Factors Effecting Ethanol Fermentation Via Simultaneous Saccharification and Fermentation

A study to determine the optimal operating conditions to convert cellulosic biomass into ethanol during enzymatic hydrolysis and microbial fermentation.

A Major Qualifying Project submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor Science

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Abstract

Biomass is becoming an increasingly popular source for alternative energy. Cellulosic biomass, an alternative to petroleum, is comprised of a molecule called lignocellulose. In order to be fermented into ethanol, lignocellulose must be broken down into glucose chains. This process requires several intermediate steps which are time consuming, costly, and relatively ineffective. This project studied simultaneous saccharification and fermentation, an alternative process to break down lignocellulose whereby all the intermediate processes were conducted simultaneously while undergoing microbial yeast fermentation. Temperature, substrate concentration and pH factors of fermentation were studied to determine the optimal operating conditions for this process. The optimal conditions were determined to be 35°C, 40 g/L of glucose, and a pH of 4.5.

Acknowledgments

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Table of Contents

Table of Figures

Executive Summary

As the world seeks to end its dependence on oil, alternative sources of fuel are being examined. Ethanol has been developed as both an additive and an alternative to fuel. It can be produced either from petroleum bases or from sources known as biomass which include wood, grass, grains, or indigestible plants. To date, switchgrass and corn are the predominant feed stock used for ethanol production from biomass.

Cellulosic biomass contains a substance called lignocellulose. This non-digestible substance is comprised of cellulose, hemicellulose, and lignin. Of the three molecules in lignocellulose, cellulose and hemicellulose are capable of being converted to ethanol. But, cellulose and hemicellulose must be converted to a glucose chain in order to be digested by yeast. Therefore to convert the biomass into ethanol, the lignocellulose has to be broken down and separated. The process to break down lignocellulose, known as hydrolysis, is difficult and costly. It requires a pretreatment to break down the tough, rigid outer cell wall of the lignocellulose and either a chemical or enzymatic treatment to break down the cellulose into glucose. Both types of hydrolysis have drawbacks. Chemical hydrolysis is costly and often requires an acid recovery system. Enzymatic hydrolysis is glucose inhibited, so as more glucose is produced the less efficient the hydrolysis becomes.

Industry created a process known as Simultaneous Saccharification and Fermentation which combines the enzymatic hydrolysis and fermentation steps into one process. Because the hydrolysis and fermentation steps occur simultaneously, the glucose yield and subsequently ethanol yield from the biomass is greater than in processes that occur individually. Also, because the two steps are combined, there is a shorter overall process time to convert the biomass into ethanol. However, optimal conditions under which simultaneous saccharification and fermentation should occur are widely debated.

This report investigates the physical factors that surround ethanol production from biomass via simultaneous saccharification and fermentation using *Saccharomyces cerevisiae*, common baker's yeast. Specifically, temperature, substrate concentration and pH were studied in relation to the ethanol yield of the reaction. A total of 13 reactions were conducted under varying conditions. Samples from each reaction were taken every 24 hours in order to monitor yeast content, ethanol content, and pH.

It was determined that substrate composition and temperature had significant affects on how well the yeasts fermented the glucose into ethanol.

The initial experiments investigated how temperature affects the fermentation reaction. These experiments suggested that the rate of the fermentation reaction was slower as temperature increased. Therefore temperature in reactions conducted after was controlled to 35°C. While hydrolysis performs better at higher temperatures, it was decided that the fermentation reaction was more important in the SSF reactions.

Substrate experiments were conducted following the temperature reactions. While the substrate variations did not show any difference in ethanol yield, it was determined that they affected how well the yeast cells reproduced. The yeast in these reactions showed signs of cell death due to ethanol inhibition and weak acid inhibition. The affects of these two inhibitory phenomena were more severe as substrate composition increased. Therefore, reactions conducted after were controlled to 40 g/L of glucose.

The reactions that investigated pH effects were less conclusive than temperature or substrate reactions. It was evident from the data that an increased pH of reaction meant that the ethanol yield from the reaction would be lower. It was hypothesized that pH affected the osmotic pressure of the yeast cells, so this report recommends future testing to further confirm this.

Introduction

Fuels and energy sources that are made from organic byproducts or naturally occurring, living organisms are known as biomass fuels (Biofuels). Paper and wood waste, grains, and decomposing organic rubbish are some of the most popular sources of biofuel. The idea of using biofuel as an alternative to coal energy has existed since the industrial revolution. When Ford designed the Model T, the original fuel source that was supposed to be used was ethanol from biomass (BioFuels).

As the world petroleum resources are being consumed at a rapid rate, biofuel and biomass energy sources have become an increasingly popular fuel alternative (History of Biofuels). Within the United States, ethanol has been an additive in gasoline since 2005 (Bioenergy). Most of the US biofuel comes from the agricultural sector of the US Economy (Mark Muller, 2007). In fact, in most of the world, ethanol is produced from either sugarcane or corn (The Differences in Ethanol) (Biofuels inthe US Transportation Sector).

Sugarcane and corn both contain readily assessable sugars within their plant walls. They are popular fuel sources because very little processing needs to be done in order to prepare the feed for fermentation. After milling the feed either through dry milling or chemical milling, such as steam explosion, yeast can be added to the mixture for fermentation.

However, the energy yield from the process is only 30% more than what's required to grow and prepare the feed for fermentation (One Molecule could cure our additction to oil). Both crops require large allotments of nutrient rich soil in order to grow. Therefore, crops have to be regularly rotated so as to prevent the soil to become nutrient deprived. After factoring the labor and energy expended to harvest the crops, mill them, and then ferment them into ethanol, the energy gain from the process can be as low as 21% (Andresss, 2002).

In addition to being an inefficient overall energy source, the corn used for ethanol production detracts from the corn being used for food sources. Nearly 41% of the corn grown in the US is being used as an ethanol source (Ethanol Fuel for the Next Generation). This high usage of corn for ethanol increases the overall price of corn around the world. As price goes up per bushel of corn, many poorer consumers aren't able to purchase the crop anymore. As seen in Mexico, the price to produce a torilla from US grown corn is steadily increasing, leaving the food source out of reach of many families in poverty (Economic Impact of Ethanol Production).

An alternative source for biofuel is from cellulosic biomass, biomass that contains high levels of lignocellulose. Crops like switchgrass, poplar trees, and straw as well as waste from paper mills or livestock such as cattle can be converted into ethanol. Lignocellulose is the non-digestible part of the plants and waste products. (Lignocellulose) It contains cellulose, hemicellulose, and lignin; the plants use this structure as a strengthening material that can withstand environmental stress. Glucose sugars can be derived from the cellulosic materials of the lignocellulose; the glucose can then be fermented into ethanol.

Cellulosic biomass is a virtually endless resource. Because grasses and waste from milling can be used to create ethanol, the availability of cellulosic ethanol is far greater than that of corn ethanol. There are major process challenges that need to be overcome before cellulosic biomass can replace corn ethanol as a prominent alternative fuel (The Differences in Ethanol)l (Cellulosic Ethanol). Process cost to convert cellulosic biomass into ethanol is more expensive than corn biomass. The cellulosic biomass needs to undergo a hydrolysis process whereby the lignocellulose is broken down and converted into glucose chains. Enzymes that are used in this conversion process are often expensive and are required in large amounts. The efficiency of the hydrolysis process isn't high enough to compete with glucose production from corns and sugars (Cellulosic Ethanol).

In an effort to better the process of ethanol conversion from cellulosic biomass, industry created a process called simultaneous saccharification and fermentation (SSF). SSF combines the hydrolysis step and the fermentation step in order to make the conversion process more efficient. Hydrolysis rates and yields are improved because the yeast and enzyme presence reduces glucose inhibition. (Takagi, 1976). However, there are problems with the SSF process. One large issue is that the optimal temperature for hydrolysis and the optimal temperature for fermentation differ by more than 15°C. Another issue is that glucose concentrations in SSF reactions need to be balanced so that the yeast can efficiently reproduce and ferment without becoming inhibited by the ethanol produced from the reaction.

Research is required to determine what the optimal conditions for SFF are. This MQP sought to begin research into these conditions in order to optimize the combined process. In order to determine what the optimal conditions are for SSF, temperature, substrate(glucose) concentration, and pH during several fermentation processes were examined. The overall ethanol yield produced during each experiment was determined. Recommendations for future experiments were discussed.

Background

Ethanol Conversion Process

In order to convert any kind of biomass into ethanol, several processes must occur. The biomass must be broken down into simple glucose chains. Cellulosic biomass undergoes the following processes (Nathan Mosier, 2005):

1. Pretreatment to break the rigid structure of the lignocellulose in order to access the lignin, hemicellulose and cellulose molecules inside the lignocellulose
2. Hydrolysis to break down the cellulose and hemicellulose into glucose chains
3. Microbial fermentation via yeast or bacteria to produce ethanol
4. Distillation to separate the products of fermentation

Yeast has long been used to ferment various substances into alcohol and bread. Common baker's yeast, *S. Cerevisiae*, can readily convert glucose molecules into ethanol. *S. cerevisiae* has the highest rate of conversion of all the yeasts found in nature. Baker's yeast can grow on simple sugars like glucose as well as complex sugars such as sucroses. The *Saccharomyces* family of yeast can best ferment in temperatures from 26-25°C. Ideally, the yeast reacts best in a slightly acidic environment (pH of 4.5). (Lin, 2006) *S. cerevisiae* has the capabilities of withstanding high concentrations of ethanol as well as producing high ethanol yields from glucose.

Pretreatment

Beneath its rigid exterior, lignocellulose is comprised of lignin, cellulose and hemicellulose which can be converted into ethanol. Because lignocellulose is so rigid and strong, pretreatment is required in order to break into the strong exterior to expose the convertible molecules. Pretreatment can be a physical or chemical process, or a combination of both.

The physical pretreatments do not use chemical agents. They typically involve some sort of process that applies an external force onto the rigid structure of the lignocellulose in order to break it down. Physical pretreatments include steam explosion, dry/wet milling, and hot water baths.

Chemical pretreatments use chemical agents to degrade the structure of the lignocellulose. Chemical pretreatment is the most predominant form of pretreatment. Processes such as catalyzed steam explosion, solvent baths using chemicals such as ozone, and acid are the most common types of chemical pretreatment.

Ultimately, the pretreatment must expose the lignin, cellulose and hemicellulose without forming any products that may inhibit hydrolysis. (Zheng, 2009)

Hydrolysis

The hydrolysis process breaks down the cellulosic molecules exposed during pretreatment into glucose molecules and short chains. Hydrolysis can be carried out chemically via acid washes or biologically via enzymatic reactions. Figure 1 depicts the 4 possible pathways involved in the conversion of lignocellulose to ethanol.

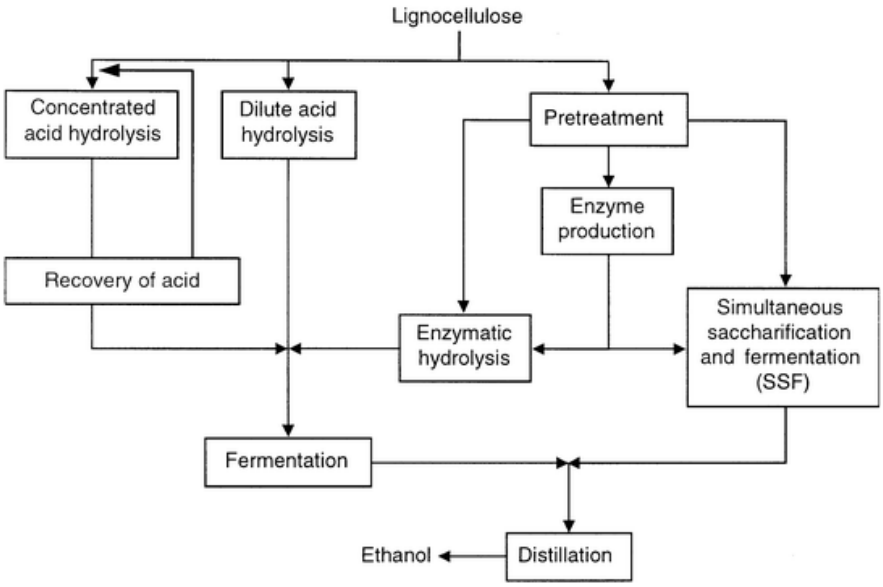

Figure 1: Hydrolysis Pathways Provided from a report by S. Carcieri et. Al (Carcieri, 2010)

Acid Hydrolysis

Acid hydrolysis occurs by exposing the cellulosic material to either a dilute or concentrated acid. The acid reacts with the cellulosic material to produce glucose molecules and short chains.

Dilute acid hydrolysis occurs under high temperature and high pressure. The process is costly to run and produces a low yield of usable glucose. The severe physical requirements that dilute acid hydrolysis occurs under subsequently decomposes the glucose as it is produced. (Lee, 1999)

Concentrated acid hydrolysis occurs at low temperatures and atmospheric pressure. The process is more efficient than its counter-part and has a high glucose yield. However, the process is time consuming, taking up to 120 hours to complete. (Moe, 2006) Additionally, the process requires an acid recovery system because any excess concentrated acid would kill yeast introduced to the product glucose.

Both processes form inhibitory byproducts; acetic acid and furfural are products of the polysaccharides breaking down into glucose. Both products inhibit ethanol production by limiting yeast growth and causing cell death.

Enzymatic Hydrolysis

Enzymatic hydrolysis occurs when enzymes are exposed to the pretreated biomass to decompose the biomass into simple sugars. The enzymes typically used are endocellulase, exocellulase, and Beta-glucosidase. The enzymes digest the lignin surface yielding cellulose. The endocellulase and exocellulase digest the cellulose into polysaccharide molecules. The polysaccharide molecules are then digested by the Beta-glucosidase yielding the final glucose product (Klass, 2008).The reaction occurs around 50°C and at a pH of about 5. Below, the figure demonstrates how the reaction path occurs.

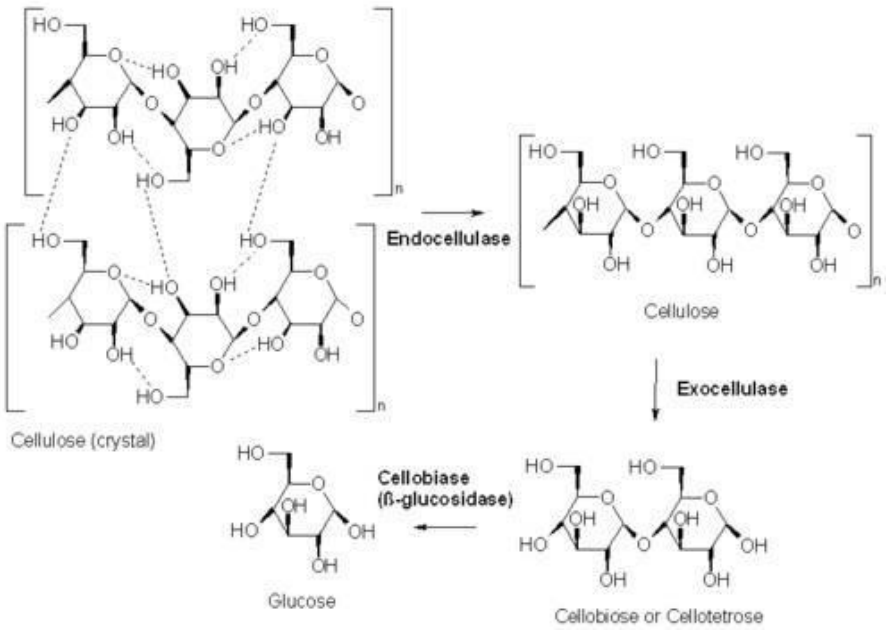

Figure 2: Reaction Route of Cellulose to Glucose (Carcieri, 2010)

However, enzymatic hydrolysis can be problematic. The hydrolysis products (glucose and cellulose chains) inhibit the ability for enzymes to convert cellulose to glucose. As more product is formed, the enzymes become more inhibited by the excess glucose present. This ultimately slows down the hydrolysis process yielding low levels of usable hydrolysis product (D'amore, 1991).

Simultaneous Saccharification and Fermentation

In order to overcome some of the problems with the hydrolysis process, hydrolysis and fermentation were combined into one step. Known as Simultaneous Saccharification and Fermentation, the process allows the glucose produced from hydrolysis to be fermented immediately. This allows the concentration of the glucose to remain low thereby allowing the hydrolysis process to continue without significant inhibition (Takagi, 1976).

In addition to the lower rate of glucose inhibition, Simultaneous Saccharification and Fermentation has other advantages. Studies are suggesting that the simultaneous fermentation process shortens the length of time required for the biomass to ethanol conversion process. The process requires less enzymes than needed in regular enzymatic hydrolysis. Because SSF combines hydrolysis and fermentation, the overall reaction time to convert biomass to ethanol is shortened. Additionally, it

reduces the chances of contamination because the process occurs at high temperatures and within the same reaction vessel (Takagi, 1976).

There exist two fundamental problems with Simultaneous Saccharification and Fermentation, however. Hydrolysis and fermentation both require specific temperature ranges for optimal operation. *S.cerevisiae* ferments best at temperatures around 25°C with a pH of between 4 and 5 (Wasungu, 1982). Any extreme of temperature during fermentation, either high or low, produces minimal concentrations of ethanol. This is partly because yeast does not grow well in temperatures much lower than 20C or much higher than 40C. The hydrolysis process, however, performs best at temperatures of about 47°C. (Palmqvist, 2000). If the temperature drops too low, the enzymes will not digest material.

The presence of the ethanol produced from the glucose fermentation during SSF has the possibility of inhibiting the fermentation reaction. As the concentration in ethanol increases, the ethanol attacks the various microorganisms in the system. Both the enzymes and the yeast undergo plasma membrane degradation as the ethanol concentration increases. Eventually, the ethanol concentration will become high enough to cause cell death in both the enzymes and the yeast. (D'amore, 1991).

Nutrients

The fermentation medium that the yeast ferments glucose in also plays a role in the effectiveness of ethanol production. Yeast has a complex nutritional requirement to undergo optimal fermentation. In general, yeast requires sugars to digest, amino acids to build proteins, vitamins and minerals to make enzymes, and phosphorus to create DNA. The exact requirements vary for different yeast types (Nutrition and Fermentation).

Vitamins are necessary in enzymatic reactions. However, yeast is not capable of digesting many essential vitamins (those in which the yeast cannot create itself); therefore, specific type of vitamins are required for fermentation. These vitamins include: biotin, nicotinic acid, vitamin B, pantothenic acid, and vitamin C. Biotin is the most important of the vitamins yeast use in fermentation. Biotin is involved in all enzymatic reactions and helps create proteins, DNA, carbohydrates and fatty acids that comprise the makeup of yeast. (Nutrition and Fermentation)

Phosphorus is a main component of DNA as well as the phospholipids in cell membranes. The yeast requires ample sources of phosphorus in order to ensure adequate cell replication both

structurally and internally. A lack of phosphorus would result in incomplete fermentation because the yeast would not replicate sufficiently. (Nutrition and Fermentation)

The minerals required for efficient yeast fermentation include potassium (K), calcium (Ca), magnesium (Mg), and Zinc (Zn). Specifically, magnesium is the most important mineral that the yeast requires. Without magnesium present, the yeast will not grow. Magnesium is a critical component in ATP development, and without it the cells would have no energy. Magnesium also acts as a strengthening device, allowing the cell to withstand stress and chemically toxic situations for longer periods of time. (Nutrition and Fermentation)

The amount of each nutrient in the slurry is dependent on the conditions of reaction within the reactor. Water quality, oxygen levels, and ethanol concentration should all be accounted for when adding nutrients into the slurry. By introducing sufficient nutrients to the fermentation process, the yeast can multiply quickly and consume glucose to produce ethanol more effectively.

Methods

Within the experiments, several physical conditions regarding ethanol fermentation were investigated. Specifically, temperature of reaction, substrate concentration, and pH were studied to determine their effect on the simultaneous saccharification and fermentation of cellulosic biomass into ethanol.

For each physical condition, three runs were conducted over a period of approximately one week. Within the three runs, each individual run tested the physical condition at a varying degree, e.g. varied substrate conditions, varied temperature of reaction, varied pH within the reaction flask. Temperature experiments were run at 30, 35, and 40°C. Substrate concentration was varied at 40, 80, and 100 g/L of glucose. pH was varied at 4.5, 5, 5.5 and 6.

In every run, *Saccharomyces Cerevisiae*, or common baker's yeast, was used.

Inoculation Media and Yeast Cultivation

The inoculation media for yeast cultivation was prepared in a 2L volumetric flask with distilled, de-ionized water; Table 1 outlines the compounds and subsequent concentrations used added to the water to create the inoculation media.

Table 1: Inoculation Medium	
Compound	**Concentration**
Glucose	20 g/L
Peptone	10 g/L
Yeast Extract	20 g/L

The solution was divided into 10 250-mL volumetric flasks that were capped with a rubber stopper. The flasks were placed into an autoclave for 45 minutes at 121°C in an effort to prevent any microorganisms other than yeast from growing. After sterilization, 0.3 mL of live yeast was transferred into each flask. The flasks were then capped with rubber stoppers and placed into a shaker at 37°C for 12±2 hours at about 160 rpm.

After the 12 hour incubation period, the flasks were removed from the shaker. The yeast was decanted from the remaining inoculation media via centrifuge. 6 tubes of approximately 50 mL in volume were spun at 4000 rpm for 3 minutes until all the yeast was separated from the inoculation

media. The yeast was then resuspended into one 50 mL tube using distilled, de-ionized water as a solution base.

The yeast solution was then analyzed to determine the actual yeast concentration in suspension. 0.1 mL of the yeast solution was diluted into a 25 mL volumetric flask. Samples from the flask were analyzed in a spectrometer. The optical density (OD) for the sample of the yeast suspension was taken at a 600 nm wavelength in a spectronic instrument. The optical density of the suspension was then used to calculate the actual yeast concentration in grams per litre using an established trendline from students at Shanghai Jiao Tong University. The equation was derived from measuring the absorbance of a fully saturated colony of yeast suspended in distilled water. The yeast was diluted down to known concentrations and then plotted against the absorbance read.

Equation 1: Trendline to determine yeast concentration

$$y = 0.14804 + 1.19501x$$

Where y is the absorbance read and x is the yeast concentration (diluted)

Once the diluted concentration is determined, the actual concentration is determined simply by back calculating from the percent dilution. In this case, the yeast concentration determined from the equation above was diluted 250 times. So, the actual yeast concentration is:

$$m = 250x$$

Where m is the actual yeast concentration and x is the diluted concentration.

Nutrient and Growth Media for Fermentation

A nutrient medium used as a supplement for fermentation was prepared using 10 L of distilled, de-ionized water. Table 2 outlines the compounds and subsequent concentrations added to the water to create the nutrient medium.

Table 2: Nutrient Medium

Compound	Concentration (g/L)
Sodium Molybdate (Na_2MoO_4)	0.00002
Ammonium Sulfate ($(NH_3)_2SO_4$)	1.0
Copper (II) Sulfate ($CuSO_4$)	0.004
Magnesium Sulfate ($MgSO_4$)	0.35
Calcium Chloride ($CaCl_2$)	0.0555

Potassium Dihydrogen Phosphate (KH_2PO_4)	0.70
Iron (II) Sulfate ($FeSO_4$)	0.004
Manganese Sulfate ($MnSO_4$)	0.002

A growth medium used for yeast fermentation was prepared using 200 mL of distilled, de-ionized water. Table 3 outlines the compounds and subsequent concentrations added to the water to create the growth medium.

Table 3: Growth Medium	
Compound	**Concentration**
Glucose	40, 80, or 100 g/L depending on the reaction
Nutrient Medium	1.0 ml / L of Solution

The growth medium was separated into three 50 mL uncontrolled volume flasks. 3 g/L of yeast was added to each 50 mL bottle after which the bottles were sealed.

Samples Taken for Ethanol Concentration and Cell Count

At zero hour and every 24 hours after that, samples were taken from the growth bottles to determine cell density and ethanol content in order to track the fermentation progress. The pH of each sample gathered was measured using a standard pH probe calibrated for a region of pH less than 7.

Cell count was measured using a spectrophotometer calibrated to 600 nm and 0% Absorbance via blank cuvette. A 2.0 mL solution sample was taken from the growth bottle and diluted in a 10 mL volumetric flask. A cuvette was then filled with the diluted sample and placed into the spectrophotometer and the absorbance was read. For accuracy, a second cuvette was filled with some diluted sample and placed into the spectrophotometer and absorbance was read. The absorbance reading was then plotted against the concentration of yeast in the sample. The concentration of yeast in the sample was determined using equation 1.

Ethanol concentration was measured using a high-performance liquid chromatograph (HPLC). 5 standard solutions were mixed with known concentrations of xylose, glucose, cellobiose, and ethanol. The standard solutions were mixed to 200, 400, 600, 800, and 1000 mg/L of each component. 1mL of each of these standard solutions was diluted in a 10 mL volumetric flask and then each tested in the HPLC. Starting with the 200 mg/L standard, 25 µL of the standard was injected into the HPLC and

analyzed. After all 5 samples were analyzed, a calibration curve was compiled. The concentration of the ethanol and glucose in the standard was plotted against the area underneath the appropriate peak of the HPLC data as shown by Figure 3 below.

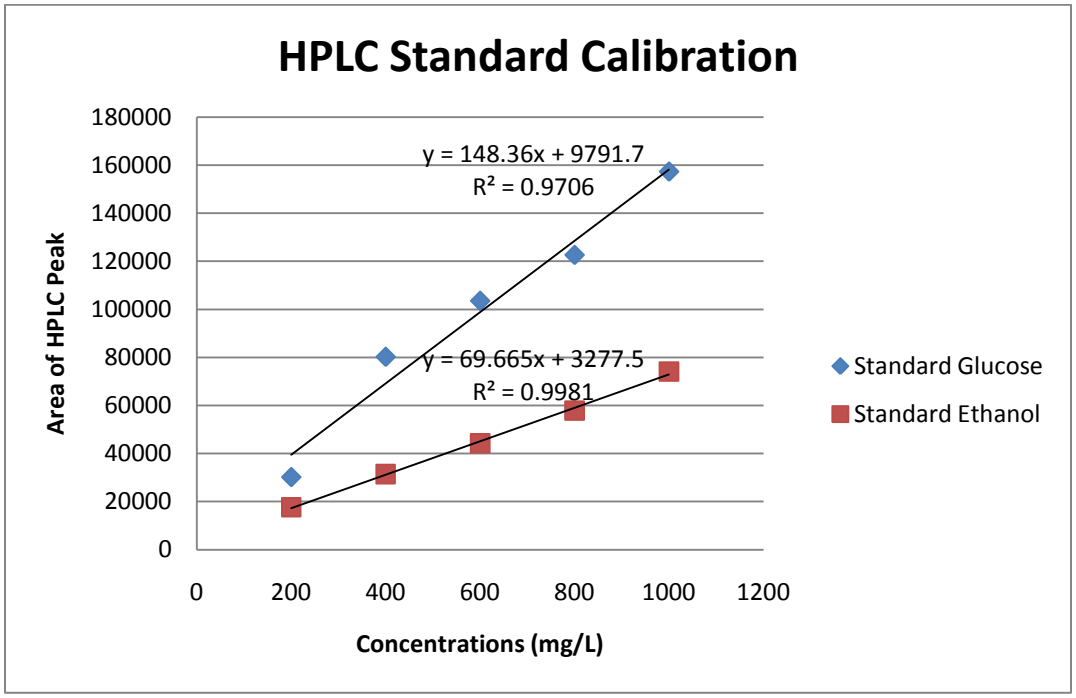

Figure 3: An Example of HPLC Standardized Data

The HPLC standard solutions must be measured once a day while the HPLC is in use. If the standard solution plots do not agree, the most current plot should be used to compare that day's data analysis.

Each sample from the growth bottles was analyzed in the HPLC. 3 mL of each sample solution was filtered through a 45 micron filter. 1 mL of the filtered solution was then diluted in a 10 mL volumetric flask. 25 µL of each diluted, filtered sample was then injected into the HPLC and analyzed. The area underneath the peak corresponding to the glucose and ethanol concentrations was recorded. Using the treadlines established from the calibration curve, the concentration of the glucose and ethanol were determined and plotted against time.

Temperature Variations

Temperature experiments were first carried out to discern what the optimal temperature for SSF was. Experiments were carried out over a range of 30 to 40 °C as this temperature range is the middle range of temperatures optimal for fermentation (25-35°C) and hydrolysis (35-47°C).

250 mL of solution was prepared using 3 g/L yeast, approximately 44 g/L substrate, and 0.4 mL of nutrient medium. The solution was divided into three 50 mL uncontrolled volume flasks; each flask was sealed with an air-tight cap and placed into a shaker at a controlled temperature. Samples were taken from the sealed flasks every 24 hours. Additionally, the flasks were emptied of any excess gas that had accumulated as a byproduct of the fermentation reaction.

Substrate Variations

Based on the results of the temperature experiments, the substrate concentration was studied to determine the effects of glucose substrate inhibition on the SSF. Experiments were carried out with a range of 40 to 100 g/L of glucose in the solution.

250 mL of solution was prepared using 3 g/L yeast, varied substrate composition, and 0.4 mL of nutrient medium. The solution was divided into three 50 mL uncontrolled volume flasks; each flask was sealed with an air-tight cap and placed into a shaker at a controlled temperature. Samples were taken from the sealed flasks every 24 hours. Additionally, the flasks were emptied of any excess gas that had accumulated as a byproduct of the fermentation reaction.

pH Variations

1L of solution was prepared using 3 g/L yeast, 40 g/L substrate composition, and 1.6 mL of nutrient medium. The solution was placed into a batch reactor at a controlled temperature of 35°C where volume was fixed and controlled to 2L. pH was controlled to 4.5, 5, 5.5, and 6 by a controller that used NaCl and NaOH to keep the pH constant. Every 24 hours, samples were taken.

Results and Discussion

Three trials were performed using common baker's yeast under varying conditions in order to determine the overall optimal conditions for SSF. The first experiment examined how temperature affected the ethanol yield by fermenting three samples at three different temperatures. The second experiment examined how the substrate composition affected ethanol yield by fermenting three samples at three different substrate compositions. The third experiment examined how the pH of the system affected ethanol yield by fermenting 4 samples at 4 fixed pHs.

Temperature Variation Results

The temperature variation experiment investigated how a range of temperatures affected the production of ethanol from yeast. It was conclusive that the temperature at which the yeast is fermented into ethanol greatly impacted the ethanol yield from the reaction. This is shown in table 7 below.

Table 7: Temperature Variation Effects

		Concentration				
	Time (h)	**0**	**24**	**48**	**72**	**96**
30 C	Glucose (mg/L)	43300	0	0	0	0
	Ethanol (mg/L)	0	20000	17200	19500	19600
	Ethanol Yield (%)	0	88.98	77.80	87.98	88.67
35 C	Glucose (mg/L)	45100	0	0	0	0
	Ethanol (mg/L)	0	18300	18500	17800	18100
	Ethanol Yield (%)	0	79.59	80.34	77.46	78.34
40 C	Glucose (mg/L)	44100	12800	10600	10000	9700
	Ethanol (mg/L)	0	13600	14600	14200	14300
	Ethanol Yield (%)	0	85.15	85.33	82.01	81.69

Equation 2: ethanol yield for 30C

$$\frac{\frac{19600mg}{L}*0.05L}{\left(\frac{43300\frac{mg}{L}*0.05L}{}\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)}*100 = 88.67\%$$

Equation 3: Ethanol yield for 35C

$$\frac{\frac{18100mg}{L}*0.05L}{\left(\frac{43300\ mg/L*0.05L}{}\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)}*100 = 78.34\%$$

Equation 4: Ethanol Yield for 40C

$$\frac{\frac{14300mg}{L}*0.05L}{\left(\frac{33600\frac{mg}{L}*0.05L}{}\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)}*100 = 81.69\%$$

Figure 4 depicts the ethanol concentration as a function of time.

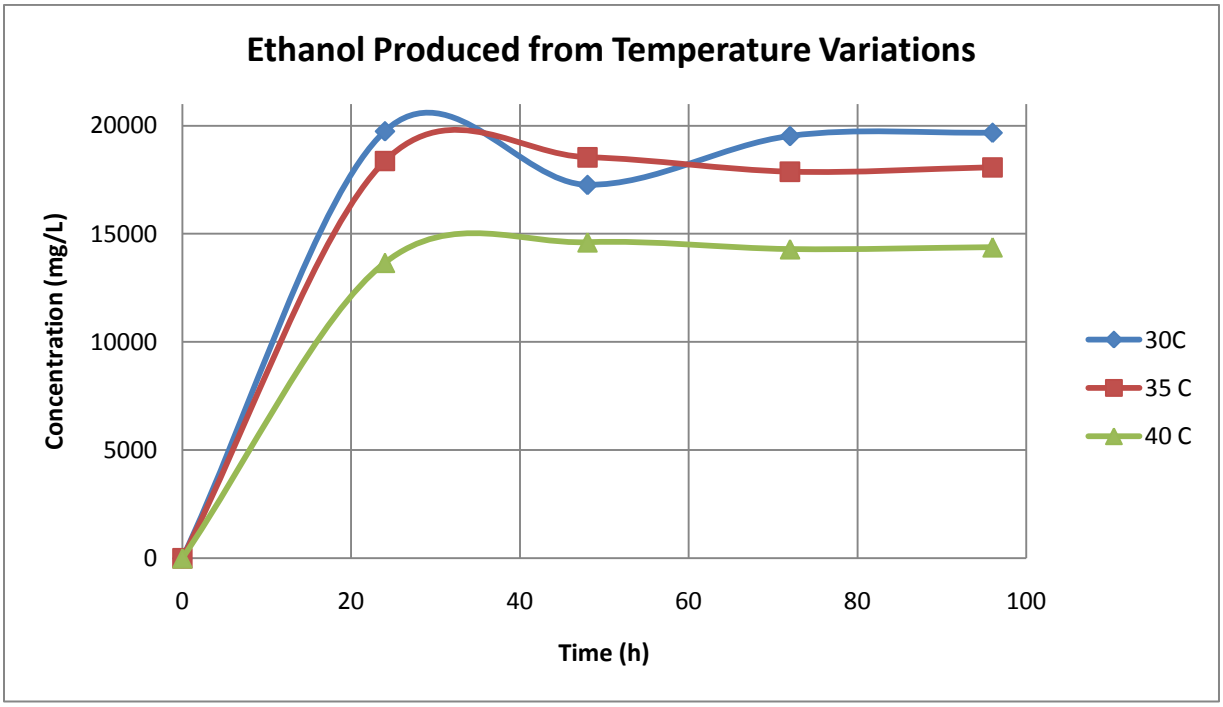

Figure 4: Ethanol production from Temperature Variations

It is evident from Table 7 and Figure 4 that the temperature at which the yeast is fermented greatly affects the ethanol yield from the reaction.

The 30°C reached reaction completion the fastest. This suggests that the yeast was under minimal stress and was not inhibited by the produced ethanol present in the flask. Therefore, the yeast cells at 30°C are presumed to be structurally sound and are capable of healthy and efficient reproduction. Given that the ethanol yield for this temperature was 88% this suggests nearly all the glucose substrate was converted into ethanol.

The 35°C experiment reached reaction completion second fastest. The kinetics support that a higher temperature affects the yeast's ability to ferment the substrate into ethanol. There were problems with this particular run, however. The shaker that this experiment was carried out in underwent a power failure for approximately 36 hours; the reaction in this time period was stationary. Because of the lack of agitation, not all the yeast was able to come in contact with the available substrate. Therefore, the ethanol yield at 35°C was lower than anticipated. If further time had been allotted to temperature trials, this temperature would have been repeated to ensure that the stationary reaction state was in fact the sole reason behind the low ethanol yield.

At 40°C, reaction kinetics were the slowest. It was assumed that the high temperature put a stress on the yeast as it reproduced. With reproduction slower and less efficient, there was less yeast to consume the available substrate. The slower kinetics played into the inhibitory affect glucose concentration has on yeast. The excess glucose present in the system slows cell growth and greatly affects cell viability.

It was therefore reasonably conclusive that lower temperatures are more favorable for ethanol fermentation. However, despite the differences in ethanol yield, all three temperatures produced a high concentration of ethanol. It was concluded that while temperature does affect fermentation kinetics and yeast viability, it does not greatly affect the system over this temperature range.

A temperature of 35°C was used in all subsequent experiments as the temperature for reaction. Given that all the glucose was consumed and ethanol was 75% of the total possible yield, this temperature did not stress the yeast enough to cause a low ethanol production. 35°C is in the middle temperature point between optimal hydrolysis and fermentation temperatures. Because SSF is a combination of the two steps, this temperature seems to be a reasonable temperature for both processes.

Substrate Variation Results

The substrate variation experiments investigated how different amounts of glucose affected the production of ethanol from yeast. Based on this experiment, it can be concluded that the glucose concentration affects several aspects of the fermentation reaction. Table 8 summarizes the ethanol yield from the reaction

Table 8: Substrate Variation Effects						
	Concentration					
	Time (h)	0	24	48	72	96
40 g/ L Base	Glucose (mg/L)	42900	6660	0	0	0
	Ethanol (mg/L)	0	6490	16500	26300	16700
	Ethanol Yield (%)	0	34.95	75.41	119.69	76.25
80 g/L Base	Glucose (mg/L)	85500	17200	0	0	0
	Ethanol (mg/L)	0	28100	36700	34400	32900
	Ethanol Yield (%)	0	80.56	84.08	78.72	75.44
100 g/L Base	Glucose (mg/L)	10900	33800	1870	0	0
	Ethanol (mg/L)	0	31300	45300	43700	42700
	Ethanol Yield (%)	0	80.84	82.30	78.02	76.28

Equation 5: ethanol yield for 40 g/L glucose

$$\frac{\frac{16700mg}{L}*0.05L}{\left(42900\frac{mg}{L}*0.05L\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)}*100 = 76.25\%$$

Equation 6: ethanol yield for 80 g/L glucose

$$\frac{\frac{32900mg}{L}*0.05L}{\left(85500\frac{mg}{L}*0.05L\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)}*100 = 75.44\%$$

Equation 7: ethanol yield for 100 g/L glucose

$$\frac{\frac{42700mg}{L}*0.05L}{\left(\frac{10900\frac{mg}{L}*0.05L}{}\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)}*100 = 76.28\%$$

The ethanol yield should decrease proportionally as the substrate composition increases; this is due largely in part to the inhibitory effects glucose has on the fermentation reaction kinetics. Additionally, the high levels of glucose should hinder yeast growth during the reaction. However, within this trial there is no noticeable effect of the glucose concentration.

Throughout this experiment pH and cell density were measured to determine how the varied glucose concentrations affected the system. Based on the absorbance curve and pH plot, Figures 5 and 6, it can be concluded that the yeast in the system experienced cell death due to weak acid inhibition.

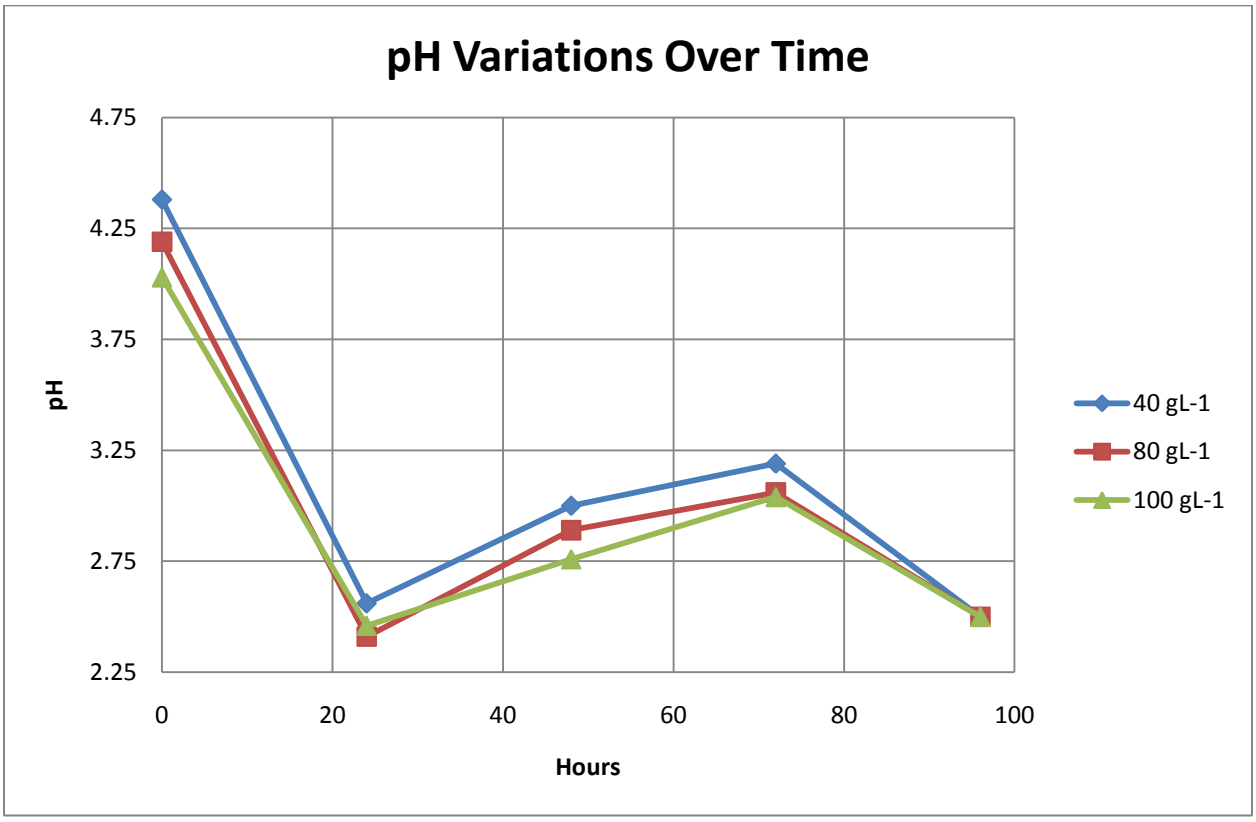

Figure 5: pH levels over substrate reactions

The pH of the reaction fluctuates as ethanol and byproducts are formed. All three variations of glucose levels followed the same trend. There is a rather steep drop in pH around the 24 hour mark. This can directly be attributed to fermentation byproducts. As the yeast digests the glucose, acetic acid and formic acid are formed as secondary byproducts. The presence of the weak acids in the system inhibits yeast production; the acids are liposoluable and therefore can diffuse across plasma membranes of yeast cells and raises the intracellular pH. The yeast cells respond to the diffusion process by expending ATP to repair the membrane and maintain a constant intracellular pH. The yeast subsequently has less ATP to devote to cell reproduction.

The presence of the weak acids and the ethanol also cause yeast cell death. The ethanol attacks the plasma membrane of the yeast. The damage done to the plasma membranes of the yeast from the ethanol and weak acid presence caused enzymes essential to reproduction to leak out of the cell wall.

Figure 6 supports this conclusion, as it shows the cell density of the ethanol over the course of the experiment. The cell densities across the three substrate concentrations remain fairly proportional; if yeast cell damage hadn't occurred, the absorbance would vary depending on the substrate concentration. Both 80 and 100 g/L concentrations would have had a proportionally lower absorbance reading because of the lack of ethanol produced in the system.

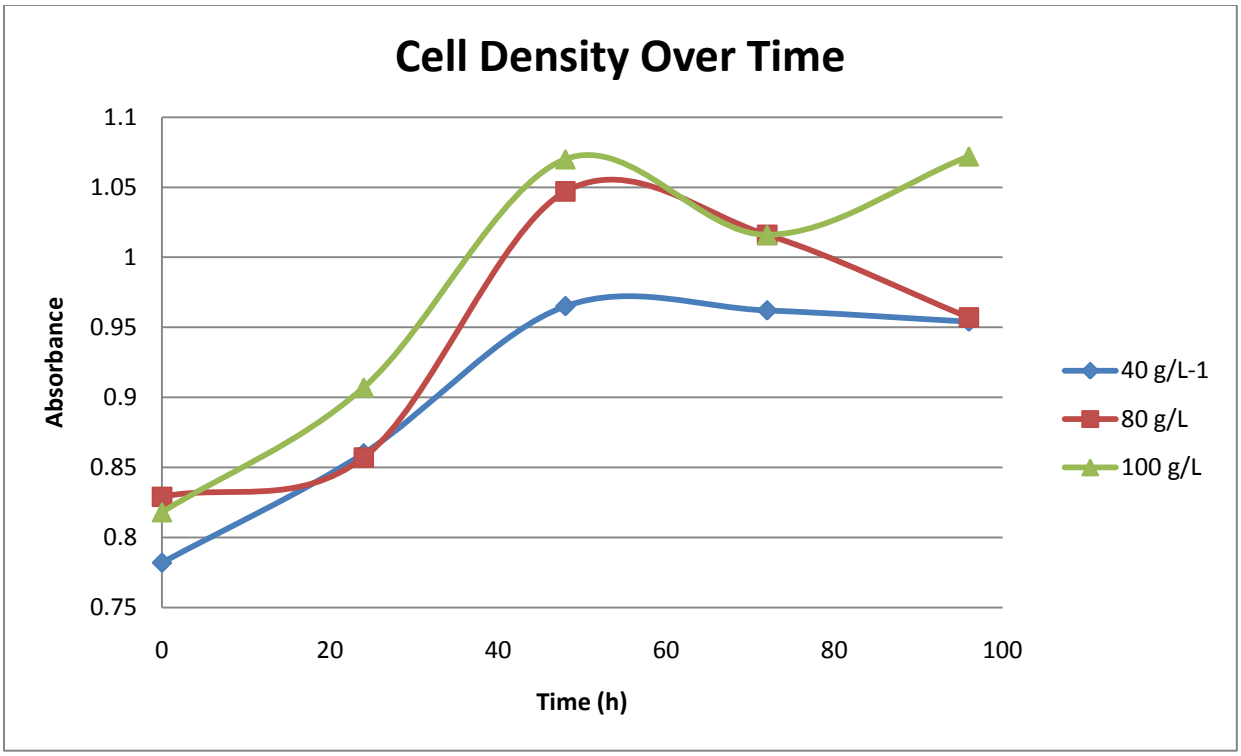

Figure 6: Cell Density over time comparing varying substrate concentrations

Figure 7 depicts the concentration of ethanol produced as a function of time. The reactions took approximately 42 too 44 hours to reach completion. This is about 10 hours slower than the time required for the temperature experiments to reach completion. The delay in the reaction time can be attributed to the cell death that was occurring during the reaction.

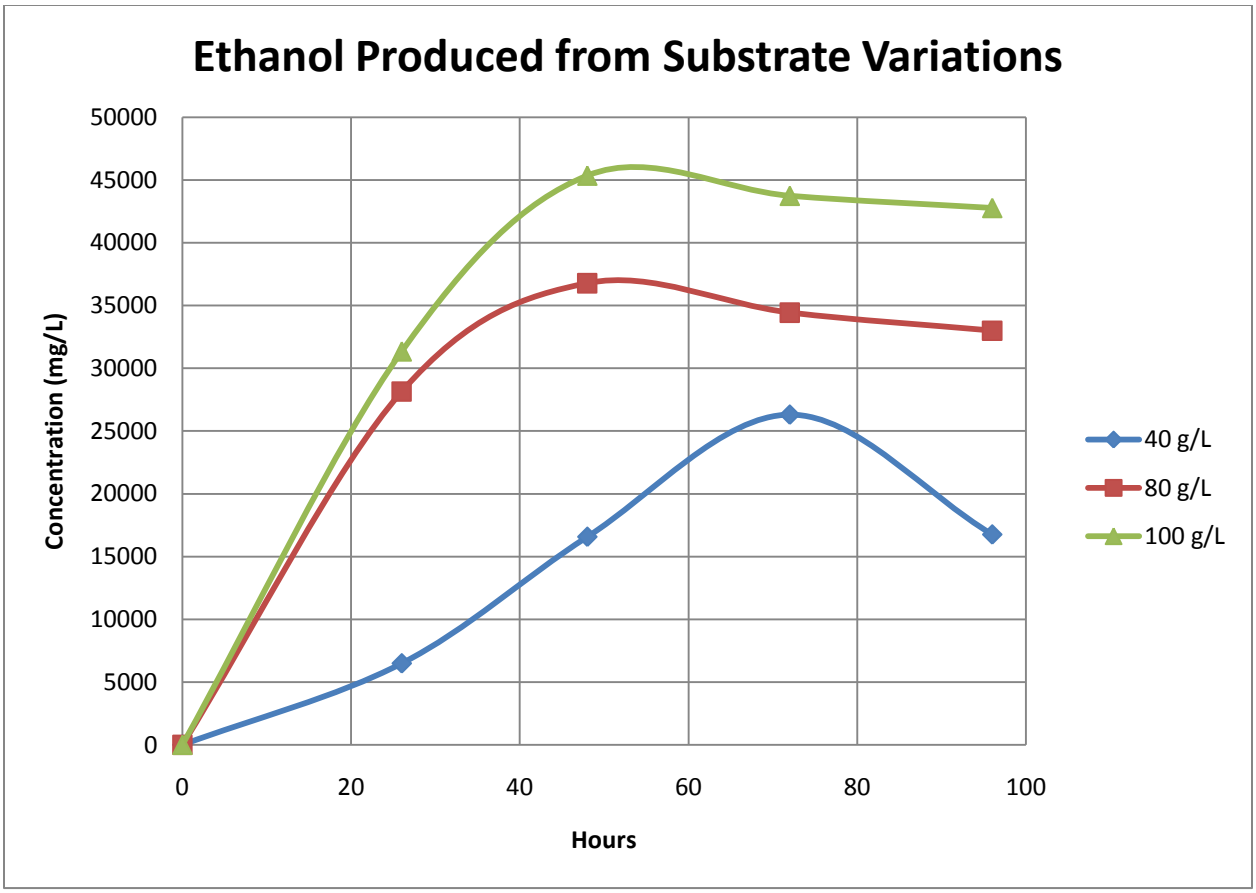

Figure 7: Ethanol Production during Substrate Variations

As yeast began to die off, there were fewer and fewer healthy yeast cells to ferment the glucose into ethanol. That means that it took a longer period of time for all of the glucose to be consumed because there was less available yeast for the reaction. Figure 6, as discussed previously, depicts that the ethanol cell density in the reactions were fairly proportional to one another. If the fermentation reaction was not inhibited by the yeast cell death, the absorbance curves would look fairly different compared to each other because the lower substrate concentration reactions would have ideally reacted faster than the higher substrate concentrations.

Based on the data from this experiment, a lower substrate concentration is desirable for fermentation to ensure that the ethanol and acids produced within the reaction don't cause too much yeast cell death. A lower substrate concentration also ensures that any substrate inhibition in future experiments that may occur would be limited.

A glucose concentration of 40 g/L was used for further experiments. Since pH can affect yeast reproduction and fermentation reaction time, the 40 g/L concentration was the best option. It had the

highest range of pHs during the experiments and completed the fermentation reaction the fastest. While the 100g/L substrate concentration produced the highest ethanol yield of 76.28%, the 40 g/L run produced just 0.03% less ethanol at 76.25%. This 0.03% difference was considered to be negligible after determining that the reaction kinetics were faster at 40 g/L substrate concentration.

pH Variation Results

The pH variation experiments investigated how the pH level of the system affected the production of ethanol from yeast. Based on this experiment, it can be concluded that pH has a noticeable effect on the fermentation of ethanol.

Table 9 summarizes the ethanol yield from the reaction.

Table 9: pH variation effects						
		Concentration				
	Time (h)	0	24	48	72	96
4.5 pH	Glucose (mg/L)	47400	0	0	0	0
	Ethanol (mg/L)	0	14900	15200	14900	14500
	Ethanol Yield (%)	0	61.48	62.77	61.72	60.24
5.0 pH	Glucose (mg/L)	48700	0	0	0	0
	Ethanol (mg/L)	0	14300	14500	14500	14100
	Ethanol Yield (%)	0	57.47	58.33	58.29	56.48
5.5 pH	Glucose (mg/L)	46900	6660	0	0	0
	Ethanol (mg/L)	0	13702.54	13400	13400	13400
	Ethanol Yield (%)	0	66.56	53.88	53.77	53.71
6.0 pH	Glucose (mg/L)	47500	8400	471	0	0
	Ethanol (mg/L)	0	10900	10300	105700	10500
	Ethanol Yield (%)	0	54.74	42.94	43.55	43.20

Ethanol yield is greatest when the pH of the reaction is fixed to a lower acidic level. As the pH increases, the reaction yield decreases. The variations in pH did not seem to affect the overall reaction kinetics of the system as can be seen in Figure 8. Instead, it appears that the pH affects the yeast's ability to convert glucose into ethanol.

Baker's yeast has an internal pH of about 5.0, therefore it favors reproduction and growth in a slightly acidic environment. pH levels of 4.5 and 5 yielded relatively similar results due to the fact that baker's yeast is naturally acidic. However, at a pH of 6, reaction yield is nearly 20% lower than a pH of 4.5, suggesting that neutral or basic pH's will be greatly inhibit overall yeast health. This can be assumed because substrate concentrations and temperature concentrations were held constant at values previously determined to be optimal conditions for fermentation. Therefore, the only variable uncontrolled during this experiment was the yeast health.

It can therefore be concluded that a slightly acidic pH, around 4.5, is optimal for yeast fermentation. Lower pH levels ensure that the yeast functions under minimal internal stress and therefore can ferment glucose into ethanol more efficiently. However, pH experiments in ranges lower than 4.5 were not investigated due to time. In order to further validate that 4.5 is the optimal pH for reaction, several runs should be conducted at values of 4, 3.5, 3, and 2.5.

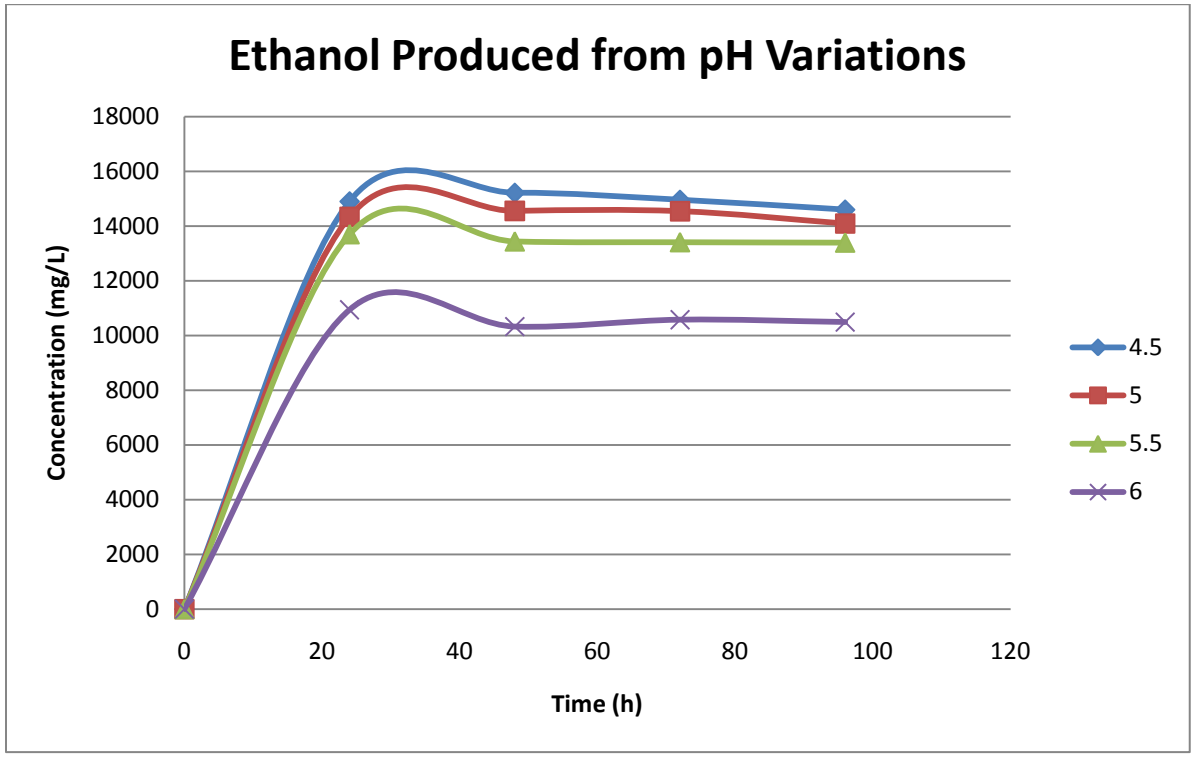

Figure 8: Ethanol Production during pH variations

A major flaw in all four of the pH reactions was that the reactor was not perfectly sealed, so the environment in the tank was not completely anaerobic. The presence of oxygen in the chamber switched the yeast reaction pathway from ethanol production to carbon dioxide and water production. Therefore, there was less ethanol produced than what was possible because some yeast and glucose produced water and CO_2. This flaw can help explain why ethanol yields in these four reactions were lower than in the reactions conducted previously. Any further reactions should be conducted in an environment that is anaerobic.

Conclusions and Recommendations

Based on the experiments run, the following conditions are considered to be the optimal conditions for SSF reactions. These conditions ensure that the possible inhibitory effects on the system are minimal. These conditions ensure that a minimum of 60% ethanol yield will be produced from the SSF reaction.

The temperature should be fixed to 35°C.

The substrate glucose concentration should be 40 g/L initially.

The pH of the reaction should be fixed to 3.5.

There are a number of experiments that should be conducted in order to confirm these results. Foremost, a complete SSF reaction should be carried out at 35°C, with a 3 g/L yeast concentration, a 40 g/L glucose concentration, and a pH controlled to 4.5. The results from that trial should fully support the conclusions drawn in this report.

Any further experiments should be conducted in completely anaerobic environments. Flasks and reaction vessels should be pumped with nitrogen gas in order to ensure that there was no oxygen present during the reaction. The presence of oxygen during the fermentation process hinders the amount of ethanol produced. The lack of oxygen in the reaction environment is actually a catalyst for the fermentation reaction. Any excess oxygen can retard inhibit the rate of fermentation.

There should be an investigation into how varying the nutrient medium that supplements the reaction affects the overall SSF process. Just as substrate concentration was varied, several trials should be conducted varying the nutrient medium. There may be inhibitory effects from too much or too little excess nutrients for the yeast. Determining what amount of additional nutrients is optimal can affect how well the yeast tolerates temperature and pH fluctuations. This could open up a wider band of physical conditions that the SSF reaction could tolerate. The greater the tolerance on physical conditions, the more applicable the process can be in industrial applications where inlet feed conditions may vary.

There should be an investigation into how osmotic pressure affects the ethanol yield. The osmotic properties of the yeast cell control how permeable the cell wall to solutions in the fermentation

solution. An increase in excess sugars or minerals will result in an increase in osmotic pressure. High osmotic stress would likely result in intracellular ethanol accumulation. This could result in a decrease of yeast growth and fermentation ability.

Several strains of yeast should also be tested to compare data and results to that of *S. cerevisiae*. While common baker's yeast is most often used in regular fermentation, other strains of yeast might prove more effective in an SSF environment.

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Appendix A: Raw Data
Temperature Data

HPLC Standard Solution Data for 35°C

Table A1: 35C, Standard Solution					
	Concentration mg/L				
	200	400	600	800	1000
Cellulobiose	34381	69448	103547	138870	163019
Glucose	34468	68446	100713	134271	158187
Xylose	37754	77577	118207	159703	193919
Ethanol	14441	28132	44024	58904	71909

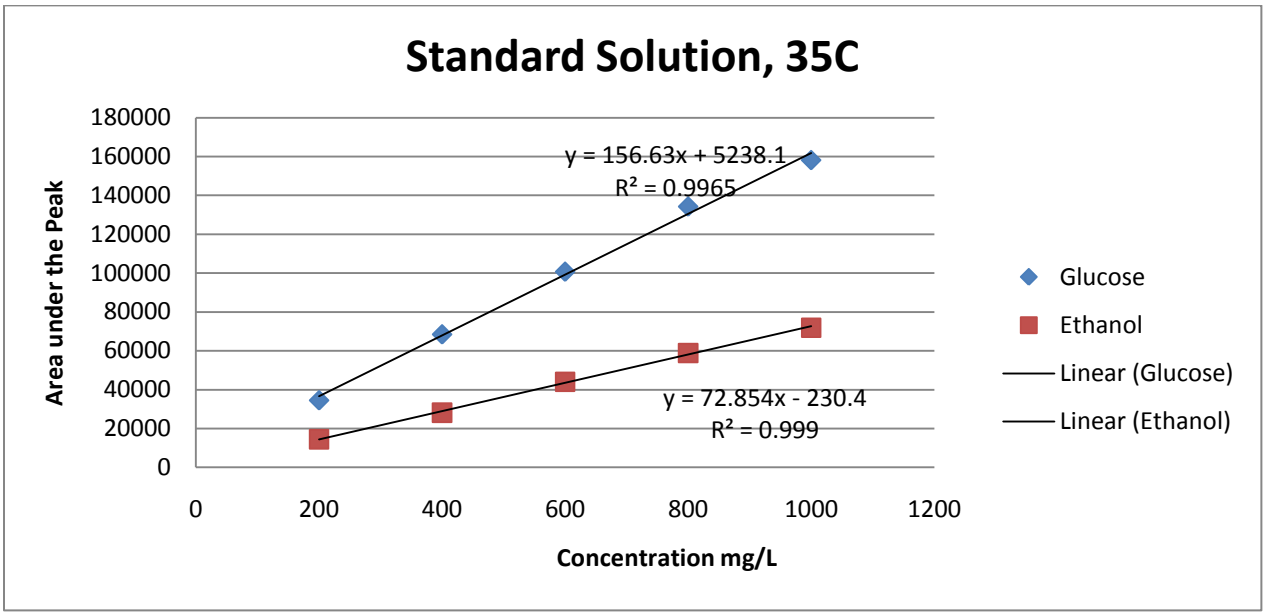

Figure A 1: Standard Solution Plot for T= 35C

HPLC Standard Solution Data for 30,40°C

Table A 2: 30, 40C StandardSolution					
	Concentration mg/L				
	200	400	600	800	1000
Cellulobiose	38166	70047	95965	136383	174563
Glucose	36568	69524	94364	133564	169887
Xylose	40176	78859	108791	152618	201600
Ethanol	16609	28727	40846	59022	71917

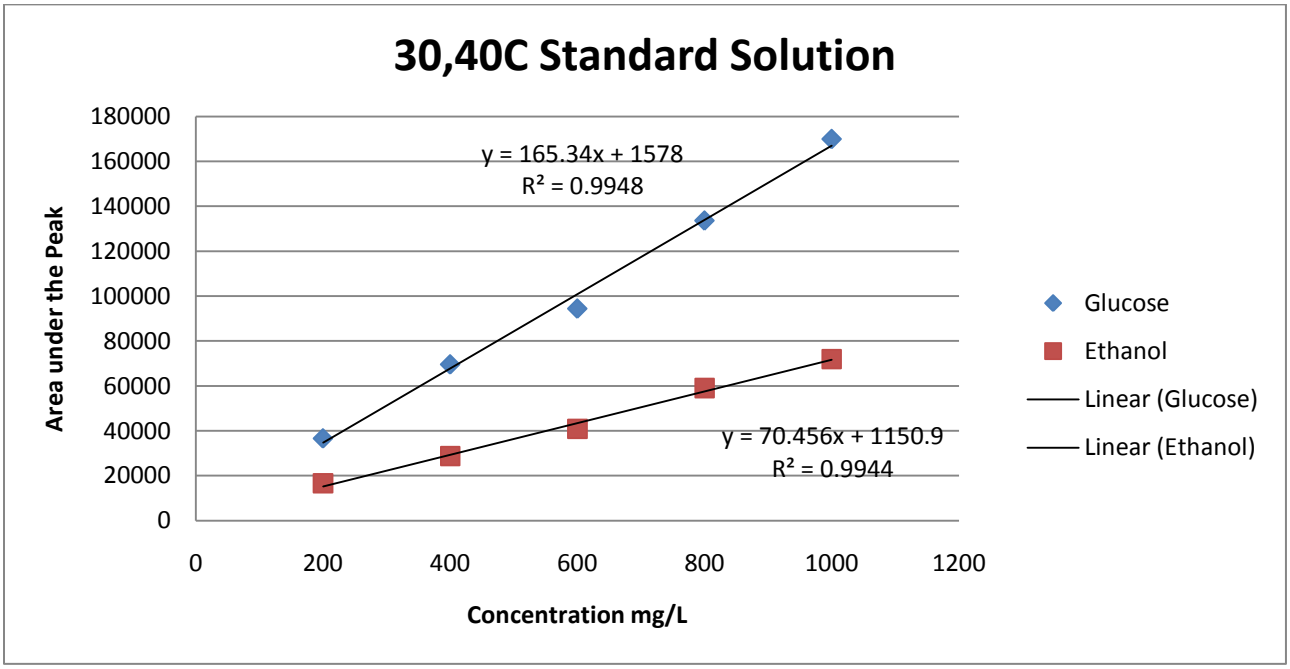

Figure A 2: HPLC STandard Solution Data for T=30,40C

Table A 3: Experimental Data for Temperature Variations						
	Time(h)	0	24	48	72	96
30C	Glucose Area	360266	0	0	0	0
	Ethanol Area	0	70709	61970	69927	70465
35C	Glucose Area	374459	1161	103	98	0
	Ethanol Area	0	68036	68666	66237	66981
40 C	Glucose Area	366280	106945	89528	84614	81759
	Ethanol Area	0	49280	52615	51493	51808

Substrate Data

Table A 4: Substrate Variation Data				
Substrate Variations with the effect on Ethanol Production				
		Bottle A	Bottle B	Bottle C
	Substrate Concentration (g/L)	40	80	100
0 hours	pH	4.38	4.19	4.03
	OD	0.782	0.829	0.818
	gas collected (mL)	0	0	0
24 hours	pH	2.56	2.41	2.46
	OD	0.86	0.857	0.907
	gas collected (mL)	32	70	85
48 hours	pH	3	2.89	2.76
	OD	0.965	1.047	1.07
	gas collected (mL)	0	24	45
72 hours	pH	3.19	3.06	3.04
	OD	0.962	1.016	1.016
	gas collected (mL)	0	0	9
96 hours	pH	2.5	2.5	2.5
	OD	0.954	0.957	1.072
	gas collected (mL)	0	0	0

HPLC Data for the Standard

Table A 5: Substrate Standard Solution					
	Concentration mg/L				
	200	400	600	800	1000
Glucose	30199	80258	103559	122663	157355
Ethanol	17689	31433	44252	57874	74133

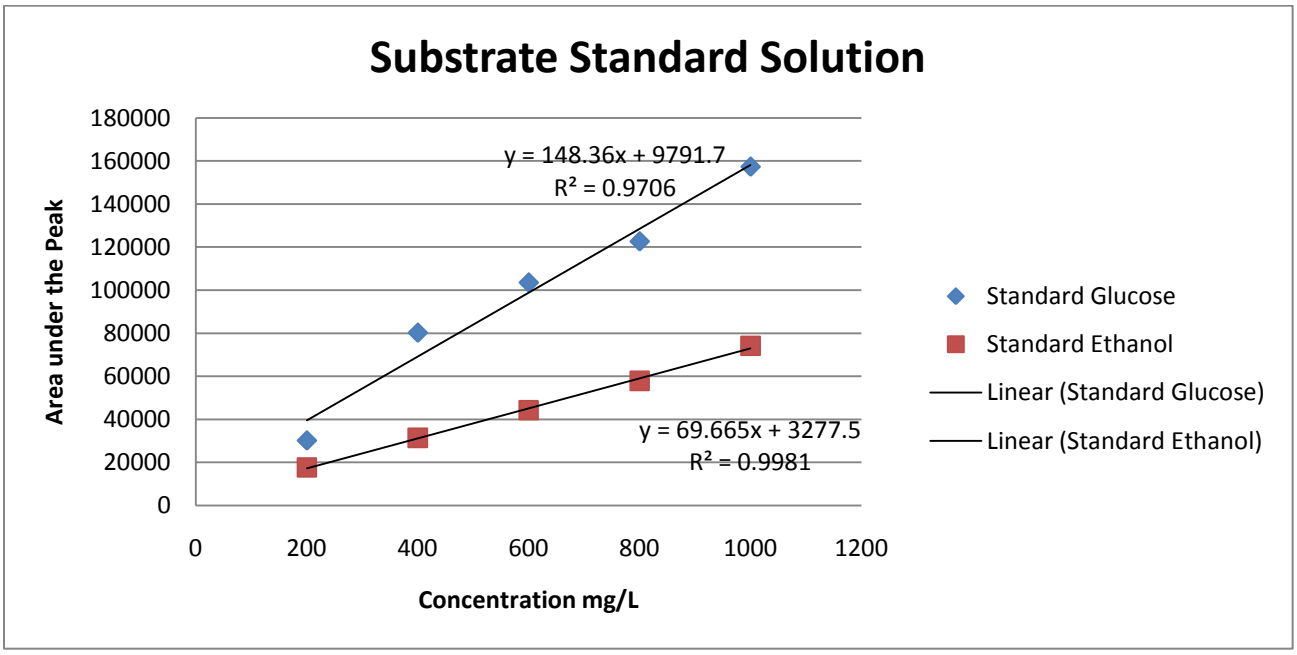

Figure B 1: HPLC Standard Plot for Substrate Variations

HPLC Data for Substrate

Table A 6: HPLC Substrate experimental data						
	time (h)	0	24	48	72	96
40 g/L	glucose area	328572	59211	1218	904	621
	ethanol area	0	25886	61013	94913	61653
80 g/L	glucose area	644104	137500	0	124	119
	ethanol area	0	101299	131369	123205	118204
100 g/L	glucose area	822908	260563	23726	130	0
	ethanol area	0	112451	161237	155631	152249

Appendix B: Sample Calculations

Determining Concentration from the HPLC Data

Using Data from Temperature Run 30C:

Standard Equations

Glucose: y = 165.43 x+ 1578

Ethanol: y = 70.456x + 1150.9

$$Glucose\ Concentration = \frac{Area\ under\ the\ Glucose\ Peak - y\ intercept\ for\ standard\ glucose\ curve}{(Slope\ of\ the\ Glucose\ Curve)} * 20$$

The equation is multiplied by 20 to correct for the 20X dilution of the actual sample for use in the HPLC

$$Ethanol\ Concentration = \frac{Area\ under\ the\ Ethanol\ Peak - y\ intercept\ for\ standard\ ethanol\ curve}{(Slope\ of\ the\ ethanol\ Curve)} * 20$$

The equation is multiplied by 20 to correct for the 20X dilution of the actual sample for use in the HPLC

Experimental Data						
	Time(h)	0	24	48	72	96
30C	Glucose Area	360266	0	0	0	0
	Ethanol Area	0	70709	61970	69927	70465

At time 0 h:

$$Glucose\ Concentration = \frac{(360266 - 1578)}{165.34} * 20 = 43387.93$$

At time 24 h:

$$Ethanol\ Concentration = \frac{(70709 - 1150.9)}{70.456} * 20 = 19745.12$$

Determining Ethanol Yield Percentage

$$Ethanol\ Theoretically\ Produced =$$

$$\left(\frac{\frac{mg}{L}\ glucose\ consumed * L\ Flask\ Volume}{}\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)$$

$$Ethanol\ Actually\ Produced = Concentration\ of\ Ethanol \frac{mg}{L} * Flask\ Volume\ L$$

$$Ethanol\ Yield = \frac{Ethanol\ Actually\ Produced}{Ethanol\ Theoretically\ Produced} * 100$$

For Temperature run 30C:

At 24h:

$$\frac{\frac{18{,}361mg}{L} * 0.05L}{\left(\frac{43387.93\frac{mg}{L} * 0.05L}{}\right)\left(\frac{mol\ glucose}{180\ g\ glucose}\right)\left(\frac{g}{1000mg}\right)\left(\frac{2mols\ Ethanol}{mol\ glucose}\right)\left(\frac{46.07\ g\ Ethanol}{mol\ ethanol}\right)} * 100 = 88.98\%$$