Least Squares Means for effect flow*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	1	2	3	4	5	6	7	8	9	10	11	12
1		0.9797	0.5115	0.0338	<.0001	<.0001	<.0001	<.0001	0.9977	0.8944	0.6476	0.0886
2	0.9797		0.4955	0.0319	<.0001	<.0001	<.0001	<.0001	0.9820	0.9145	0.6295	0.0842
3	0.5115	0.4955		0.1326	0.0002	<.0001	<.0001	<.0001	0.5097	0.4308	0.8411	0.2853
4	0.0338	0.0319	0.1326		0.0133	0.0004	<.0001	<.0001	0.0336	0.0249	0.0901	0.6537
5	<.0001	<.0001	0.0002	0.0133		0.2062	<.0001	<.0001	<.0001	<.0001	<.0001	0.0041
6	<.0001	<.0001	<.0001	0.0004	0.2062		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0003	<.0001	<.0001	<.0001	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003		<.0001	<.0001	<.0001	<.0001
9	0.9977	0.9820	0.5097	0.0336	<.0001	<.0001	<.0001	<.0001		0.8966	0.6456	0.0881
10	0.8944	0.9145	0.4308	0.0249	<.0001	<.0001	<.0001	<.0001	0.8966		0.5558	0.0675
11	0.6476	0.6295	0.8411	0.0901	<.0001	<.0001	<.0001	<.0001	0.6456	0.5558		0.2062
12	0.0886	0.0842	0.2853	0.6537	0.0041	<.0001	<.0001	<.0001	0.0881	0.0675	0.2062	
13	0.0561	0.0531	0.1994	0.8191	0.0074	0.0002	<.0001	<.0001	0.0558	0.0420	0.1398	0.8257
14	0.0113	0.0106	0.0531	0.6497	0.0392	0.0015	<.0001	<.0001	0.0112	0.0081	0.0341	0.3684
15	<.0001	<.0001	<.0001	0.0012	0.3744	0.7011	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003
16	<.0001	<.0001	<.0001	<.0001	0.0189	0.2519	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	0.9055	0.9235	0.4806	0.0435	<.0001	<.0001	<.0001	<.0001	0.9075	1.0000	0.5981	0.1006
18	0.9678	0.9496	0.5842	0.0614	0.0001	<.0001	<.0001	<.0001	0.9657	0.8735	0.7124	0.1363
19	0.5637	0.5485	0.9919	0.1743	0.0006	<.0001	<.0001	<.0001	0.5620	0.4869	0.8656	0.3335
20	0.0061	0.0058	0.0267	0.3589	0.1727	0.0152	<.0001	<.0001	0.0061	0.0045	0.0174	0.1903
21	<.0001	<.0001	<.0001	0.0010	0.2196	0.9225	<.0001	<.0001	<.0001	<.0001	<.0001	0.0003
22	<.0001	<.0001	<.0001	0.0002	0.0788	0.5164	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001
23	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.5876	0.0053	<.0001	<.0001	<.0001	<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0004	<.0001	<.0001	<.0001	<.0001

LSMeans for Main and Interaction Affects
Ethanol and Acetate Production

The GLM Procedure
Least Squares Means

Least Squares Means for effect flow*time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: acetate

i/j	13	14	15	16	17	18	19	20	21	22	23	24
1	0.0561	0.0113	<.0001	<.0001	0.9055	0.9678	0.5637	0.0061	<.0001	<.0001	<.0001	<.0001
2	0.0531	0.0106	<.0001	<.0001	0.9235	0.9496	0.5485	0.0058	<.0001	<.0001	<.0001	<.0001
3	0.1994	0.0531	<.0001	<.0001	0.4806	0.5842	0.9919	0.0267	<.0001	<.0001	<.0001	<.0001
4	0.8191	0.6497	0.0012	<.0001	0.0435	0.0614	0.1743	0.3589	0.0010	0.0002	<.0001	<.0001
5	0.0074	0.0392	0.3744	0.0189	<.0001	0.0001	0.0006	0.1727	0.2196	0.0788	<.0001	<.0001
6	0.0002	0.0015	0.7011	0.2519	<.0001	<.0001	<.0001	0.0152	0.9225	0.5164	<.0001	<.0001
7	<.0001	<.0001	<.0001	0.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0001	0.5876	<.0001
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0053	0.0004
9	0.0558	0.0112	<.0001	<.0001	0.9075	0.9657	0.5620	0.0061	<.0001	<.0001	<.0001	<.0001
10	0.0420	0.0081	<.0001	<.0001	1.0000	0.8735	0.4869	0.0045	<.0001	<.0001	<.0001	<.0001
11	0.1398	0.0341	<.0001	<.0001	0.5981	0.7124	0.8656	0.0174	<.0001	<.0001	<.0001	<.0001
12	0.8257	0.3684	0.0003	<.0001	0.1006	0.1363	0.3335	0.1903	0.0003	<.0001	<.0001	<.0001
13		0.4955	0.0006	<.0001	0.0675	0.0934	0.2461	0.2635	0.0005	<.0001	<.0001	<.0001
14	0.4955		0.0043	<.0001	0.0169	0.0247	0.0806	0.6068	0.0031	0.0006	<.0001	<.0001
15	0.0006	0.0043		0.1292	<.0001	<.0001	<.0001	0.0343	0.6597	0.3231	<.0001	<.0001
16	<.0001	<.0001	0.1292		<.0001	<.0001	<.0001	0.0009	0.3518	0.7021	<.0001	<.0001
17	0.0675	0.0169	<.0001	<.0001		0.8845	0.5254	0.0089	<.0001	<.0001	<.0001	<.0001
18	0.0934	0.0247	<.0001	<.0001	0.8845		0.6237	0.0129	<.0001	<.0001	<.0001	<.0001
19	0.2461	0.0806	<.0001	<.0001	0.5254	0.6237		0.0412	<.0001	<.0001	<.0001	<.0001
20	0.2635	0.6068	0.0343	0.0009	0.0089	0.0129	0.0412		0.0209	0.0058	<.0001	<.0001
21	0.0005	0.0031	0.6597	0.3518	<.0001	<.0001	<.0001	0.0209		0.6140	<.0001	<.0001
22	<.0001	0.0006	0.3231	0.7021	<.0001	<.0001	<.0001	0.0058	0.6140		<.0001	<.0001
23	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	

NOTE: To ensure overall protection level, only probabilities associated with pre-planned comparisons should be used.

Appendix F.4: **SAS Analysis of *C. autoethanogenum*: Gas Composition over Time**

```
Title1 'C. autoethanogenum on Syngas 06/27/2006';
Title2 'LSMeans for Main and Interaction Affects';
Title3 'Gas Composition over Time';


Data practice;
Input H2 N2 CO CO2 Flow Time ;
Cards;
9.8   48.52 20.27 19.96 5     1
9.71  48.8  20.32 20.76 5     1
9.88  48.54 20.3  19.81 5     1
9.89  48.47 20.42 19.93 7.5   1
9.65  49.91 20.31 18.94 7.5   1
10.25 48.94 20.33 19.49 7.5   1
9.69  48.63 20.19 19.99 10    1
9.68  48.67 20.27 19.93 10    1
8.77  50.23 19.78 19.13 10    1
9.63  48.46 20.16 20.14 5     2
9.53  48.5  19.98 20.91 5     2
9.44  49.05 19.91 19.6  5     2
9.67  48.87 20.01 19.89 7.5   2
9.31  49.15 19.83 19.5  7.5   2
9.24  49.85 19.31 19    7.5   2
9.66  48.43 20.21 19.91 10    2
9.57  48.77 20    19.77 10    2
9.48  49.21 19.82 19.58 10    2
9.68  48.05 19.98 20.15 5     3
9.91  48.24 20.11 20.69 5     3
9.44  48.66 19.92 19.75 5     3
9.83  48.7  19.91 19.9  7.5   3
9.73  48.42 20.22 19.87 7.5   3
9.78  48.39 20.06 20.03 7.5   3
9.83  47.84 20.12 20.2  10    3
9.28  48.79 19.85 19.96 10    3
9.68  48.35 20.13 19.85 10    3
9.65  47.99 19.72 20.24 5     4
9.66  48.27 19.68 22.46 5     4
9.74  48.16 19.89 20.06 5     4
9.17  49.73 18.5  19.48 7.5   4
9.42  48.96 19.52 19.32 7.5   4
9.5   49.07 19.25 20.02 7.5   4
9.37  48.96 19.68 20.17 10    4
9.79  48.21 20.2  20.09 10    4
9.7   47.99 19.79 20.06 10    4
9.54  48.09 19.4  22.32 5     5
9.26  48.41 19.27 20.61 5     5
9.44  48.46 19.71 20.44 5     5
9.11  49.78 18.82 18.88 7.5   5
9.19  49.16 19.04 20.12 7.5   5
8.3   51.56 17.29 18.31 7.5   5
9.34  48.31 19.79 20.65 10    5
9.55  48.12 19.89 20.01 10    5
9.09  49.15 19.04 20.12 10    5
```

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9.42  48.58 19.49 20.78 5     6
9.83  48.7  19.71 21.26 5     6
9.34  48.84 19.41 20.42 5     6
9.69  48.33 20.14 20.13 7.5   6
9.58  48.69 19.82 20.67 7.5   6
9.52  48.69 19.71 20.19 7.5   6
9.05  49.33 19.34 20.65 10    6
9.29  48.8  19.57 20.48 10    6
9.47  48.79 19.9  19.9  10    6
;
OPTIONS NODATE PAGENO = 1;
Proc GLM Data = practice;
Class Time Flow;

model N2 H2 CO CO2/*put in desired gas to analyze*/ = Time|Flow ;
lsmeans Time Flow Time*Flow /pdiff stderr;
/* pdiff gives pair-wise differences b/w factors */;
/* stderr gives standard error for each average */;
run;
end;
```

C. autoethanogenum on Syngas 06/27/2006
LSMeans for Main and Interaction Affects
Gas Composition over Time

The GLM Procedure

Class Level Information

Class	Levels	Values
Time	6	1 2 3 4 5 6
Flow	3	5 7.5 10

Number of Observations Read	54
Number of Observations Used	54

C. autoethanogenum on Syngas 06/27/2006
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Gas Composition over Time

The GLM Procedure

Dependent Variable: N2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	12.39329815	0.72901754	2.75	0.0052
Error	36	9.53140000	0.26476111		
Corrected Total	53	21.92469815			

R-Square	Coeff Var	Root MSE	N2 Mean
0.565267	1.055057	0.514549	48.76981

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	5	2.69007593	0.53801519	2.03	0.0974
Flow	2	4.37255926	2.18627963	8.26	0.0011
Time*Flow	10	5.33066296	0.53306630	2.01	0.0610

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	5	2.69007593	0.53801519	2.03	0.0974
Flow	2	4.37255926	2.18627963	8.26	0.0011
Time*Flow	10	5.33066296	0.53306630	2.01	0.0610

Dependent Variable: H2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	2.94920000	0.17348235	2.83	0.0043
Error	36	2.20793333	0.06133148		
Corrected Total	53	5.15713333			

R-Square	Coeff Var	Root MSE	H2 Mean
0.571868	2.601690	0.247652	9.518889

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	5	1.49155556	0.29831111	4.86	0.0017
Flow	2	0.21090000	0.10545000	1.72	0.1936
Time*Flow	10	1.24674444	0.12467444	2.03	0.0585

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	5	1.49155556	0.29831111	4.86	0.0017
Flow	2	0.21090000	0.10545000	1.72	0.1936
Time*Flow	10	1.24674444	0.12467444	2.03	0.0585

C. autoethanogenum on Syngas 06/27/2006
LSMeans for Main and Interaction Affects
Gas Composition over Time

The GLM Procedure

Dependent Variable: CO

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	11.02634259	0.64860839	5.80	<.0001
Error	36	4.02880000	0.11191111		
Corrected Total	53	15.05514259			

R-Square	Coeff Var	Root MSE	CO Mean
0.732397	1.692575	0.334531	19.76463

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	5	6.81094259	1.36218852	12.17	<.0001
Flow	2	0.89610370	0.44805185	4.00	0.0269
Time*Flow	10	3.31929630	0.33192963	2.97	0.0080

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	5	6.81094259	1.36218852	12.17	<.0001
Flow	2	0.89610370	0.44805185	4.00	0.0269
Time*Flow	10	3.31929630	0.33192963	2.97	0.0080

Dependent Variable: CO2

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	17	14.37159259	0.84538780	2.51	0.0099
Error	36	12.11653333	0.33657037		
Corrected Total	53	26.48812593			

R-Square	Coeff Var	Root MSE	CO2 Mean
0.542567	2.888751	0.580147	20.08296

Source	DF	Type I SS	Mean Square	F Value	Pr > F
Time	5	3.30672593	0.66134519	1.96	0.1076
Flow	2	7.82838148	3.91419074	11.63	0.0001
Time*Flow	10	3.23648519	0.32364852	0.96	0.4921

Source	DF	Type III SS	Mean Square	F Value	Pr > F
Time	5	3.30672593	0.66134519	1.96	0.1076
Flow	2	7.82838148	3.91419074	11.63	0.0001
Time*Flow	10	3.23648519	0.32364852	0.96	0.4921

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Time	H2 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	9.70222222	0.08255065	<.0001	1
2	9.50333333	0.08255065	<.0001	2
3	9.68444444	0.08255065	<.0001	3
4	9.55555556	0.08255065	<.0001	4
5	9.20222222	0.08255065	<.0001	5
6	9.46555556	0.08255065	<.0001	6

Least Squares Means for effect Time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: H2

i/j	1	2	3	4	5	6
1		0.0971	0.8798	0.2171	0.0001	0.0501
2	0.0971		0.1296	0.6573	0.0141	0.7481
3	0.8798	0.1296		0.2769	0.0002	0.0689
4	0.2171	0.6573	0.2769		0.0045	0.4458
5	0.0001	0.0141	0.0002	0.0045		0.0303
6	0.0501	0.7481	0.0689	0.4458	0.0303	

Time	CO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	20.2433333	0.1115104	<.0001	1
2	19.9144444	0.1115104	<.0001	2
3	20.0333333	0.1115104	<.0001	3
4	19.5811111	0.1115104	<.0001	4
5	19.1388889	0.1115104	<.0001	5
6	19.6766667	0.1115104	<.0001	6

Least Squares Means for effect Time
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: CO

i/j	1	2	3	4	5	6
1		0.0442	0.1913	0.0002	<.0001	0.0010
2	0.0442		0.4558	0.0415	<.0001	0.1403
3	0.1913	0.4558		0.0069	<.0001	0.0299
4	0.0002	0.0415	0.0069		0.0081	0.5484
5	<.0001	<.0001	<.0001	0.0081		0.0016
6	0.0010	0.1403	0.0299	0.5484	0.0016	

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Flow	CO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	19.8461111	0.0788498	<.0001	1
7.5	19.5827778	0.0788498	<.0001	2
10	19.8650000	0.0788498	<.0001	3

Least Squares Means for effect Flow
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: CO

i/j	1	2	3
1		0.0237	0.8664
2	0.0237		0.0159
3	0.8664	0.0159	

Flow	CO2 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
5	20.5755556	0.1367419	<.0001	1
7.5	19.6483333	0.1367419	<.0001	2
10	20.0250000	0.1367419	<.0001	3

Least Squares Means for effect Flow
Pr > |t| for HO: LSMean(i)=LSMean(j)

Dependent Variable: CO2

i/j	1	2	3
1		<.0001	0.0072
2	<.0001		0.0593
3	0.0072	0.0593	

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Time	Flow	H2 LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5	9.79666667	0.14298191	<.0001	1
1	7.5	9.93000000	0.14298191	<.0001	2
1	10	9.38000000	0.14298191	<.0001	3
2	5	9.53333333	0.14298191	<.0001	4
2	7.5	9.40666667	0.14298191	<.0001	5
2	10	9.57000000	0.14298191	<.0001	6
3	5	9.67666667	0.14298191	<.0001	7
3	7.5	9.78000000	0.14298191	<.0001	8
3	10	9.59666667	0.14298191	<.0001	9
4	5	9.68333333	0.14298191	<.0001	10
4	7.5	9.36333333	0.14298191	<.0001	11
4	10	9.62000000	0.14298191	<.0001	12
5	5	9.41333333	0.14298191	<.0001	13
5	7.5	8.86666667	0.14298191	<.0001	14
5	10	9.32666667	0.14298191	<.0001	15
6	5	9.53000000	0.14298191	<.0001	16
6	7.5	9.59666667	0.14298191	<.0001	17
6	10	9.27000000	0.14298191	<.0001	18

Least Squares Means for effect Time*Flow
Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: H2

i/j	1	2	3	4	5	6	7	8	9
1		0.5138	0.0466	0.2011	0.0617	0.2697	0.5566	0.9348	0.3292
2	0.5138		0.0100	0.0576	0.0138	0.0835	0.2183	0.4630	0.1080
3	0.0466	0.0100		0.4532	0.8958	0.3537	0.1510	0.0556	0.2911
4	0.2011	0.0576	0.4532		0.5350	0.8571	0.4830	0.2304	0.7559
5	0.0617	0.0138	0.8958	0.5350		0.4245	0.1902	0.0731	0.3537
6	0.2697	0.0835	0.3537	0.8571	0.4245		0.6011	0.3059	0.8958
7	0.5566	0.2183	0.1510	0.4830	0.1902	0.6011		0.6125	0.6947
8	0.9348	0.4630	0.0556	0.2304	0.0731	0.3059	0.6125		0.3706
9	0.3292	0.1080	0.2911	0.7559	0.3537	0.8958	0.6947	0.3706	
10	0.5786	0.2304	0.1423	0.4630	0.1797	0.5786	0.9739	0.6355	0.6708
11	0.0389	0.0081	0.9348	0.4061	0.8315	0.3136	0.1300	0.0466	0.2561
12	0.3881	0.1340	0.2430	0.6708	0.2984	0.8061	0.7809	0.4340	0.9088
13	0.0660	0.0150	0.8700	0.5566	0.9739	0.4435	0.2011	0.0781	0.3706
14	<.0001	<.0001	0.0156	0.0022	0.0113	0.0013	0.0003	<.0001	0.0009
15	0.0259	0.0051	0.7935	0.3136	0.6947	0.2367	0.0920	0.0312	0.1902
16	0.1956	0.0556	0.4630	0.9869	0.5457	0.8443	0.4729	0.2243	0.7435
17	0.3292	0.1080	0.2911	0.7559	0.3537	0.8958	0.6947	0.3706	1.0000
18	0.0133	0.0024	0.5898	0.2011	0.5034	0.1466	0.0518	0.0162	0.1149

Dependent Variable: H2

i/j	10	11	12	13	14	15	16	17	18
1	0.5786	0.0389	0.3881	0.0660	<.0001	0.0259	0.1956	0.3292	0.0133
2	0.2304	0.0081	0.1340	0.0150	<.0001	0.0051	0.0556	0.1080	0.0024
3	0.1423	0.9348	0.2430	0.8700	0.0156	0.7935	0.4630	0.2911	0.5898
4	0.4630	0.4061	0.6708	0.5566	0.0022	0.3136	0.9869	0.7559	0.2011
5	0.1797	0.8315	0.2984	0.9739	0.0113	0.6947	0.5457	0.3537	0.5034
6	0.5786	0.3136	0.8061	0.4435	0.0013	0.2367	0.8443	0.8958	0.1466
7	0.9739	0.1300	0.7809	0.2011	0.0003	0.0920	0.4729	0.6947	0.0518
8	0.6355	0.0466	0.4340	0.0781	<.0001	0.0312	0.2243	0.3706	0.0162
9	0.6708	0.2561	0.9088	0.3706	0.0009	0.1902	0.7435	1.0000	0.1149
10		0.1223	0.7559	0.1902	0.0003	0.0862	0.4532	0.6708	0.0483
11	0.1223		0.2125	0.8061	0.0190	0.8571	0.4152	0.2561	0.6472
12	0.7559	0.2125		0.3136	0.0007	0.1555	0.6589	0.9088	0.0920
13	0.1902	0.8061	0.3136		0.0104	0.6708	0.5676	0.3706	0.4830
14	0.0003	0.0190	0.0007	0.0104		0.0290	0.0023	0.0009	0.0537
15	0.0862	0.8571	0.1555	0.6708	0.0290		0.3213	0.1902	0.7809
16	0.4532	0.4152	0.6589	0.5676	0.0023	0.3213		0.7435	0.2067
17	0.6708	0.2561	0.9088	0.3706	0.0009	0.1902	0.7435		0.1149
18	0.0483	0.6472	0.0920	0.4830	0.0537	0.7809	0.2067	0.1149	

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Time	Flow	CO LSMEAN	Standard Error	Pr > |t|	LSMEAN Number
1	5	20.2966667	0.1931417	<.0001	1
1	7.5	20.3533333	0.1931417	<.0001	2
1	10	20.0800000	0.1931417	<.0001	3
2	5	20.0166667	0.1931417	<.0001	4
2	7.5	19.7166667	0.1931417	<.0001	5
2	10	20.0100000	0.1931417	<.0001	6
3	5	20.0033333	0.1931417	<.0001	7
3	7.5	20.0633333	0.1931417	<.0001	8
3	10	20.0333333	0.1931417	<.0001	9
4	5	19.7633333	0.1931417	<.0001	10
4	7.5	19.0900000	0.1931417	<.0001	11
4	10	19.8900000	0.1931417	<.0001	12
5	5	19.4600000	0.1931417	<.0001	13
5	7.5	18.3833333	0.1931417	<.0001	14
5	10	19.5733333	0.1931417	<.0001	15
6	5	19.5366667	0.1931417	<.0001	16
6	7.5	19.8900000	0.1931417	<.0001	17
6	10	19.6033333	0.1931417	<.0001	18

Least Squares Means for effect Time*Flow
Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: CO

i/j	1	2	3	4	5	6	7	8	9
1		0.8368	0.4328	0.3122	0.0407	0.3009	0.2900	0.3986	0.3414
2	0.8368		0.3237	0.2257	0.0255	0.2169	0.2083	0.2954	0.2491
3	0.4328	0.3237		0.8180	0.1918	0.7992	0.7806	0.9517	0.8653
4	0.3122	0.2257	0.8180		0.2794	0.9807	0.9613	0.8653	0.9517
5	0.0407	0.0255	0.1918	0.2794		0.2900	0.3009	0.2125	0.2539
6	0.3009	0.2169	0.7992	0.9807	0.2900		0.9807	0.8463	0.9324
7	0.2900	0.2083	0.7806	0.9613	0.3009	0.9807		0.8274	0.9132
8	0.3986	0.2954	0.9517	0.8653	0.2125	0.8463	0.8274		0.9132
9	0.3414	0.2491	0.8653	0.9517	0.2539	0.9324	0.9132	0.9132	
10	0.0587	0.0375	0.2539	0.3599	0.8653	0.3725	0.3854	0.2794	0.3295
11	<.0001	<.0001	0.0009	0.0017	0.0277	0.0018	0.0019	0.0011	0.0014
12	0.1452	0.0985	0.4911	0.6456	0.5297	0.6630	0.6807	0.5297	0.6030
13	0.0041	0.0024	0.0293	0.0489	0.3536	0.0516	0.0543	0.0336	0.0429
14	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	0.0119	0.0071	0.0718	0.1133	0.6030	0.1186	0.1242	0.0812	0.1008
16	0.0085	0.0050	0.0543	0.0874	0.5141	0.0917	0.0962	0.0618	0.0773
17	0.1452	0.0985	0.4911	0.6456	0.5297	0.6630	0.6807	0.5297	0.6030
18	0.0156	0.0094	0.0895	0.1389	0.6807	0.1452	0.1518	0.1008	0.1242

Dependent Variable: CO

i/j	10	11	12	13	14	15	16	17	18
1	0.0587	<.0001	0.1452	0.0041	<.0001	0.0119	0.0085	0.1452	0.0156
2	0.0375	<.0001	0.0985	0.0024	<.0001	0.0071	0.0050	0.0985	0.0094
3	0.2539	0.0009	0.4911	0.0293	<.0001	0.0718	0.0543	0.4911	0.0895
4	0.3599	0.0017	0.6456	0.0489	<.0001	0.1133	0.0874	0.6456	0.1389
5	0.8653	0.0277	0.5297	0.3536	<.0001	0.6030	0.5141	0.5297	0.6807
6	0.3725	0.0018	0.6630	0.0516	<.0001	0.1186	0.0917	0.6630	0.1452
7	0.3854	0.0019	0.6807	0.0543	<.0001	0.1242	0.0962	0.6807	0.1518
8	0.2794	0.0011	0.5297	0.0336	<.0001	0.0812	0.0618	0.5297	0.1008
9	0.3295	0.0014	0.6030	0.0429	<.0001	0.1008	0.0773	0.6030	0.1242
10		0.0186	0.6456	0.2741	<.0001	0.4911	0.4121	0.6456	0.5617
11	0.0186		0.0059	0.1840	0.0139	0.0853	0.1107	0.0059	0.0683
12	0.6456	0.0059		0.1242	<.0001	0.2539	0.2040	1.0000	0.3009
13	0.2741	0.1840	0.1242		0.0004	0.6807	0.7806	0.1242	0.6030
14	<.0001	0.0139	<.0001	0.0004		0.0001	0.0002	<.0001	<.0001
15	0.4911	0.0853	0.2539	0.6807	0.0001		0.8940	0.2539	0.9132
16	0.4121	0.1107	0.2040	0.7806	0.0002	0.8940		0.2040	0.8086
17	0.6456	0.0059	1.0000	0.1242	<.0001	0.2539	0.2040		0.3009
18	0.5617	0.0683	0.3009	0.6030	<.0001	0.9132	0.8086	0.3009	

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Least Squares Means

Time	Flow	CO2 LSMEAN	Standard Error	LSMEAN Pr > |t|	Number
1	5	20.1766667	0.3349479	<.0001	1
1	7.5	19.4533333	0.3349479	<.0001	2
1	10	19.6833333	0.3349479	<.0001	3
2	5	20.2166667	0.3349479	<.0001	4
2	7.5	19.4633333	0.3349479	<.0001	5
2	10	19.7533333	0.3349479	<.0001	6
3	5	20.1966667	0.3349479	<.0001	7
3	7.5	19.9333333	0.3349479	<.0001	8
3	10	20.0033333	0.3349479	<.0001	9
4	5	20.9200000	0.3349479	<.0001	10
4	7.5	19.6066667	0.3349479	<.0001	11
4	10	20.1066667	0.3349479	<.0001	12
5	5	21.1233333	0.3349479	<.0001	13
5	7.5	19.1033333	0.3349479	<.0001	14
5	10	20.2600000	0.3349479	<.0001	15
6	5	20.8200000	0.3349479	<.0001	16
6	7.5	20.3300000	0.3349479	<.0001	17
6	10	20.3433333	0.3349479	<.0001	18

Least Squares Means for effect Time*Flow
Pr > |t| for HO: LSMean(i)=LSMean(j)
Dependent Variable: CO2

i/j	1	2	3	4	5	6	7	8	9
1		0.1355	0.3046	0.9332	0.1408	0.3774	0.9666	0.6106	0.7166
2	0.1355		0.6302	0.1158	0.9833	0.5305	0.1253	0.3177	0.2532
3	0.3046	0.6302		0.2677	0.6451	0.8833	0.2857	0.6009	0.5036
4	0.9332	0.1158	0.2677		0.1205	0.3345	0.9666	0.5535	0.6551
5	0.1408	0.9833	0.6451	0.1205		0.5442	0.1303	0.3277	0.2618
6	0.3774	0.5305	0.8833	0.3345	0.5442		0.3556	0.7062	0.6009
7	0.9666	0.1253	0.2857	0.9666	0.1303	0.3556		0.5817	0.6856
8	0.6106	0.3177	0.6009	0.5535	0.3277	0.7062	0.5817		0.8833
9	0.7166	0.2532	0.5036	0.6551	0.2618	0.6009	0.6856	0.8833	
10	0.1253	0.0038	0.0131	0.1463	0.0040	0.0187	0.1355	0.0444	0.0609
11	0.2367	0.7480	0.8723	0.2060	0.7639	0.7586	0.2210	0.4949	0.4079
12	0.8833	0.1763	0.3774	0.8177	0.1829	0.4606	0.8504	0.7166	0.8285
13	0.0533	0.0012	0.0044	0.0636	0.0012	0.0065	0.0582	0.0166	0.0236
14	0.0296	0.4648	0.2287	0.0244	0.4522	0.1785	0.0268	0.0883	0.0655
15	0.8613	0.0972	0.2314	0.9276	0.1013	0.2919	0.8944	0.4949	0.5913
16	0.1829	0.0066	0.0217	0.2109	0.0069	0.0305	0.1965	0.0694	0.0933
17	0.7480	0.0724	0.1807	0.8123	0.0756	0.2314	0.7800	0.4079	0.4949
18	0.7270	0.0684	0.1721	0.7907	0.0714	0.2210	0.7586	0.3925	0.4775

Dependent Variable: CO2

1	0.1253	0.2367	0.8833	0.0533	0.0296	0.8613	0.1829	0.7480	0.7270
2	0.0038	0.7480	0.1763	0.0012	0.4648	0.0972	0.0066	0.0724	0.0684
3	0.0131	0.8723	0.3774	0.0044	0.2287	0.2314	0.0217	0.1807	0.1721
4	0.1463	0.2060	0.8177	0.0636	0.0244	0.9276	0.2109	0.8123	0.7907
5	0.0040	0.7639	0.1829	0.0012	0.4522	0.1013	0.0069	0.0756	0.0714
6	0.0187	0.7586	0.4606	0.0065	0.1785	0.2919	0.0305	0.2314	0.2210
7	0.1355	0.2210	0.8504	0.0582	0.0268	0.8944	0.1965	0.7800	0.7586
8	0.0444	0.4949	0.7166	0.0166	0.0883	0.4949	0.0694	0.4079	0.3925
9	0.0609	0.4079	0.8285	0.0236	0.0655	0.5913	0.0933	0.4949	0.4775
10		0.0088	0.0946	0.6703	0.0005	0.1721	0.8340	0.2210	0.2314
11	0.0088		0.2982	0.0029	0.2950	0.1763	0.0148	0.1355	0.1287
12	0.0946	0.2982		0.0387	0.0411	0.7480	0.1408	0.6401	0.6204
13	0.6703	0.0029	0.0387		0.0001	0.0767	0.5260	0.1026	0.1083
14	0.0005	0.2950	0.0411	0.0001		0.0197	0.0009	0.0138	0.0129
15	0.1721	0.1763	0.7480	0.0767	0.0197		0.2449	0.8833	0.8613
16	0.8340	0.0148	0.1408	0.5260	0.0009	0.2449		0.3078	0.3210
17	0.2210	0.1355	0.6401	0.1026	0.0138	0.8833	0.3078		0.9777
18	0.2314	0.1287	0.6204	0.1083	0.0129	0.8613	0.3210	0.9777	